A PHYTOCHEMICAL EXPLORATION OF GRIFFONIA SIMPLICIFOLIA SEEDS AND LEAVES

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ABSTRACT OF THE THESIS

A Phytochemical Exploration of *Griffonia simplicifolia* Seeds and Leaves

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Griffonia simplicifolia is a legume indigenous to western region of Sub-Saharan Africa. While the seeds of this plant are known for being the most abundant natural source of 5-hydroxtryptophan (5-HTP), a widely consumed alternative treatment for conditions involving a serotonin imbalance, many phytochemical characteristics of this species remain unreported. In order to further characterize the phytochemical composition of *Griffonia simplicifolia*, the 5-HTP, nitrile glycoside, polyphenol, antioxidant, protein, flavonoid, mineral, fatty acids, and tocopherol content of several different populations of griffonia seeds and leaves collected from various regions of Ghana and Liberia were evaluated. For comparison, the 5-HTP content of 18 commercial products on the nutraceutical supplement market was quantified in order to verify the declared amount on their labels match their measured amount. Total polyphenol content of griffonia leaves was 21.16 ± 0.48 GAE/g and antioxidant capacities of leaves and seeds were 12.31 ± 0.45 TEAC/g and 216.50 ± 13.88 TEAC/g, respectively. Protein content of griffonia leaves was 6.89 ± 0.47 BSAE/gram and protein content of seeds was 33.58 ± 1.52 BSAE/gram. Average fatty acid, α-tocopherol, and γ-tocopherol content of seeds were 269.54 ± 2.77 mg/g, 22.62 ± 1.54 µg/g, and 86.58 ± 4.01 µg/g, respectively.

Average flavonoid content of griffonia leaves was 9182.02 ± 140.90 µg/g. The average 5-HTP content from all the sampled seed populations was 120.84 ± 9.00 mg/g and total nitrile glycosides averaged 14.25 ± 1.65 mg/g. Griffonia seeds were found to be a source of manganese but not calcium, and a high source of iron, zinc, magnesium, copper, and phosphorus. Among the various populations, the most substantial differences in secondary metabolite accumulation was observed in polyphenol, antioxidant, and flavonoid content of leaves as well as nitrile glycoside and tocopherol content of seeds. Pasteurizing seeds with dry heat does not significantly affect the 5-HTP content and seeds with black colored endosperms contained lower amounts of 5-HTP and nitrile glycosides than seeds with yellow endosperms. The 5-HTP content of all 18 dietary supplements was within ± 15% of the amounts declared on their labels.

DEDICATION

To my parents for their unwavering support throughout this journey and who instilled in me the virtues of integrity, hard work, and tenacity that made it possible for me to complete this degree. To my older brother, Jason, who has always provided me with invaluable guidance- I couldn’t have asked for a better role model.

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1.1 Overview of *Griffonia simplicifolia*

1.1.1. Description and Traditional Uses

Griffonia simplicifolia (formerly known as *Bandeiraea simplicifolia)*, a member of the Leguminosae family, is a "stout, woody, climbing shrub, woody vine or liana" (Brendler et al., 2010) that reaches approximately three meters in length and is found in the forests of Sub-Saharan West Africa ranging from Liberia to Gabon (**Figure 1-1a**) (Brendler et al., 2010). The leaves and flowers of this plant have a green color (**Figure 1-1b**) while the seeds have a flat, black, circular appearance that resemble wisteria seeds, which weigh approximately 0.5 grams and average 1.7 cm in diameter (**Figure 1-1c**) (Brendler et al., 2010). Griffonia is known throughout Africa by several different names, including atooto, gbogbotri, kajya, kanya, and kwakuo-aboto (Brendler et al., 2010) and has been used as a traditional medicine for a variety of ailments. The leaves are used to treat skin injuries and kidney disorders (Brendler et al., 2010), the bark is used to treat skin ulcerations (Brendler et al., 2010), and infusions of the leaves and twigs are used to treat vomiting, diarrhea and as an aphrodisiac (Neuwinger, 2000; Brendler et al., 2010). Roots and leaves have also been used to treat sickle cell disease (Larmie and Poston, 1991).

1.1.2. Phytochemistry

Griffonia seeds have attracted commercial interest because they contain an unusually high amount of a rare amino acid known as 5-hydroxytryptophan (5-HTP) (Lemaire and Adosraku, 2002). 5-HTP is a widely sought after natural treatment for conditions involving an imbalance of serotonin such as depression, insomnia (Kim et al., 2009), insatiety (Del Corral and Pacak, 2005), and fibromyalgia (Birdstall, 1998). The unusually high 5-HTP content is thought to protect griffonia seeds from attack by certain feeding insects (Bell, 1975). 5-HTP has also been found to occur naturally in the fruits and seeds of other species such as *Ananas comosus* (pineapple),

Juglans regia (walnut), *Musa sapientum* (banana), *Persea americana* (avocado), *Solanum lycopersicum* (tomato), *Solanum melongena* (eggplant), (Hendler and Rorvik, 2001), *Citrullus lanatus* (watermelon) (Fellow and Bell, 1966), and *Hypericum perforatum* (St. John's Wort) (Kang et al., 2007). However, none of these sources accumulate high enough amounts of 5-HTP to be viewed as viable commercial sources given the high accumulation of 5-HTP (up to ~20% (w/w) (Lemaire and Adosraku, 2002)) appears to be exclusive to the genus *Griffonia* (Fellow and Bell, 1975). Other alkaloids (**Figure 1-2**) such as trigonelline, serotonin, 5-hydroxy-3-(2-hydroxyethyl) indole, 5-hydroxyindole-3-carboxaldehyde, hyrtiosulawesine, hyrtioerectine B, 3-carboxy-6-hydroxy-β-carboline, griffonine (Wang et al., 2013), indole-3-acetyl aspartic acid, and 5'-hydroxyindole-3-acetic acid (Fellow and Bell, 1970) have been found in the seeds in much lower content (generally less than 23 μg/g). Trigonelline is a relatively non-toxic metabolite of vitamin B_3 that is found in high abundance in *Trigonella foenum-graecum* (fenugreek) (Zhou et al., 2012) and genus *Coffea* (Coffee). This compound may also protect against type 2 diabetes (Lang et al., 2013) as well as central nervous system disease (Zhou et al., 2012). While the indoles found in griffonia commonly occur in the plant and animal kingdoms, the β-carboline derivatives are much less common. Hyrtioerectine B and hyrtiosulawesine were first isolated from the Red Sea sponge *Hyrtios erectus* (Salmoun et al., 2002; Yamanokuchi, 2012). Hyrtioerectine B has since been found in the related marine sponge *Hyrtios reticulatus* (Yamanokuchi et al., 2012) while hyrtiosulawesine is known to occur in only one other higher plant: *Alocasia macrorrhiza* (Araceae), which is used traditionally as an analgesic and antiphlogistic in subtropical Asia (Zhu et al., 2012). 3-Carboxy-6-hydroxy-β-carboline and griffonine are novel compounds that have never been isolated elsewhere in nature. 5-Hydroxyindole acetic acid is believed to act as a hormone (Fellow and Bell, 1970) while these other secondary metabolites found in griffonia likely defend the plant from feeding insects and microbes. Carbolines similar in structure to hyrtiosulawesine, hyrtioerectine B, 3-carboxy-6-hydroxy-β-carboline, and griffonine have been found to be toxic to some insects (Nenaah, 2011;

Marques et al., 2005; Zeng, 2010). Additionally, Hyrtiosulawesine, hyrtioerectine B, and griffonine have been found to inhibit the growth of HepG2 cells in the low micromolar range *in vitro* which indicates that these compounds may be effective anti-cancer agents (Wang et al., 2013).

Figure 1-1. African countries in which *Griffonia simplicifolia* is found (Brendler et al., 2010) (A). Live *Griffonia simplicifolia* plant (B). *Griffonia simplicifolia* seeds (C).

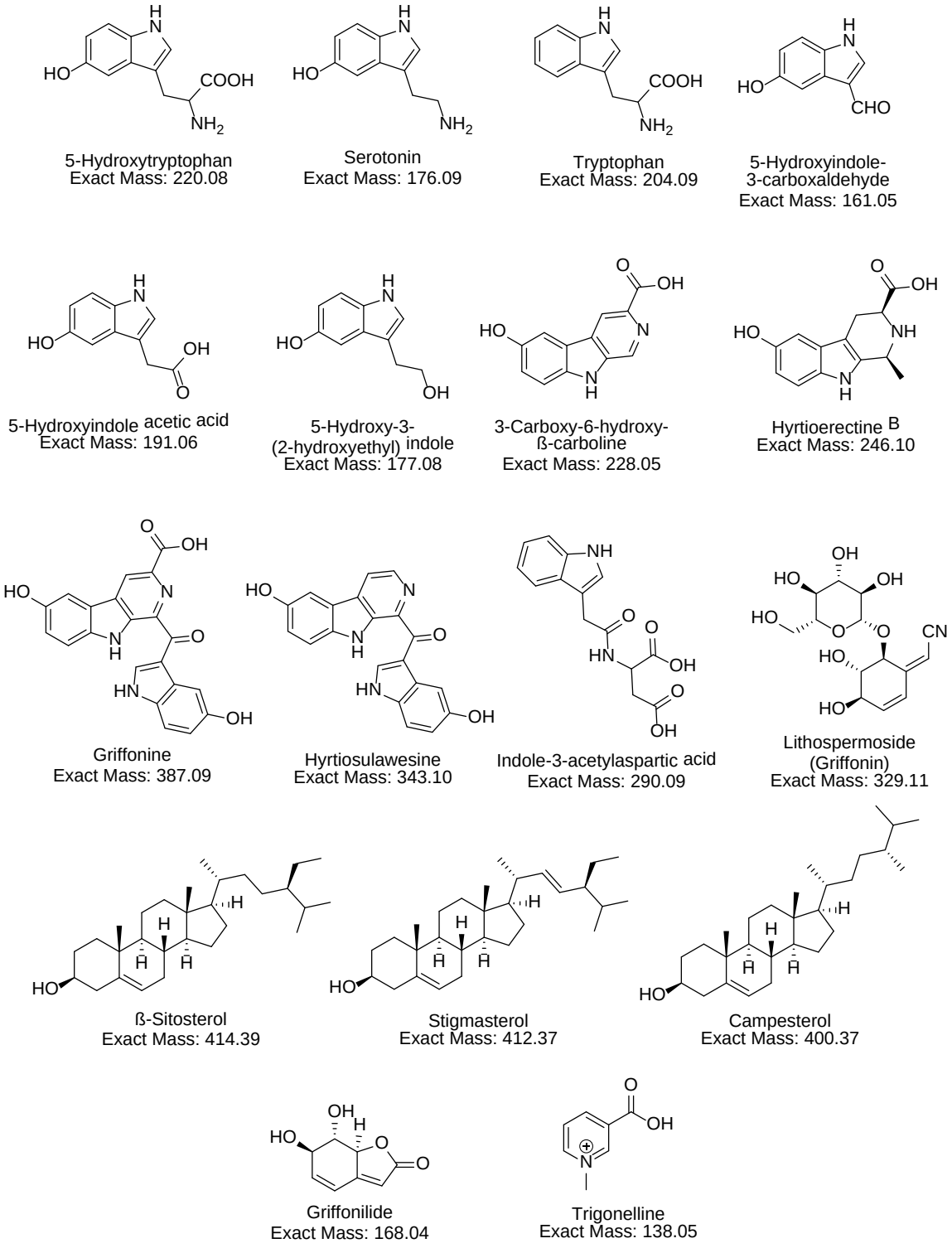

Figure 1-2. Secondary metabolites isolated from *Griffonia simplicifolia*.

Fatty acids are important to the vitality of both plant and animal cells. This is because a large amount of energy is stored in the fatty acid molecule's carbon-carbon bonds and this energy can be released to drive the cell's metabolic processes. Energy for metabolic processes can also be obtained by breaking down sugars and proteins, however, these molecules yield only about half the amount of energy per gram than fatty acids due to the fact that most of the carbons in sugars and proteins are already partially oxidized (Nelson and Cox, 2006). Fatty acids are also important precursors in the production of phospholipids, pigments, cofactors, detergents, transporters, hormones, chemical messengers, and anchors for membrane proteins (Lehninger et al., 1992). Linoleic and α-linolenic acids are two fatty acids that are essential to human health that the body cannot produce and must be obtained through plant sources. These two fatty acids serve as precursors to prostaglandins (Garrett and Grisham, 2006) which act as signaling hormones that help regulate vasodilation, blood platelet aggregation, inflammation, and smooth muscle contraction (Garrett and Grisham, 2006).

Myristic, palmitic, stearic, linoleic, oleic, and arachidic acids are the most commonly encountered fatty acids in the plant kingdom and all these have been reported to occur in griffonia seeds (Ramazanov and Petkov, 2003). Linoleic acid accounts for 53-60% of the fatty acid content of griffonia seeds and enrichment of seed oils to 95% linoleic acid have been successful (Ramazanov and Petkov, 2003) highlighting this species as an excellent source of this vital nutrient. In comparison to other legumes, linoleic acid accounts for 21-53% of the total free fatty acid content (Grela and Gunter, 1995; Ryan et al., 2007) and members of the legume subfamily Caesalpinioideae of which griffonia is a member of, are known to have a higher content of this fatty acid (Bagci and Sahin, 2004).

The phytosterols is a diverse class of compounds found in plants. The primary function of these molecules is to control the permeability and elasticity of cell membranes by restricting the motion

of the phospholipids of which they are composed (Hac-Wydro et al., 2007). Proper fluidity of cell membranes is not only important in maintaining the structural integrity of the cell, but is also "crucial for optimal enzymatic activity, ion and metabolite transport or channeling, protein–protein and protein–lipid interactions, signal transduction and finally to face fluctuating environmental conditions" (Schaller, 2003). Phytosterols also serve as precursors to other important biomolecules which include saponins, glycoalkaloids, cardenolides, and brassinosteroids (Piironen et al., 2000). *Beta*-sitosterol, stigmasterol, and campesterol the three sterols found in griffonia seeds (Ramazanov and Petkov, 2003), are also the three most common sterols in the plant kingdom (Piironen et al., 2000). These sterols are formed from squalene, a triterpene that is produced by aggregating isoprene units which are derived from the mevalonic acid pathway (Heinrich et al., 2012). β-Sitosterol, stigmasterol, and campesterol, are generally restricted to the plant kingdom and are not found in animal cells. Instead, animal cells rely mainly on cholesterol to modulate fluidity. In addition to this function, cholesterol is also a precursor to hormones and vitamin D (Berg et al., 2002), and derivatives of cholesterol also partake in food digestion (Berg et al., 2002; Garrett and Grisham, 2006). Phytosterols such as β-sitosterol are widely consumed as a dietary supplements to treat hypercholesterolemia. Being nearly identical in chemical structure to cholesterol, it is believed that the phytosterols lower blood cholesterol levels by blocking its absorption in the intestine (Hendriks et al., 1999). A 2006 meta-analysis concluded that supplementing the diet with plant sterols is useful in lowing LDL levels in both normocholesterolemic and hypercholesterolemic patients (Moruisi et al., 2006). Although phytosterols can reduce LDL by 8-15%, regular exercise, healthy diet, and/or drug treatment are still necessary because most hypercholesterolemic patients require an LDL reduction by 40% (Moruisi et al., 2006). In contrast to cholesterol, phytosterols do not appear to cause atherosclerosis except for in those with a rare genetic disorder that inhibits the liver's ability to excrete plant sterols from the blood known as phytosterolemia (Hendriks et al., 1999). Many metric tons of griffonia seeds are collected and processed each year for the natural 5-HTP content

which is then added into a variety of dietary supplements. The waste material following the extraction of 5-HTP is discarded. Given the presence of phytosterols, depending upon their yields, developing an extraction protocol for phytosterols in processed seed materials could be a commercial consideration. β-Sitosterol, stigmasterol, and campesterol have been reported in griffonia seeds however their absolute concentrations have not been reported.

The tocopherols are a class of four naturally occurring lipophilic compounds with potent antioxidant properties that play an integral role in human nutrition. Collectively with the tocotrienols, these compounds make up the class of nutrients commonly known as vitamin E. "Tocopherols are ubiquitously synthesized in all photosynthetic organisms" (Hussain et al., 2013) with the highest concentrations in plants occurring in seed oils (Zingg, 2007). However, tocotrienols have only been identified in specific areas of the plant kingdom (Hussain et al., 2013). Tocopherols and tocotrienols are beneficial because they can quench free radicals and thus prevent them from oxidatively damaging tissues. Oxidative damage has been linked to several serious health conditions such as cancer, diabetes, and atherosclerosis (Machado-Sanchez et al., 2006). While all tocopherols have similar *in vitro* antioxidant activity, α-tocopherol is the most biologically active of all tocopherol species (Zingg, 2007) and this has more to do with pharmacokinetics rather than differences in free radical quenching capacities (Zingg, 2007). The distribution of tocopherols into the bloodstream is believed to be mediated for the most part by α-tocopherol transport protein (α-TTP) and as its name suggests, this protein preferentially uptakes α-tocopherol (Zingg, 2007; Rigotti, 2007) resulting in α-tocopherol being the dominant tocopherol found throughout the human body with plasma concentrations averaging approximately 10 times greater than that of γ-tocopherol and 100 times greater than δ-tocopherol (Zingg, 2007). To date, the tocopherol composition of griffonia seeds has not been reported. If the tocopherol content in griffonia seeds is significant, it could be worth developing a process to extract these compounds from the seed material that is discarded after 5-HTP extraction.

In 1976, Dwuma Badu et al. isolated two compounds from griffonia roots, which were named griffonilide and griffonin. Griffonilide was a novel chemical structure while griffonin turned out to be identical to lithospermoside, a compound isolated from the unrelated species *Lithospermum purpureocaeruleum* that was characterized by Sosa et al. the same year and has since been found to occur in a small selection of various plant species including *Lithospermum officinale* (Sosa et al., 1977), *Coldenia procumbens* (Niranjan et al., 2013) *Cowania mexicana* (Ito et al., 1999), *Semiaquilegia adoxoides* (Han et al., 2001), *Lophiria alata* (Tih et al., 2003), *Ochna schweinfurthiana* (Ndongo et al., 2015), as well as several members of the genus *Thalictrum* (Wu et al., 1979; Erdemgil et al., 2003). Several species closely related to griffonia in the Leguminosae family also contain lithospermoside. They are *Cercis siliquastrum* (Plouvier, 1977), *Tylosema esculentum* (Mazimba et al., 2011), *Bauhinia fassoglensis* (Fort et al., 2001), *Bauhinia sirindhorniae* (Athikornkulchai et al., 2003) and *Cercis chinensis* (Li et al., 2005) which along with griffonia, are all members of the tribe Cercideae. This could provide further evidence that the genera *Griffonia* and *Bauhinia* are paraphyletic (Sinou et al., 2009). Other analogues of lithospermoside have been identified as well and like lithospermoside, these other compounds which are collectively known as the nitrile glycosides are rare occurrences that appear in seemingly unrelated corners of the plant kingdom. The toxicological properties of lithospermoside and its isomers (**Figure 2-6 and Tables 2-1 and 2-2**) have not been widely explored. While these molecules possess a cyano feature, this moiety is likely not cleaved from the molecule under physiological conditions or by endogenous enzymes thus it likely poses no threat to the normal function of cytochrome c oxidase, which is inhibited by cyanide although toxicological studies in humans should be conducted before making strong conclusions about the toxicity of these molecules. Lithospermoside has shown *in vitro* anti-sickling activity (Larmine and Poston, 1991) and anti-tumor promoting activity (Itoh et al., 1994; Ito et al., 1999). Other structural analogues have been found to have insecticidal, antifungal (Abbassy et al., 2007),

antiviral (Geng et al., 2012), and anorexic (Chida et al., 1991) activities. In this study, lithospermoside and an isomer known as riachin were detected in griffonia seeds of Liberian origin and this appears to be the first reported occurrence of these compounds in the seeds of this plant.

Griffonilide is a butenolide that often occurs alongside lithospermoside and does not have an established biological role although similar structures are known to induce nyctinastic leaf movements in other legumes (Urakawa et al 2004; McNulty et al., 2007). The medicinal activity of griffonilide itself has never been studied however similar compounds have shown anti-cancer (McNulty et al., 2007) and antibacterial (Wenderska et al., 2011) activity.

Polyphenolic compounds are a diverse class of secondary metabolites that include flavonols, flavones, flavanols, isoflavonoids, the phenolic acids, stilbenes, procyanidins, catechols, lignans, lignins, anthrocyanidins, tannins, and catechins. These compounds are known to occur in plants and some microorganisms (Robards, 2003). The polyphenols serve a variety of roles in plants where they act as pigments, signaling molecules, regulators of cell and plant growth, and also protect the species from UV light and predation by insects, animals, and other harmful organisms (Siqueria, 1991). Phenolic compounds are often linked to a wide range of health benefits as many of these compounds have been shown to behave as potent antioxidants *in vitro*, and these properties potentially offer protection against inflammation, heart disease, and cancer (Ferguson, 2001). There is also some evidence to suggest that excessive consumption of these compounds may have negative health effects as well (Ferguson, 2001); phenolics have been shown to inhibit topoisomerase, prostanoid biosynthesis, and signal transduction and in addition, behave as pro-oxidants which may lead to mutagenic effects *in vivo* (Ferguson, 2001). Despite the risk of these negative side effects, dietary intake of moderate amounts of botanical phenols is generally

recommended (Robards et al., 1999). To data, the polyphenol content and antioxidant capacities of griffonia seeds and leaves have not been reported.

The Leguminosae family is a unique member of the plant kingdom because many of the species it encompasses produce seeds that are exceptionally high in protein content. This is mainly due to an increased availability of nitrogen to these plants provided by several species of *Rhizobia*, a genus of bacteria that dwells in their roots (Friedman and Brandon, 2001). In exchange for fixed carbon molecules derived from photosynthesis (Kaschuk et al., 2009), *Rhizobia* bacteria provide the plant with ammonium (NH_4^+) produced from atmospheric nitrogen (Charles and Trainer, 2006). Not only is the plant provided with the nitrogen needed to produce copious amounts of proteins, but also allows for the plant to thrive in soils regardless of nitrate levels (Charles and Trainer, 2006). This is likely a significant contributing factor to the success and diversity of the Leguminosae family which encompasses over 19,000 species making it the third largest plant family (Veitch, 2010). In regard to human nutrition, proteins from plants are vital sources of nutrients as they contain nine amino acids that are essential to human health that the body cannot produce. Currently, no reports regarding the protein content of griffonia seeds exist. Griffonia being a member of the legume family likely produces seeds high in protein content therefore it may be worth investigating the nutritional quality of these proteins. The quantities of these amino acids can vary widely across plant species for example, corn tends to supply insufficient quantities of tryptophan and lysine while legumes such as soy often lack in methionine (Friedman and Brandon, 2001). Although this griffonia seeds and leaves are not traditionally consumed, the quantity of amino acids available from this legume may provide incentive to use this crop as a non-conventional source of protein.

Flavonoids are a class of chromones that are believed to protect the plants from ultraviolet radiation, insects (Romanelli et al., 2010), fungi, and also act as signaling molecules (Shirely,

1996). In legumes, flavonoids also serve as a chemical messengers between the plant and the root dwelling *Rhizobia* bacteria (Broughton et al., 2000). Like other polyphenols, flavonoids are widely distributed throughout the plant kingdom and are very potent antioxidants that have shown promise in reducing the risk of cancer (Marchand, 2002), heart disease (Cook and Samman, 1996), and thrombosis (Siddhuraju and Becker, 2003). Flavonoids have been shown to be more potent antioxidants *in vitro* than vitamins C and E (Rice-Evans et al., 1997) and they are more water soluble than vitamin E which may allow for them to have a wider range of activity throughout the body although there are strong differences between the various flavonoids in intestinal absorption between these nutrients (Manach et al., 2005). To date, no studies have been published regarding the flavonoid profile of griffonia leaves.

Minerals are inorganic nutrients that both plants and animals require for healthy growth, cellular and metabolic function. While rare in the developed world, mineral deficiency is relevant in many areas of Sub-Saharan Africa were undernourishment is common. Since griffonia is common in some of these countries, this plant could potentially be used as a non-traditional source of minerals if it contains large enough amounts of these nutrients and if there are no significant amounts of concurrent antinutrients. Currently, the mineral content of griffonia seeds and leaves have not been reported.

1.2 Serotonin

1.2.1 Serotonin in the Plants

In the plant kingdom, serotonin is generally formed in a two-step process. Tryptophan is first decarboxylated by tryptophan decarboxylase to give tryptamine followed by hydroxylation of tryptamine via tryptamine 5-hydroxylase to form serotonin (Pelagio-Flores et al., 2011). One exception is *Hypericum perforatum* (St. John's Wort) in which serotonin is formed via a 5-HTP intermediate (Kang et al., 2007). Serotonin is known to occur in griffonia seeds and leaves as well as many other species and its role in the plant kingdom is not entirely clear although it is believed by some that it has a role in senescence, blooming of flowers, shoot development, and protection from predators **(**Ramakrishna et al., 2011; Kang et al., 2009**)**. Others have hypothesized that integration of nitrogen into serotonin may also be a way to protect the plant from excess ammonia (Schroder et al., 1999).

1.2.2 Serotonin in Humans

Serotonin is a neurotransmitter that is produced in animals in two steps beginning with the conversion of tryptophan to 5-HTP by tryptophan hydroxylase followed by decarboxylation of 5-HTP to give serotonin via aromatic amino acid decarboxylase (**Figure 1-3**) (Pelagio-Flores et al., 2011). Serotonin is believed to contribute to feelings of well-being and euphoria. It controls mood by acting as an agonist to a family of seven receptors known as the 5-HT receptors in the central nervous system (CNS) (Barnes and Sharp 1999). 5-HT receptors are also found outside the CNS thus it is believed that serotonin has a role in other physiological functions as well (Hoyer et al., 1994). Neurological activity of serotonin is restricted to that which is produced in the CNS as this compound cannot cross the blood brain barrier (Birdstall, 1999). There has been evidence to suggest that 5-HTP itself may act as a 5-HT receptor agonist due to its structural similarity to

serotonin. However, it has not yet to be determined which 5-HT receptor(s) it binds to (Hendler and Rorvik, 2001).

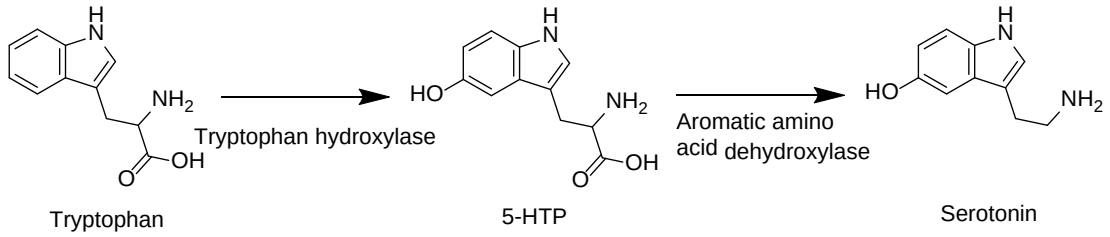

Figure 1-3. Serotonin pathway from tryptophan in humans (Pelagio-Flores et al., 2011).

1.2.3 Serotonin and Depression

By 1957 serotonin was only known to have a role in vasoconstriction and smooth muscle contraction. Serotonin was known to exist in the brain however, its function and link to mood remained undiscovered (Whitiker-Azmitia, 1999). In the following years, several researchers made observations that would implicate neurological serotonin imbalance in the pathology of depression. Researchers observed that some patients being treated for tuberculosis with the drug iproniazid also experienced an improvement in mood. Since iproniazid also acts as a monoamine oxidase inhibitor (MAOI), it was supposed that the mood improvement was due to this drug blocking the destruction of some endogenous amines (Owens and Nemeroff, 1994). In 1965, Coppen et al. suggested a link between serotonin and depression when it was observed that some people suffering from depression had a diminished ability to convert 5-HTP to serotonin and a decade later, Asberg et al. (1976) found that some patients with depression had lower amounts of 5-hydroxyindole acetic acid (5-HIAA) in their cerebral spinal fluid (CSF) (Owens and Nemeroff, 1994). 5-HIAA is the main metabolite of serotonin and studies have shown that a decrease in 5-HIAA does correlate with a decrease in serotonin as well (Owens and Nemeroff, 1994). Other

studies that followed also concluded that depressed patients suffered from serotonin deficiency in the brain (Owens and Nemeroff, 1994). It should be noted that some patients suffering from depression have also shown normal serotonin levels indicating that serotonin deficiency is not always causes depression (Owens and Nemeroff, 1994).

1.3 5-HTP

1.3.1 5-HTP in Humans

The primary purpose of 5-HTP in humans is to serve as an intermediate in the production of serotonin. The first experimental evidence of this role in animals appeared in 1953 when Udenfriend and co-workers observed that the kidney cells of dogs and guinea pigs readily converted 5-HTP to serotonin under aerobic conditions *in vitro* while the same cells were unable to metabolize tryptophan and tryptamine to serotonin (Whitaker-Azmitia, 1999). A few years later Udenfriend's findings were reinforced by a study in which a significant increase in serotonin concentration in the brain as well as in other various tissues was observed when 5-HTP was administered to rabbits, dogs, and rats (Udenfriend et al., 1957). Besides serving as an intermediate in serotonin synthesis, 5-HTP may be a useful antioxidant due to the fact it contains a hydroxyl substituted aromatic system. Aside from this, it is unknown if 5-HTP has any other biological roles.

1.3.2 5-HTP for Depression

The discovery of serotonin's role in mood prompted many studies aimed at evaluating the usefulness of 5-HTP as a clinical treatment for depression. Many of these studies have been criticized for insufficient sample size, lack of placebo control, and no patient blinding and many of these studies contradicted each other (Shaw et al., 2009). Thus, many believe that there is insufficient evidence to support the clinical efficacy of 5-HTP against depression. Authors of

some studies have suggested that further studies are needed to sufficiently conclude their findings (Hinz et al., 2012). The failure of 5-HTP to display broad efficacy could be due to a combination of factors. One study found that the penetration of 5-HTP through the blood brain barrier may be hindered in depressed patients (Agren et al., 1991). As mentioned earlier, another study found that 40% of depressed individuals had normal serotonin levels indicating that serotonin deficiency may not always be the sole cause of depression in some patients (Owens and Nemeroff, 1994). Another factor could have been that most 5-HTP was being converted to serotonin outside the CNS where it is unable to have an effect on mood. Researchers have tried to block this by co-administering 5-HTP with other compounds known to hinder its peripheral metabolism. The co-administration of 5-HTP with carbidopa was one such combination that was examined. Carbidopa (Lodosyn), a decarboxylase inhibitor, is primarily used in conjunction with levodopa (L-DOPA) to treat Parkinson's disease. In one small study of 12 subjects, 5-HTP combined with carbidopa has been found to give a 15-fold increase in plasma 5-HTP concentrations compared to 5-HTP alone (Gijsman et al., 2002). Disappointingly and despite these significant increases in blood 5-HTP concentrations, improvement of depressive symptoms did not appear (Gijsman et al., 2002). Research into using 5-HTP for depression reached its height in the mid 80's. However in 1987, fluoxetine the first of a new class of antidepressants known as selective serotonin reuptake inhibitors (SSRI's), was approved by the FDA which caused a shift in research away from 5-HTP to SSRIs (Iovieno et al., 2011). Despite the lack of strong clinical evidence of efficacy, 5-HTP is still a widely consumed natural remedy for depression with most interest is based on anecdotal claims of effectiveness. Interestingly, many antidepressants in today's pharmaceutical market were never shown to have a statistically significant effects compared to placebo either (Turner et al., 2006). Since its introduction as a treatment for depression, 5-HTP has been reintroduced for other indications such as insomnia (Birdstall, 1998), obesity (Yamada et al., 1999), and fibromyalgia syndrome (FMS) (Birdstall, 1998).

1.3.3 5-HTP for Obesity

Since increased appetite has been associated with decreased intrasynaptic serotonergic activity (Blundell and Halford, 1998; Del Corral and Pacak, 2005), 5-HTP was explored as a possible anorectic for overweight patients who struggle to control their food intake (Del Corral and Pacak, 2005). It is believed that serotonin suppresses appetite by activating 5-HT_{2C} receptors found in the hypothalamus (Yamada et al., 1999). A six week study concluded that 900 mg 5-HTP per day was effective at reducing caloric intake in 28 obese patients (Cangiano et al., 1992). Since most 5-HTP is metabolized prior to entering the CNS and since this peripheral serotonin cannot cross the blood brain barrier and stimulate intracranial 5-HT_{2C} receptors that regulate appetite, it brings into question whether or not these results are valid. A 1999 follow up study found that 5-HTP supplementation likely is effective at suppressing appetite because an increase in plasma serotonin causes fat cells to secrete a hormone known as leptin into the bloodstream (Yamada et al., 1999). Leptin from the bloodstream then crosses the blood brain barrier and stimulates the release of intracranial serotonin resulting in a sensation of satiety (Yamada et al., 2003; Yamada et al., 1999).

1.3.4 5-HTP for Fibromyalgia

Fibromyalgia (FMS) is a poorly understood chronic disorder which is defined by heightened sensitivity to painful stimulus. People suffering from FMS often experience a multitude of symptoms which include muscle pain, fatigue, tenderness, insomnia, and morning stiffness (Birdstall, 1998). Thought to be a psychosomatic illness (Hawkins, 2013), recent evidence indicates that the pathogenesis of FMS involves a combination of factors. Genetics (Maletic and Raison, 2009), neurotransmitter, and neuroendocrine imbalances as well as environmental triggers including stress and physical illness are believed to result in the central nervous system becoming overly sensitive (Mease, 2005). The efficacy of 5-HTP against fibromyalgia was

explored on the basis that pain perception is inversely proportional to plasma serotonin levels (Juhl, 1998) and that patients suffering from FMS were found to have low plasma serotonin and tryptophan levels and low levels of tryptophan and 5-HTP in their cerebral spinal fluid (Del Corral and Pacak, 2005; Juhl, 1998). Studies have shown that SSRI and tricyclic antidepressants were effective in treating patients with FMS and subsequently, 5-HTP was evaluated for efficacy as well (Birdstall, 1998). In the three studies conducted, 5-HTP was found to significantly improve FMS symptoms. In one placebo controlled study involving 200 FMS patients, it was found that 5-HTP alone was as effective as tricyclic antidepressants and MAOI's in mitigating symptoms and 5-HTP in combination with MAOI's had the most significant impact on symptoms (Birdstall, 1998).

1.3.5 Pharmacokinetics of 5-HTP

5-HTP readily enters the blood stream through the small intestines where up to 25% is converted mostly to 5-HIAA upon first hepatic pass (Das et al., 2004). Although there is dispute over its degree of efficacy (Hinz et al., 2012), there is little disagreement that in theory, ingestion of 5-HTP would be more effective in treating depression than ingesting tryptophan and serotonin. This is because tryptophan and serotonin are unable to unilaterally penetrate the blood brain barrier (Del Corral and Pacak, 2005; Hinz et al., 2010). Only with the assistance of a transport molecule is it possible for tryptophan to enter the CNS and it is believed that only 1% of dietary tryptophan will be carried across the blood brain barrier (Birdstall, 1998). Of this small percentage of tryptophan that enters the CNS, only a fraction of will be converted to serotonin as tryptophan is also used to produce niacin, kynurenine, and integrated into proteins (Birdstall, 1998). In contrast to tryptophan, animal studies have shown that approximately 7% of dietary 5-HTP enters the CNS (Del Corral and Pacak, 2005) and this molecule is not consumed in the production niacin, kynurenine, and proteins (Birdstall, 1998). There is also evidence to suggest that depression may worsen with increased levels of tryptophan due to biochemical feedback inhibition (Hinz et al.,

2012). Another theoretical advantage to 5-HTP supplementation is that it bypasses the rate limiting step in serotonin production which is the conversion of tryptophan to 5-HTP by tryptophan hydroxylase (Turner et al., 2006). Stress, Type II diabetes, vitamin B6 deficiency, and low magnesium (Iovieno et al., 2011) have all been found to decrease activity of tryptophan hydroxylase which may explain the widespread co-occurrence of depression with these conditions. These same factors appear to increase the production of kynurenine from tryptophan which decreases its availability for conversion to 5-HTP (Iovieno et al., 2011). 5-HTP intake was also been associated with increased levels of melatonin, dopamine, norepinephrine, and β-endorphin (Iovieno et al., 2011).

1.3.6 Safety of 5-HTP and "Peak X"

5-HTP is well tolerated and side effects such as nausea, vomiting, diarrhea, insomnia, headache and heart palpitations have rarely been reported (Turner et al., 2006). Gastrointestinal effects are likely due to the fact that the GI tract becomes stimulated by the increase of peripheral serotonin (Turner et al., 2006). It is recommended that 5-HTP be avoided by people taking SSRIs or MAOIs because of a possible risk of patients developing serotonin syndrome by this combination (Iovieno, 2011) although to date there have been no reported cases of this disorder associated with any 5-HTP and prescription antidepressant combination (Turner et al., 2006). Clinical studies have also concluded that there is very little risk associated with combining 5-HTP with SSRIs, MAOIs and tricyclic antidepressants (Turner et al., 2006). However, each of these studies consisted of less than 30 subjects (Turner et al., 2006), thus these findings may not be conclusive. Serotonin syndrome has however been reported in patients taking tryptophan with the SSRI fluoxetine (Prozac) (Turner et al., 2006). Excess serotonin causes the CNS to become overly excited (Boyer and Shannon, 2009) resulting in hypertension, hyperthermia, hyperreflexia, dizziness, flushing, disorientation, and involuntary muscle twitching (Turner et al., 2006).

In 1989, 37 people died and 1500 were sickened with an unusual disorder known as eosinophilia-myalgia syndrome (EMS) (Turner et al., 2006). The symptoms of this disorder include weakness, oral ulcers, abdominal pain, skin rash, increased serum aldolase, muscle pain, and above average eosinophil count (Michelson et al., 1994). The cause of this illness was traced to tainted tryptophan supplements. These supplements were produced from a batch of tryptophan that was produced by a bacterial fermentation method and was inadequately purified after production (Das et al., 2004). One of the principle contaminates was the toxin 1,1'-ethylidenebis(tryptophan). Subsequent studies found that 1,1'-ethylidenebis(tryptophan) is able to activate splenic T cell production of interleukin-5(IL-5) as well as upregulating of the amount of IL-5 receptors in normodense eosinophils (Yamaoka et al., 1994; Barth et al., 2001) which may explain the increase in eosinophils associated with EMS. Another study found that 1,1'-ethylidenebis(tryptophan) was able to induce similar types of muscle tissue abnormalities associated with EMS (Emslie-Smith et al., 1994). 3-(N-Phenylamino)alanine was also found in case-implicated tryptophan supplements and metabolites of this compound resemble toxins linked to a disorder similar to EMS known as toxic oil syndrome (Martínez-Cabot and Messeguer, 2007). Over 60 other impurities were detected in the case-implicated tryptophan supplements (Adachi et al., 2000) and the physiological effects of many of these contaminants are still unknown. Thus, there may be more contaminants that contribute to symptoms of EMS. In 1994, a suspected case of EMS occurred in a mother and her two children after coming into contact with an infusion of 5-HTP, tetrahydrobiopterin, L-dopa, and carbidopa. This infusion was being administered to the two children by their mother as a treatment for tetrahydrobiopterin deficiency (Michelson et al., 1994). The mother's symptoms included muscle soreness, joint pain, fatigue, dyspnea, edema, and eosinophilia while the only symptoms experienced by her two children were slight eosinophilia, leukocytosis and elevated aldolase (Michelson et al., 1994). Due to the structural similarities between tryptophan and 5-HTP and the patients' eosinophilia, it was assumed that similar contaminants to those found in tainted tryptophan were to blame for her

illness. An investigation of the case-implicated 5-HTP revealed a minor contaminant labeled peak X. The structure of peak X has never been firmly established as only two independent research groups have detected peak X and each group reported two very different identities for this peak. Michelson et al. (1994) argued that peak X was likely 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline while Johnson et al. claimed that peak X was a cluster of compounds of mass 235, one of which being a possible neurotoxin known as 4,5-tryptophan dione (Johnson et al., 1999; Klarskov et al., 2003; Dryhurst and Wrona, 2001). There is currently no consensus as to whether this woman and her children's EMS-like symptoms were caused by consuming peak X contaminated 5-HTP supplements or if this was a purely coincidental event. Of the three people implicated in this case, the mother developed the most severe symptoms despite the fact that she did not ever consume this infusion and only had skin contact with it. Aside from her muscle soreness and eosinophilia, the rest of her symptoms (fatigue, dyspnea, and edema) are not features of EMS (Das et al., 2004). The two children who consumed the infusion did not display any EMS symptoms except for a mild eosinophilia and elevated aldolase which resolved after switching to a different supply of 5-HTP that did not contain the peak X impurity (Michelson et al., 1994). This may suggest that peak X was the cause of this outbreak however many others have consumed 5-HTP supplements, some of which were found to contain this contaminant, yet no additional EMS like illnesses have been reported (Das et al., 2004; Michelson et al., 1994). Michelson et al. (1994) also cites a specific case of another child being treated for tetrahydrobiopterin deficiency with peak X-contaminated 5-HTP who did not develop any symptoms related to EMS (Michelson et al., 1994). A 1980 case in which a single patient developed scleroderma after taking 5-HTP and carbidopa is often cited as another case of EMS linked to 5-HTP supplementation however none of this patient's symptoms were characteristics of EMS (Sternberg et al., 1980). Eosinophilia is usually associated with parasitic infections and there are also numerous other causes such as hay fever (Newsholme and Doherty, 2005), and reactions to other allergens and prescription drugs (Valent, 2009). Therefore the presence of mild

eosinophilia without any other EMS symptoms in two children with other health issues may not a strong indicator of this disorder. Unlike her two children, the mother's eosinophilia did not resolve after terminating her exposure to peak X-contaminated supplements (Das et al., 2004; Michelson et al., 1994). It is also important to note that the children's father was also exposed to this infusion numerous times and did not develop any symptoms (Das et al, 2004; Michelson et al., 1994). Another factor that has not been explored is whether or not the other components of the infusion which included tetrahydrobiopterin, L-dopa, carbidopa and possibly any excipients contributed to these symptoms as they were never examined for impurities (Das et al., 2004). Furthermore Das et al. conducted a critical review of all three studies regarding peak X and concluded that the peak X family of contaminants are likely artifacts from a previous HPLC run. Das et al. pointed out that the peak X compounds were present in such small amounts that it was not possible to accurately determine their structures or quantitate them by the most sophisticated mass spectroscopic techniques (available at the time) and also that to date, no other labs have been able to detect the peak X class of compounds. Das et al. also points out that one of the peak X molecules was believed to be the suspected neurotoxin 4,5-tryptophan dione however this molecule is very unstable in acid and is likely destroyed upon ingestion. This molecule is also not very water soluble which would severally hamper its distribution throughout the human body (Das et al., 2004). Despite these facts, 5-HTP supplement suppliers are still very watchful of the product they distribute as most 5-HTP is inspected for peak X.

1.3.7 Synthetic 5-HTP

5-HTP can be created through traditional synthetic chemistry techniques. However, extraction from griffonia seeds is much more economical. This is because griffonia seeds yield a relatively high amount of L-5-HTP (as much as 200 mg/g of seed or ~20% dry weight) and the product can

be easily extracted and purified. Seeds are grinded and combined with mixtures of methanol and water which is then filtered and then purified by recrystallization. This extraction process requires much less labor, energy, and time, and results in much less chemical waste and has a lower environmental impact compared to synthetic procedures. Another flaw with synthetic procedures is that they will likely yield a mixture of DL-5-HTP as the final product. D-5-HTP likely has a much lower turnover to serotonin than its enantiomer L-5-HTP (Carter et al., 1978) which means that a larger dosage of DL-5-HTP mixture must be taken to have the desired effect. These enantiomers could be separated however this is a difficult task because enantiomers have the same physical and chemical properties. Conversion of DL-HTP to diastereomers would be necessary to resolve these enantiomers by chromatography in an achiral environment or by crystallization. The first total synthesis of 5-HTP was reported in 1954 which was a 13 step process that yielded a racemic mixture of 5-HTP (Witkop, 1954). In 1959 a four-step method employing the Fischer indole synthesis was developed. However, this also gave a mixture of D and L enantiomers (Frangatos and Chubb, 1959). In 2014, a method of obtaining 1.1-1.2 g/L of enantiomerically pure L-5-HTP from glucose was developed using genetically engineered microbes (Lin et al., 2014). This technique of accessing 5-HTP could potentially compete with griffonia suppliers in Africa if this process could be scaled up.

1.4 Thesis Objective

Griffonia seeds are well known as the most abundant natural source of 5-HTP however certain other phytochemical characteristics about this species remain unreported. This study sought to profile and quantitate the 5-HTP levels as well as characterize the other secondary metabolites such as flavonoids, tocopherols, and inorganic minerals found in this species and to quantitate the nitrile glycosides, proteins, antioxidants, total polyphenols, and fatty acids, among ten griffonia populations originating in various regions of Ghana and Liberia. As 5-HTP is widely sought after, finding populations that produce high amounts of this commodity could have a significant

economic impact on locals who supply these seeds to the world. Likewise, if seeds and leaves are found to be viable sources of these other valuable compounds, a new use for this crop could arise causing a new demand that further increases the economic value of harvesting this crop for local populations. In addition, 18 dietary supplements containing 5-HTP were analyzed for content of this amino acid and it was found that the measured content in each supplement was within ± 15% of the amount declared on the supplements label thus all of them were found to be in accordance with U.S. Pharmacopoeia guidelines (Tunna and Patel, 2013).

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Chapter 2. Riachin and Lithospermoside: Identification in *Griffonia simplicifolia* Seeds

2.1 Introduction

Griffonia simplicifolia is a legume found in many Sub-Saharan African countries that include Nigeria, The Ivory Coast, Ghana, Liberia, and Gabon (Brendler et al., 2010). Griffonia is of interest because of it is the richest source of 5-HTP. Yet, preliminary studies showed that the HPLC/UV chromatogram of Liberian griffonia seeds contained two additional peaks that are not present in specimens of Ghanaian origin (**Figure 2-1**) .The proximity of these two peaks to the 5-HTP peak could suggest the presence of two indole compounds. However, the UV spectra of both peaks displayed a single λ_{max} around 260 nm (**Figures 2-2 and 2-3**) which is much different than the characteristic absorbance associated with indole derivatives which would be expected to include two λ_{max} around 220 nm and 275 nm (Meng et al., 2012). Additionally, mass spectroscopy revealed a mass of 329 daltons (**Figures 2-4 and 2-5**) for both compounds which does not match any known derivatives of indole. This mass does however match that of a compound previously isolated from griffonia roots named lithospermoside (Dwuma Badu et al., 1976). After isolating peaks and subjecting them to NMR, the unknown peaks were identified as riachin and lithospermoside (**Figure 2-6**).

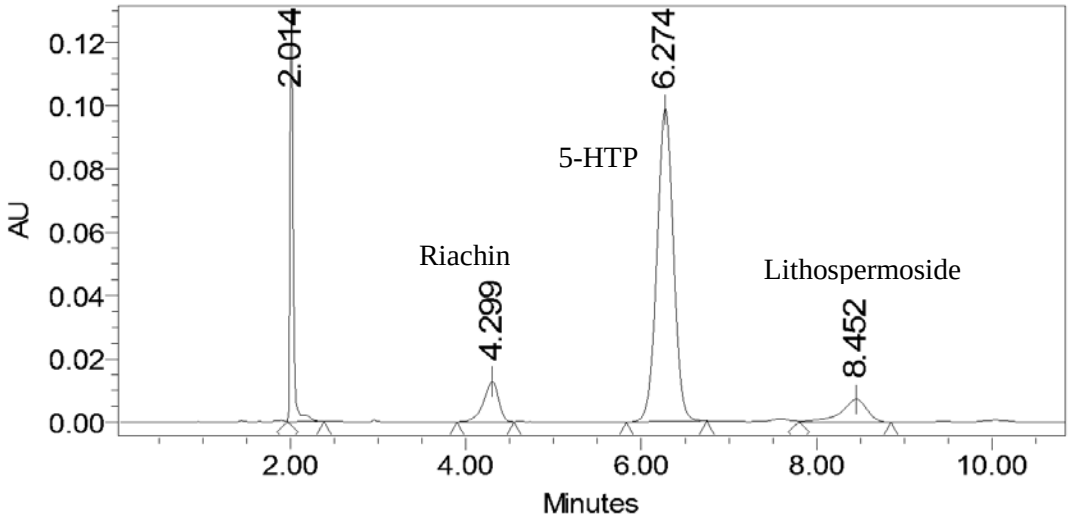


Figure 2-1. Representative HPLC-UV chromatogram (270nm) of griffonia seeds from Liberia

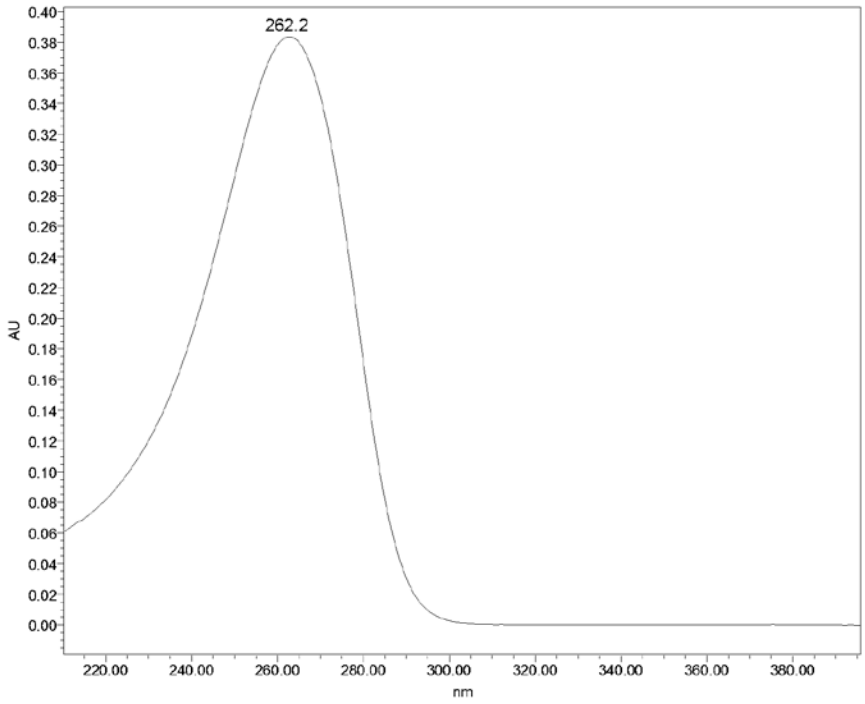


Figure 2-2. UV spectrum of riachin

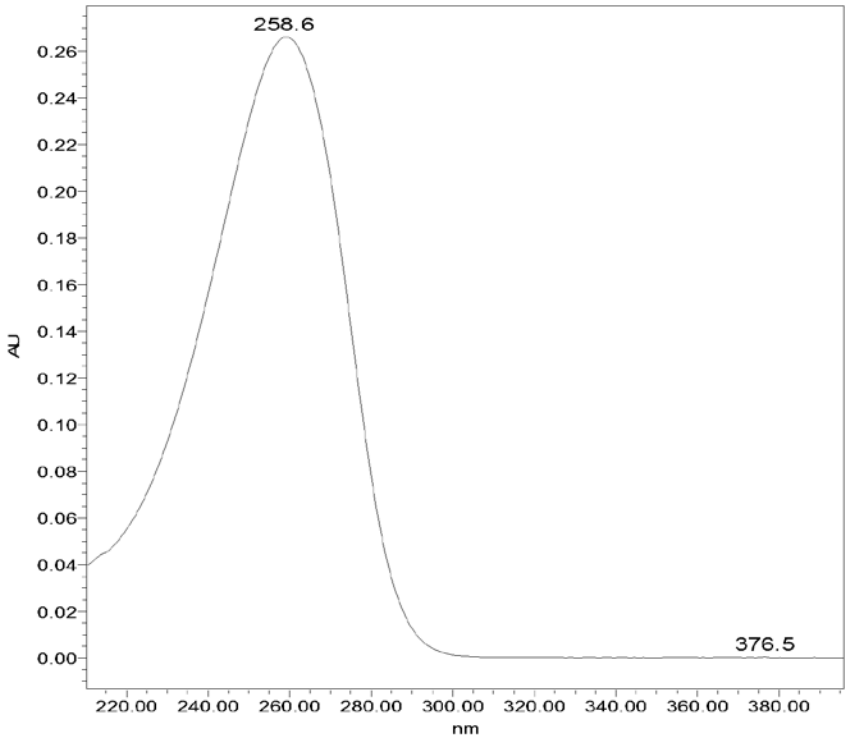


Figure 2-3. UV spectrum of lithospermoside

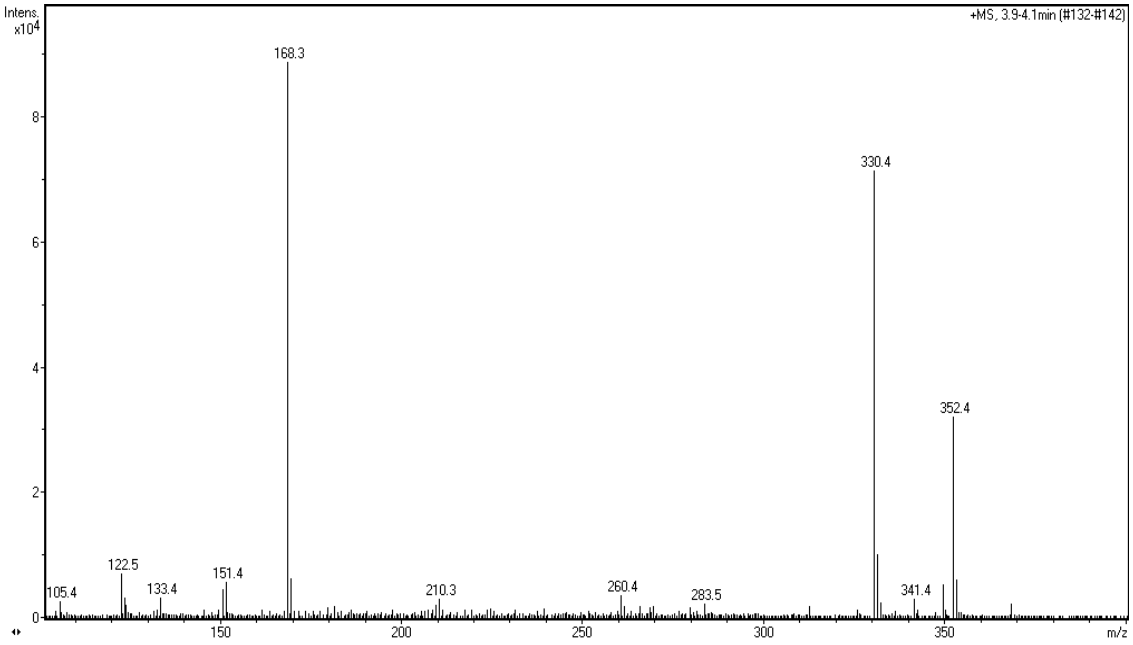


Figure 2-4. Mass spectrum of riachin

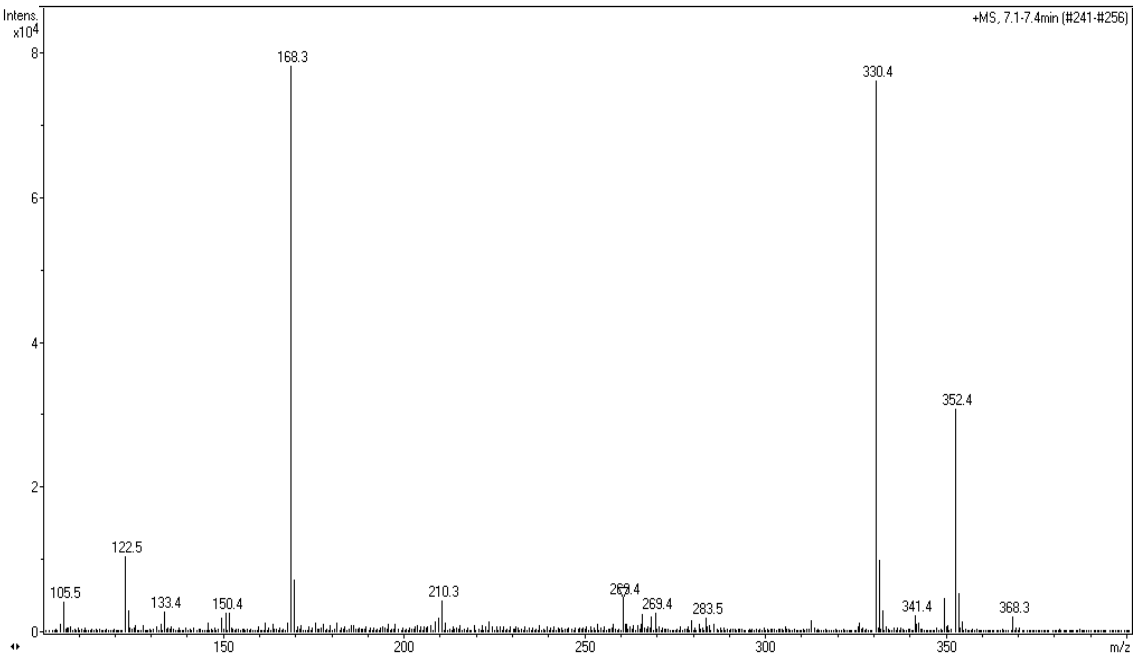


Figure 2-5. Mass spectrum of lithospermoside

Lithospermoside which is sometimes referred to as griffonin, was first isolated in 1976 from the roots of the unrelated species *Lithospermum purpureocaeruleum* (Boraginaceae) and later that year, it was found to occur in griffonia roots. Lithospermoside was found later in several closely related species to griffonia including *Cercis chinensis*, a legume commonly found in southeast Asia which is widely used as a traditional medicine for a range of indications (Li et al., 2005); *Cercis silquastrum*, a plant that grows throughout Europe and Asia (Plouvier, 1978); *Bauhinia fassoglensis*, a legume native to Tanzania (Fort et al., 2000); *Bauhinia sirindhorniae* a tree climbing shrub and legume found throughout the old world tropics (Athikornkulchai et al., 2003); and *Tylosemia esculentum*, a crop indigenous to the Kalahari desert of Botswana that has spread to neighboring Namibia and South Africa (Mazimba et al., 2011). Lithospermoside and several of its isomers (**Figure 2-6 and Tables 2-1 – 2-6**) have also been isolated from other plant families that are genetically distant from griffonia. These include several members of the Ranunculaceae family including *Thalictrum rugosum*, *Thalictrum revolutum* (Fort et al., 2000), *Thalictrum orientale* (Erdemgil et al., 2003), and *Semiaquilegia adoxoides* (Han et al., 2001). In addition, species in the Boraginaceae family: *Lithospermum officinale* (Joisen-Lefebvre and LeDrian, 2003), *Ehretia philippinensis* (Simpol et al., 1994) and *Coldenia procumbens* (Niranjan et al., 2013) also were found to contain lithospermoside. Lithospermoside also occurs in two members of the Ochnaceae family, *Lophira alata* (Tih et al., 2003) and *Ochna schweinfurthiana* (Ndongo et al., 2015) as well as one member of Rosaceae, *Cowana mexicana* (Ito et al., 2007; Ito et al., 1999). The 4-epimer of lithospermoside known as dasycarponin **(Figure 2-6**) has been identified in the roots of *Cercis chinensis* (Li et al., 2005) and *Thalictrum dasycarpum* (Wu et al., 1979). The 5-epimer of lithospermoside **(Figure 2-6**) has been isolated from the berries of the holly species *ilex warburgii* (Aquifoliaceae) (Ueda et al., 1983). Riachin which is a diasteriomer of lithospermoside, has been recently identified from the root bark of the closely related legume *Bauhinia pentandra* (Silva et al., 2013). Collectively referred to as either the nitrile glycosides or nitrilosides, analogues of lithospermoside including menisdaurin, purshinin, bauhinin, the

simmondsins, the lophirosides, the Ehretiosides, the lanceolins, the campylosides, as well as a few of their aglycones (**Figure 2-6, Tables 2-1 and 2-2**) are found throughout the plant kingdom however their occurrences are very rare. It is interesting to note that four griffonia populations from Ghana were included in this study and not a trace of lithospermoside and riachin were detected. The genus *Griffonia* contains four species and it is unknown whether nitrile glycosides occur in any of the other three species. The biogenesis of the nitrile glycosides is still unclear as their production has never been the subject of an extensive study. Researchers have proposed that the nitrile glycosides are formed from tyrosine through a p-hydroxyphenylacetonitrile intermediate in a similar manor to that of cyanogenic analogues of lithospermoside such as dhurrin and taxiphyllin (**Figure 2-7**) (Zintchem et al., 2014).

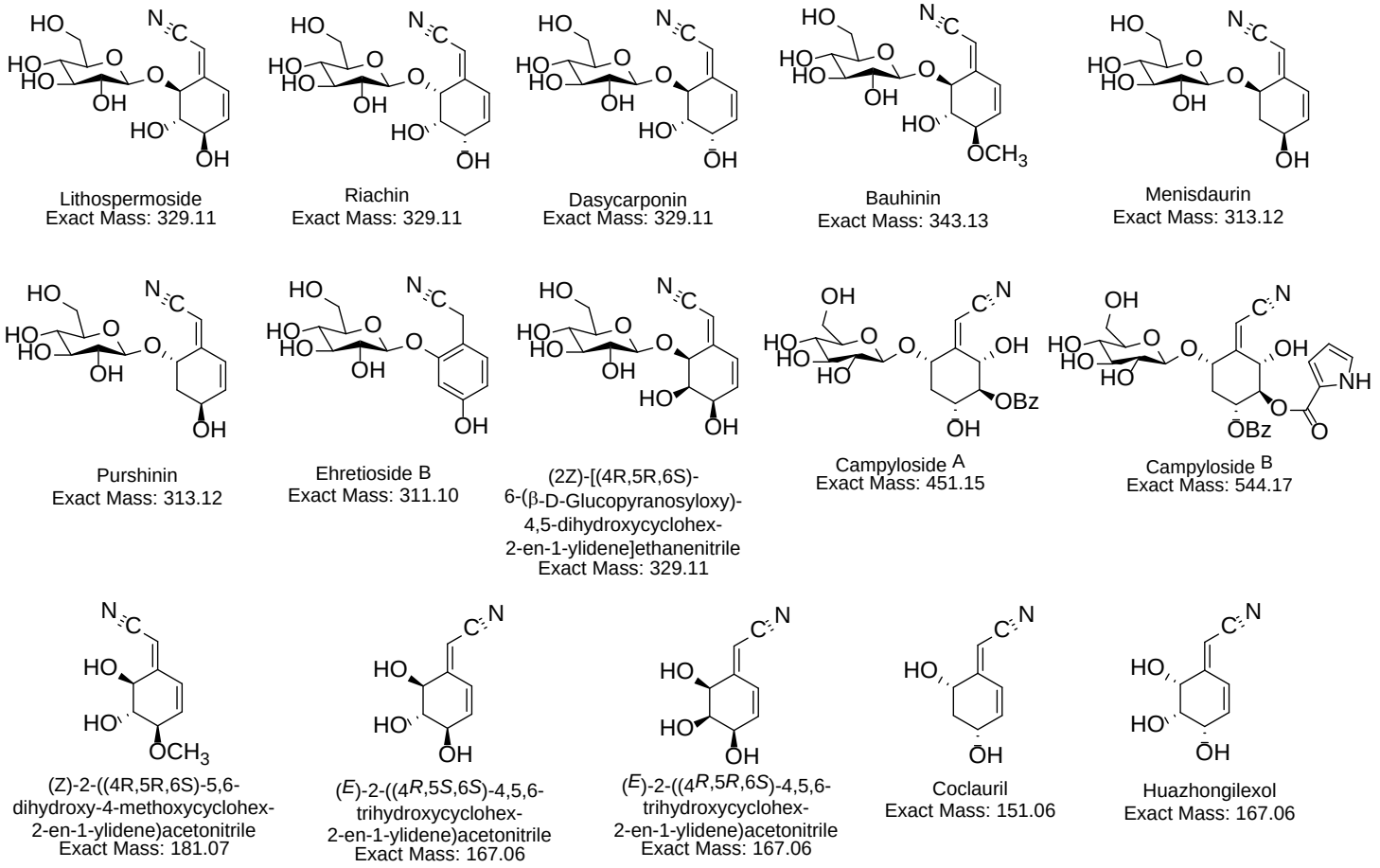


Figure 2-6. Structures of known nitrile glycosides. Campylosides A and B are only known to occur in the stem roots of *Campylospermum glaucum* (Ochnaceae) (Zintchem et al., 2008).

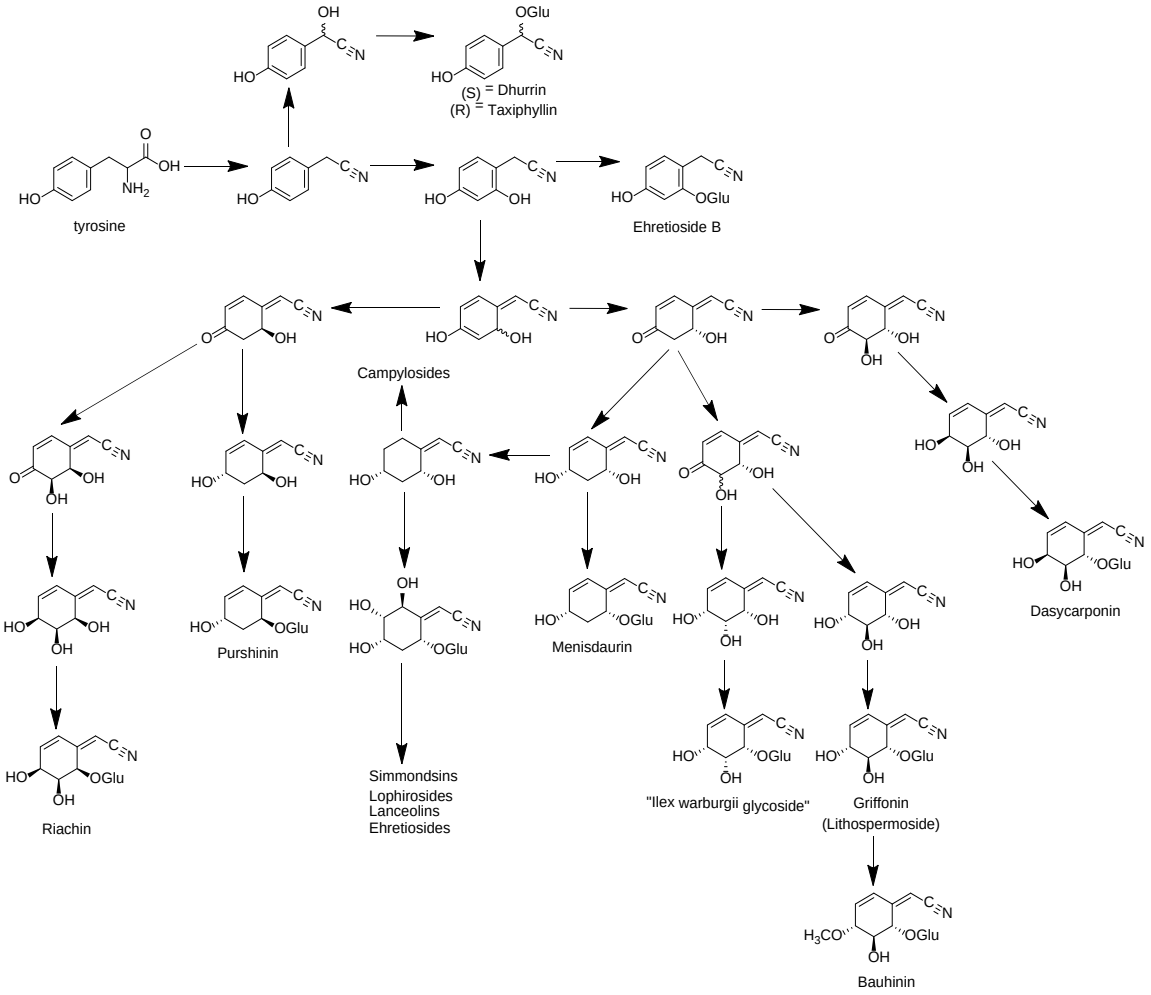
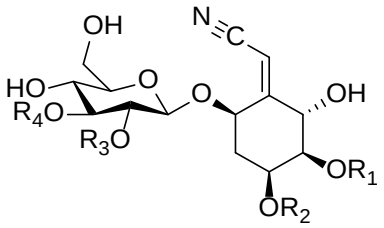


Figure 2-7. A proposed biosynthetic pathway for nitrile glycosides adapted from Seigler et al., 2005 and Zintchem et al., 2014.

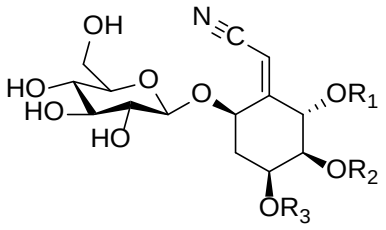


General structure of simmondsins

Table 2-1. Simmondsin and derivatives

R_1	R_2	R_3	R_4	Name	Exact Mass
CH3	CH3	H	H	Simmondsin	375.15
H	CH3	H	H	4-demethylsimmondsin	361.14
CH3	H	H	H	5-demethylsimmondsin	361.14
H	H	H	H	Didemethylsimmondsin	347.12
CH3	CH3	Ferulate	H	Simmondsin 2'-ferulate	551.20
CH3	CH3	H	Ferulate	Simmondsin 3'-ferulate	551.20
H	CH3	Ferulate	H	4-demethylsimmondsin 2'-ferulate	537.18
CH3	H	Ferulate	Ferulate	5-demethylsimmondsin 2'-ferulate	537.18

Simmondsin itself has been isolated from only three species: the seeds and leaves of *Simmondsia chinensis* (Buxeceae) (Elliger et al., 1974; Benzioni et al., 2005), the bark of *Ehretia philippinensis* (Simpol et al., 1994) and leaves of *Ehretia dentata* (Boraginaceae) (Thuy et al., 2007). Derivatives of simmondsin are only known to occur in *Simmondsia chinensis* (Van Boven et al., 1996; Van Boven et al., 2000).



General structure of lophirosides, lanceolins, and ehretiosides

Table 2-2. Lophirosides, Lanceolins, and Ehretiosides.

R_1	R_2	R_3	Name	Exact Mass
H	Benzoyl	Benzoyl	Lophiroside A1	555.17
Benzoyl	H	Benzoyl	Lophiroside A2	555.17
H	Cinnamate	Benzoyl	Lophiroside B1	581.19
Cinnamate	H	Benzoyl	Lophiroside B2	581.19
Benzoyl	H	H	Lanceolin A	451.15
H	H	Benzoyl	Lanceolin B	451.15
H	Benzoyl	H	Lanceolin C	451.15
H	CH_2=CHCO	H	Ehretioside A1	429.16
H	H	CH_2=CHCO	Ehretioside A2	429.16
CH_2=CHCO	H	H	Ehretioside A3	429.16

Lophirosides and Lanceolin C are only known to occur in the bark of *Lophira alata* (Ochnaceae). (Murakami et al., 1993; Messanga et al., 1998). Lanceolin A and B are only known to occur in the stem heart wood of *Lophira lanceolata* (Ochnaceae) (Tih, 1994). Ehretiosides A1, A2, A3, and B1 were first found to occur in *Ehretia philippinensis* (Simpol et al., 1994). Ehretioside A1 has since been identified in the leaves of *Ehretia dentata* (Thuy et al., 2007) and the aerial parts of *Coldenia procumbens* (Niranjan et al., 2013) (Boraginaceae). Ehretioside B is also found in the roots of *Semiaquilegia adoxoides* (Ranunculaceae) (Zhang et al., 2004) and *Glechoma longituba* (Lamiaceae) (Liu et al., 2014)

Table 2-3. Natural occurences of lithospermoside (griffonin)

Fabaceae	Ranunculaceae	Boraginaceae	Ochnaceae	Rosaceae
Griffonia simplicifolia roots (Dwuma Badu et al., 1976)	*Thalictrum rugosum* roots (Wu et al., 1979)	*Lithospermum purpureocaeruleum*m roots (Sosa et al., 1976)	*Lophira alata* leaves (Tih et al., 2003)	*Cowana mexicana* Leaves and stems (Ito et al., 2007), aerial parts (Ito et al., 1999)
Cercis siliquastrum bark (Plouvier 1977)	*Thalictrum revolutum* roots (Wu et al., 1979)	*Lithospermum officinale* roots (Sosa et al., 1976)	*Ochna schweinfurthiana* stem bark (Ndongo et al., 2015)	
Cercis chinensis roots (Li et al., 2005)	*Thalictrum orientale* roots (Erdemgil et al., 2003)	*Coldenia procumbens* aerial parts (Niranjan et al., 2013)		
Bauhinia fassoglensis roots (Fort et al., 2001)	*Semiaquilegia adoxoides* roots (Han et al., 2001)			
Bauhinia sirindhorniae roots (Athikornkulchai et al., 2003)				
Tylosemia esculentum Husks (Mazimba et al., 2011)				

Table 2-4. Natural occurences of menisdaurin

Ochnaceae	Fabaceae	Boraginaceae	Rosaceae	Menispermaceae	Aquifoliaceae	Ranunculaceae	Saxifragaceae
Ochna calodendron leaves (messanga et al., 2002)	*Cercis chinensis* roots (Li et al., 2005)	*Ehretia longiflora* stem bark (Hoang et al., 2009)	*Purshia tridentata* stems (Nakanishi et al., 1994)	*Menispermum dauricum* vines (Takahashi et al., 1978)	*Ilex warburgii* fruits (Ueda et al., 1983)	*Semiaquilegia adoxoides* roots (Han et al., 2001)	*Saniculiphyllum guangxiense* whole plant (Geng et al., 2014)
Ouratea turnarea roots (Bikobo et al., 2013)	*Bauhinia rufescens* stem bark (Usman et al., 2013)	*Tiquilia plicata* leaves (Seigler et al., 2005)	*Cowana mexicana* Leaves and stems (Ito et al., 2007) aerial parts (Ito et al., 1999)	*Sinomenium acutum* rhizomes and caulises (Otsuka et al., 1993)	*Ilex aquifolium* fruits (Nahrstedt and Wray, 1990)		
Campylospermum densiflorum leaves (Bikobo et al., 2011)	*Bauhinia sirindhorniae* stems and roots (Athikornkulchai et al., 2003)	*Tiquilia canescens* leaves (Seigler et al., 2005)					
Campylospermum oliverianum leaves (zintchem et al., 2014)							
Campylospermum sulcatum leaves (zintchem et al., 2014)							

Table 2-5. Other nitrile glycosides natural occurrences

Bauhinin	**Purshianin**		**Dasycarponin**		**(2Z)-[(4R,5R,6S)-6-(β-D-Glucopyranosyloxy)-4,5-dihydroxycyclohex-2-en-1-ylidene]ethanenitrile**		**Riachin**
Fabaceae	**Rosaceae**	**Saxifragaceae**	**Ranunculaceae**	**Fabaceae**	**Ranunculaceae**	**Aquifoliaceae**	**Fabaceae**
Bauhinia championii roots (Chen et al., 1985)	*Purshia tridentata* stems (Nakanishi et al., 1994)	*Saniculiphyllum guangxiense* whole plant (Geng et al., 2014)	*Thalictrum dasycarpum* roots (Wu et al., 1979)	*Cercis chinensis* roots (Li et al., 2005)	*Semiaquilegia adoxoides* roots (Niu et al., 2006)	*Ilex warburgii* fruits (Ueda et al., 1983)	*Bauhinia pentandra* root bark (Silva et al., 2013)
Bauhinia aurea stems (Shang et al., 2012)	*Cowania mexicana* aerial parts (Ito et al., 2007)						

Table 2-6. Nitrile glycoside aglycones natural occurrences

Coclauril	**(Z)-2-((4R,5R,6S)-5,6-dihydroxy-4-methoxycyclohex-2-en-1-ylidene)acetonitrile**	**Huazhongilexol**	**(E)-2-((4R,5S,6S)-4,5,6-trihydroxycyclohex-2-en-1-ylidene)acetonitrile**	**(E)-2-((4R,5S,6S)-4,5,6-trihydroxycyclohex-2-en-1-ylidene)acetonitrile**
Menispermaceae	**Fabaceae**	**Aquifoliaceae**	**Euphorbiaceae**	**Ranunculaceae**
Cocculus lauriforius leaves (Yogo et al., 1990)	*Bauhinin aurea* stems (Shang, et al., 2012)	*Ilex centrochinensis* leaves (Lin, et al., 1995)	*Thecacoris annobonae* stem bark (Guedem et al., 2012)	*Semiaquilegia adoxoides* Roots (Zhang et al., 2004)

A small number of bioactivity reports do exist for these compounds and in most cases the nitrile glycosides failed to display strong medicinal activity both *in vivo* and *in vitro*. Many plants sources of these compounds are used as traditional treatments and while some extracts have shown *in vitro* activity, more often than not, the nitrile glycoside(s) in the extracts do not appear to be the active component. This suggests that these compounds are either inactive or are only active in conjunction with other components. Several cancer cell lines have been evaluated for reactivity to lithospermoside and in all cases no cytotoxic effects were observed. A study by Ndongo et al., found that cervical adenocarcinoma (HeLa) cells were unreactive toward lithospermoside (Ndongo et al., 2015) and A 2012 study by Lee et al., found that lithospermoside was ineffective against eight cancer cell lines with IC_{50} values greater than 100 µg/mL (Lee et al., 2012). The cell lines assayed in this study included two lung cancer (A549 and H129), one breast cancer (MDA-MB-231), one ovarian cancer (SKOV3), two colon cancer (HCT116 and HT29), one pancreatic cancer (AsPC-1) and one oral cancer (Ca922). In the same study, lithospermoside was also tested for inhibitory activity against the release of superoxide anions and elastase from neutrophils. These two chemical species are released by neutrophils in response to an active infection. Prolonged exposure to these species can be very damaging to bodily tissue resulting in complications that often leads to death. Unfortunately, this *in vitro* study found that lithospermoside was insufficient in treating this condition (Lee et al., 2012). Lithospermoside has shown to have positive activity against sickle cell anemia. An *in vitro* study by Larmie and Poston found that lithospermoside may be useful in treating sickle cell anemia by helping to increase the intracellular sodium content of sickled cells. The increase in sodium content is believed to cause an inflow of fluid into the cells via osmosis causing the sickled erythrocytes to adapt a more regular shape (Larmie and Poston, 1991). Other studies have suggested that lithospermoside and several of its isomers have anti-tumor promoting properties. *In vitro* studies

have found that lithospermoside was able to strongly inhibit the production of Epstein-Barr Virus Early Antigen (EBV-EA) from “EBV genome-carrying lymphoblastoid cells (Raji cells derived from Burkitt's lymphoma)” (Ito et al., 1999) despite being in the presence of the strong tumor promotor 12O-tetradecanoylphorbol-13-acetate suggesting that lithospermoside may have useful anti-tumor promoting properties (Itoh et al., 1994; Ito et al., 1999). Other Epstein-Barr viruses are associated with nasopharyngeal and gastric carcinomas, Hodgkin and Burkitt lymphomas, and mononucleosis (Cohen et al., 2013) thus lithospermoside and its isomers may have activity against these conditions as well. It is interesting to note that in the same study menisdaurin, is the 5-dehydroxy analogue of lithospermoside, displayed comparatively much weaker anti-tumor promoting properties (Itoh et al., 1994; Ito et al., 1999).

Riachin (**Figure 2-6**) is a newly discovered compound and as such, the bioactivity has yet to be extensively studied. Riachin exhibited limited antibacterial properties on its own. However, De Farias et al. (2015a) reported that riachin is able to enhance the activity of clindamycin against *Staphylococcus aureus* and amikacin against *Pseudomonas aeruginosa*.

The biological activity of menisdaurin (**Figure 2-6)** has been more extensively studied than lithospermoside and riachin. A 2013 study by Muhammad and Sarat found that menisdaurin had antityrosinase activity. In humans, tyrosinase plays an important role in the production of melanin; tyrosinase catalyzes the conversion L-DOPA to L-dopaquinone followed by the conversion of L-leucodopachrome to L-dopachrome (Queen Mary University of London Online). This means that menisdaurin and other nitrile glycosides may be useful for treating conditions of hyperpigmentation such as melasma (formation of tan discolorations on skin), smoker’s melanosis (darkening of the oral tissues due to smoking), and acanthosis nigricans (formation of

dark velvet like skin caused mainly by obesity or endocrine disorders). Tyrosinase is also believed that have a role in wound healing and sclerotization in insects (Kim and Uyama, 2005) which indicates that menisdaurin may have some applications in pest management. Studies aimed at determining the antibacterial properties of menisdaurin have yielded mixed results. A 2013 study by Usman et al. used a disc diffusion method to measure the potency of menisdaurin against four bacteria strains: *Bacillus subtilis*, *Corynaebacterium* spp., *Escherichia coli* and *Shigella dysenteriae*. Menisdaurin was very potent against these strains; the efficacy of this compound was similar to ciprofloxacin, erythromycin, and gentamicin (Usman et al., 2013). In contrast, a similar disc diffusion experiment by Zintchem et al. found that menisdaurin had no activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* (Zintchem et al., 2014). Muhammad and Sarat also assayed menisdaurin for antibacterial and antifungal activity using a broth micro dilution method and it was found that this compound had no activity against *Bacillus subtilis* (ATCC6633), *Staphylococcus aureus* (ATCC29737), *Enterococcus faecalis* (ATCC19433), *Escherichia coli* (ATCC10536), *Kleibsiella pneumonia* (ATCC13883), *Pseudomonas putida* (ATCC49128), *Aspergillus niger* (ATCC16888), *Candida glabrata* (ATCC2001), and *Saccharomyces cerevisiae* (ATCC7754) (Muhammad and Sarat, 2013). As mentioned earlier, menisdaurin has been screened for anti-tumor promoting activity (Itoh et al., 1994; Ito et al., 1999), HIV (Nakanishi et al., 1994) and hepatitis B. Hepatitis B DNA replication was inhibited by menisdaurin with an IC_{50} value of 0.32 mM (Geng et al., 2012). In the same study, menisdaurin was found to be ineffective in inhibiting secretions of HBsAg or HBeAg (geng et al., 2012). Menisdaurin did not have any significant activity against HIV and only slight activity as an anti-tumor promoter (Itoh et al., 1994; Ito et al., 1999).

There have been two studies in which purshianin was screened for bioactivity. Purshianin (**Figure 2-6)** failed to display any inhibitory activity against HIV-1 reverse transcriptase (Nakanishi et al., 1994; Nakanishi et al., 1993) and interestingly, was inactive against hepatitis B viral replication despite the fact that purshianin is a diasteriomer of menisdaurin (Geng et al., 2012).

Simmondsin and several of its derivatives (**Table 2-1**) have been studied for their insecticidal, antifungal, antifeedant (adverse effects on insects or animals that feed on them), and satiety-inducing properties. Simmondsin and simmondsin 2'-ferulate were found to be highly toxic to the third instar larvae of *Spodoptera littoralis* with LD_{50} values of 1.49 µg/larvae and 2.58 µg/larvae, respectively (Abbassy et al., 2007). Simmondsin and simmondsin 2-ferulate also showed some activity against four strains of fungi that are pathogenic to plants: *Rhizoctonia solani*, *Pythium debaryanum*, *Botrytis fabae*, and *Fusarium oxysporum*. Simmondsin had a slightly lower LD_{50} value than simmondsin 2-ferulate against all four strains of fungi (Abbassy et al., 2007). Simmondsin may also be useful against the fungal strains *Fusarium solani* and *Rhizoctonia solani* as simmondsin enriched extracts from *Simmondsia chinensis* were found to be effective at inhibiting their mycelial growth (Mansour and El-Sharkawy, 2014). Abbassy and co-workers also found Simmondsin and simmondsin 2'-ferulate had antifeedant properties against *Spodoptera littoralis*; concentrations of 1 mg/mL of simmondsin and simmondsin '2-ferulate deterred feeding of 92.12% and 90.0% of larvae, respectively (Abbassy et al., 2007). Simmondsin appears to have anorexic properties in animals as well. These effects may be partially due to adverse flavor. Analogues of simmondsin such as menisdaurin and the lophirosides are known to have a bitter flavor similar to quinine (Murakami et al., 1993). Lievens et al. theorizes that simmondsin is able to induce satiety and other physical effects that deters feeding (Lievens et al., 2009). It is interesting to note that simmondsin is a slightly stronger anorexic than its '2-*trans*-ferulate (Flo et al., 1998) and that the demethylated simmondsin analogues do not have any anorexic activity (Flo et al., 1998; Harry-O'kuru 2000). Two synthetic analogues of simmondsin, simmondsinamide

and hydroxymethoxyphenylacetonitrile, were found to not have any anorexic properties (Flo et al., 1998).

Simpol et al. (1994) determined that the ehretiosides (**Table 2-2**) lack antihistamine activity and in the same publication, it was reported that the lophirosides did not display any activity against several unspecified microbes or insects with one exception: the lophirosides displayed weak antibacterial activity against *Micrococcus luteus* with a minimum inhibitory concentration of 100 µg. Campylosides A and B did not exhibit any activity against *Enterococcus spp*. P054, *Enterococcus hirae* ATCC 9790, *Staphylococcus aureus* ATCC 85923, *Staphylococcus aureus* U271, *Staphylococcus saprophyticus, Escherichia coli, Klebsiella spp., Serratia marsescens, Pseudomonas aeruginosa, Acinetobacter baumannii, Candida spp., Cryptococcus neoformans, Aspergillius spp*., and *Tricophyton spp*. (Zintchem et al., 2008). To date, no publications regarding the bioactivity of dasycarponin, (2Z)-[(4R,5R,6S)-6-(β-D-glucopyranosyloxy)-4,5-dihydroxycyclohex-2-en-1-ylidene]ethanenitrile, (E)-2-((4R,5S,6S)-4,5,6-trihydroxycyclohex-2-en-1-ylidene)acetonitrile, huazhongilexol, coclauril, bauhinin aglycone, and the lanceolins exist.

The toxicological properties of the nitrile glycosides have not been thoroughly investigated. Unlike analogues such as dhurrin and taxiphyllin, the nitrile glycosides are not known to be cyanogenic although these compounds may damage proteins and alkylate DNA as they have structural features that allow them to act as Michael acceptors (Schultz et al., 2007; Wondrousch et al., 2010). A study by Zintchem et al. (2014) found that menisdaurin is highly toxic to brine shrimp (*Artemia salina*) suggesting these compounds may be cytotoxic to animal cells. The toxicity of simmondsin and its ‘2-ferulate have been investigated in mice and it was found that doses as high as 500 mg/kg caused no outward signs of toxicity although effects on enzymes and hormone concentrations were observed (Abbassy et al., 2008). Bauhinin also appears to be relatively non-toxic as doses as high as 300 mg/kg did not cause any outward signs of toxicity in

mice (Chen et al., 1985). An *in silico* study conducted by Farias et al. estimates that the LD_{50} of riachin as 310 mg/kg (Farias et al. 2015b). Based on these few studies, it is very difficult to draw strong conclusions about the toxicity of the nitrile glycosides.

The purpose of this study was to identify, characterize, and quantitate the content of two peaks observed in the HPLC-UV chromatogram of griffonia seeds collected from various regions of Liberia. We determined that the compounds corresponding to these two peaks were riachin and lithospermoside, using different spectrometric methods including UV-VIS, NMR, and mass spectroscopy. Additionally, the content of each of these two compounds was evaluated in seeds based on endosperm coloring to see if this feature can be used as an indicator of riachin and lithospermoside content and also to determine if the formation of these compounds is triggered by a response to infection or deteriorating seed health (e.g. stress inducible). If the concentrations of these two compounds are significant, griffonia seeds from Liberia could be a useful source of these compounds for future biological studies.

2.2 Experimental

2.2.1 Materials

Griffonia seeds of Liberian origin (for collection sites and sample sizes, see **Table 5-1**), Silica gel particle size 32-63 from Selecto Scientific (Georgia, USA), HPLC grade water, acetonitrile, methanol, and chloroform from Fischer Scientific (Fair Lawn, NJ), 95% formic acid, Sephadex LH20, C-18 Silica 90, and DMSO-d_6 from Sigma Aldrich (St Louis, MO), D_2O from Cambridge Isotope Labs Inc. (Andover, MA).

2.2.2 Analytical Equipment

Quantitation of riachin and lithospermoside was conducted using Waters 2695 HPLC separation module equipped with a Waters 2996 photo diode array detector. Separation was performed on a Phenomenex Luna phenyl hexyl column (150mm x 4.6mm, 5µm).

Mass spectra of riachin and lithospermoside were obtained using an Agilent 1100 Series LC-MSD system with electrospray ionization (ESI) using MS detection in positive mode. Nebulizer was set at 40 L/min, drying gas at 9.00 L/min, the drying gas temperature at 350 ºC, the compound stability at 100%, the capillary voltage at 4.5kV, Skim 1 at 27.6 V, skim 2 at 6.0 V, capillary exit offset at 70.4 V, octopole 1 at 2.37 V, octopole delta at 2.40 V, octopole RF at 150.0 Vpp, lens 1 at -5 V, lens 2 at -60.0 V, the trap drive level was at 23.5, and the scan range was from 100 to 1000 m/z.

NMR was conducted using a Bruker Avance III 400MHz NMR spectrometer with 5 mm diameter multinuclear probe. Topspin software (Bruker, version 3.5) was used to process data.

2.2.3 HPLC Conditions

The mobile phase consisted of an isocratic mixture of 99% solvent A (ddH_2O, 0.1% Formic acid) and 1% solvent B (acetonitrile, 0.1% formic acid) for 10 minutes. The flow rate was set at 1.0 mL/min with UV detection set at 262 nm for riachin and at 258 nm for lithospermoside. The injection volume was 10 µL and analysis occurred at room temperature.

2.2.4 Methods

2.2.4.1 Isolation of Riachin and Lithospermoside

Griffonia seeds were collected by ASNAPP-Ghana, an NGO registered in Ghana, whose team members are medicinal plants/botanical experts working in Ghana and Liberia on griffonia and other Non-Timber Forest Products (NTFP). The ASNAPP-Ghana team collected griffonia seeds from the forest floor from various regions of Liberia (**Table 5-1**). The seeds were collected, dried under the sun on drying racks off the ground, and then shipped to Rutgers University (New Brunswick, NJ) for chemical analysis. First the seed coats were isolated manually from the seeds and were found to not contain any riachin or lithospermoside (data not presented) and as such were removed to avoid adding additional contaminants to the mixture. Bare endosperms (seed coat removed) were grinded to a yellow powder and 44.2 grams of this powder was subjected to soxhlet extraction with hexane for 20 hours to remove fatty materials. This yielded 21.2 grams of a yellowish white powder in the thimble. This material was then combined with 23 grams of silica gel. 17.7 grams of this mixture was subjected to column chromatography with a $CHCl_3$ and MeOH as a mobile phase. This yielded 451.5 mg of a yellow powder that contained riachin and lithospermoside as well as other impurities. This orange powder was decolored using sephadex LH20 and then riachin and lithospermoside were separated from one another using flash chromatography with C18 as the stationary phase and 100% water as the mobile phase. This resulted in 14.8 mg of pure riachin and 24.0 mg of pure lithospermoside. Purity was confirmed by NMR.

2.2.4.2 Quantitation of Riachin and lithospermoside

Approximately one gram of seed powder was dissolved in 100 mL of 50% methanol in water and was sonicated for 15 minutes giving an opaque white solution which was then allowed to sit at room temperature for three hours. A 1.5 mL aliquot of this solution was centrifuged for 5

minutes. After centrifugation, a one milliliter aliquot was diluted with 10 mL of 50% methanol in water and then placed in an amber HPLC vial for analysis. Each sample was prepared and analyzed in triplicate.

2.2.4.3 Riachin and lithospermoside by endosperm coloring

Endosperms were inspected and placed into one of two groups based on their coloring. The first group consisted of seeds that had a bright yellow colored endosperm without any patches of black. The second group consisted of seeds with nearly or completely black endosperm color (for color photographs see **Figure 3-2**). These endosperms along with their seed coats were prepared for analysis in an identical manner as described in section 2.2.4.2 with one exception; instead of being diluted with 10 mL, samples were diluted to 10 mL. Each sample was prepared and analyzed in triplicate.

2.2.5 Calibration Curves

2.2.5.1 Calibration Curve A: Riachin

Riachin (9.2 mg) isolated from griffonia seeds (see 2.2.4.1) was dissolved in 10 mL of water and was sonicated for 5 minutes. One milliliter of this stock solution was used to make dilutions of 1/2x, 1/4x, 1/8x, 1/16x, 1/32x, 1/64x, 1/128x, 1/256x, 1/512x and 1/1024x to cover ranges of 0.90 µg/mL to 460 µg/mL. Each dilution was very pure and yielded a well-shaped peak. Each dilution was injected three times and peaks areas were averaged together to insure instrument reliability. The R^2 value was 1, showing excellent linearity (**Figure 2-8**).

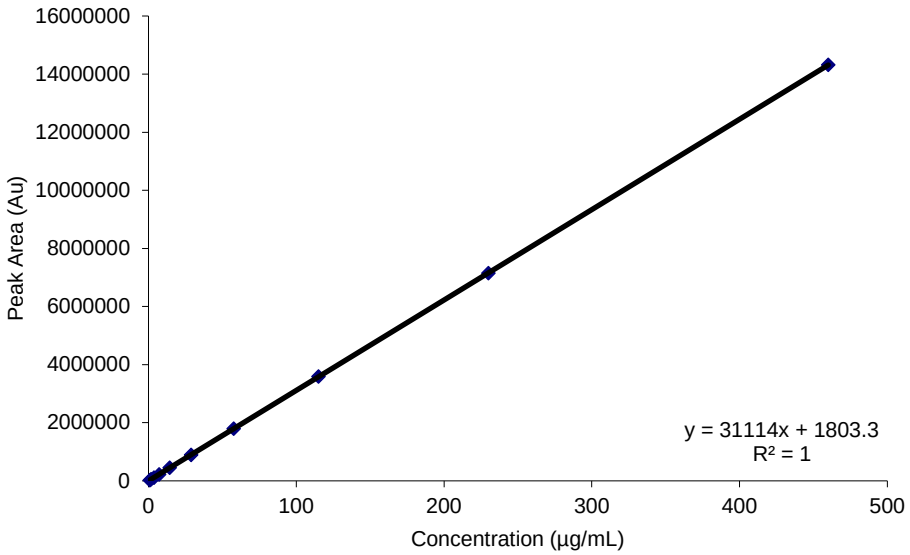


Figure 2-8. Calibration curve for riachin

2.2.5.2 Calibration Curve B: lithospermoside

Lithospermoside (12.5 mg) isolated from griffonia seeds (see 2.2.4.1) was dissolved in 10 mL of water and was sonicated for 5 minutes. One milliliter of this stock solution was used to make dilutions of 1/2x, 1/4x, 1/8x, 1/16x, 1/32x, 1/64x, 1/128x, 1/256x and 1/512x to cover ranges of 2.44 µg/mL to 625 µg/mL. Each dilution was very pure yielded a well-shaped peak. Each dilution was injected three times and peak areas were averaged together to insure instrument reliability. The R^2 value was 1, showing excellent linearity (**Figure 2-9**).

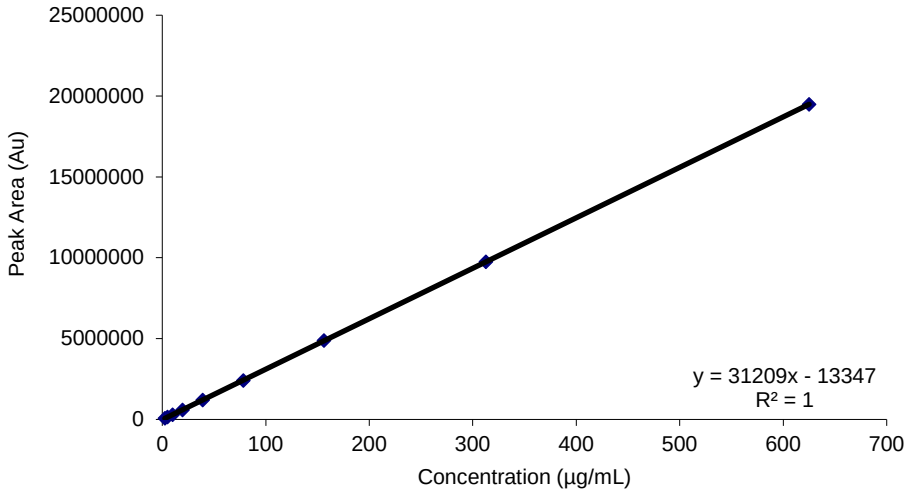


Figure 2-9. Calibration curve for lithospermoside

2.3 Results and Discussion

The intent of this study was to isolate and identify two compounds detected in HPLC-UV (**Figure 2-1**) in griffonia seeds of Liberian origin as well as determine their abundance in each population. Column chromatography separation technique led to the isolation of each of the two compounds with excellent purity as indicated as ^{13}C NMR data. UV profile of both compounds (**Figures 2-2 and 2-3**) indicated that neither compound was an indole derivative despite having close retention times to 5-HTP. Mass spectroscopy specified that both compounds had the same mass of 329 Daltons (**Figures 2-4 and 2-5**), which is identical to the mass of lithospermoside, a compound previously isolated from griffonia roots (Dwuma Badu et al., 1976). A comparison with the ^{13}C NMR data to lithospermoside analogues previously reported in the literature led to the identification of these two compounds as riachin and lithospermoside. (Silva et al., 2013; Joisen-Lefebvre and Le Drian; 2003)

Riachin and lithospermoside content in the seeds of the six Liberian populations were quantitated by HPLC-UV. Riachin content ranged from 1.41 ± 0.01 mg/g to 6.43 ± 0.63 mg/g and averaged 4.59 ± 0.95 mg/g (**Table 2-7 and Graph 2-1**). The two griffonia seed populations with the least amount of riachin came from Grand Gedeh County which is to the southeast of Nimba County, where the other four populations studied originated. The population from Nimba County that contained the least amount of riachin came from Tapita in the southern portion of the county and it contained 4.93 ± 0.19 mg/g. The population from Upper Nimba County contained 5.56 ± 0.59 mg/g of riachin and the two griffonia populations from Nimba County unspecified sites 1 and 2 contained 6.30 ± 0.45 mg/g and 6.43 ± 0.63 mg/g riachin, respectively. Lithospermoside content of seed populations ranged from 7.39 ± 0.81 mg/g to 14.48 ± 0.32 mg/g and averaged 9.65 ± 1.35 mg/g (**Table 2-7 and graph 2-1**). Lithospermoside content of populations from Nimba County was approximately 38% than that of populations from Grand Gedeh County. In all cases, the lithospermoside content was greater than the riachin content.

Endosperm coloring does appear to be indicative of riachin and lithospermoside content (**Table 2-8**). Dark colored endosperms which could be associated with rot due to insects and/or microbial infections, contained the least amount of riachin and lithospermoside with 3.91 ± 0.06 mg/g and 3.37 ± 0.01 mg/g, respectively. Riachin and lithospermoside content of healthy yellow endosperms was 5.78 ± 0.36 mg/g and 5.51 ± 0.34 mg/g, respectively.

Riachin: ESI-MS: M/z 352 $[M+Na]^+$, 330 $[M+1]^+$, 168.2 $[M+1-162]^+$; ^{1}H NMR (400MHz, D_2O) 6.31 (d, *J* = 10.16, 1H), 6.02 (d, *J* = 10.16, 1H), 5.67 (s, 1H), 4.84 (d, *J* = 4.24, 1H), 4.64 (brs, 2H), 4.26 (brs, 1H), 3.86 (m, 1H), 3.69 (m, 1H), 3.29-3.50 (m, 3H), 3.17 (t, *J* = 8.62, 1H); ^{13}C (100 MHz) 152.82, 137.39, 126.00, 117.50, 102.94, 100.33, 77.10, 76.19, 75.73, 73.03, 69.46, 68.73, 65.64, 60.59. λ_{max}: 262nm

In d_6-DMSO: ^{1}H NMR (400MHz) 6.18 (d, *J* = 9.92, 1H), 5.92 (d, *J* = 10.04, 1H), 5.72 (s, 1H), 4.56 (d, *J* = 4.12, 1H), 4.45 (brs, 1H), 4.39 (d, *J* = 7.80, 1H), 4.05 (brs, 1H), 3.66 (m, 1H), 3.50 (m, 1H), 3.05-3.19 (m, 3H), 2.89 (t, *J* = 8.26, 1H); ^{13}C (100 MHz) 154.13, 139.60, 124.93, 117.54, 103.03, 98.52, 76.79, 76.68, 76.55, 73.28, 69.90, 68.54, 65.24, 61.18.

Lithospermoside: ESI-MS: m/z 352 $[M+Na]^+$, 330 $[M+1]^+$, 168.2 $[M+1-162]^+$;^{1}H NMR (400MHz, D_2O) 6.29 (dd, *J* = 10.10, *J* = 1.34, 1H), 6.07 (dd, *J* = 10.00, *J* = 3.08, 1H), 5.58 (s, 1H), 4.84 (d, *J* = 7.8, 1H), 4.80 (dd, *J* = 8.22, *J* = 1.54, 1H), 4.23-4.27 (m, 1H), 3.91 (dd, *J* = 8.20, *J* = 6.12, 1H), 3.86 (dd, *J* = 12.36, *J* = 1.80, 1H), 3.69 (dd, *J* = 12.32, *J* = 5.24, 1H), 3.31-3.52 (m, 4H); ^{13}C (100 MHz) 155.35, 136.20, 127.02, 117.73, 102.57, 97.13, 76.14, 75.89, 75.79, 73.95, 72.80, 69.97, 69.72, 60.89. λ_{max}: 258 nm

In d_6-DMSO: ^{1}H NMR (400MHz) 6.23 (dd, *J* = 10.12, *J* = 1.20, 1H), 6.03 (dd, *J* = 9.90, *J* = 3.14, 1H), 5.70 (s, 1H), 4.62 (d, *J* = 7.36, 1H), 4.57 (dd, *J* = 7.84, *J* = 1.36, 1H), 4.03 (m, 1H), 3.66-3.74 (m, 2H), 3.40-3.48 (m, 1H), 3.12-3.21 (m, 3H), 3.00-3.07 (m, 1H); ^{13}C (100 MHz) 155.25, 138.16, 125.80, 117.06, 102.27, 96.64, 76.90, 76.48, 75.27, 73.54, 73.02, 70.14, 69.74, 61.50.

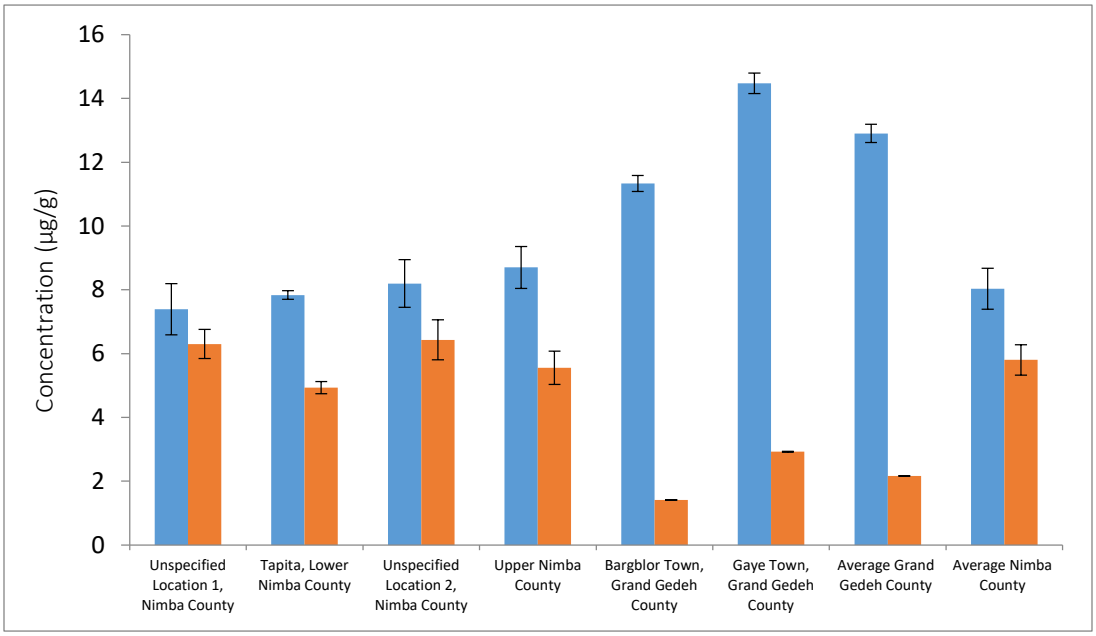
Table 2-7. Lithospermoside and riachin content of griffonia populations from Liberia

Grand Gedeh County

Collection Site	lithospermoside content (mg/g)	riachin content (mg/g)
Bargblor Town, Grand Gedeh County	11.33 ± 0.25	1.41 ± 0.01
Gaye Town, Grand Gedeh County	14.48 ± 0.32	2.92 ± 0.02
Average, Grand Gedeh County	12.90 ± 0.29	2.17 ± 0.01
Average, all Liberian populations	9.65 ± 1.35	4.59 ± 0.95

Nimba County

Collection Site	lithospermoside content (mg/g)	riachin content (mg/g)
Tapita, Lower Nimba County	7.84 ± 0.14	4.93 ± 0.19
Unspecified Site 1, Nimba County	7.39 ± 0.81	6.30 ± 0.45
Unspecified Site 2, Nimba County	8.20 ± 0.75	6.43 ± 0.63
Upper Nimba County	8.70 ± 0.65	5.56 ± 0.59
Average, Nimba County	8.03 ± 0.64	5.81 ± 0.48
Average, all Liberian populations	9.65 ± 1.35	4.59 ± 0.95



Graph 2-1. Lithospermoside (blue) and riachin content (red) of griffonia populations (mg/g)

Table 2-8. Riachin and lithospermoside content by endosperm color

	riachin content (mg/g)	lithospermoside content (mg/g)
Nearly or completely black endosperms	3.91 ±0.06	3.37±0.01
Yellow (no discoloration)	5.78±0.36	5.51±0.34
Control (no sorting)	5.52±0.04	5.75±0.15

2.4 Conclusions

Griffonia seeds appear to be useful sources of riachin and lithospermoside averaging 4.59 ± 0.95 mg/g and 9.65 ± 1.35 mg/g, respectively and ranging from 1.41 ± 0.01 mg/g to 6.43 ± 0.63 mg/g and 7.39 ± 0.81 mg/g to 14.48 ± 0.32 mg/g respectively. The amount of riachin varied widely between populations with seeds from Grand Gedeh County containing less than those from Nimba County. While lithospermoside content did not vary widely among populations from the same county, populations from Nimba County were found to contain approximately 38% less of this compound than populations from Grand Gedeh County. These variations could be a function of differences in genetics and/or access to sunlight, water, and soil nutrients, water stress, and disease state.

This study also found that seeds with black endosperms contain less riachin and lithospermoside than seeds with yellow endosperms. Whether or not infection or another biotic or abiotic reduces accumulation of these compounds is unknown.

These compounds likely serve to protect the plant from feeding insects and outside of this, it appears that riachin, lithospermoside and its structural analogues have poor bioactivity profiles although more bioactivity studies should be completed before drawing strong conclusions. Based on *in vivo* testing of simmondsin, its simmondsin 2‘-ferulate (Abbassy et al., 2008) and bauhinin (Chen et al., 1985), the toxicity of riachin and lithospermoside is likely low as the nitrile feature

of this molecule is likely not liberated in the body. However, *in vivo* animal studies with riachin and lithospermoside should be conducted to further confirm this.

2.5 References

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Chapter 3. 5-Hydroxytryptophan Content of *Griffonia simplicifolia* Seeds from Various Regions in Ghana and Liberia and Factors Impacting Content

3.1 Introduction

Griffonia simplicifolia seeds are the main commercial source of 5-hydroxytryptophan (5-HTP), which is a widely used alternative treatment for symptoms related to serotonin imbalance such as depression, anxiety, insomnia (Kim et al., 2009), as well as insatiety (Del Corral and Pacak, 2005). 5-HTP is the immediate biological precursor to serotonin (see **Figure 1-2**), a neurotransmitter believed to contribute to feelings of happiness, euphoria, and satiety. Serotonin is formed in mammals from the essential amino acid tryptophan in a two-step process. In the first step, tryptophan is hydroxylated by tryptophan hydroxylase to form 5-HTP. 5-HTP is then decarboxylated by aromatic-l-amino acid decarboxylase to form serotonin (Popova, 2006).

5-HTP has several pharmacokinetic properties that lead many to believe that taking it as a dietary supplement will have a positive influence on mood. Unlike tryptophan, 5-HTP readily crosses the blood brain barrier without the help of a transporter and it is not used in the production of niacin, kynurenine, and proteins (Birdstall, 1998). Despite these seemingly promising pharmacokinetic properties, the efficacy of 5-HTP in treating depression is highly debatable (Hinz et al., 2012). Although many clinical trials have concluded that 5-HTP is effective at mitigating symptoms of depression, most of these studies suffer from several flaws including insufficient sample size, lack of placebo control, and no patient blinding and therefore may not be reliable indicators of efficacy (Shaw et al., 2009). Many coordinators of these trials have also suggested that further studies should be conducted to sufficiently conclude their findings (Hinz et al., 2012). One reason why 5-HTP may not be effective *in vivo* is that it is quickly metabolized by peripheral enzymes before it has the chance to enter the CNS. For example, 5-HTP is a substrate for L-amino acid decarboxylase which is found in high concentrations in both the CNS and in the kidneys (Bertoldi, 2014). Unlike 5-HTP, serotonin produced in the periphery is unable to influence mood because it is unable to cross the blood brain barrier. Researchers have tried to remedy this by co-administering 5-HTP with a decarboxylase inhibitor to prevent it from being turned over to

serotonin in the bloodstream. While a dramatic increase in serum 5-HTP levels was observed, depressive symptoms did not subside (Gijsman et al., 2002). This could indicate that 5-HTP could be hindered from entering the CNS in depressed patients (Argen et al., 1991) or other endogenous chemicals may have an unknown role in mood (Owens and Nemeroff, 1994).

The purpose of the study was to quantitate and compare the 5-HTP content of griffonia seeds from ten populations across Ghana and Liberia (**Table 5-1**) as well as to determine if a seed's endosperm color could be an indicator of its 5-HTP content. In addition, the effect of dry heat pasteurization with two temperatures (90 ˚C and 125 ˚C respectively) on the content of 5-HTP in griffonia seeds were also examined. In nature it is common for plants of the same species to have very different phytochemical characteristics including composition of bioactive compounds. This can be due to differences in genetics and/or the expression of those genes due to growth rates, edaphic features, access to sunlight and water, post-harvest handling (Figueiredo et al., 2008), biotic and abiotic stress (Atkinson and Urwin, 2012), as well as varying growing temperatures (Aksit et al., 2013), therefore we hypothesized that the level of 5-HTP could vary by population and/or region in West Africa. Given that no genetic studies have been conducted to establish phenotype differences, we sought to identify the regions, conditions, and populations that favor higher 5-HTP yield by collecting seeds from ten different regions across Ghana and Liberia and quantifying their 5-HTP content by high performance liquid chromatography (HPLC).

The endosperm color of griffonia seeds often varies based on maturity; mature seeds are usually yellow while less mature seeds have a light green color (Kim et al., 2009). Importers of griffonia seeds check for both levels of 5-HTP, the integrity of the whole seed, and other physical characteristics among seeds that they perceive to provide an overall indicator of the 5-HTP yield and quality. While diameter, weight, and moisture content provide at best a weak indication of 5-HTP content, endosperm color is often the most accurate indicator (Kim et al., 2009). Partially or

totally black endosperms can be a sign of seed deterioration due to fungus or insects. Fully mature griffonia seeds are known to contain slightly more 5-HTP than less mature seeds (Kim et al., 2009) while the average 5-HTP content of partially or totally discolored seeds is unknown. For this reason, we decided to measure the 5-HTP content of griffonia seeds based on endosperm coloring. It is important to equate endosperm color to 5-HTP content in order to have a reliable predictor of natural product content as this bioactive compound is the product of commerce. This information will also be used to help farmers and suppliers of griffonia seeds improve the marketability of their crops.

Heat is often applied to many food and beverages in order to reduce or completely disinfect them from harmful microorganisms and their spores as well as to extend their shelf life. Sterilization is a term used to describe the complete inactivation of all viable microbes from a food or beverage using high heat, pressure, irradiation and/or chemicals (Talaro, 2007). When simply using heat, sterilization is often achieved by heating products at 170 ºC for 60 minutes although using steam and pressure allows for sterilization to occur at lower temperatures for a shorter time period (Talaro, 2007). While sterilization can successfully eliminate all harmful microbes and their spores, the extreme temperatures and pressures can have a significantly negative impact on the aroma, flavor, and texture of a food or beverage (Talaro, 2007). In order to preserve their favorable taste, aroma, and texture, foods and beverages are often subjected to milder temperature and pressures with the intent to severely reduce number of viable microbes rather than completely eliminate all of them, and this is called pasteurization (Talaro, 2007). Griffonia seeds are sometimes subjected to elevated temperatures in order to kill microbes that degrade seeds and contaminate them with toxins. Since heat can also catalyze the transformation and destruction of chemical species, we decided to measure the impact dry heat pasteurization has on the 5-HTP content of griffonia seeds. The temperatures and time periods used for this experiment (90 ºC and 125 ºC for 180 minutes and 60 minutes, respectively) were chosen because they are expected to

be more than sufficient to significantly reduce the numbers of most harmful microbes while not causing a significant change in seed mass by a loss of volatiles (Talaro, 2007; Santhirasegaram et al., 2013; Mesias et al., 2015). While it is unlikely that 5-HTP would be unstable under these conditions, it is possible that prolonged exposure to these temperatures may activate enzymes within the seeds that degrade this commodity. Likewise, exposure to elevated temperatures could also inactivate degradation enzymes thus prolonging the stability of 5-HTP in intact seeds (Asiamah, 2006). Griffonia seeds are vulnerable to contamination by toxin-producing microbes because the nature of its harvest and colleting system is such that the griffonia pods drop to the forest floor when mature. These pods are thus collected from the forest floor immediately or after time and as such the seeds are exposed to a wide range of microbiological organisms, some of which could be pathogenic to humans. However, this issue is not generally relevant for griffonia as nearly all seeds are subjected to processing to extract the 5-HTP. A relevant issue is that in the weeks to months between the time seeds are harvested and shipped to processors, microbial growth on seeds can degrade seed quality and ultimately lower 5-HTP yield. Another issue is that uncontrolled microbial growth can lead to the accumulation of harmful toxins. For example, *Aspergillus* is a genus of fungi that includes several species that are infectious to humans and can thrive on some legumes (Benford et al., 2010). While these pathogenic species are usually killed during processing the highly toxic and carcinogenic compounds they leave behind known as aflatoxins (Benford et al., 2010) are not necessary removed or destroyed. Exposure to Aflatoxin B1 (**Figure 3-1a**) can cause a mutation to occur in the p53 tumor-suppressing gene resulting in heptocelluar carcinomas especially in those infected with hepatitis B (Benford et al., 2010). Depending on the extraction and purification process, these toxins may or may not be entirely removed from the final product leaving one susceptible to illness. An example of this occurred in the 1990's when 37 people died and 1500 were sickened by an unusual illness known as eosinophilia-myalgia syndrome (EMS). The cause of this illness was linked to a batch of tryptophan that contained over 60 impurities (Adachi et al., 2000) two of which being known

toxins 1,1'-ethylidenebis(tryptophan) (**Figure 3-1b**) (Yamaoka et al., 1994; Barth et al., 2001; Emslie-Smith et al., 1994) and 3-(N-Phenylamino)alanine (**Figure 3-1c**) (Martínez-Cabot and Messeguer, 2007). These impurities were side products of a bacterial fermentation process used to produce the tryptophan. The failure of the purification process to sufficiently remove these contaminants from the final product not only resulted in the death of many consumers, it also caused the FDA to ban the sale of tryptophan supplements for several years. The safety of 5-HTP supplements once came under scrutiny when it was reported that a mother and her two children became ill with "EMS-like symptoms" after being exposed to 5-HTP supplements. Subsequent investigation of the case implicated 5-HTP supplements revealed a miniscule series of impurities labeled "peak X" and it was assumed that these impurities were the cause of the outbreak (Johnson et al., 1999; Klarskov et al., 2003) however whether or not this was the cause of this outbreak is debatable as these impurities have been found in other 5-HTP supplements and no one else was or has been reported to become ill since (Michelson et al., 1994; Das et al., 2004).

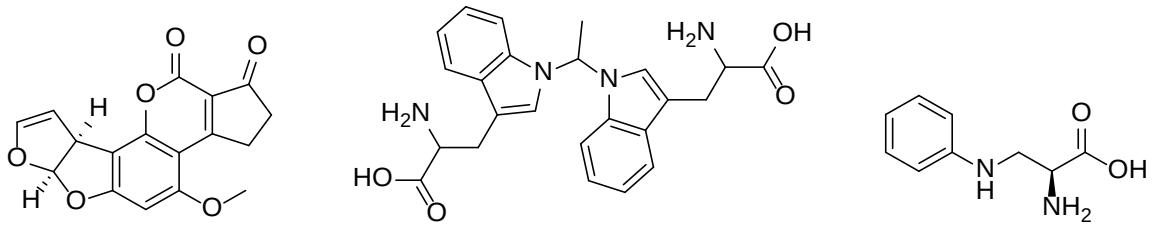


Figure 3-1. Contaminants known to cause harmful health effects.
(A) Aflatoxin B1, a carcinogen produced by fungi that can thrive on many legumes. (B) 1,1'-ethylidenebis(tryptophan) and (C) 3-(N-Phenylamino)alanine, two harmful contaminants found on tryptophan supplements linked to the EMS outbreak.

3.2 Experimental

3.2.1 Materials

HPLC grade water, acetonitrile, and methanol from Fisher Scientific (Fair Lawn, NJ) and 95% formic acid from Sigma Aldrich (St. Louis, MO). Griffonia seeds from various regions of Ghana and Liberia were used in this study (for collection sites and sample sizes, see **Table 5-1**).

3.2.2 Equipment

A Waters 2695 separation module equipped with a Waters 2996 photo diode array detector was used for HPLC separation. Separation was carried out using a Phenomenex Luna phenyl hexyl column (150 mm x 4.6 mm, 5 μm).

3.2.3 HPLC Conditions

The mobile phase consisted of an isocratic mixture of 99% solvent A (H_2O, 0.1% formic acid) and 1% solvent B (acetonitrile, 0.1% formic acid) for 10 minutes. The flow rate was set at 1.0 mL/min with UV detection set at 270nm. The injection volume was 10 μL and analysis occurred at room temperature.

3.2.4 Standards

Chromatographic grade 5-HTP (98%) from Sigma Aldrich (St. Louis, MO)

3.2.5 Methods

3.2.5.1 Method A: 5-HTP Content of Seeds from Various Origins

Griffonia seeds were collected by ASNAPP-Ghana team from various regions of Ghana and Liberia (**Table 5-1**). Seeds were manually harvested from fallen seed pods and collection from the forest floor, seeds removed, cleaned and air/sun dried off the ground prior to shipment.

Twenty seeds that were representative of each location in mass and endosperm color were selected and grinded to a powder and approximately one gram of this powder was extracted with 100 mL of 50% methanol. This was sonicated for 15 minutes giving an opaque white solution which was then allowed to sit at room temperature for three hours. A 1.5 mL aliquot of this solution was transferred to an eppendorf tube and centrifuged for 5 minutes. After centrifugation, a one milliliter aliquot of sample was diluted to 11 mL with 50% methanol in water and then placed in an amber HPLC vial for analysis. Each sample was measured in triplicate and results were adjusted for moisture content.

3.2.5.2 Method B: 5-HTP Content of Seeds Based on Endosperm Color

Endosperms were inspected and placed into one of two groups based on their coloring (**Figure 3-2**). The first group consisted of seeds that had a bright yellow colored endosperm without any patches of black. The second group consisted of seeds with nearly or completely black endosperm color. These endosperms along with their seed coats were ground to a fine powder and approximately one gram of this powder was extracted with 100 mL of 50% methanol in water. Solution was sonicated for 15 minutes which was then allowed to sit at room temperature for three hours. A 1.5 mL aliquot of this solution was transferred to an Eppendorf tube and centrifuged for 5 minutes. After centrifugation, a one milliliter aliquot of sample was diluted to 10 mL with 50% methanol in water and then placed in an amber HPLC vial for analysis. Each sample was analyzed in triplicate and results were adjusted for moisture content.

Figure 3-2. Photos of griffonia endosperms separated by condition. (A) Perfect yellow endosperms. (B) Nearly or completely black endosperms

3.2.5.3 Method C: Effect of Dry Heat Pasteurization on 5-HTP content

For the dry heat pasteurization experiment at 90 ºC, seeds were ground to a fine powder and approximately 100 mg of this powder was placed in each of six beakers and all were placed at once in an oven preset to 90 ºC. One beaker was removed from the oven every 30 minutes and allowed to cool to room temperature prior to extraction.

For the dry heat pasteurization experiment at 125 ºC, seeds were ground to a fine powder and approximately 100 mg of this powder was placed in each of six beakers and all were placed at once in an oven preset to 125 ºC. One beaker was removed from the oven every 10 minutes and allowed to cool to room temperature prior to extraction.

After heating, powder was extracted with 100 mL of 50% methanol in water and sonicated for 15 minutes. After sonication, solution was then allowed to sit at room temperature for three hours. Approximately 1.5 mL of this solution was transferred to an Eppendorf tube and centrifuged for 5 minutes. After centrifugation, samples were placed in an amber HPLC vial and analyzed. Each sample was analyzed in triplicate and results were adjusted for moisture content.

3.2.5.4 Method D: 5-HTP Content of Seed Coat vs. Endosperm

In order to calculate the proportion of 5-HTP contained by the seed coats and ultimately how much would be lost if seed coats were removed, we first had to determine the seed coat's average mass. This was done by selecting 30 representative seeds from unspecified Site 1, Nimba County and individually weighing them and then removing and weighing each seed coat. Griffonia seed coats ranged from 72.0 mg to 153.4 mg and weighed on average 97.3 mg. In terms of percentages, griffonia seed coats were found to range from 9.8-17.8% the seeds total mass, and averaged approximately 13.1%.

Seed coats were ground by mortar and pestle and sieved (500 µM) while endosperms were ground to a fine powder using a coffee grinder. Approximately 1 gram of each material was extracted with 100 mL of 50% methanol in water and sonicated for 15 minutes. After sonication, samples were allowed to sit at room temperature for three hours. A 1.5 mL aliquot of this solution was transferred to an Eppendorf and centrifuged for 5 minutes. After centrifugation, a one milliliter aliquot of each sample was diluted to 10 mL of 50% methanol in water and then placed in an amber HPLC vial for analysis. Samples were analyzed in triplicate and results were adjusted for moisture content.

3.2.6 Calibration Curves

3.2.6.1 Calibration Curve A: 5-HTP Content of Seeds from Various Origins

12.2 mg of 5-HTP was dissolved in 10 mL of 50% methanol in water and vortexed. Then, 0.75 mL of this stock solution was used to make dilutions of 1/4x, 1/8x, 1/16x, 1/32x, 1/64x, 1/128x, 1/256x, and 1/512x to cover concentrations ranging from 2.38 µg/mL to 305 µg/mL. All dilutions yielded a well-shaped peak. The peak area of each dilution was analyzed twice to ensure instrument reliability. R^2 value was 0.9994, showing excellent linearity (**Figure 3-3**).

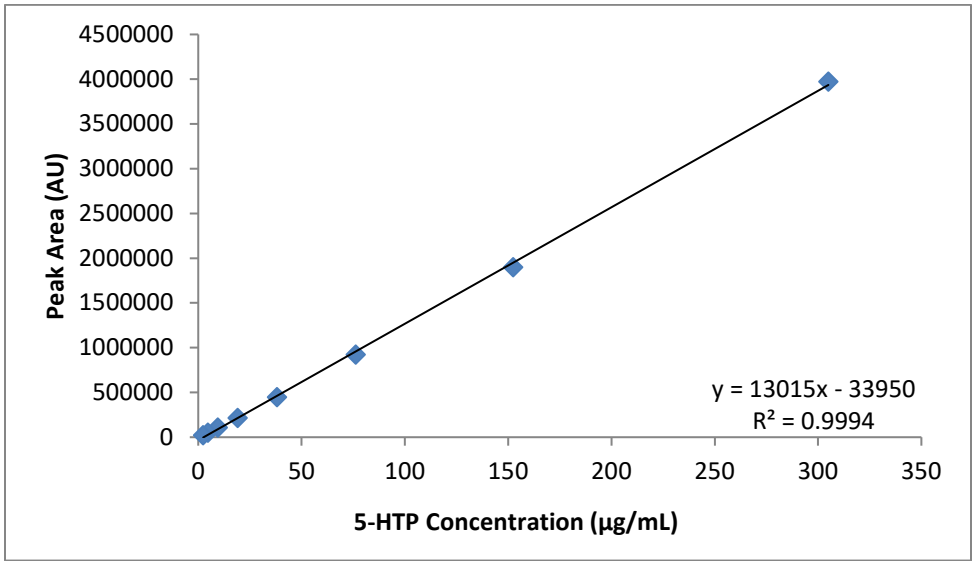


Figure 3-3. Calibration curve used to quantitate 5-HTP content of griffonia seeds from various regions of Ghana and Liberia

3.2.6.2 Calibration Curve B: 5-HTP Content by Endosperm Color and Seed Part

5-HTP (104.2 mg) was dissolved in 100 mL of 50% methanol in water in a volumetric flask. A one milliliter aliquot of this stock solution was used to make dilutions of 1/2x, 1/4x, 1/8x, 1/16x, 1/32x, and 1/64x to cover concentrations ranging from 16.28 µg/mL to 521µg/mL. All dilutions yielded a well-shaped peak. The peak area of each dilution was analyzed twice to ensure instrument reliability. R^2 value was 1, showing excellent linearity (**Figure 3-4**).

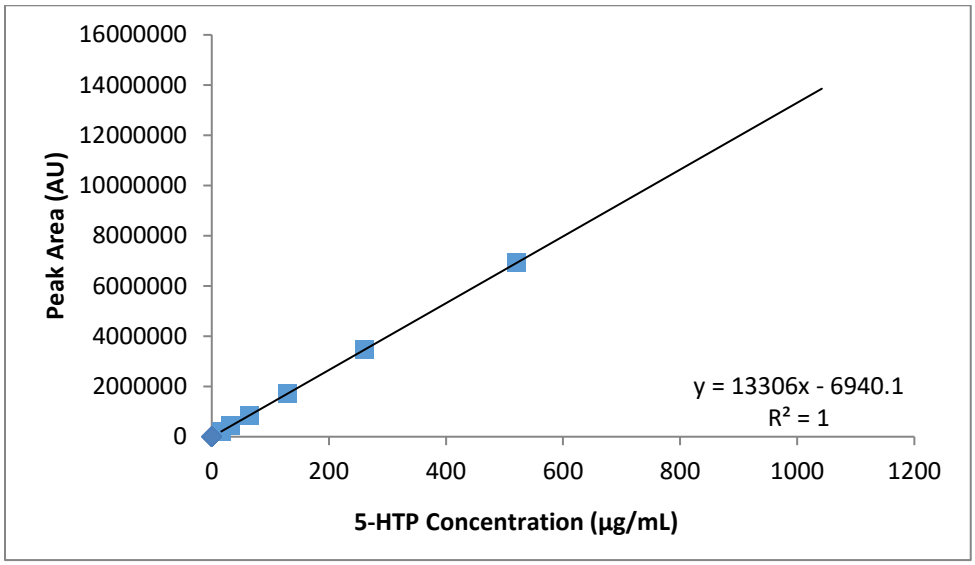


Figure 3-4. Calibration curve used to quantitate 5-HTP content of griffonia seeds based on endosperm color

3.2.6.3 Calibration Curves C and D: Effect of dry heat pasteurization on 5-HTP Content

12.5 mg of 5-HTP was dissolved in 25 mL of 50% methanol in water and this stock solution was used to make calibration curves for both pasteurization experiments. A one milliliter aliquot of the stock solution was used to make dilutions of 1/2x 1/4x, 1/8x, 1/16x, 1/32x, 1/64x, 1/128x,

1/256x, and 1/512x to cover concentrations ranging from 0.976 µg/mL to 250 µg/mL. Calibration curve used for pasteurization experiment at 90ºC yielded an R^2 value was 0.9999, showing excellent linearity (**Figure 3-5**). Calibration curve used for pasteurization experiment at 125 ºC yielded an R^2 value was 0.9999, showing excellent linearity (**Figure 3-6**).

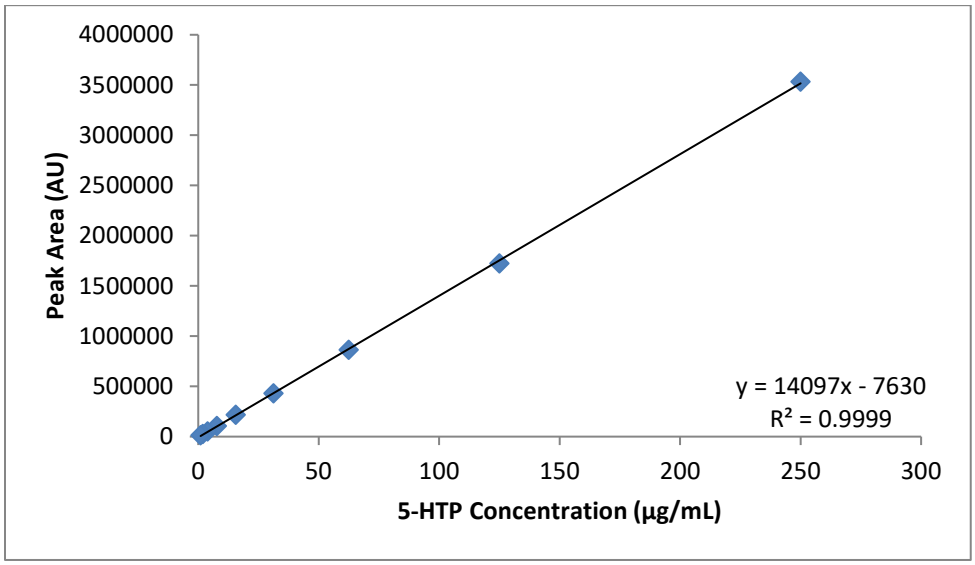


Figure 3-5. Calibration curve used to quantitate 5-HTP content of griffonia seeds for dry heat pasteurization experiment at 90 ˚C for 180 minutes

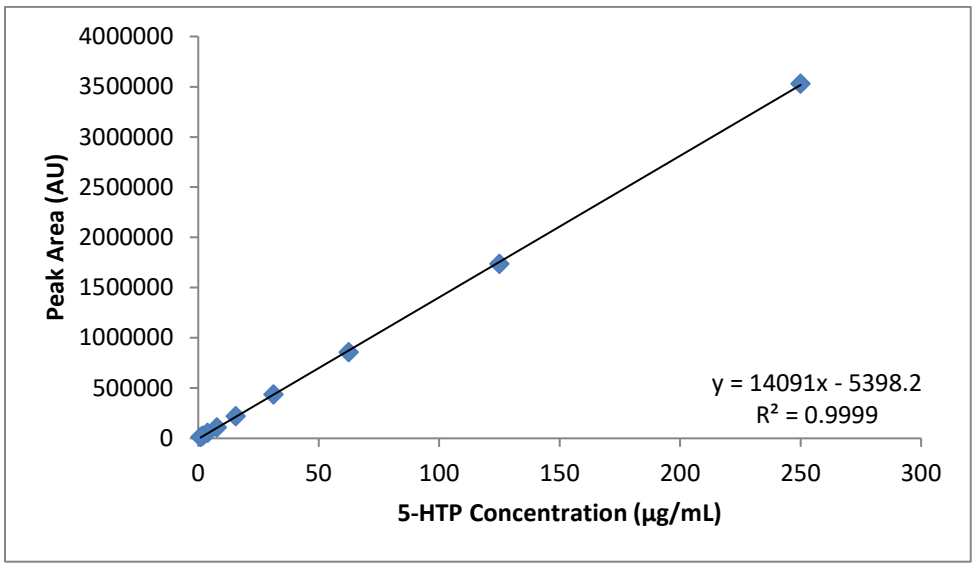


Figure 3-6. Calibration curve used to quantitate 5-HTP content of griffonia seeds for dry heat pasteurization experiment at 125 ˚C for 60 minutes

3.3 Results and Discussion

The HPLC method used provided excellent separation for 5-HTP (**Figure 3-7**). 5-HTP content of seeds from ten populations across Ghana and Liberia were very similar ranging from 110.23 ± 1.10 mg/g to 137.04 ± 9.06 mg/g and averaged 120.84 ± 9.00 mg/g (**Table 3-1 and Graph 3-1**). The average 5-HTP content of seeds from Ghana was 129.27 ± 12.39 mg/g while the 5-HTP content of Liberian seeds was 115.23 ± 5.73 mg/g. Variation was relatively high in seeds from Brong Ahafo and Ashanti regions indicating seeds from these regions may vary in 5-HTP content. While no population was found to contain excessively large amounts of 5-HTP, seeds from each region yield a high enough percentage of 5-HTP (6-10%) to attract commercial interest (Lemaire and Adosraku, 2002).

Seeds with perfect endosperm coloring contained 110.96 ± 6.46 mg/g while seeds that were nearly or completely black contained 70.37 ± 2.01 mg/g 5-HTP (**Table 3-2**). This proves that seeds with black endosperms contain less 5-HTP than healthy seeds although there still maybe useful amounts of 5-HTP available as seeds as low as 6% 5-HTP by mass have been known to attract commercial interest (Lemaire and Adosraku, 2002).

Pasteurization of griffonia seeds at 90 ºC and 125 ºC under dry heat for 180 minutes and 60 minutes respectively did not catalyze the destruction of 5-HTP (**Tables 3-3 and 3-4, Figures 3-8 and 3-9**). In fact, a slight increase in 5-HTP content was observed. This could be due to several factors that include loss of moisture and other volatiles from the seeds or quite possibly the increase in temperature could increase the activity of enzymes that produce 5-HTP. This is the first report that shows that griffonia seeds can be exposed to temperatures of 90 ºC and 125 ºC for 180 minutes and 60 minutes respectively without any significant effect on 5-HTP content.

Endosperms contained 132.79 ± 2.02 mg/g of 5-HTP while seed coats contained 80.36 ± 1.84 mg/g of 5-HTP (**Table 3-5 and Graph 3-2**). Griffonia seed coats were found to range from 72.0 mg to 153.4 mg in mass implying that each seed coat could contain anywhere from 5.78 mg to 12.33 mg of 5-HTP. As griffonia seeds are most often secured in kilograms, removing the seed coats to remove possible deposits of toxins on the surface would have a significant economic impact. Therefore, a better strategy to mitigate the risks associated with microbial growth and surface toxins is simply to pasteurize seeds.

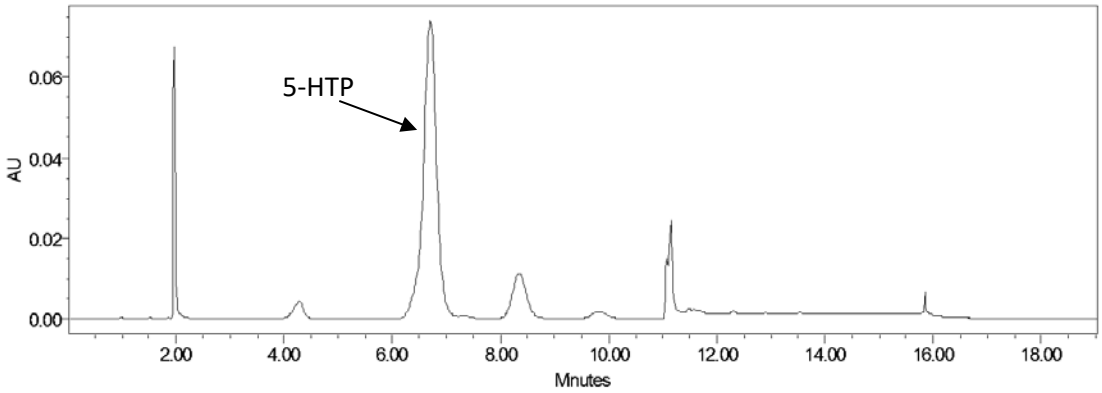
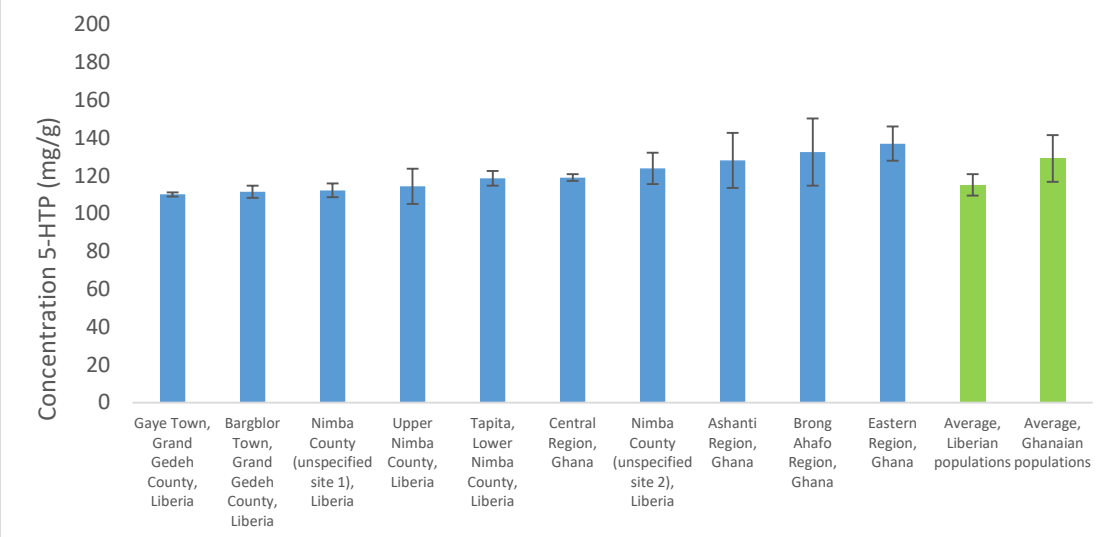


Figure 3-7. A representative HPLC-UV chromatogram of Liberian griffonia seeds at 270nm

Table 3-1. 5-HTP Content of griffonia seeds from various wild populations from Ghana and Liberia

Liberia	
Collection site	**5-HTP Content (mg/g)**
Bargblor Town, Grand Gedeh County	111.62 ± 3.25
Gaye Town, Grand Gedeh County	110.23 ± 1.10
Tapita, Lower Nimba County	118.73 ± 3.96
Unspecified Site 1, Nimba County	112.31 ± 3.64
Unspecified Site 2, Nimba County	123.96 ± 8.32
Upper Nimba County	114.52 ± 9.32
Average, Liberian populations	**115.23 ± 5.73**
Average, all populations	**120.84 ± 9.00**

Ghana	
Collection site	**5-HTP Content (mg/g)**
Ashanti Region	128.23 ± 14.56
Brong Ahafo Region	132.65 ± 17.80
Central Region	119.15 ± 1.72
Eastern Region	137.04 ± 9.06
Average, Ghanaian populations	**129.27 ± 12.39**
Average, all populations	**120.84 ± 9.00**



Graph 3-1. 5-HTP Content of griffonia seeds from various wild populations from Ghana and Liberia

Table 3-2. Content of 5-HTP in griffonia seeds based on endosperm color

Endosperm color	5-HTP content (mg/g)
Nearly or completely black endosperms	70.37 ± 2.01
Yellow (no discoloration)	110.96 ± 6.46
Control (no sorting)	104.07 ± 1.63

Table 3-3. 5-HTP content of seeds for dry heat pasteurization experiment at 90°C

Time period (minutes)	5-HTP content (mg/g)
0	113.84 ± 2.52
30	112.21 ± 0.43
60	113.59 ± 2.16
90	115.89 ± 0.83
120	114.67 ± 1.44
150	116.23 ± 0.92
180	116.52 ± 1.55

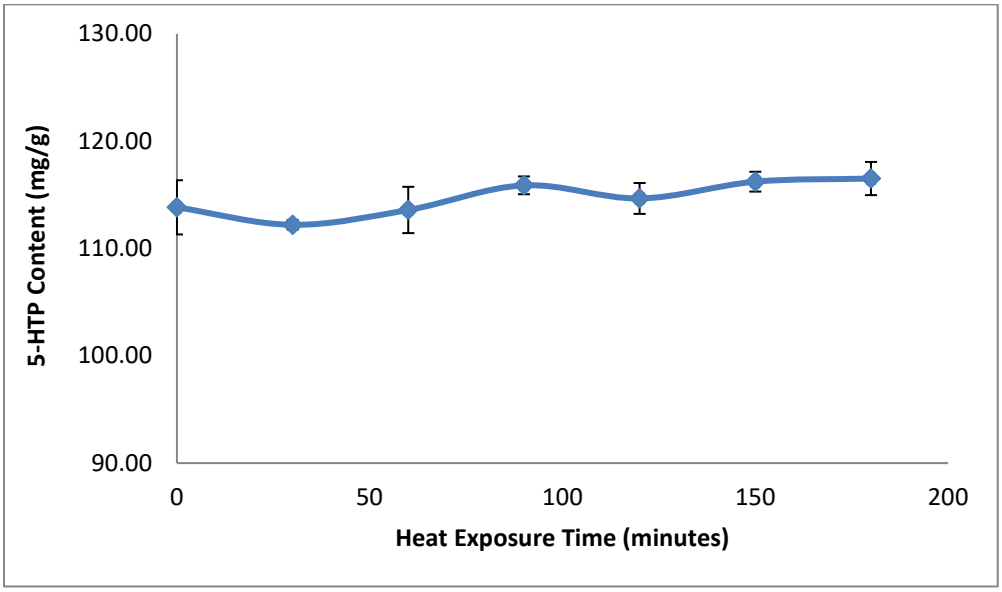


Figure 3-8. 5-HTP content of seeds for dry heat pasteurization experiment at 90°C

Table 3-4. 5-HTP content of seeds for dry heat pasteurization experiment at 125°C

Time period (minutes)	5-HTP content (mg/g)
0	112.53 ± 0.50
10	113.28 ± 3.50
20	112.14 ± 1.06
30	111.92 ± 0.61
40	114.36 ± 1.99
50	113.91 ± 1.79
60	115.72 ± 1.12

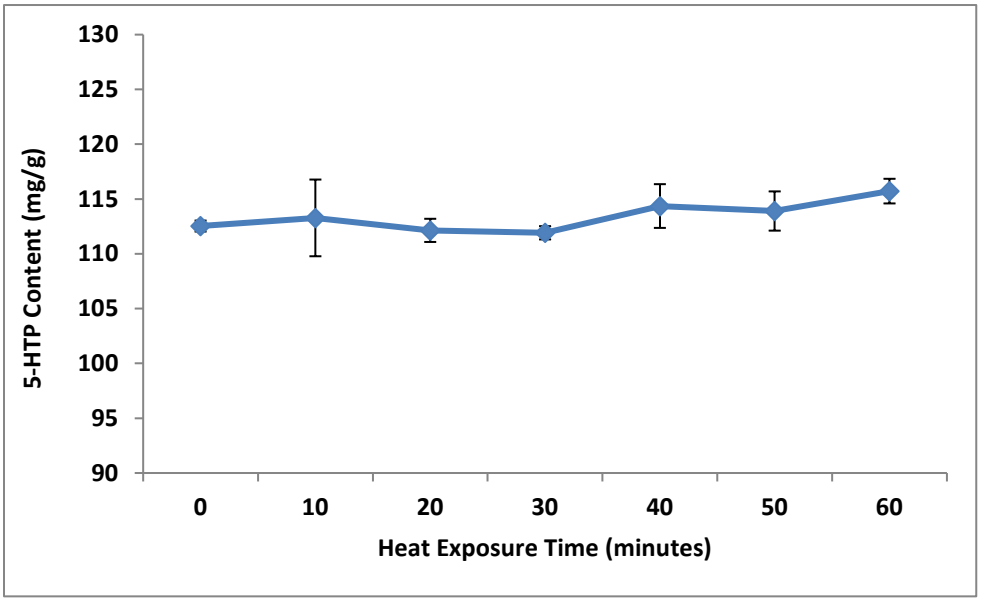
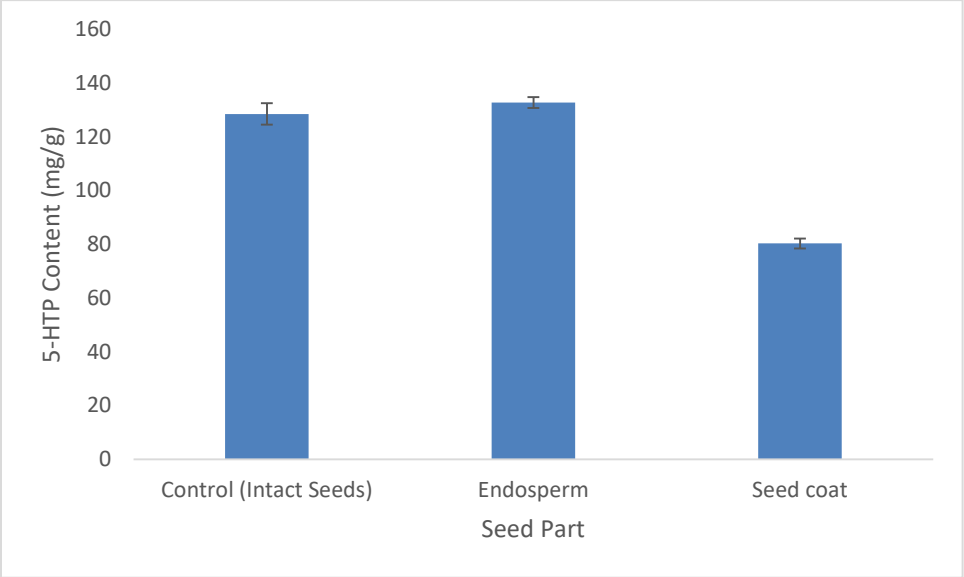


Figure 3-9. 5-HTP content of seeds for dry heat pasteurization experiment at 125°C

Table 3-5. Masses of seed components and average 5-HTP content in seed coats vs. endosperms

Seed component	Mass range (mg)	Average Mass (mg)	Average % of total seed mass	5-HTP content (mg/g)
Endosperms	444.3 – 1126.6	651.94	86.9	132.79 ± 2.02
Seed Coats	72.0 - 153.4	97.34	13.1	80.36 ± 1.84
Control (Intact seeds)	501.1 - 1280.0	749.28		128.55 ± 3.97



Graph 3-2. 5-HTP content by seed part

3.4 Conclusions

5-HTP content varied little between the various griffonia populations in Ghana and Liberia which ranged from 10.9% to 13.7% of total seed mass. 5-HTP content of griffonia seeds generally range from 6% to 20% by weight and seeds as low as 6% 5-HTP content are known to be still of interest to commercial exporters (Lemaire and Adosraku, 2002). Therefore, while no populations were found to be exceptionally high in 5-HTP, all populations investigated in this study contain sufficient 5-HTP to attract commercial interest, and thus for Liberia as a potential new source of this natural product these results are quite promising. Differences in 5-HTP accumulation among the populations can be influenced by a variety of factors which include differences in genetics and maturity, access to soil nutrients, water, and sunlight as well as biotic and abiotic stressors, post-harvest handling and possibly the health of the *Rhizobia* bacteria that dwell in the legume's roots. Further studies should be aimed at evaluating the impact of each of these factors and use

this information to maximize the production of 5-HTP of these populations in order to increase their economic value.

In this study, we found that seeds that were completely black contained appoximtaley 36% less 5-HTP than healthy colored seeds. While seeds with discolored and completely black endosperms can still be useful sources of 5-HTP, proper harvesting and post-harvesting practices can have a significant effect on 5-HTP yield and ultimately marketability of the seeds.

We also found that exposing griffonia seeds to temperatures as high as 125 ºC for an hour does not negatively impact 5-HTP content. Therefore, pasteurizing seeds after harvesting is a useful strategy to eliminate microbes that degrade seeds over time and can be a source of toxins and ensure food safety issues are addressed. Exposing intact griffonia seeds to 70 ºC for 30 minutes is believed to denature enzymes responsible for the degradation of 5-HTP (Asiamah et al., 2006). Therefore, pasteurizing seeds should be expected to extend the shelf life of the 5-HTP contained by these seeds. Refrigeration of griffonia seeds should be avoided as in the same study by Asiamah et al., it was found that cooler temperatures likely activate enzymes that catalyze the breakdown of 5-HTP (Asiamah et al., 2006). Lastly, removing seed coats to eliminate surface microbes and contaminants will have a strongly negative economic impact as we found that each seed coat could contain anywhere from 5.78 mg to 12.33 mg of 5-HTP.

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Chapter 4. 5-HTP Content of 18 Commercial Supplements

4.1 Introduction

In 1994, Americans spent approximately $5.8 billion dollars on dietary supplements and since, the nutritional supplement industry has grown to an estimated $35 billion a year industry and it is believed to grow even larger in the years to come (FDA Voice, 2016). Despite the increasing popularity of these products, regulation of supplements is not as rigorous as regulation of pharmaceuticals; unlike drugs, distributors of dietary supplements are not required to seek approval from, or submit evidence of efficacy to the FDA prior to marketing (NIH Office of Dietary Supplements Website, 2016). Manufacturers and distributors are required to insure products are safe prior to marketing and are required to follow good manufacturing practices (GMP) when formulating their supplements and they must insure that there is some evidence efficacy to back any label claims. Manufacturers and distributors must also be prepared to submit this evidence of efficacy, safety, and GMP to the FDA when requested (NIH Office of Dietary Supplements Website, 2016). 5-HTP is often marketed as an alternative treatment for conditions involving an imbalance of serotonin such as depression, insatiety, and fibromyalgia. While several clinical trials have indicated that 5-HTP may be useful for the treatment of these conditions (Cangiano et al., 1992; Birdstall 1998), there is still skepticism regarding the efficacy of 5-HTP in the treatment of depression. This is partially due to several flaws in the clinical trials assessing the efficacy of 5-HTP that include insufficient sample size, lack of placebo control, and no patient blinding (Shaw et al., 2009). Authors of some studies have suggested that further studies are needed to sufficiently conclude their findings (Hinz et al., 2012). One reason why 5-HTP fails to show indisputable efficacy against depression may be due to the fact that upon ingestion, most 5-HTP is quickly converted to serotonin (and other metabolites), and serotonin produced outside the CNS is unable to cross the blood brain barrier to effect mood (Gijsman et al., 2002). Other studies have found that 40% of depressed individuals had normal serotonin levels (Owens and Nemeroff, 1994) and that when co-administered with a drug that blocks its conversion to serotonin in the periphery, 5-HTP was ineffective against depression despite a 15

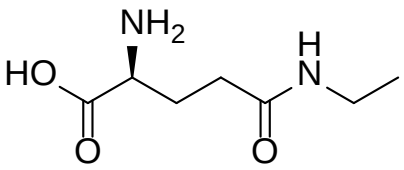
fold increase in plasma levels which indicate that an imbalance of serotonin may not be the sole cause of depression (Gijsman et al., 2002). Another study has suggested 5-HTP may be an ineffective treatment because the penetration of 5-HTP through the blood brain barrier may be hindered in depressed patients (Agren et al., 1991).

5-HTP is well tolerated and side effects such as nausea, vomiting, diarrhea, insomnia, headache and heart palpitations have rarely been reported (Turner et al., 2006). Gastrointestinal effects are likely due to the fact that the GI tract becomes stimulated by the increase of peripheral serotonin (Turner et al., 2006). It is recommended that 5-HTP be avoided by people taking SSRIs or MAOIs because of a possible risk of patients developing serotonin syndrome by this combination (Iovieno, 2011) although to date there have been no reported cases of this disorder associated with any 5-HTP and prescription antidepressant combination (Turner et al., 2006). Clinical studies have also concluded that there is very little risk associated with combining 5-HTP with SSRIs, MAOIs and tricyclic antidepressants (Turner et al., 2006). However, each of these studies consisted of less than 30 subjects (Turner et al., 2006), thus these findings may not be conclusive. Serotonin syndrome has however been reported in patients taking tryptophan with the SSRI fluoxetine (Prozac) (Turner et al., 2006). Excess serotonin causes the CNS to become overly excited (Boyer and Shannon, 2009) resulting in hypertension, hyperthermia, hyperreflexia, dizziness, flushing, disorientation, and involuntary muscle twitching (Turner et al., 2006). While 5-HTP supplementation is regarded as safe, the safety of 5-HTP was questioned when two children being treated with 5-HTP for tetrahydrobiopterin deficiency and their mother became ill. Their symptoms were partly similar to eosinophilia-myalgia syndrome (EMS), an illness caused by tryptophan supplements contaminated with over 60 impurities (Adachi et al., 2000). Michelson et al. (1994) reported the presence of a contaminant named “peak X” in case implicated 5-HTP and a later study by Johnson et al. (1999) would characterize Peak X as a

cluster of impurities with the same mass one of which was believed to be 4,5-tryptophan dione, a possible neurotoxin (Johnson et al., 1999; Klarskov et al., 2003). This suspected case EMS has caused a great concern among 5-HTP distributors many of which have responded by routinely having their supplies investigated for peak X contamination. A review by Das et al., (2004) argues however that there are several strong reasons to believe that this suspected outbreak was not related to contaminated 5-HTP. The case implicated infusion which included 5-HTP also included tetrahydrobiopterin, L-dopa, carbidopa and possibly excipients yet neither of these substances were examined for impurities (Das et al., 2004). The only EMS symptoms the mother experienced was muscle soreness and eosinophilia. Her other symptoms were are not features of EMS. The mother also had previous medical conditions which could have also played a role in her symptoms (Das et al., 2004). The children who ingested the 5-HTP and had suffered from other medical conditions as well (Das et al., 2004; Michelson et al., 1994) and only displayed mild eosinophilia and elevated aldose. Eosinophilia is associated with parasitic infections, hay fever (Newsholme and Doherty, 2005), and allergies to prescription drugs (Valent, 2009) therefore the presence of mild eosinophilia could be due to other causes. An analysis of 5-HTP supplements from various sources by Michelson et al. found that peak X was present in many other 5-HTP formulations including that of another child being treated for tetrahydrobiopterin deficiency yet no one else, including this other patient, has been reported to have become ill with any EMS symptoms (Das et al., 2004; Michelson et al., 1994). Lastly, while the mother was being hospitalized and examined, the children's father was also exposed to this infusion numerous times and did not develop any symptoms (Das et al., 2004; Michelson et al., 1994). After critically reviewing the three published reports characterizing peak X, Das et al. concluded that the peak X cluster of compounds was likely an artifact from a previous HPLC run. It was pointed out that the peak X compounds were present in such small amounts that it was not possible to accurately determine their structures or quantitate them by the most sophisticated mass spectroscopic techniques (available at the time) and also to date, no other reports of peak X being detected. Das

et al. also points out that one of the peak X molecules was believed to be the suspected neurotoxin 4,5-tryptophan dione, a molecule that is very unstable in acid and is likely destroyed upon ingestion. This molecule is also not very water soluble which would severally hamper its distribution throughout the human body (Das et al., 2004). Despite these facts, 5-HTP supplement suppliers are still very watchful of the product they distribute as most 5-HTP is inspected for peak X.

5-HTP is often formulated in dietary supplements with other compounds such as Vitamin B6, Vitamin B12, Folic Acid, and L-theanine (**Figure 4-1**). Mixtures of B vitamins have been reported to increase brain serotonin levels (Dakshinamurti et al., 1990). Vitamin B6 is believed to act as a cofactor for L-amino acid decarboxylase, which is the endogenous enzyme that converts 5-HTP to serotonin (Hendler, 2001). L-theanine has been reported to have anxiety relieving properties which may potentiate the effect of 5-HTP (Kimura et al., 2007; Juneja et al., 1999). It is interesting to note that there are conflicting reports regarding the effect of L-theanine on brain serotonin levels; animals studies have suggested that L-theanine suppresses serotonin production (Yokogoshi et al. 1995; Yokogoshi et al. 1998) while another study suggests the opposite (Lu et al., 2006). Folic acid deficiency is commonly observed in depressed patients although a strong correlation and mechanism between folic acid deficiency and depression has yet to be established (Lazarou and Kapsou, 2010). There is some evidence to suggest that folic acid can increase efficacy of certain selective serotonin re-uptake inhibitors (Christensen et al., 2011).



L-Theanine
Exact Mass: 174.10

Figure 4-1. Chemical structure of L-Theanine

The purpose of this study was to measure the amount of 5-HTP present in commercial dietary supplements in the US marketplace, either available in stores or online in order to verify their declared amounts and see if the products meet their own label claim. Since dietary supplements are not required to be as heavily monitored as pharmaceuticals, there is more potential for these commodities to contain inaccurate amounts of active constituents.

4.2 Experimental

4.2.1 Materials

HPLC grade acetonitrile, water, and methanol from Fisher Scientific Inc. (Fair Lawn, NJ). Formic acid and 5-Hydroxytryptophan purchased from Sigma-Aldrich (St. Louis, MO). 18 commercial supplements that were obtained from online sources and from local stores in Bergen County, New Jersey

4.2.2 Equipment

Quantitation of 5-HTP in supplements was conducted using a Waters 2695 separation module equipped with a Waters 2996 photo diode array detector. Separation was performed on a Luna hexyl-phenyl column 250 x 4.6 mm, 5 μm (Phenomenex Inc. Torrance, CA).

4.2.3 HPLC Conditions for Quantitation of 5-HTP in Supplements

The mobile phase consisted of a gradient that included solvent A (ddH_2O, 0.1% formic acid) and solvent B (acetonitrile, 0.1% formic acid). Gradient consisted of 1% to 5% B from 0 to 10 minutes, 5% to 25% from 10 to 30 minutes, 25% to 40% from 30 to 40 minutes, and from 40 to 50 minutes gradient was held constant at 40%. The flow rate was set at 1 mL/min with UV detection set at 270 nm. The injection volume was 10 µL and the running time was 55 minutes. Analysis occurred at room temperature.

4.2.4 Standards

5-Hydroxytryptophan (≥98% purity) was purchased from Sigma Aldrich Co. (St. Louis, MO).

4.2.5 Preparation

To ensure the identity of the manufacturers remained anonymous, a person not affiliated with the analysis removed the pills from their original containers and placed them in plastic bags. The only information recorded on the bags was the amounts of 5-HTP each pill contained as declared by the manufacturer. In order to obtain a representative sample for each manufacturer, five capsules all from the same manufacturer, were randomly selected, emptied, and mixed together. The total mass was recorded and this number divided by five was used as the average mass per pill for calculations. Approximately 100 mg of this mixture was extracted with 25mL of 50% methanol in water and was sonicated for 10 minutes and left to extract at room temperature for 2 hours. Samples were centrifuged for 5 minutes and then a one milliliter aliquot of this solution was diluted with 25 mL of 50% methanol in water. Samples were then transferred into HPLC vials for HPLC analysis. Analysis was done in triplicate with the average of these runs reported below.

4.2.6 Calibration Curve

12.0 mg of chromatographic grade 5-HTP was dissolved in 25 mL of 50% methanol/water and one millimeter of this stock solution was diluted to concentrations of 1/2x, 1/4x, 1/8x, 1/16x, 1/32x, 1/64x, 1/128x, 1/256x, and 1/512x to cover a range of 0.94 µg/mL to 240 µg/mL. Each data point was obtained by triplicate injections. R^2 value was 0.9999 indicating excellent linearity (**Figure 4-2**).

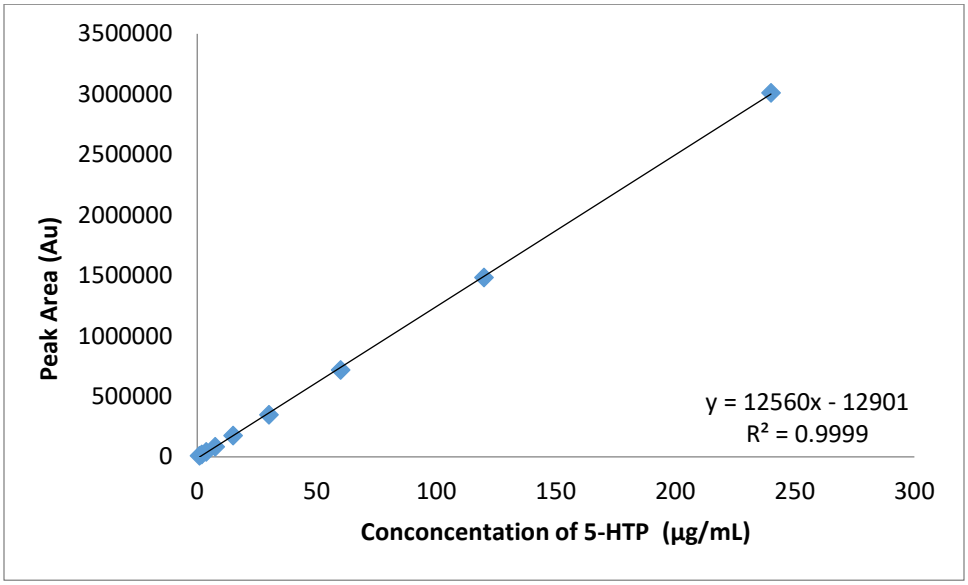


Figure 4-2. Calibration curve used to quantitate 5-HTP in commercial products

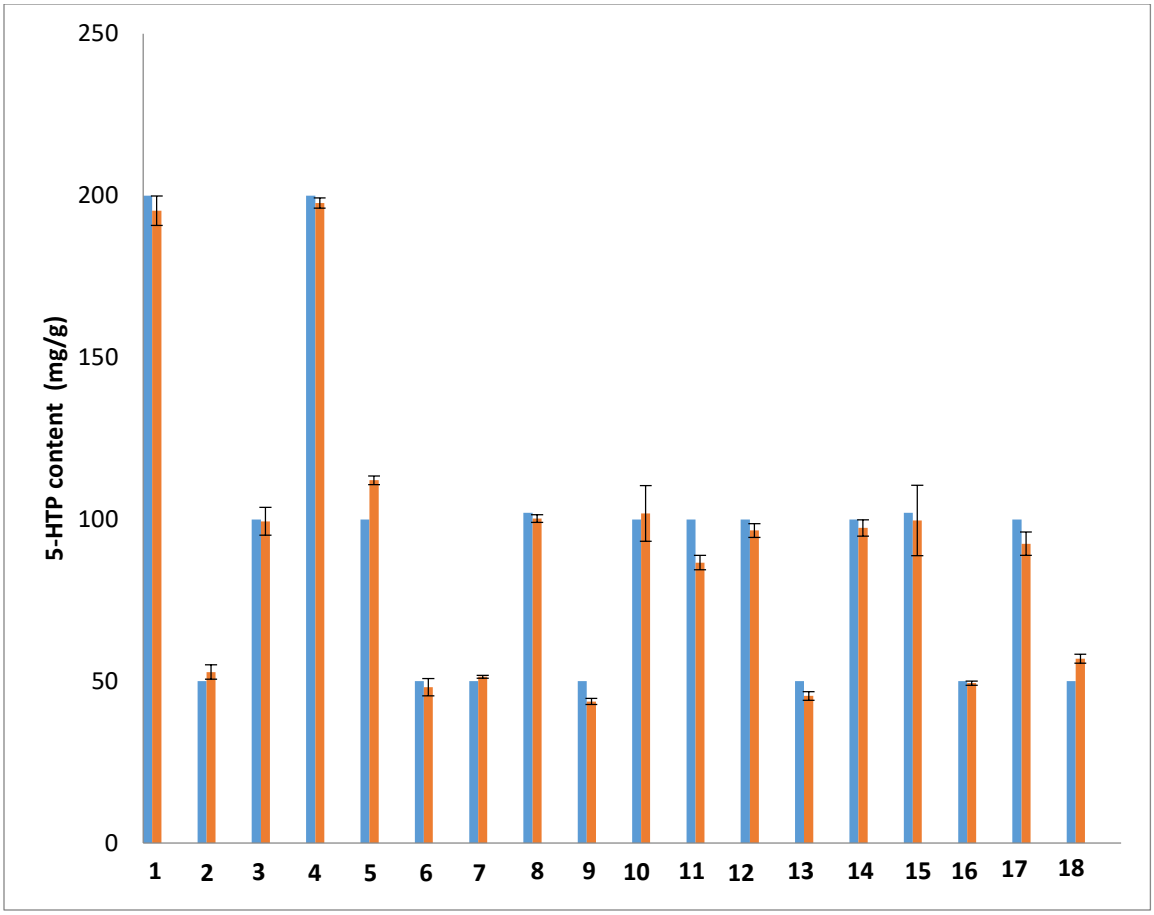
4.3 Results and Discussion

The purpose of this study was to verify the actual content of 5-HTP per pill, which should be between 85-115% the declared amount to be in accordance with US Pharmacopoeia guidelines (Tunna and Patel, 2013). The measured amount of 5-HTP in supplements from each supplier was found to fall within 85-115% of their declared amounts although the margin of error for 9, 11, and 13 falls below the lower limit while the margin of error for 18 reaches above the upper limit (**Table 4-1 and Graph 4-1**).

Table 4-1. 5-HTP content of dietary supplements

Supplement number*	declared amount of 5-HTP per capsule (mg)	measured amount of 5-HTP per capsule by HPLC (mg)	Measured amount/declared amount (%)
1	200	195.32 ± 2.31	97.66% ± 6.30%
2	50	52.85 ± 4.18	105.7% ± 7.91%
3	100	99.39 ± 4.36	99.39% ± 4.39%
4	200	197.65 ± 0.81	98.825% ± 0.41%
5	100	112.07 ± 1.18	112.07% ± 1.05%
6	50	48.16 ± 5.50	96.32% ± 11.42%
7	50	51.39 ± 0.86	102.78% ± 1.67%
8	100	100.27 ± 1.18	100.27% ± 1.18%
9	50	43.77 ± 2.24	87.54% ± 5.12%
10	100	101.82 ± 8.46	101.82% ± 8.31%
11	100	86.65 ± 2.61	86.65% ± 3.01%
12	100	96.58 ± 2.21	96.58% ± 2.29%
13	50	45.45 ± 2.97	90.9% ± 6.53%
14	100	97.36 ± 2.57	97.36% ± 2.64%
15	100	99.66 ± 10.89	97.71% ± 10.93%
16	50	49.41 ± 1.27	98.82% ± 2.57%
17	100	92.48 ± 3.92	92.48% ± 4.24%
18	50	56.98 ± 2.39	113.96% ± 4.19%

*Supplement number refers to a single supplement bottle obtained from a unique commercial source. This was done to protect the identities of commercial vendors. A list of vendors can be requested by reaching out to us through our website: http://newuseag.rutgers.edu/



Graph 4-1. Content of 5-HTP of each commercial supplement. Bars to the left (blue) indicate declared 5-HTP content from the product label while bars to the right (red) indicate actual measured amount

4.4 Conclusions

The objective of this study was to verify the declared amount of 5-HTP on multiple commercial supplement labels. The actual amount of 5-HTP in each of the 18 supplements investigated were within 85% to 115% of their declared amount and thus all are in accordance with USP guidelines. This study did not screen for non-natural (synthetic) 5-HTP because it is highly unlikely to be incorporated into any supplements. Synthesizing 5-HTP is very costly and can only be accessed by a large number of steps some of which have poor yields. This leaves little incentive to acquire this amino acid by synthetic method when it is available in large amounts at a lower cost from griffonia seeds.

Many 5-HTP suppliers claim to be “peak X” free. However, it is debatable whether or not this impurity even exists and if it does, the evidence implicating this minuscule contaminant as the cause of any illness is weak (Das et al., 2004). For this reason, “peak X’ was not specifically searched for in any of the commercial supplements studied. It has been over 20 years since 5-HTP was last suspected to be involved with a clinical illness which should speak to the safety of this dietary supplement.

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Chapter 5. Antioxidant Capacity, Polyphenol, and Protein Content of *Griffonia simplicifolia* Seeds and Leaves

5.1 Introduction

Polyphenols are a diverse class of compounds that include simple and complex naturally occurring derivatives of phenol. Some examples of polyphenols include the anthocyanidins, flavonols, flavones, the phenolic acids, stilbenes, procyanidins, catechols, lignans, lignins, tannins, and catechins (Blainski et al., 2013). These compounds are generally water soluble, reactive toward oxidation, and there presence is ubiquitous throughout the plant kingdom. These compounds serve a variety of purposes within the plant such as protection from ultraviolet radiation, attack from microbial invaders (Blainski et al., 2013) and insects (Rimando and Duke, 2006) as well as acting as attractants for pollinators (Joule and Mills, 2010). Epidemiological evidence and scientific studies have suggested that diets rich in polyphenols can protect against inflammation (Mukhtar and Ahmad, 2000), cancer, and cardiovascular disease (Manach et al., 2005) and the protective effects of polyphenols against these conditions have been largely attributed to their antioxidant properties (Rice-Evans et al., 1996).

Free radicals, molecules with an unpaired electron in their valence, are highly reactive. Some examples of molecules that readily form into free radicals include diatomic oxygen, hydroxide, and peroxides, nitric oxide, nitrogen dioxide, and halides. These compounds are present in the environment and can enter the body through natural means or they can be formed in the body as a byproduct of normal metabolic or chemical processes. High frequency radiation from the sun such as gamma rays can cleave water molecules in the body to form the hydroxyl radical ($\cdot OH$) and ultraviolet light can cause O_2 to form peroxide (H_2O_2) which can split to form $2(\cdot OH)$ (Halliwell, 1996). Additionally, certain electrophilic ions of copper and iron found in the body can also catalyze the formation of H_2O_2 from superoxide, which can subsequently break down into two hydroxide radicals (Halliwell, 1996). To go to a lower state of energy and thus become more stable, a free radical will abstract an electron from a nearby molecule. When free radicals abstract an electron from lipids or lipoproteins, lipid peroxidation can result which contributes to

hardening or the arteries and cardiovascular disease (Halliwell, 1996; Bagchi et al., 2000). When free radicals are formed near DNA, they attack the deoxyribose sugars and the purine and pyrimidine bases. If this occurs in certain parts of the DNA molecule, mutations can result that ultimately lead to cancer (Halliwell, 1996; Bagchi et al., 2000). Polyphenols protect these important biomolecules by readily neutralizing free radicals without themselves becoming dangerous free radicals. When an electron is abstracted from an alkoxide substituent of a polyphenol by a free radical, the adjacent electron rich π-system stabilizes the electronic vacancy by delocalizing unpaired electron. This results in a polyphenol radical that is relatively stable and thus much less reactive than free radicals towards these important biomolecules. Results from *in vitro* studies showed that polyphenols are stronger antioxidants than tocopherols (Blokhina et al., 2003) although it should be noted that strong *in vitro* activity is not necessarily a strong predictor of *in vivo* efficacy. That is because polyphenols are a structurally diverse class of compounds and as such, there are strong differences in absorbability and pharmacokinetics (bioavailability) among them. For example, studies have found that anthocyanidins, the antioxidant compounds that give wine and grape juice their color, have low bioavailability while the bioavailability of flavonols appears to differ from person to person possibly due to genotypic differences in intestinal enzymes and transporters (Manach et al., 2005). Bioavailability of the catechins which are abundant in tea, appears to vary greatly with increased galloyl substitutions reducing the absorption of this class of compounds. To date, epigallocatechin gallate is the polyphenol found in the highest bioavailability with plasma concentrations ranging from 77-90% (Manach et al., 2005). Tannic acid is a unique member of the polyphenol class because in addition to acting as an antioxidant, this compound can also suppress formation of hydroxide radicals by chelating to and sequestering Fe(II) ions which catalyze the formation of these dangerous radicals from H_2O_2 (Lopes et al., 1999).

While some phenolics such as flavonoids and cinnamates can be easily detected and quantified by LC/MS, and the structural complexity of many others phenolics such as tannins and lignans make their quantitation by LC/MS timely and expensive. As such in industry, polyphenols are usually estimated using the Folin-Ciocalteu assay instead of LC/MS. In this assay, polyphenols are reacted with a mixture of phosphomolybdate, phosphotungstate and sodium carbonate to produce a blue colored solution. This color change is likely the result of a reductive process between Mo(VI) and the polyphenols to produce Mo(V) (Everette et al., 2010). The absorbance of this blue solution is measured at 765 nm and ideally, this value is proportional to the polyphenol concentration of the solution. A major disadvantage to the Folin-Ciocalteu assay is that it is susceptible to interference as the Folin-Ciocalteu reagent is also reactive toward amino acids, tocopherols, sugars, phenolic amines, ascorbic acid and other organic acids, Fe(II) (Prior et al., 2005), thiol derivatives, guanine, and proteins (Everette et al., 2010), and thus polyphenol content is often over estimated. Since the introduction of this assay, several researchers have proposed modifications to the Folin-Ciocalteu protocol to make it more selective toward polyphenols such as removing interfering reactants by solid phase extraction (SPE) or by oxidation of interfering chemical species with H_2O_2. While these methods have yielded accurate results, these extra steps add to the cost and time required thus these modified protocols have not been adopted by most researchers (Sanchez-Rangel et al., 2013).

A major deficiency of the Folin-Ciocalteu assay is that the scope of this assay is limited to water soluble antioxidants which are stable at pH of 10 (Huang et al., 2005). As such other assays measuring the antioxidant activity of plant extracts have also been developed. The ABTS assay also known as the trolox equivalence antioxidant capacity assay (TEAC) was developed in order to account for a wider range of water and fat soluble antioxidants present in an extract such as tocopherols, tocotrienols, and carotenoids. The ABTS assay uses a radical formed by combining 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) with potassium persulfate and this radical

has an intense blue color. This blue colored radical is then combined with an aliquot of plant extract. The ABTS radical is then neutralized by the antioxidants in the plant extract causing the solution to lose some its color and the new shade of the solution is proportional to the antioxidant content. The solution's absorbance is then measuring using a spectrophotometer and the antioxidant capacity is then be calculated using a calibration curve based on a water soluble form of vitamin E known as trolox with results expressed as trolox equivalence. The Trolox Equivalence Assay was designed to account for a broader range of antioxidants than the Folin-Ciocalteu assay yet, it too has similar flaws. Like polyphenols, there is a wide range of structural variability among antioxidants and with this structural diversity comes differences in reaction kinetics between the antioxidant and ABTS radical (Schaich and Tian, 2015). This means that the reaction between the ABTS radical and antioxidants may not be entirely complete at time of measurement and this measurement may not reflect the potency of these compounds in the body (Karadag et al., 2009).

The Oxygen Radical Absorbance Capacity (ORAC) assay is another widely popular method of quantifying the antioxidant capacities of botanical extracts. In this assay, the extract's antioxidant capacity is correlated with its ability to protect a fluorescent molecule from degradation (Schaich and Tian, 2015). An azide compound such as 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) (Balboa et al., 2013) is decomposed into one molar equivalent of diatomic nitrogen and two molar equivalents of the AAPH radical (Schaich and Tian, 2015). These AAPH radicals instantly combine with O_2 to form two peroxyl radicals which then react with a fluorescent molecule such as fluorescein. Upon quenching the peroxyl radicals either by hydrogen atom transfer or radical addition, the fluorescent molecule loses its fluorescent properties. When a plant extract is added to this mixture, the antioxidants protect the fluorescent molecule from degradation. (Schaich and Tian, 2015). The degradation of fluorescein is monitored over time and the antioxidant capacity of the plant extract is then correlated to this loss

of fluorescence (Schaich and Tian, 2015). Like the ABTS assay, the ORAC assay can be adjusted to detect a variety of polar and non-polar antioxidants present in an extract (Schaich and Tian, 2015). In addition, the ORAC assay may also be a better model of an extract's *in vivo* antioxidant efficacy as the peroxyl radical is one the most common endogenous species (Schaich and Tian, 2015). The DPPH and ABTS assays on the other hand, utilize unnatural radicals that are quenched by a different mechanism (electron transfer) and thus have different reaction rates and kinetics (Schaich and Tian, 2015). Meticulous control of temperature, oxygen content, reagent, and samples concentrations are critical parameters for accurate and reproducible results which is regarded as a disadvantages to this assay (Schaich and Tian, 2015).

Proteins from plant sources are important for a healthy diet because they can supply the body with nine vital amino acids that the body cannot self-produce. The body catabolizes dietary proteins to amino acids and then uses these amino acids to build its own proteins. If intake of these amino acids is insufficient, the body will resort to digesting its own muscles and proteins in order to compensate for the deficit. Proteins also play an important role in keeping iron and copper ions sequestered. As already mentioned, an encounter between metal ions and singlet oxygen can result in the formation peroxide that can decay into cancer causing hydroxide radicals (Halliwell, 1996). As is the case with polyphenols, protein quantitation by LC/MS is very time consuming and costly due to the complex and structurally diverse nature of these compounds. A more economical alternative to quantitating protein content of plant extracts is the Bradford protocol. In this protocol, proteins are extracted and combined with the Bradford reagent which is a dye known as Coomassie Brilliant Blue. The proteins bind to the dye causing the color of the solution to turn blue. The absorbance of the blue colored solution is then measured at 595 nm. Unlike the Folin-Ciocalteu and ABTS reagents, the Bradford reagent is not susceptible to interference from other chemical species with the exception of detergents, sugars, and polymers

(Silverio et al., 2012). A disadvantage to using this procedure is that the Bradford reagent is only linear in a narrow range of concentrations (Zor and Selinger, 1996).

The purpose of this experimental study was to determine the total polyphenol content, antioxidant capacity, and protein content of griffonia leaves and seed using the Folin-Ciocalteu, ABTS, and Bradford assays, respectively. Tryptophan is a known interferent of the Folin-Ciocalteu reagent (Sanchez-Rangel et al., 2013) therefore it is likely that this assay will yield inflated results when applied to griffonia seeds because of their high 5-HTP content. For this reason, griffonia seeds were excluded from the Folin-Ciocalteu assay in this study. The legume family encompasses many species that are known for their high protein content. This is because many species in this family foster a type of bacteria in their roots that is able to "fix" atmospheric nitrogen to be integrated into proteins (Charles and Trainer, 2006). Since griffonia is a member of this family, it would be interesting to determine how the protein content of this species compares to other legumes. This information may also be useful in understanding the phylogenetic placement of this species in relation to other species within the legume family. Although griffonia seeds and leaves are not widely consumed by humans, a relatively high phenolic, antioxidant, and protein content may attract interest in using this plant as an alternative source of these nutrients in Sub-Saharan Africa where some lack access to protein. Further studies investigating the content of amino acids available from these proteins should be conducted because not all proteins provide a complete array of amino acids necessary for a healthy diet. Examples of this includes corn proteins which lacks tryptophan and lysine (Friedman and Brandon, 2001) and soybeans which lack the vital amino acid methionine (Friedman and Brandon, 2001).

5.2 Experimental

5.2.1 Materials

Griffonia seeds and leaves collected by ASNAPP-Ghana team from various regions of Ghana and Liberia (**Table 5-1 and Figure 5-1**). Seeds were manually harvested from fallen seed pods and collection from the forest floor, seeds removed, cleaned and air/sun dried off the ground prior to shipment. Leaves were also simply dried prior to shipment. Polystyrene 96-well plate from CytoOne (Ocala, Florida), Folin-Ciocalteu reagent from MP Biomedical (Solon, OH), sodium carbonate, gallic acid, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulfate, trolox, bradford reagent, Tris (tris(hydroxymethyl)aminomethane HCl and bovine serum albumin from Sigma Aldrich (St. Louis, MO), HPLC grade water, methanol, and ethanol from Fisher Scientific (Fair Lawn, NJ)

Table 5-1. Origins of griffonia seeds and leaves from Ghana and Liberia and sample sizes

Liberia

Collection site	Seeds sample size (grams)	Leaves sample size (grams)
Bargblor Town, Grand Gedeh County	44.8	24.0
Gaye Town, Grand Gedeh County	138.7	1.8
Tapita, Lower Nimba County	112.6	4.1
Unspecified Site 1, Nimba County	~1300	-
Unspecified Site 2, Nimba County	~1300	-
Upper Nimba County	78.8	6.7

Ghana

Collection site	Seeds sample size (grams)	Leaves sample size (grams)
Ashanti Region	49.2	69.9
Brong Ahafo Region	54.8	86.5
Central Region	84.8	35.6
Eastern Region	57.9	48.8

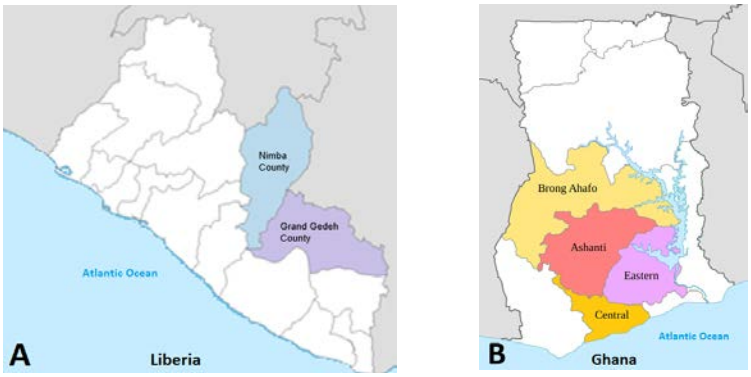


Figure 5-1. Counties and regions in the West African countries of Liberia and Ghana where griffonia seeds and leaves were collected. (A). Counties in Liberia where griffonia seeds and leaves were collected. (B). Regions in Ghana where griffonia seeds were collected.

5.2.2 Equipment

Synergy HT spectrophotometer from BioTek (Winooski, VT)

5.2.3 Folin-Ciocalteu Polyphenol Assay

A stock solution of Folin-Ciocalteu reagent was prepared by diluting 5 mL of preformulated reagent obtained from MP Biomedical (Solon, OH) with 50 mL of HPLC grade water. 15% sodium carbonate (w/v) was prepared by dissolving 15 grams of sodium carbonate in 100 mL of HPLC grade water. Approximately 80 mg of ground leaf was extracted with 5 mL of 60% methanol/water (v/v) and mixture was allowed sonicate for 5 minutes. After sonication, mixture was centrifuged and 40 µL of this solution was combined with 900 µL of diluted Folin-Ciocalteu reagent and allowed to sit at room temperature for 5 minutes. A 400 µL aliquot of 15% sodium carbonate was then added and allowed to react for 45 minutes. After time elapsed, mixture was centrifuged for five minutes and 200 µL of this solution was transferred to a polystyrene 96-well plate and absorbance was measured at 752 nm. Each sample was analyzed in triplicate in order to insure instrument reliability.

5.2.4 ABTS Assay

ABTS radical stock solution was prepared by dissolving 38.5 mg of ABTS and 8.0mg of sodium persulfate in 10 mL of HPLC grade water. Mixture was then left to equilibrate in the dark for 19 hours. After equilibration time elapsed, 3 mL of this solution was diluted to an absorbance of 0.73 with approximately 100 mL of denatured ethanol. Approximately 100 mg of ground leaf material was extracted in 2 mL of ethanol and 50 mg of seed material was extracted with 25 mL of denatured ethanol. Mixtures were allowed to sonicate for 5 minutes and then an aliquot of each stock solution was placed in an Eppendorf tube and centrifuged for 5 minutes. After centrifugation, 10 µL of solution was mixed with 990 µL of ABTS stock solution and mixture was left to react at room temperature for 30 minutes. After reaction time elapsed, 200 µL of each solution was placed in a polystyrene 96-well plate and UV absorbance was measured at 734 nm. Antioxidant capacities was calculated as a percent inhibition using the equation % inhibition = $[(\text{Absorbance}_{\text{Radical}} - \text{Absorbance}_{\text{sample}})/\text{Absorbance}_{\text{Radical}}] \times 100$. Each sample was analyzed in triplicate in order to insure instrument reliability.

5.2.5 Bradford Protein Assay

Approximately 50 mg of seed material was extracted with 50 mM Tris (tris(hydroxymethyl)aminomethane HCl (bris-tris glycerol HCl). Approximately 125 mg of Ghanaian leaf material was extracted with 10 mL of 50 mM bris-tris glycerol HCl. Due to limited amounts of available sample material, approximately 30 mg of Liberian leaf material was extracted with 2.5 mL of Tris. Mixtures were allowed to sonicate for 5 minutes and centrifuged for 5 minutes. After centrifugation, 600 µL was combined with 3mL of Bradford reagent and mixture was allowed to react for 20 minutes. After reaction time elapsed, UV absorbance was measured at 595 nm. Each sample was analyzed in triplicate in order to insure instrument reliability.

5.2.6 Calibration Curves

5.2.6.1 Polyphenol Assay

Calibration curve used to quantitate polyphenols in griffonia leaves was constructed by dissolving 7.8 mg of gallic acid in 25 mL of 60% methanol and mixture was sonicated for 5 minutes. A one milliliter aliquot of this stock solution was used to make dilutions of 1/2x, 1/4x, 1/8x, 1/16x, 1/32x, and 1/64x, to cover a range of 4.875 µg/mL to 312 µg/mL. The R^2 value was 0.9998, showing excellent linearity (**Figure 5-2**).

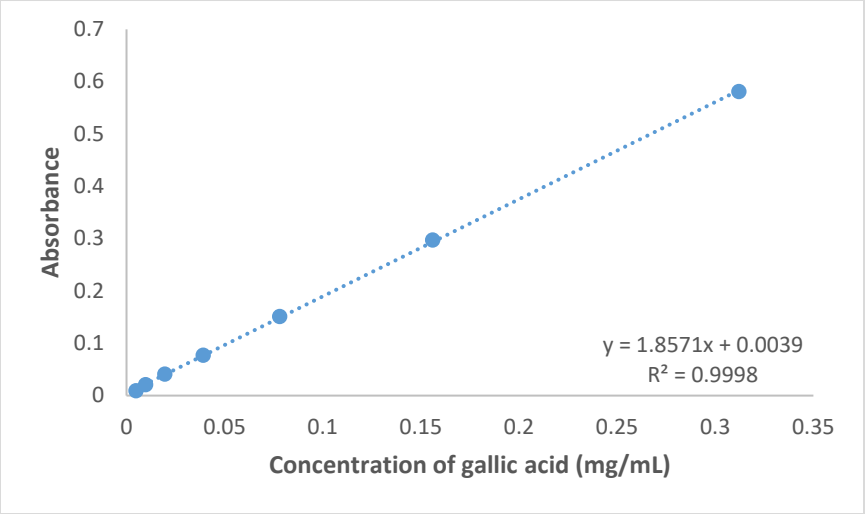


Figure 5-2. Calibration curve used to quantitate total polyphenols of all griffonia leaf populations

5.2.6.2 ABTS Assay

To quantitate the antioxidant capacities of griffonia leaves, 17.6 mg of trolox was dissolved in 25 mL of denatured ethanol and mixture was sonicated for 5 minutes. A one milliliter aliquot of this stock solution was used to make dilutions of 1/2x, 1/4x, 1/8x, and 1/16x, to cover ranges of 44 µg/mL to 704 µg/mL. . The R^2 value was 0.9982, showing excellent linearity (**Figure 5-3**).

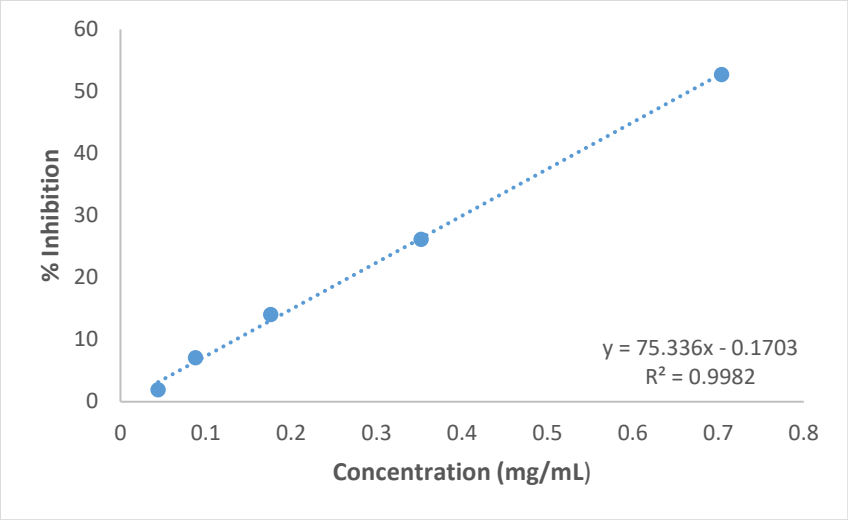


Figure 5-3. Calibration curve used to quantitate antioxidant capacities of all griffonia leaf populations

To quantitate the antioxidant capacities of griffonia seeds, 17.2 mg of Trolox was dissolved in 25 mL of denatured ethanol and was sonicated for 5 minutes. A one milliliter aliquot of this stock solution was used to make dilutions of 1/2x, 1/4x, 1/8x, 1/16x, and 1/32x to cover ranges of 21.5 µg/mL to 688 µg/mL. The R^2 value was 0.9984, showing excellent linearity (**Figure 5-4**).

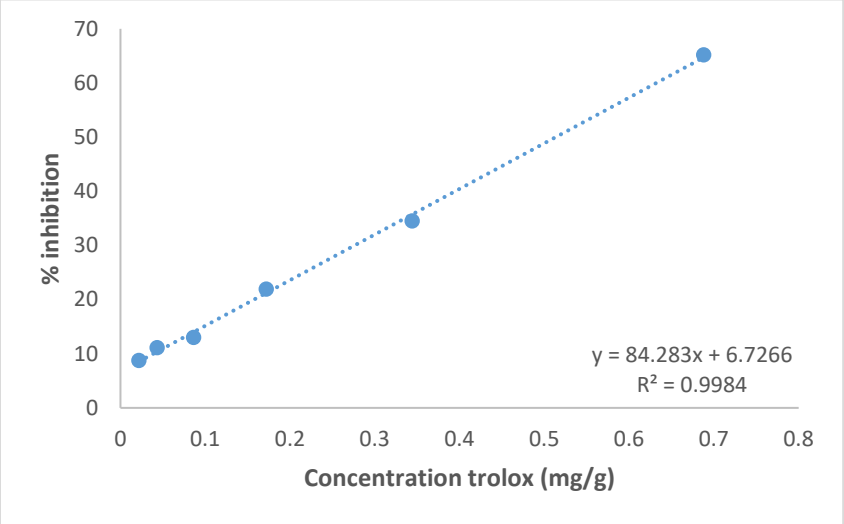


Figure 5-4. Calibration curve used to quantitate antioxidant capacities of all griffonia seed populations

5.2.6.3 Bradford Protein Assay

Calibration curve used to quantitate protein content of griffonia leaves was created by dissolving 27.8 mg of bovine serum albumin in 10 mL of 50 mM bris-tris glycerol HCl and mixture was sonicated for 5 minutes. A one milliliter aliquot of this stock solution was used to make dilutions of 1/8x, 1/16x, 1/32x, 1/64x, 1/128x, and 1/256x to cover ranges of 10.9 µg/mL to 347.5 µg/mL. The R^2 value was 0.9889, showing excellent linearity (**Figure 5-5**).

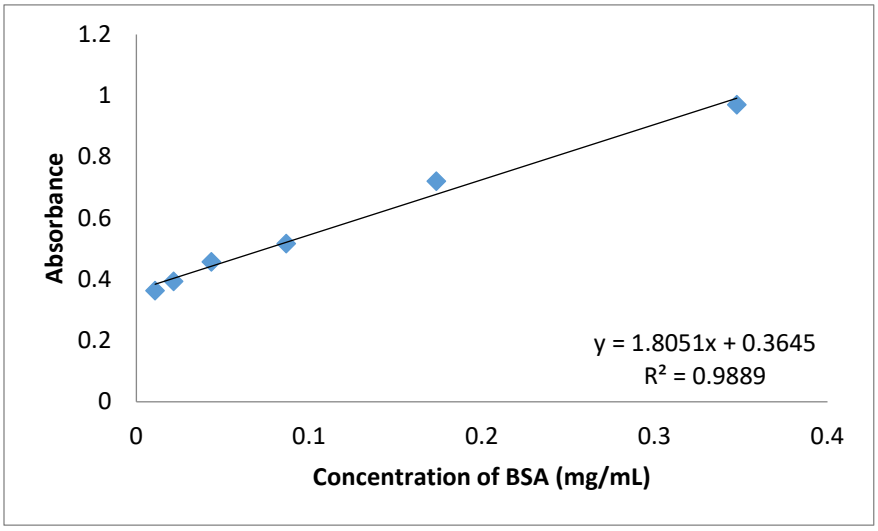


Figure 5-5. Calibration curve used to quantitate protein content of all griffonia leaf populations

Calibration curve used to quantitate protein content of griffonia seeds was created by dissolving 30.8 mg of bovine serum albumin in 10mL of 50mM bris-tris glycerol HCl and was sonicated for 5 minutes. A one milliliter aliquot of this stock solution was used to make dilutions of 1/8x, 1/16x, 1/32x, 1/64x, 1/128x, 1/256x, and 1/512x to cover ranges of 6.0 µg/mL to 385 µg/mL. The R^2 value was 0.9928, showing excellent linearity (**Figure 5-6**).

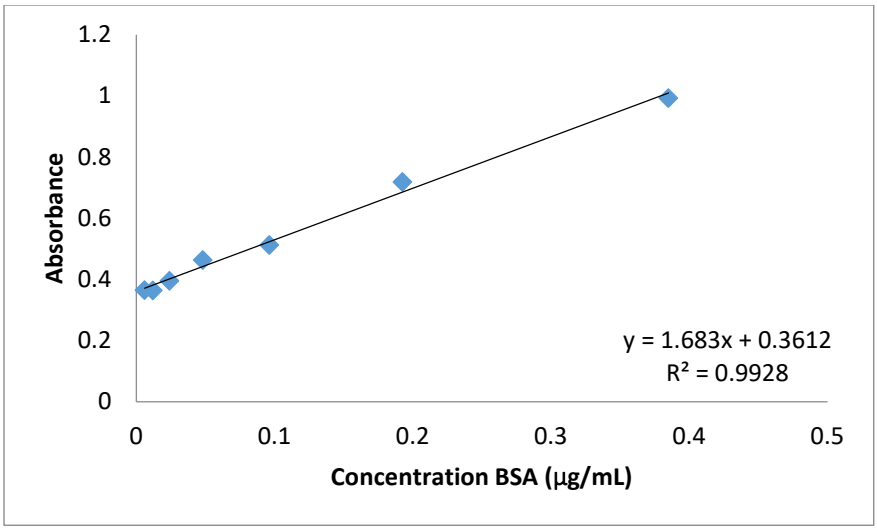


Figure 5-6. Calibration curve used to quantitate protein content of all griffonia seed populations

5.3 Results and Discussion

Polyphenol content of griffonia leaves in gallic acid equivalence (GAE) as determined by the Folin-Ciocalteu assay, ranged from 7.83 ± 0.49 GAE/gram to 39.86 ± 0.60 GAE/gram with all populations averaging 21.16 ± 0.48 GAE/gram (**Table 5-2 and Graph 5-1**). Polyphenol content of Ghanaian leaf populations ranged from 9.78 ± 0.21 GAE/gram to 29.83 ± 0.69 GAE/gram and averaged 17.10 ± 0.21 GAE/gram with specimens from Brong Ahafo being significantly greater in polyphenol content than the other three Ghanaian populations. Polyphenol content of Liberian populations ranged from 7.83 ± 0.49 GAE/gram to 39.86 ± 0.60 GAE/gram and averaged 25.22 ± 0.53 GAE/gram with leaves from both Nimba County populations being significantly greater in polyphenol content than populations from Grand Gedeh County. The polyphenol content of griffonia leaves compares to other closely related species as follows: in a study by Chew et al. it was determined using the Folin-Ciocalteu assay that the polyphenol content of the 75% methanolic extract of *Bauhinia kockiana* leaves was 4.44 GAE/gram (Chew et al., 2009). In another study by Mazimba et al. (2011), the butanol extract of the husks of another closely related legume *Tylosema esculentum* (marama beans), was determined to contain 235.11 GAE/gram.

Few reports exist regarding the polyphenol content of leaves in this area of the plant kingdom therefore it is not possible to make more detailed comparisons between griffonia and its closest relatives at this time. The polyphenol content of green tea leaves was determined in a 2007 study by Chan et al. to be 75.86 ± 19.95 GAE/gram indicating that griffonia leaves contain approximately one quarter the amount of polyphenols as green tea.

The antioxidant capacities of all griffonia leaf populations in trolox equivalence (TEAC) as determined by the trolox equivalence antioxidant capacity assay ranged from 5.14 ± 0.19 TEAC/gram to 22.15 ± 0.64 TEAC/gram and averaged 12.13 ± 0.45 TEAC/gram (**Table 5-3 and Graph 5-2**). Antioxidant capacities of Ghanaian leaf populations ranged from 6.80 ± 0.38 TEAC/gram to 19.37 ± 0.54 TEAC/gram and averaged 13.09 ± 0.51 TEAC/gram with leaf populations from Ashanti and Brong Ahafo regions being significantly greater in antioxidant capacities than populations from Central and Eastern Regions. The antioxidant capacities of Liberian leaf populations ranged from 5.14 ± 0.19 TEAC/gram to 22.15 ± 0.64 TEAC/gram and averaged 11.54 ± 0.38 TEAC/gram. Leaf population from Tapita, Nimba County was significantly higher in antioxidant capacity than the other three Liberian leaf populations. Overall the antioxidant capacities of Ghanaian leaf specimens were slightly higher than Liberian leaf specimens. The antioxidant capacity of griffonia leaves is comparable to that of the 40% ethanolic extract of the leaves of the closely related species *Bauhinia microstachya* which contain 32.77 TEAC/gram (Da Silva et al., 2007). TEAC of griffonia leaves were significantly lower than that of *Bauhinia tomentosa* seeds (Krishnaswamy et al., 2013) and Korean grown green tea leaves (Ku et al., 2010). The antioxidant capacities of all griffonia seed specimens ranged from 163.65 ± 15.46 TEAC/gram to 257.35 ± 11.43 TEAC/gram and averaged 216.50 ± 13.88 TEAC/gram (**Table 5-4 and Graph 5-3**). Ghanaian seed populations were similar in antioxidant capacities ranging from 233.13 ± 6.64 TEAC/gram to 257.35 ± 11.43 TEAC/gram and averaged 247.06 ± 18.61 TEAC/gram. Liberian seed populations ranged from 163.65 ± 15.46 TEAC/gram to 226.47

± 7.1 TEAC/gram and averaged 196.13 ± 9.51 TEAC/gram with populations from Nimba County being slightly greater in antioxidant capacity than populations from Grand Gedeh County. Overall, Ghanaian seed populations had a higher antioxidant capacity than Liberian seed populations. Krishnaswamy et al. reported that one gram of the 50% methanolic extract of *Bauhinia tomentosa* seeds has the antioxidant power of 658.1 ± 484.4 µmol of trolox which equates to 164.72 ± 121.24 mg/g of trolox (Krishnaswamy et al., 2013) which is slightly more potent than griffonia seed extracts. The antioxidant capacity of ethanolic extracts of griffonia seeds is comparable to that of some green tea leaves. In 2010, Ku et al. concluded that the TEAC of the MeOH/H_2O/$CHCl_3$ (2.5/1/1) extract of Korean grown green tea leaves was 785 ± 14 mM/gram which equates to 196.48 ± 3.50 TEAC/gram of extract therefore the antioxidant capacities of griffonia leaves from all populations studied are significantly lower than that of tea leaves.

Protein content of all griffonia leaf populations in bovine albumin serum equivalence (BSAE) as determined by the Bradford assay ranged from 2.12 ± 0.36 BSAE/gram to 10.73 ± 0.65 BSAE/gram and averaged 6.89 ± 0.47 BSAE/gram (**Table 5-5 and Graph 5-4**). Protein content among Ghanaian leaf populations ranged from 2.12 ± 0.36 BSAE/gram to 6.75 ± 0.14 BSAE/gram and averaged 5.46 ± 0.47 BSAE/gram. Leaves from Ashanti Region were significantly lower in protein content than the other three Ghanaian populations. Protein content of Liberian leaf populations ranged from 7.03 ± 0.31 BSAE/gram to 10.73 ± 0.65 BSAE/gram and averaged 8.33 ± 0.47 BSAE/gram. Leaves from Tapita, Nimba County were significantly greater in protein content than the Liberian leaf populations. Liberian leaf populations were higher in protein content than populations from Ghana. The protein content of all griffonia leaves are similar to that of corn kernels which ranges from 7.2 to 8.4 BSA/gram (Boyes et al., 1997). Protein content of all griffonia seed populations ranged from 28.36 ± 0.48 BSAE/gram to 36.51 ±

0.53 BSAE/gram and averaged 33.58 ± 1.52 BSAE/gram (**Table 5-6 and Table 5-5**). Protein content of Ghanaian seed populations ranged from 28.36 ± 0.48 BSAE/gram to 36.51 ± 0.53 BSAE/gram and averaged 33.74 ± 1.46 BSAE/gram. Protein content of Liberian seed populations ranged from 31.25 ± 0.61 BSAE/gram to 36.26 ± 0.84 BSAE/gram and averaged 33.48 ± 1.56 BSAE/gram. No significant regional differences in protein content among Liberian and Ghanaian seeds were observed. On average, Ghanaian and Liberian griffonia seeds were nearly identical in protein content. It is difficult to make comparisons between protein content of griffonia seeds and leaves and other legumes as few reports using the Bradford protocol to quantitate proteins exist. The protein content of the closely related marama beans (*Tylosema esculentum*) has been found to be comparable to that of soybeans (Holse et al., 2010) although to date no study has been conducted that allows for a direct comparison between protein contents of griffonia, soybeans, and marama beans. Future studies could focus on determining their nutritional quality of these proteins as proteins from legumes tend to be lower in sulfur containing amino acids (Ortega-Nieblas, 1994; Friedman and Brandon, 2001). Molecules that hinder the digestibility of these proteins such as lectins (Friedman and Brandon, 2001) are also present and their effects should be investigated if griffonia seeds are ever to be consumed as a source of protein.

Differences in polyphenol content, antioxidant capacities, and protein contents could be due to a number of factors that include differences in genetics and maturity, access to sunlight, water, and soil nutrients as well as post-harvesting handling practices and the presence of biotic and abiotic stressors. If interest in using griffonia seeds and leaves as a non-traditional source of these nutrients, the effect of these factors should be studied in order to maximize accumulation of these secondary metabolites.

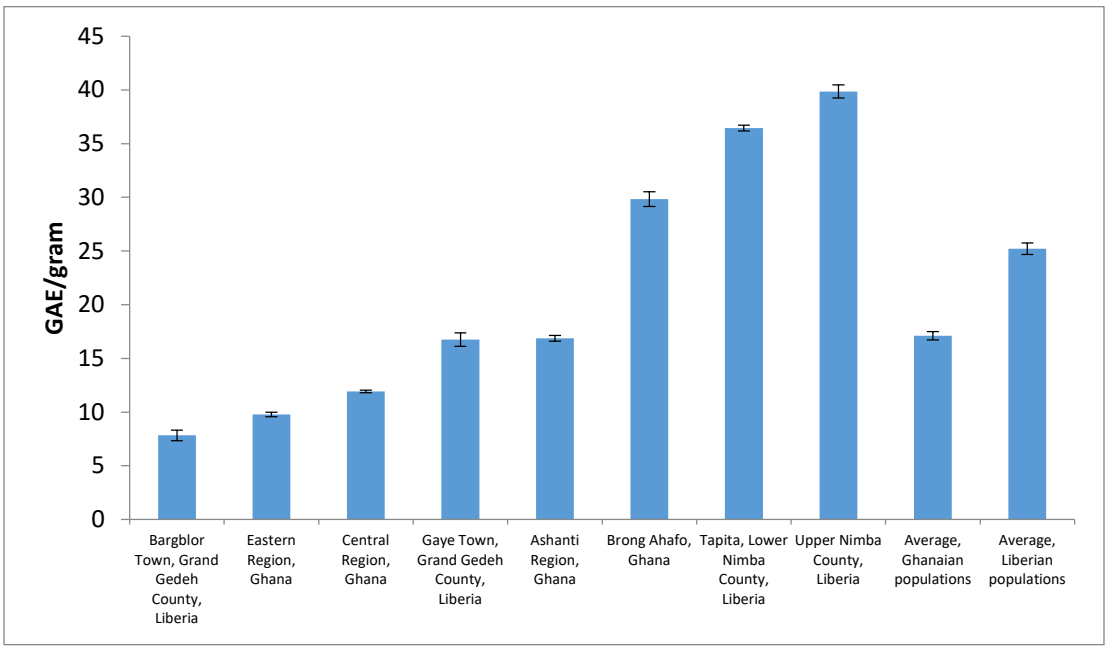
Table 5-2. Total polyphenol content of griffonia leaf populations from Liberia and Ghana and comparison with other species.

Ghana	
Collection site	**GAE/gram**
Ashanti Region	16.86 ± 0.27
Brong Ahafo Region	29.83 ± 0.69
Central Region	11.92 ± 0.12
Eastern Region	9.78 ± 0.21
Average, all Ghanaian griffonia leaf populations	**17.10 ± 0.39**

Liberia	
Collection site	**GAE/gram**
Bargblor Town, Grand Gedeh County	7.83 ± 0.49
Gayetown, Grand Gedeh County	16.76 ± 0.63
Tapita, Lower Nimba County	36.44 ± 0.27
Upper Nimba County	39.86 ± 0.60
Average, all Liberian griffonia leaf populations	**25.22 ± 0.53**

Polyphenol content griffonia leaves compared to other species	**GAE/gram**
Average, all griffonia leaf populations	**21.16 ± 0.48**
Bauhinia kockiana leaves (Chew et al., 2009)	4.44
Green tea leaves (Chan et al., 2007)	75.86 ± 19.9
Raisins (Williamson and Carughi, 2010)	10.7 ± 1.6
Tylosema esculentum husks (marama beans) ((Mazimba et al., 2011)	235.11

GAE = Gallic acid equivalence



Graph 5-1. Total polyphenol content of griffonia leaf populations from Liberia and Ghana

Table 5-3. Antioxidant capacities of griffonia leaf populations from Liberia and Ghana and comparison with other species.

Ghana

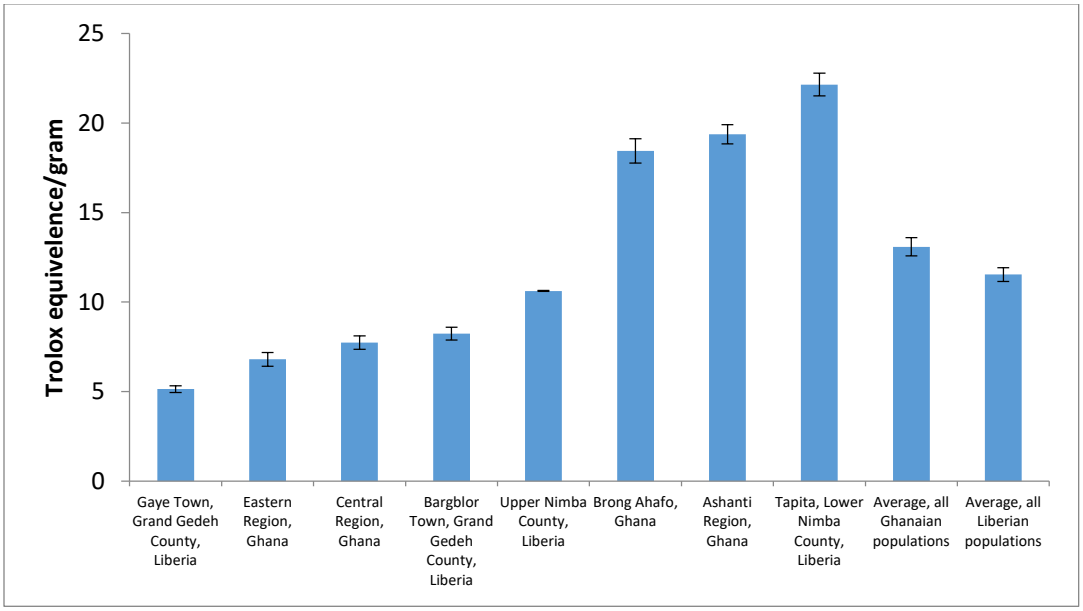
Collection site	**TEAC/gram**
Ashanti Region	19.38 ± 0.54
Brong Ahafo Region	18.45 ± 0.68
Central Region	7.74 ± 0.38
Eastern Region	6.81 ± 0.38
Average, all Ghanaian griffonia leaf populations	**13.09 ± 0.51**

Liberia

Collection site	**TEAC/gram**
Bargblor Town, Grand Gedeh County	8.24 ± 0.36
Gayetown, Grand Gedeh County	5.14 ± 0.19
Tapita, Lower Nimba County	22.15 ± 0.64
Upper Nimba County	10.62 ± 0.03
Average, all Liberian griffonia leaf populations	**11.54 ± 0.38**

Antioxidant capacities of griffonia leaves compared to other species	**TEAC/gram**
Average, all griffonia leaf populations	**12.32 ± 0.45**
Bauhinia microstachya leaves (Da Silva et al., 2007)	32.77
Bauhinia tomentosa seeds (Krishnaswamy et al., 2013)	164.72 ± 121.24
Korean green tea leaves (Ku et al., 2010)	196.48 ± 3.50

TEAC = Trolox equivalence antioxidant capacity



Graph 5-2. Antioxidant capacity of griffonia leaf populations from Liberia and Ghana

Table 5-4. Antioxidant capacities of griffonia seed populations from Liberia and Ghana and comparison with other species.

Ghana

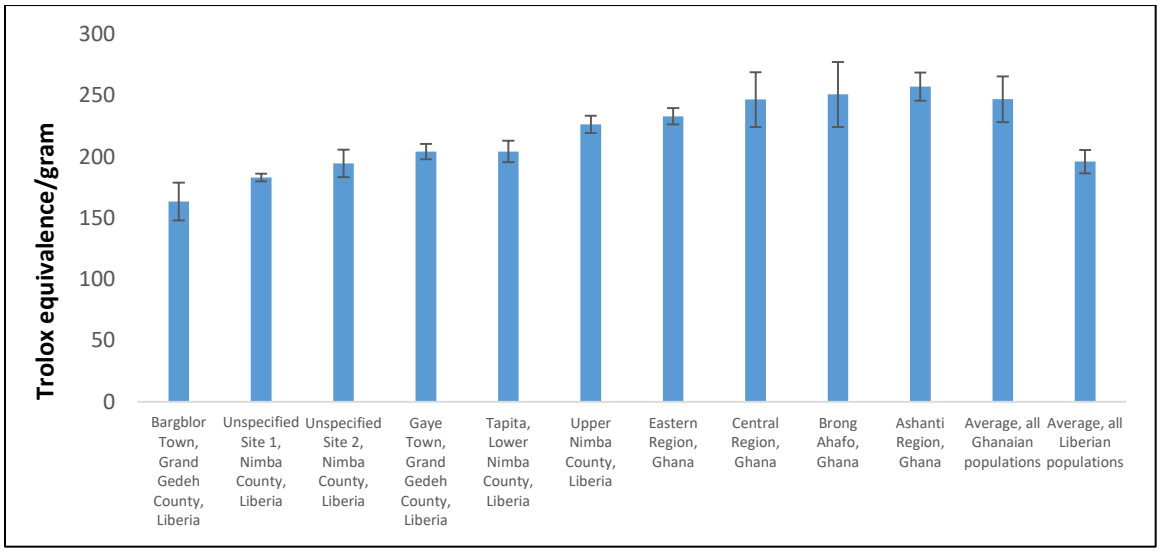
Collection site	**TEAC/gram**
Ashanti Region	257.36 ± 11.43
Brong Ahafo Region	250.98 ± 26.6
Central Region	246.81 ± 22.42
Eastern Region	233.15 ± 6.64
Average, all Ghanaian griffonia seed populations	**247.07 ± 18.61**

Liberia

Collection site	**TEAC/gram**
Bargblor Town, Grand Gedeh County	163.65 ± 15.46
Gayetown, Grand Gedeh County	204.24 ± 6.29
Tapita, Lower Nimba County	204.47 ± 8.83
Unspecified Site 1, Nimba County	183.24 ± 3.15
Unspecified Site 2, Nimba County	194.76 ± 11.23
Upper Nimba County	226.48 ± 7.1
Average, all Liberian griffonia seed populations	**196.14 ± 9.51**

Antioxidant capacities of griffonia seeds compared to other species	**TEAC/gram**
Average, all griffonia seed populations	**216.51 ± 13.88**
Bauhinia microstachya leaves (Da Silva et al., 2007)	32.77
Bauhinia tomentosa seeds (Krishnaswamy et al., 2013)	164.72 ± 121.21
Korean green tea leaves (Ku et al., 2010)	196.48±3.50

TEAC = Trolox equivalent antioxidant capacity



Graph 5-3. Antioxidant capacities of griffonia seed populations from Liberia and Ghana

Table 5-5. Total proteins of griffonia leaf populations from Liberia and Ghana and comparison with other species.

Ghana

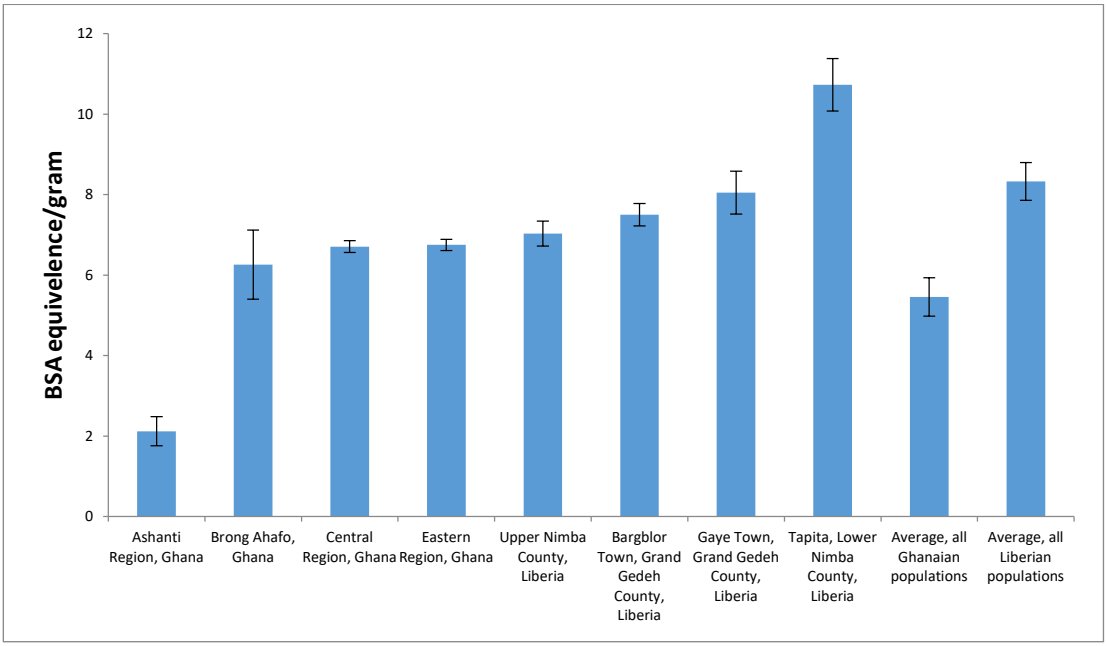
Collection site	**BSAE/gram**
Ashanti Region	2.12 ± 0.36
Brong Ahafo	6.26 ± 0.86
Central Region	6.71 ± 0.15
Eastern Region	6.75 ± 0.14
Average, all Ghanaian griffonia leaf populations	**5.46 ± 0.47**

Liberia

Collection site	**BSAE/gram**
Bargblor Town, Grand Gedeh County	7.5 ± 0.28
Gayetown, Grand Gedeh County	8.05 ± 0.53
Tapita, Lower Nimba County	10.73 ± 0.65
Upper Nimba County	7.03 ± 0.31
Average, all Liberian griffonia leaf populations	**8.33 ± 0.47**

Protein content of griffonia leaves compared to other species	**BSAE/gram**
Average, all griffonia leaf populations	**6.89 ± 0.47**
Corn kernels (Boyes et al., 1997)	7.2-8.4

BSAE = Bovine serum albumin equivalents



Graph 5-4. Total proteins in griffonia leaf populations from Liberia and Ghana

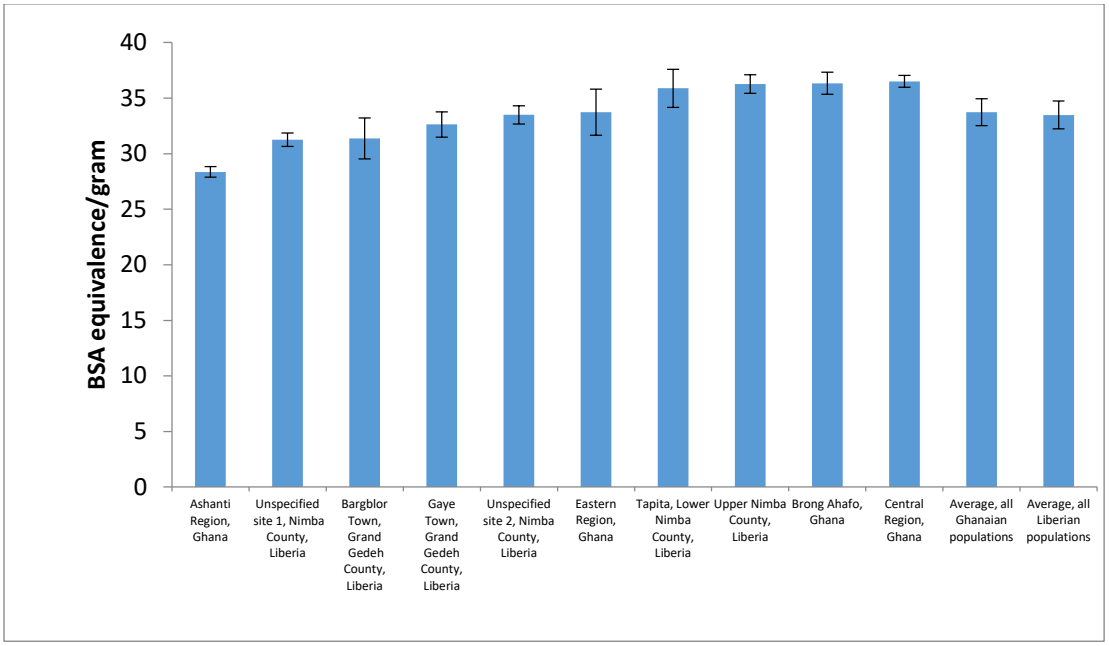
Table 5-6. Total proteins of griffonia seed populations from Liberia and Ghana and comparison with other species.

Ghana	
Collection site	**BSAE/gram**
Ashanti Region	28.36 ± 0.48
Brong Ahafo	36.34 ± 1.00
Central Region	36.51 ± 0.53
Eastern Region	33.73 ± 2.08
Average, all Ghanaian griffonia seed populations	**33.74 ± 1.46**

Liberia	
Collection site	**BSAE/gram**
Bargblor Town, Grand Gedeh County, Liberia	31.37 ± 1.85
Gayetown, Grand Gedeh County, Liberia	32.63 ± 1.14
Tapita, Lower Nimba County, Liberia	35.88 ± 1.70
Unspecified Site 1, Nimba County, Liberia	31.25 ± 0.61
Unspecified Site 2, Nimba County, Liberia	33.49 ± 0.81
Upper Nimba County, Liberia	36.26 ± 0.84
Average, all Liberian griffonia seed populations	**33.48 ± 1.56**

Protein content of griffonia seeds compared to other species	**BSAE/gram**
Average, all griffonia seed populations	**33.58 ± 1.52**
Corn kernels (Boyes et al., 1997)	7.2-8.4

BSAE = Bovine serum albumin equivalents



Graph 5-5. Total proteins in griffonia seed populations from Liberia and Ghana

5.4 Conclusions

The purpose of this study was to determine the content of total polyphenols, antioxidants and proteins in the seeds and leaves of selected griffonia populations from Liberia and Ghana. The amounts of polyphenols and antioxidants in leaves and seeds varied significantly across all populations studied. Griffonia seed populations and most griffonia leaf populations were very similar in protein content. Among Ghanaian populations, leaves from Eastern Region, Central Region, and Ashanti Region were significantly lower in polyphenol content than leaves from the Brong Ahafo Region. Among Liberian populations, both leaf populations from Grand Gedeh County were significantly lower in polyphenol content than the two populations from Nimba County. Overall, leaves of Liberian origin were significantly greater in polyphenol content than leaves from Ghanaian populations. Leaves from Tapita, Nimba County had a much greater antioxidant capacity than the leaves from the other three Liberian populations. The antioxidant capacities of leaves from Eastern and Central Regions, Ghana were significantly lower than leaves from Ashanti Region and Brong Ahafo Region. Overall, Liberian leaf populations were slightly lower in antioxidant capacities than leaf populations from Ghana. Ghanaian seed populations were similar in antioxidant capacities with Ashanti region being the greatest and Eastern region being the lowest. The antioxidant capacities among Liberian seed populations did not vary widely with populations from Nimba County being only slightly greater in antioxidant capacity than populations from Grand Gedeh County. Overall, the antioxidant capacities of Ghanaian seed populations were about 25% greater than Liberian populations. With the exception of the populations from Ashanti Region which was significantly lower in content, the amount of protein among Ghanaian griffonia leaf populations did not significantly vary. Likewise, Liberian griffonia leaf populations were similar in protein content with the exception of the population from Lower Nimba County which was significantly more concentrated in protein content. Liberian leaf populations were slightly greater in protein content overall than Ghanaian leaf populations. The protein content of griffonia seeds was similar across all Ghanaian and Liberian

populations, with the average protein content of Ghanaian populations being nearly identical to that of the Liberian populations. There are many factors that could contribute to the large variability in polyphenol, protein, and antioxidant content that include genetics, soil nutrients, access to sunlight and water, and maturity. Griffonia leaves appear to contain only about 1/3 the amount of total polyphenols as Malaysian green tea (Chan et al., 2007), and 1/16 the antioxidant capacity of Korean green tea (Ku et al., 2010). The average antioxidant capacity of griffonia seeds is similar to that of Korean green tea however it is possible that this value is inflated by the high amount of 5-HTP and proteins, which can act as an antioxidants. The protein content of griffonia leaves and seeds are slightly lower than, and five times greater than the amount provided by corn kernels (Boyes et al., 1997), respectively. Potential nutritional benefits from a crop should not necessarily be measured by quantity of nutrients as the bioavailabilities of different polyphenols and antioxidants can widely vary (Manach et al., 2005). Apigenin-C-glycosides have been detected in griffonia leaves in concentrations of approximately 9.2 mg/g (see **chapter 6**) however animal studies have indicated that C-glycosides may be poorly absorbed (Courts and Williamson, 2015; Angelino et al., 2013; Buqui et al., 2015). Therefore, conclusions regarding nutritional value of griffonia seeds and leaves should not be based solely on the quantities polyphenols, antioxidants, and amino acids afforded from griffonia leaves and seeds, but eventually upon the bioavailability of each unique nutrient.

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Chapter 6. Quantification of Flavonoids in *Griffonia simplicifolia* Leaves

6.1 Introduction

Flavonoids are a class of chromones that have an important role in the physiological well-being of plants and they offer many nutritional benefits to humans. These secondary metabolites are ubiquitous throughout the plant kingdom and their main function is believed to be to protect these organisms from insect damage, attack from microbial invaders, ultraviolet sunlight (Blainski et al., 2013; Romanelli et al., 2010; Rimando and Duke, 2006) as well as acting as attractants for pollinators (Joule and Mills, 2010; Heinrich et al., 2012). In legumes, flavonoids also serve an additional role as chemical messengers between the plant and the root dwelling *Rhizobia* bacteria that provide the plant with a usable form of atmospheric nitrogen (Broughton et al., 2000). Flavonoids contain three aromatic features that allow for these molecules absorb a wide range of ultraviolet radiation. The A ring is responsible for absorbance between 240-285 nm while the C ring produces a characteristic band between ranging from 300-550 nm depending on the substituents (DeRijke et al., 2006). Thus, it is no surprise that an increased accumulation of these protective molecules is often observed in plants under increased exposure to light (Manach et al., 2004). An increase in production of these compounds has also been observed in plants battling infection and further investigation found that flavonoids have antimicrobial properties as well (Kumar and Pandey, 2013; Abad and Bedoya et al., 2012). Over 4000 flavonoid derivatives exist and approximately 28% of all known flavonoid aglycones occur in the Fabaceae/Leguminosae family (Hegnauer and Grayer-Barkmeijer, 1993). Plants produce flavonoids from intermediates derived from the polyketide and shikimic acid pathways and can be further derived to benzopyrylium compounds known anthrocyanins (Heinrich et al., 2012). Flavonoids are believed to defend against heart disease and cancer and these health benefits are likely a result of their antioxidant properties that can protect DNA and cellular membranes from damage by free radicals (Heinrich et al., 2012). Studies have shown that flavonoids are more potent antioxidants than vitamins C and E (Rice-Evans et al., 1997). However, vitamins C and E have transporters that guarantee these molecules a wide distribution through the body (Savini et al., 2008; Rigotti,

2007) while phenolic compounds do not. Significant differences in gastrointestinal absorbability amongst the various flavonoids have been observed and it is likely that the health benefits associated with many are restricted to the GI tract (Manach et al., 2004; Manach et al., 2005).

To date, no studies have been published characterizing the flavonoids in griffonia leaves. Therefore the purpose of this study is to identify and quantitate the abundances of flavonoids in griffonia leaves from various Ghanaian and Liberian populations. As noted previously, flavonoids are potent antioxidants and many epidemiological studies have detailed various health benefits associated with diets rich in these compounds. A new interest in using griffonia leaves for nutraceutical or cosmetic purposes could arise if this crop is a significant source of these nutrients. We selected populations of griffonia from two counties in order to capture a wider genetic and environmental impact.

6.2 Experimental

6.2.1 Materials

Griffonia leaves were collected by ASNAPP-Ghana team in both Ghana and Liberia (for sample sizes and collection sites, see **Table 5-1**). HPLC grade water, acetonitrile, and methanol from Fischer Scientific (Fair Lawn, NJ). 95% formic acid from Sigma Aldrich (St Louis, MO). Chromatographic grade vitexin (**Figure 6-3**) ≥95% from Sigma Aldrich (St. Louis, MO)

6.2.2 Equipment

Qualitative identification was carried out using a Hewlett Packard Agilent 1100 series LC/MS (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, quaternary pump system, DAD detector, degasser, and MSD with electrospray ionization source (ESI) in negative mode.

Quantitation of flavonoids was conducted using a Waters 2695 separation module equipped with a Waters 2996 photo diode array detector. Separation was performed on a Phenomenex luna phenyl hexyl column (150mm x 4.6mm, 5µm 100Å).

6.2.3 HPLC Conditions

The mobile phase consisted of a gradient of solvent A (ddH_2O with 0.1% Formic acid) and solvent B (acetonitrile with 0.1% formic acid). Gradient was from 0 to 10 minutes 1% to 5 %B, and from 10 to 30 minutes 5 to 30% B. The flow rate was set at 1.0 mL/min with UV detection set at 330 nm. The injection volume was 10 µL and analysis occurred at room temperature.

6.2.4 LC/MS Conditions

LC conditions were identical to those described in detail in section **6.2.3**. A post column splitter was used to decrease flow into mass spectrometer by 3:1. Nebulizer was set at 40 L/min, drying gas at 9.00 L/min, the drying gas temperature at 350ºC, compound stability at 100%, the capillary voltage at 4.5kV, Skim 1 at -39.4 V, skim 2 at -6.0 V, capillary exit offset at -75.3 V, octopole 1 at -2.56 V, octopole delta at -2.40 V, octopole RF at 150.0 Vpp, lens 1 at 5 V, lens 2 at 60.0 V, trap drive level at 41.0, and scan range was 100 m/z to 800 m/z.

6.2.5 Sample Preparation

For both qualitative identification and quantitative analysis, approximately 100 mg of dried leaf material was ground and extracted with 10 mL of 50% methanol in water and sonicated for 15 minutes. After sonication, and aliquot of this mixture was placed in an Eppendorf tube and centrifuged for 5 minutes. After centrifugation, approximately 1.5 mL of sample was then transferred to an amber HPLC vial for analysis. Each sample was prepared and analyzed in triplicate.

6.2.6 Calibration Curve

Quantitation of flavonoids was based on a calibration curve constructed from chromatographic grade vitexin from Sigma Aldrich (St. Louis, MO) and all analytes were calculated using molecular ratios to correct for differences in mass. Vitexin (5.4 mg) (**figure 6-3**) was dissolved in 25 mL of a 60% methanol in water (499.97 nmol/mL) and mixture and vortexed. A one milliliter aliquot of this stock solution was diluted to 5 mL and 2 mL of this solution was used to make dilutions of 1/5x, 1/10x, 1/20x, 1/40x, 1/80x, 1/160x, 1/320x, and 1/640x to construct a calibration curve covering concentrations of 1.56 nmol to 499.97 nmol. The peak area of each dilution was measured twice to insure instrument reliability. To maximize linearity, the 1/5x, 1/10x, and 1/20x were excluded. The R^2 value was 1, showing excellent linearity (**Figure 6-1**).

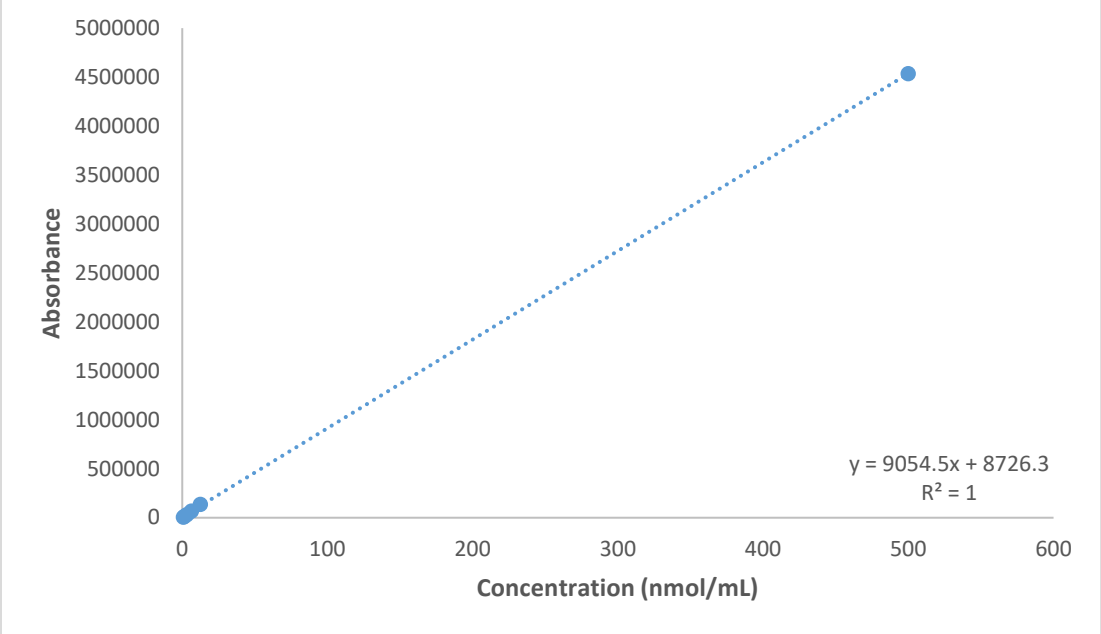


Figure 6-1. Calibration curve of vitexin used to quantitate flavonoids in griffonia leaves

6.3 Results and Discussion

6.3.1 Qualitative Identification

The purpose of this study was to characterize and quantitate the flavonoids in griffonia leaves from various populations across Ghana and Liberia. The HPLC-UV chromatogram of the 50% methanolic extract griffonia leaves revealed the presence eight flavonoids and six of which have been tentatively identified as apigenin-6,8-diglycosides while the remaining two are apigenin-C-monoglycosides that are likely vitexin and isovitexin (**Figure 6-3**) based on mass spectroscopy and UV data. All eight peaks displayed an almost identical UV profile with λmax absorbance at approximately 215, 270, and 335 nm (**Figure 6-2**). This absorbance pattern suggests these compounds are flavones (DeRijke et al., 2006). Mass spectroscopy revealed masses for each peak that match those of several known apigenin C-glycosides. Mass spectroscopy also revealed that all eight flavone peaks were C-glycosides as $[M-H-60]^-$, $[M-H-90]^-$, and $[M-H-120]^-$ were the dominant fragment ions observed (Zhou et al., 2012) (**Table 6-1** and **Figure 6-4**). Peak A was found to have a *m/z* of 593 and with fragment ions $[M-H-90]^-$, and $[M-H-120]^-$. The presence of these ions and the lack of $[M-H-60]^-$ is highly suggestive that the sugar moieties of this apigenin glycoside are both likely to be hexoses. One possible identity of this peak is vicenin-2 (**Figure 6-5**). The mass spectra of peaks B, C, D, and E all contained $[M-H-60]^-$, $[M-H-90]^-$, and $[M-H-120]^-$ fragment ions which indicates that each peak has one pentose and one hexose moiety. Possible identities of these peaks include vicenin-1, vicenin-3, schaftoside, neoschaftoside and isoschaftoside (**Figure 6-5**). Peak F was found to have a *m/z* of 533 which matches the mass of apigenin-dipentoside (**Figure 6-3**). Peaks G and H both had the same *m/z* of 431, matching the masses of vitexin and isovitexin. Comparison of retention times between peak H and an authentic vitexin reference standard was used to further assert the identity of this peak. Unfortunately, the sugar moieties and substituted position of these flavonoid glycosides cannot be characterized by mass spectroscopy. Peaks will need to be isolated and subjected to NMR spectroscopy to confirm their absolute identities. Despite this, the UV and mass spectroscopy data presented is strongly

indicative that the eight dominating flavonoids found in griffonia leaves are apigenin-C-glycosides. Similar apigenin-C-glycosides have been identified in the closely related species *Schnella glabra* (Farag et al., 2015) although among other members of the Cercideae tribe for which there is flavonoid data, the occurrence of C-glycosides appears to be a nearly exclusive feature of genus *Griffonia*. It should be noted that the flavonoid profiles of many other closely related species within the tribe Cercideae such as those within the genus *Adenolobus* have yet to be identified. *Piliostigma thonningii* which is a close relative to griffonia, has been found to contain several C-methyl derivatives of quercetin and kaempferol (Ibewuike et al., 1996) (**Figure 6-6**). Several species within genus *Cercis* are known to contain a variety of *O*-glycosides of kaempferol, quercetin, myricetin (Salatino et al., 2000), and syringetin (Na et al., 2009) (**Figure 6-6**) and species within genus *Bauhinia* have been found to contain a similar variety of O-glycosides as well as several O-glycosides of apigenin and isorhamnetin (Salatino et al., 1999; Farag et al., 2015) (**Figure 6-6**). A similar assortment of apigenin-C-glycosides has been identified in *Passiflora incarnata*, a member of the passion flower family that has been used as a natural sedative throughout the Americas (Raffaelli et al., 1997).

6.3.2 Quantitation of the Flavonoids

Quantitative analysis showed that Ghanaian populations had on average higher amounts of flavonoids than Liberian populations and that across all populations, griffonia leaves on average contained 9182.02 ± 140.90 μg/g (**Table 6-2 and Graphs 6-1 to 6-9**). Among Ghanaian populations, total flavonoid content ranged from 5480 ± 95.34 μg/g to 14003.19 ± 171.33 μg/g, and averaged 10264.13 ± 177.68 μg/g. Leaves from Ashanti Region were significantly lower in peaks C, D, and F as well as total flavonoid content than the other three Ghanaian populations which were relatively similar in total flavonoid content to each other. Leaves from Brong Ahafo Region were significantly greater in vitexin and isovitexin than the other Ghanaian populations. Leaves from Central Region were significantly greater in peak E and F content than any other

Ghanaian region. There was a relatively wide degree of variation in peak F among all Ghanaian populations. Total flavonoid content among Liberian populations ranged from 720.87 ± 14.31 μg/g to 11481.97 ± 147.90 μg/g, and averaged 8099.91 ± 90.18 μg/g. Leaves from Bargblor Town, Grand Gedeh County were significantly lower in all peaks and thus total flavonoids than the other three Liberian populations. Population from Upper Nimba County was much more concentrated in peaks C and D than the other Liberian populations. Population from Tapita, Nimba County was significantly greater in vitexin and isovitexin than the other three Liberian populations. Peaks D, E, F, vitexin and isovitexin had a high degree of variation among all four Liberian populations. Overall, Ghanaian populations were significantly more abundant in peaks A, B, E, and F than Liberian populations and Liberian populations were slightly more abundant in peaks D, E, vitexin, and isovitexin than Ghanaian populations. In this study, dried griffonia leaves were found to be slightly less concentrated in flavonoids than dried spinach leaves which according to a 2005 study by Bergquist et al., contain 13-23 mg/g of these vital nutrients. Griffonia leaves also appear to be a significantly lower source of flavonoids than that of dry green tea leaves, the content of which was found to be 140.66 mg/g in a 2005 study by Peterson et al. Griffonia leaves appear to be approximately two times greater in flavonoid content than dried celery based on one study in which content was found to be 4.22 mg/g (Lin et al., 2007) although in the same study, flavonoid content of another variety (Chinese celery) was found to be 16.02 mg/g. Flavonoid content of fermented rooibos leaves was determined by Bramati et al. (2003) to be 5.5 mg/g thus in comparison to this source, griffonia leaves appear to be approximately twice as rich in these compounds. *Passiflora incarnata*, a member of the passionflower family that contains a similar assortment of apigenin-C-glycosides, was found to contain on average 17.47 mg/g of these compounds (Marchart et al., 2003) thus compared to this source, griffonia leaves are approximately half as rich in these compounds.

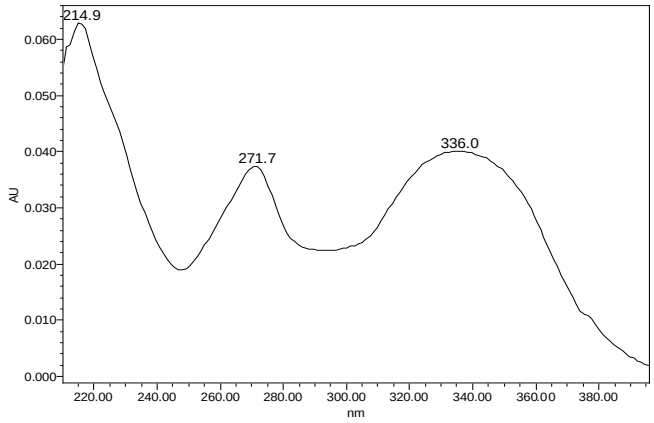


Figure 6-2. Representative UV spectrum of flavonoid peaks A - H

Table 6-1. Ions and tentative identities of flavonoid peaks

Peak ID	Rt (mins)	*m/z*	MS/MS data	Tentative identity
A	22.93	593	593, 502.9, 472.9, 383, 353	Apigenin-dihexose
B	24.02	563	563, 544.9, 503, 473, 443, 383.1, 353.1	Apigenin-pentose-hexose
C	24.46	563	563, 544.9, 473, 443, 383, 352.9	Apigenin-pentose-hexose
D	24.77	563	563, 544.8, 503, 472.9, 443, 425, 383, 353	Apigenin-pentose-hexose
E	25.16	563	563, 545.9, 503, 472.9, 443, 383.1, 353.1	Apigenin-pentose-hexose
F	26.43	533	516, 474, 444, 384, 354	Apigenin-dipentose
G	27.11	431	431, 340.8, 310.9	Isovitexin
H	27.40	431	431, 340.8, 310.9, 283	Vitexin

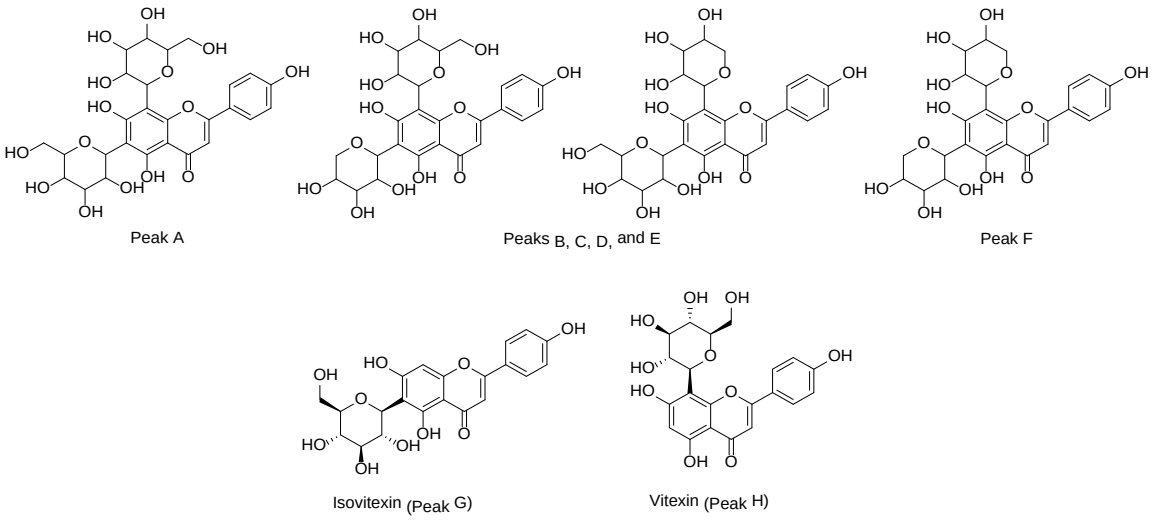


Figure 6-3. Tentative assignments of flavonoids in griffonia leaves based on mass spectroscopy data

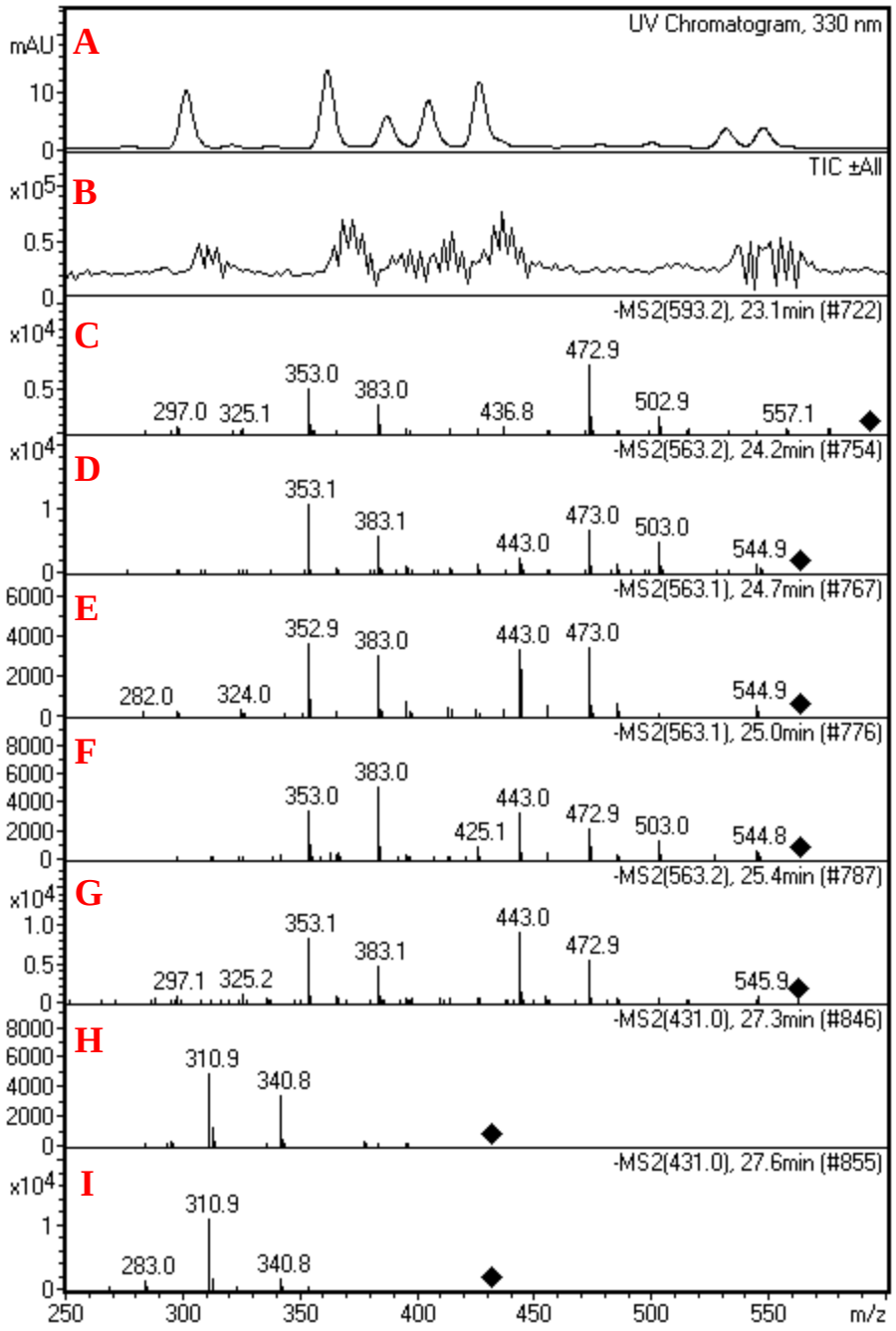


Figure 6-4. HPLC-UV/MS chromatograms and MS/MS spectra of griffonia leaves depicting flavonoid peaks A-E, isovitexin and vitexin. (A). HPLC-UV chromatogram at 330 nm of leaves from Brong Ahafo Region, Ghana. (B). Total Ion MS/MS Chromatogram of leaves from Brong Ahafo Region, Liberia. (C), MS/MS spectra peak A, (D) peak B, (E) peak C, (F) peak D, (G) peak E, (H) isovitexin, and (I) vitexin.

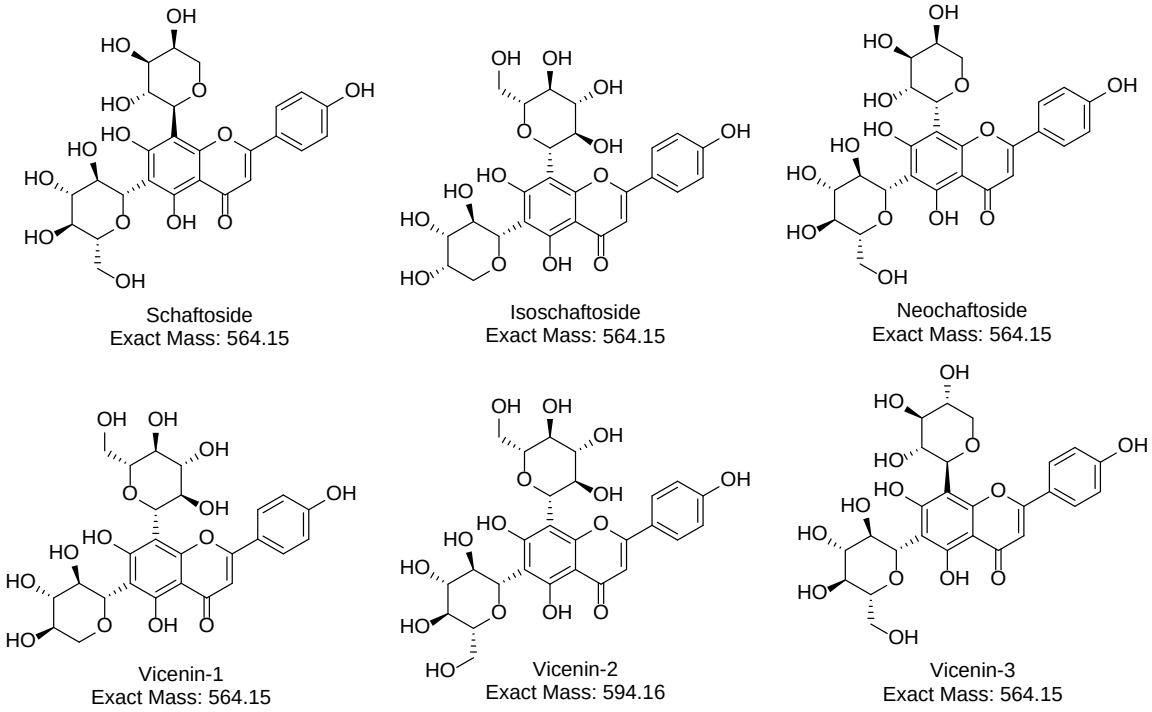


Figure 6-5. Possible identity of peak A (vicenin-2) and of flavonoid peaks B – E (schaftoside, isoschaftoside, neoschaftoside, vicenin-1, and vicenin-3)

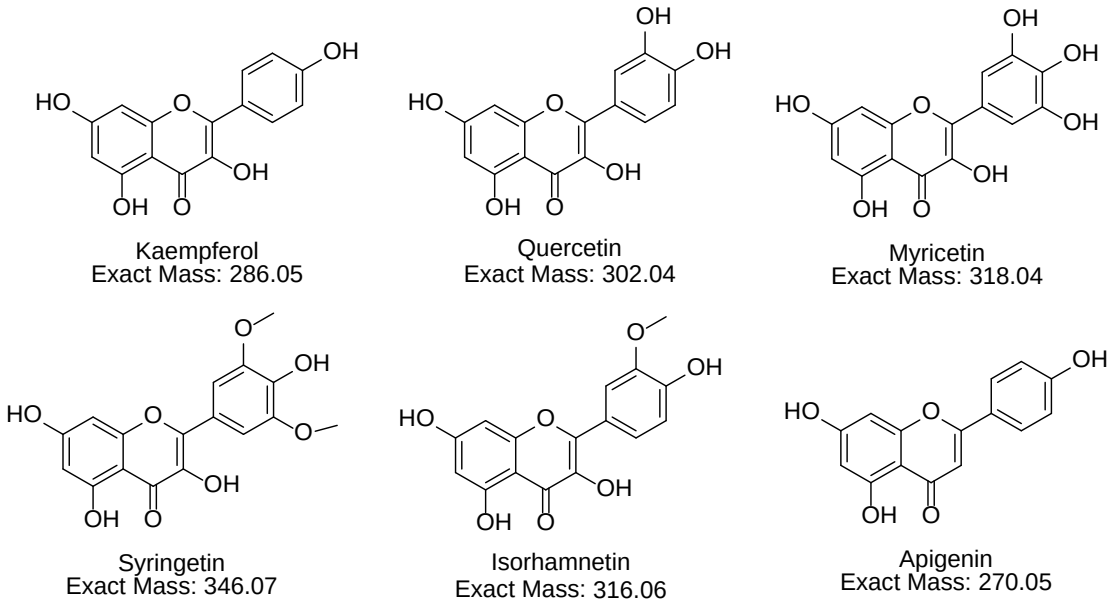


Figure 6-6. Flavonoid aglycones found in several of griffonia's closest relatives

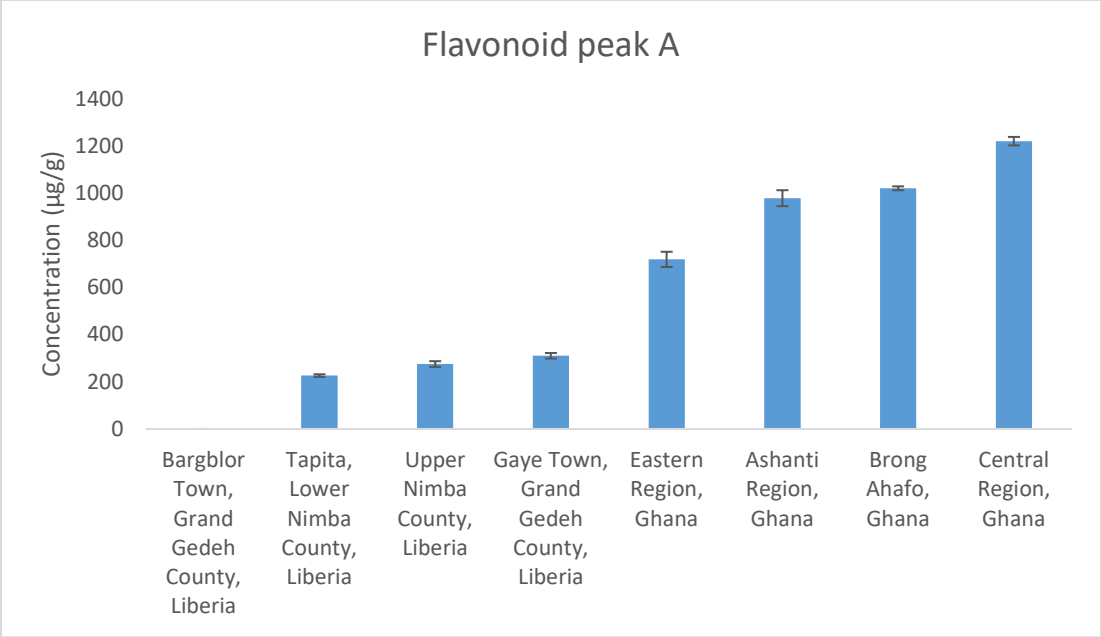
Table 6-2. Content of flavonoid peaks A-F, isovitexin and vitexin (µg/g)

Ghana

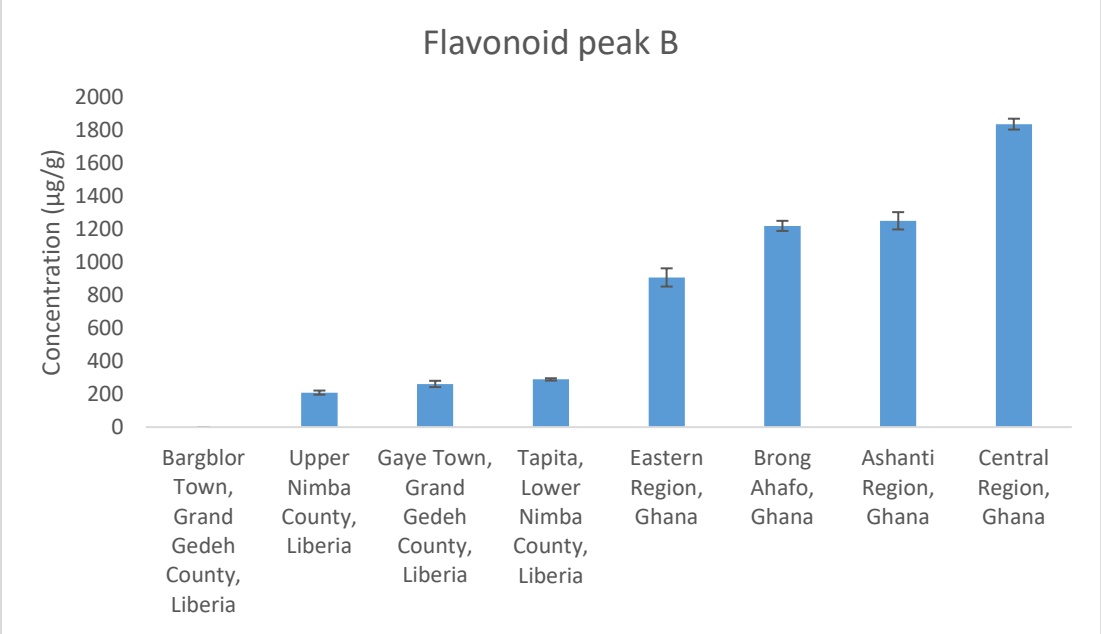
	Flavonoid peak A (µg/g)	Flavonoid peak B (µg/g)	Flavonoid peak C (µg/g)	Flavonoid peak D (µg/g)	Flavonoid peak E (µg/g)	Flavonoid peak F (µg/g)	Flavonoid peak G (isovitexin) (µg/g)	Flavonoid peak H (vitexin) (µg/g)	Total flavonoids (µg/g)
Ashanti Region,	978.78 ± 33.88	1250.05 ± 52.41	568.1 ± 17.16	852.65 ± 34.69	1184.98 ± 54.83	56.86 ± 17.97	293.73 ± 13.85	295.48 ± 13.28	5480.64 ± 95.34
Brong Ahafo,	1020.76 ± 8.26	1219.23 ± 30.65	1440.81 ± 9.07	2195.05 ± 30.30	1146.75 ± 95.17	1089.73 ± 10.59	1590.88 ± 17.07	1309.83 ± 10.66	11013.04 ± 107.62
Central Region,	1220.62 ± 18.02	1835.81 ± 32.89	2681.18 ± 56.19	3720.54 ± 80.39	1730.09 ± 126.75	2133.51 ± 45.42	261.35 ± 12.28	420.11 ± 7.00	14003.20 ± 171.33
Eastern Region,	719.40 ± 32.23	906.84 ± 55.21	2480.95 ± 151.51	3513.75 ± 197.42	815.21 ± 42.30	1399.11 ± 86.20	304.26 ± 14.95	420.15 ± 28.16	10559.66 ± 276.14
Ghana average	**984.89 ± 25.40**	**1302.98 ± 44.21**	**1792.76 ± 81.38**	**2570.50 ± 109.04**	**1219.26 ± 86.48**	**1169.80 ± 49.82**	**612.55 ± 14.64**	**611.39 ± 16.82**	**10264.13 ± 177.68**
All griffonia populations average	**594.01 ± 19.02**	**746.44 ± 32.38**	**1961.98 ± 67.03**	**2870.43 ± 90.09**	**694.24 ± 61.87**	**919.20 ± 37.25**	**701.99 ± 16.65**	**693.73 ± 18.44**	**9182.02 ± 140.90**

Liberia

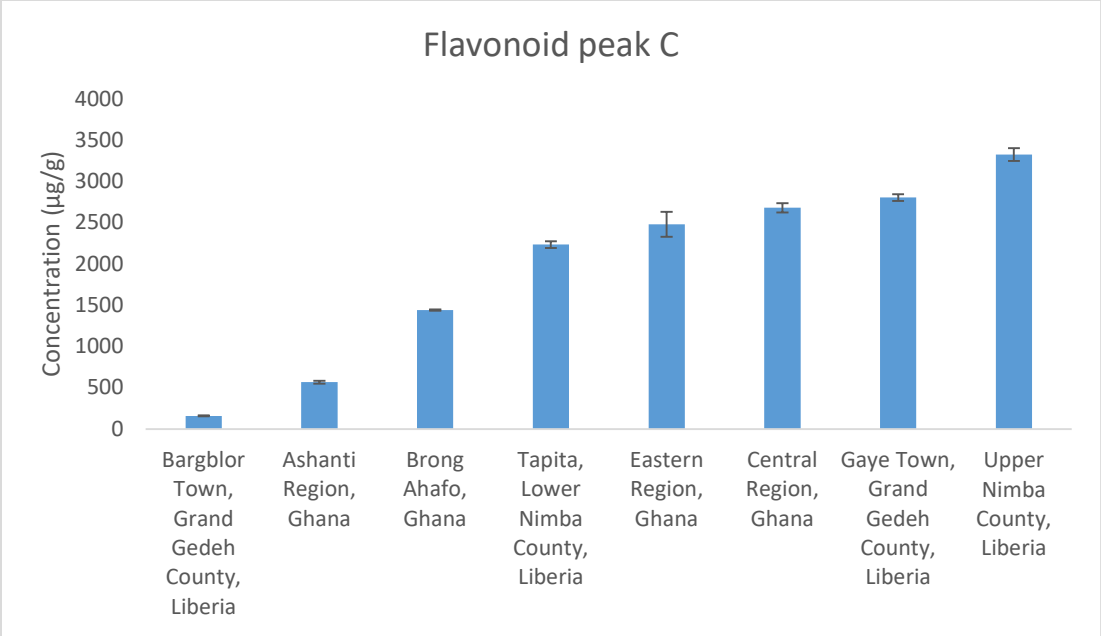
	Flavonoid peak A (µg/g)	Flavonoid peak B (µg/g)	Flavonoid peak C (µg/g)	Flavonoid peak D (µg/g)	Flavonoid peak E (µg/g)	Flavonoid peak F (µg/g)	Flavonoid peak G (isovitexin) (µg/g)	Flavonoid peak H (vitexin) (µg/g)	Total flavonoids (µg/g)
Bargblor Town, Grand Gedeh Co,	Trace	Trace	159.47 ± 5.29	339.26 ± 6.55	Trace	63.72 ± 10.80	58.19 ± 2.36	100.24 ± 3.41	720.87 ± 14.31
Gaye Town, Grand Gedeh Co.	310.57 ± 11.73	261.55 ± 19.09	2805.16 ± 40.67	3995.13 ± 36.82	254.51 ± 15.86	1098.59 ± 15.94	792.17 ± 6.74	909.04 ± 8.66	10426.72 ± 64.32
Tapita, Lower, Nimba Co.	226.44 ± 5.52	289.00 ± 7.03	2234.61 ± 41.54	3097.77 ± 47.83	155.81 ± 4.47	612.07 ± 12.10	1741.62 ± 32.23	1412.74 ± 31.87	9770.07 ± 79.46
Upper Nimba Co.	275.48 ± 12.04	209.04 ± 12.48	3325.59 ± 77.77	5249.30 ± 116.95	266.55 ± 20.84	900.05 ± 25.58	573.73 ± 16.48	682.23 ± 22.08	11481.97 ± 147.90
Liberia average	**203.12 ± 8.85**	**189.90 ± 11.93**	**2131.21 ± 48.62**	**3170.37 ± 65.89**	**169.22 ± 13.28**	**668.61 ± 17.11**	**791.43 ± 18.44**	**776.06 ± 19.40**	**8099.91 ± 90.18**
All griffonia populations average	**594.01 ± 19.02**	**746.44 ± 32.38**	**1961.98 ± 67.03**	**2870.43 ± 90.09**	**694.24 ± 61.87**	**919.20 ± 37.25**	**701.99 ± 16.65**	**693.73 ± 18.44**	**9182.02 ± 140.90**



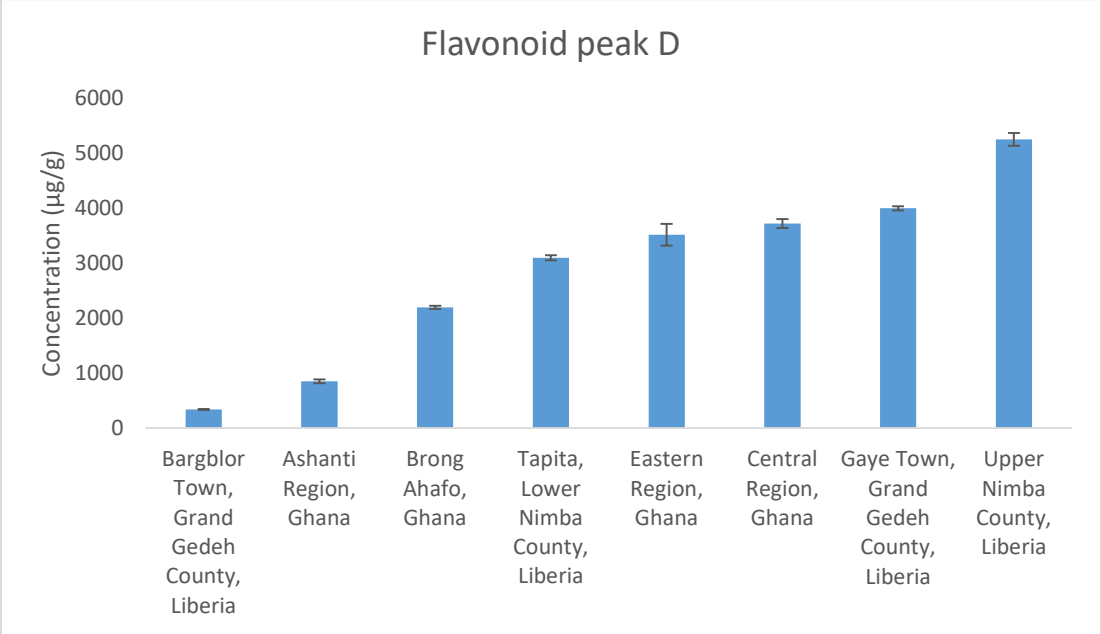
Graph 6-1. Content of flavonoid peak A (µg/g) in leaves of each griffonia population.



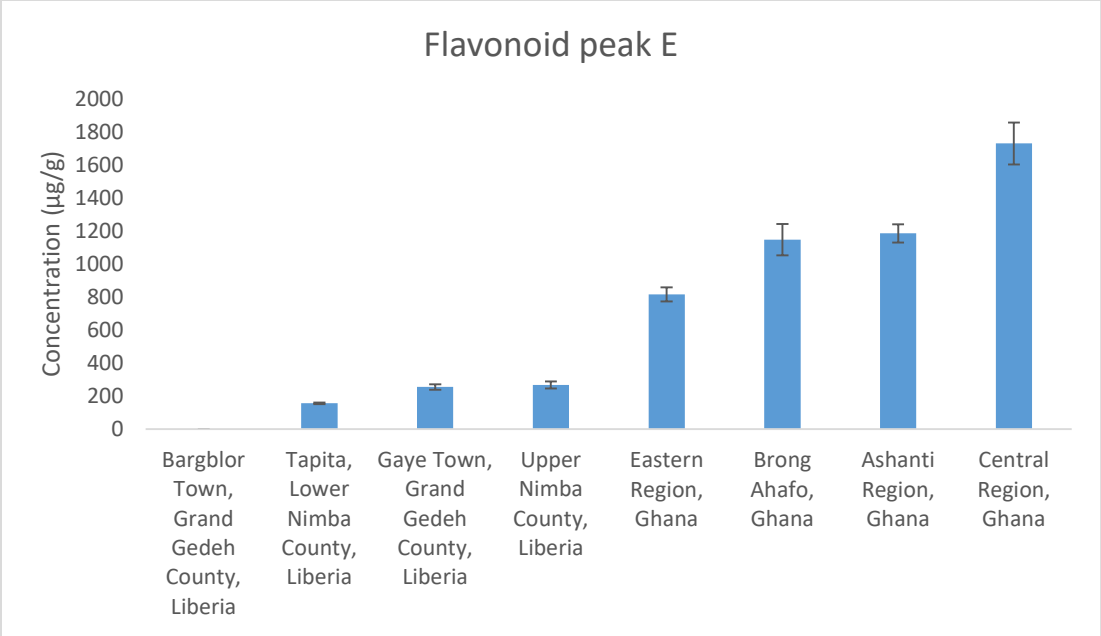
Graph 6-2. Content of flavonoid peak B (µg/g) in leaves of each griffonia population



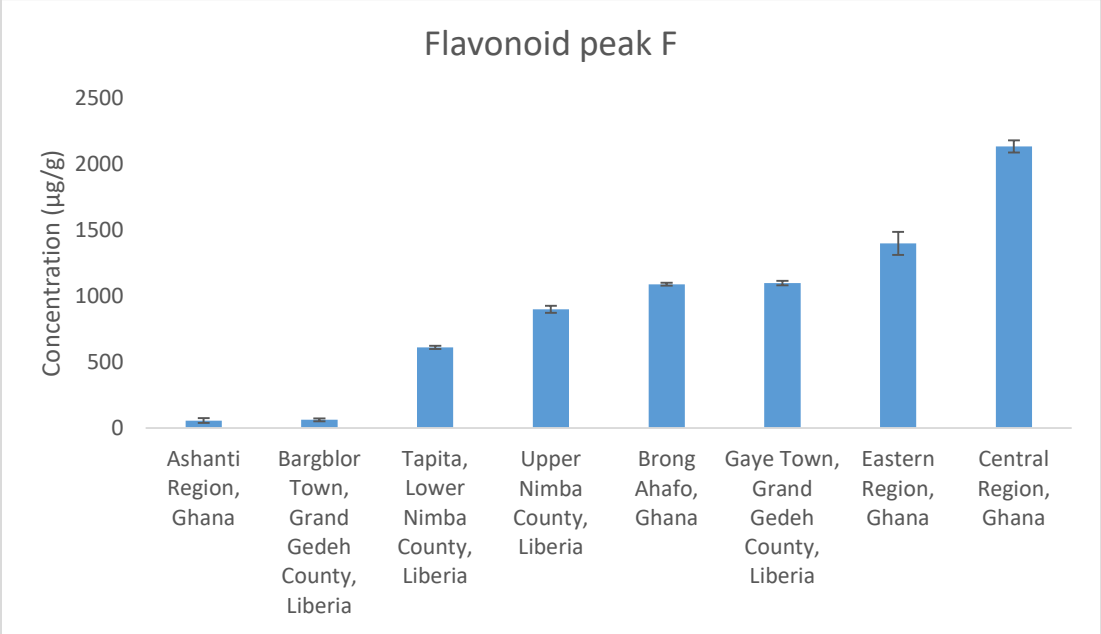
Graph 6-3. Content of flavonoid peak C (µg/g) in leaves of each griffonia population



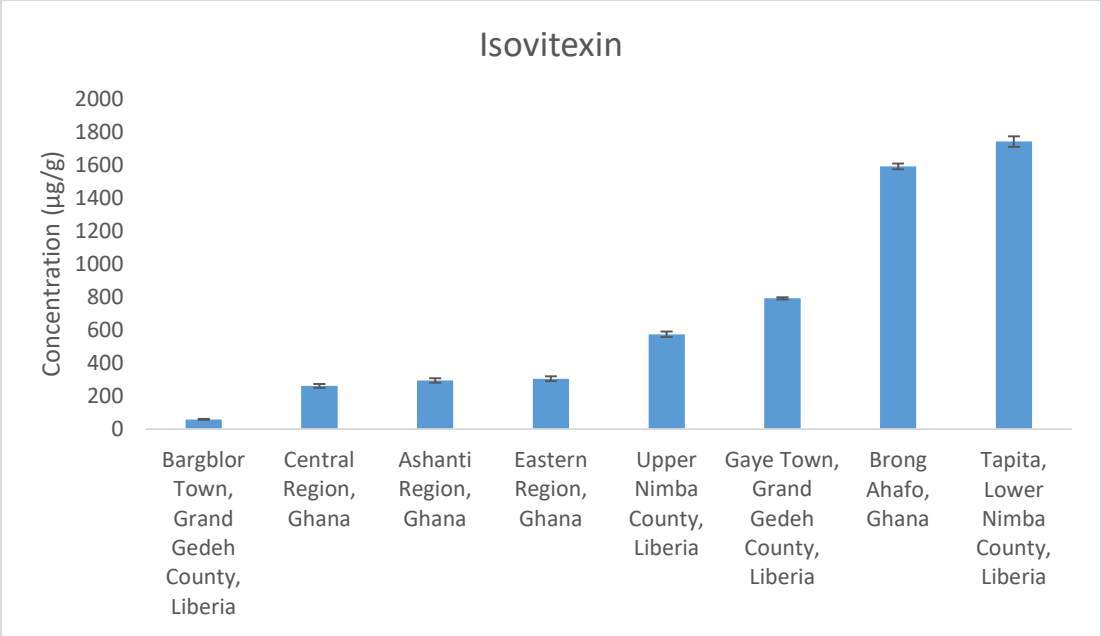
Graph 6-4. Content of flavonoid peak D (µg/g) in leaves of each griffonia population



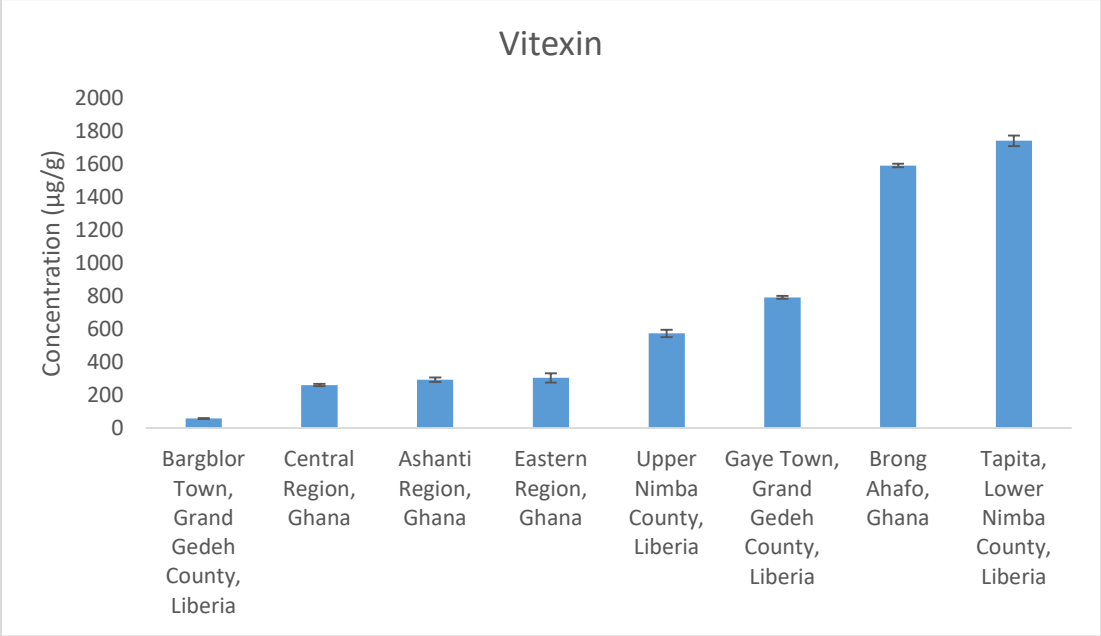
Graph 6-5. Content of flavonoid peak E (µg/g) in leaves of each griffonia population



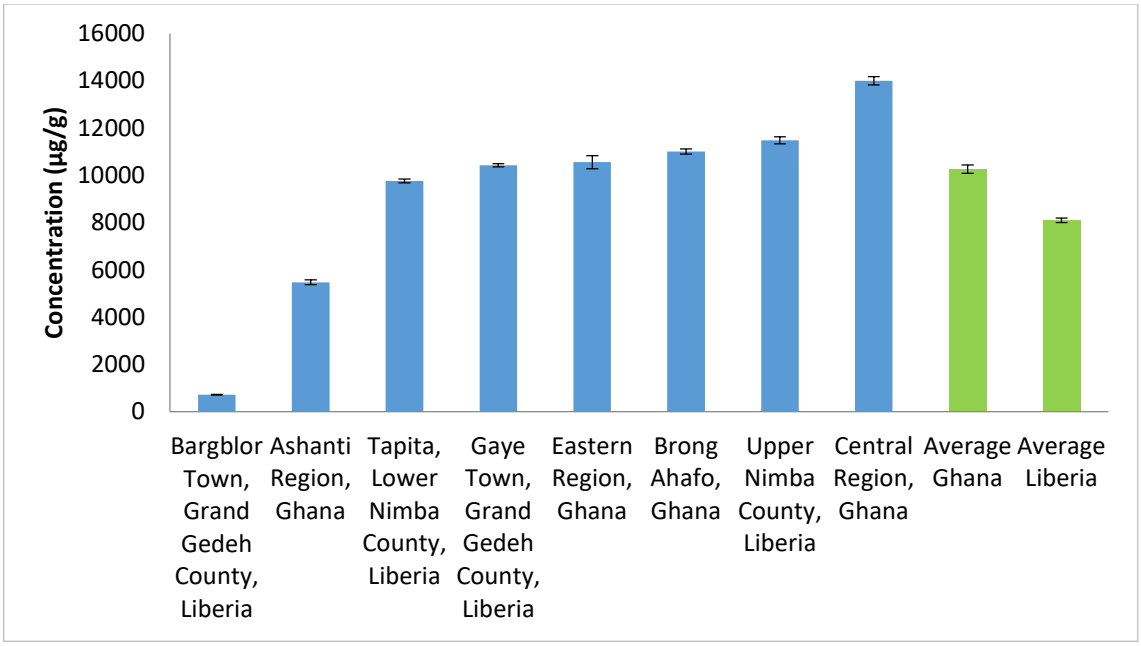
Graph 6-6. Content of flavonoid peak F (µg/g) in leaves of each griffonia population



Graph 6-7. Content of isovitexin (µg/g) in leaves of each griffonia population



Graph 6-8. Content of vitexin (µg/g) in leaves of each griffonia population



Graph 6-9. Content of total flavonoids (µg/g) of each griffonia population

6.4 Conclusions

The purpose of this experimental study was to identify and compare the abundances of flavonoids found in griffonia leaves from various populations across Ghana and Liberia. Mass spectroscopy data suggests that all eight major peaks represent apigenin-C-glycosides (**Figure 6-3**). Unfortunately, the chirality of the sugar moieties and substituted positions could not be discerned by mass spectroscopy and these compounds will have to be isolated and subjected to NMR spectroscopy in future studies for absolute structural characterization. A similar assortment of apigenin-C-glycosides has been identified in *Passiflora incarnata*, a member of the passionflower (Raffaelli et al., 1997). To date, flavonoid data for griffonia's closest relatives remain fragmentary. Among closest living genera to griffonia to have been evaluated for flavonoids, *Cercis* and *Bauhinia* have been found to contain mainly O-glycosides of kaempferol, quercetin, and myricetin (Salatino et al., 2000; Farag et al., 2015) (**Figure 6-6)** while C-methyl derivatives of quercetin and kaempferol have been identified in a species of *Piliostigma* (Ibewuike et al., 1996) (**Figure 6-6)**. Flavonoid data is often at least partially useful in complementing morphological data (Hegnauer, 1993) therefore this data may be interesting to plant taxonomists interested in studying this area of the plant kingdom.

Using an authentic vitexin standard, each of eight flavonoid peaks in eight griffonia leaf populations from various regions in Ghana and Liberia were quantitated by HPLC-UV. On average, total flavonoid content of griffonia leaves was 9182.02 ± 140.90 μg/g and Ghanaian populations were higher in flavonoid content than Liberian populations. We also observed that there was a wide degree of variation in flavonoid content among the various populations. The reason for these differences could be related to differences in genetics, soil nutrition, biotic and abiotic stress, disease state, access to sunlight (Haytowitz et al., 2013), as well as the health of the node dwelling *rhizobia* bacteria. Apigenin-C-glycosides are strong antioxidants and as such, these compounds could protect DNA and cellular membranes from damage by free radicals thus

mitigating the risk of cancer and heart disease. Griffonia leaves appear not to be as rich of a source of flavonoids as dry spinach (Bergquist et al., 2005) and green tea leaves (Peterson et al. 2005). Griffonia leaves appear to be similar in flavonoid content to dried celery (Lin et al., 2007). To fully assess the potential nutritional benefits of griffonia leaves, future studies should focus on the bioavailabilities of these apigenin-C-glycosides.

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Chapter 7. Quantification of Fatty Acids and Tocopherols in *Griffonia simplicifolia* Seeds of Various Origins

7.1 Introduction

Fatty acids are a primary source of energy for both plants and animals. While energy is also sourced from carbohydrates and proteins, the catabolism of fatty acids yields nearly twice as much energy (per gram) than these other biomolecules (Nelson and Cox, 2008). This is because sugars and proteins are generally composed of partially oxidized carbons which yield less energy when catabolized. (Garrett and Grisham, 2006). It's therefore expected that the seeds of many plant species including griffonia, contain large amounts of fatty acids to provide the developing organism with the needed energy for the seed to germinate and emerge as it matures into an autotrophic organism. In germinating seedlings, Acetyl-CoA which is a by-product of β-oxidation, is converted to oxaloacetate by the glyoxylate cycle which is then converted to glucose by gluconeogenesis. The glucose produced by this pathway is then used to produce nucleotides, amino acids, polysaccharides, and other metabolic intermediates (**Figure 7-1**) (Nelson and Cox, 2008). Aside from acting as a primary storage for energy, the human body also uses fatty acids to produce phospholipids, pigments, cofactors, transporters, hormones, chemical messengers, and anchors for membrane proteins (Lehninger et al., 1992). Thus, fatty acid intake is essential to maintaining proper nutrition. Fatty acids such as linoleic and linolenic acids are important precursors to prostaglandins and thromboxane that the body cannot self-produce (Garrett and Grisham, 2006), therefore dietary intake of these compounds from plant sources is essential.

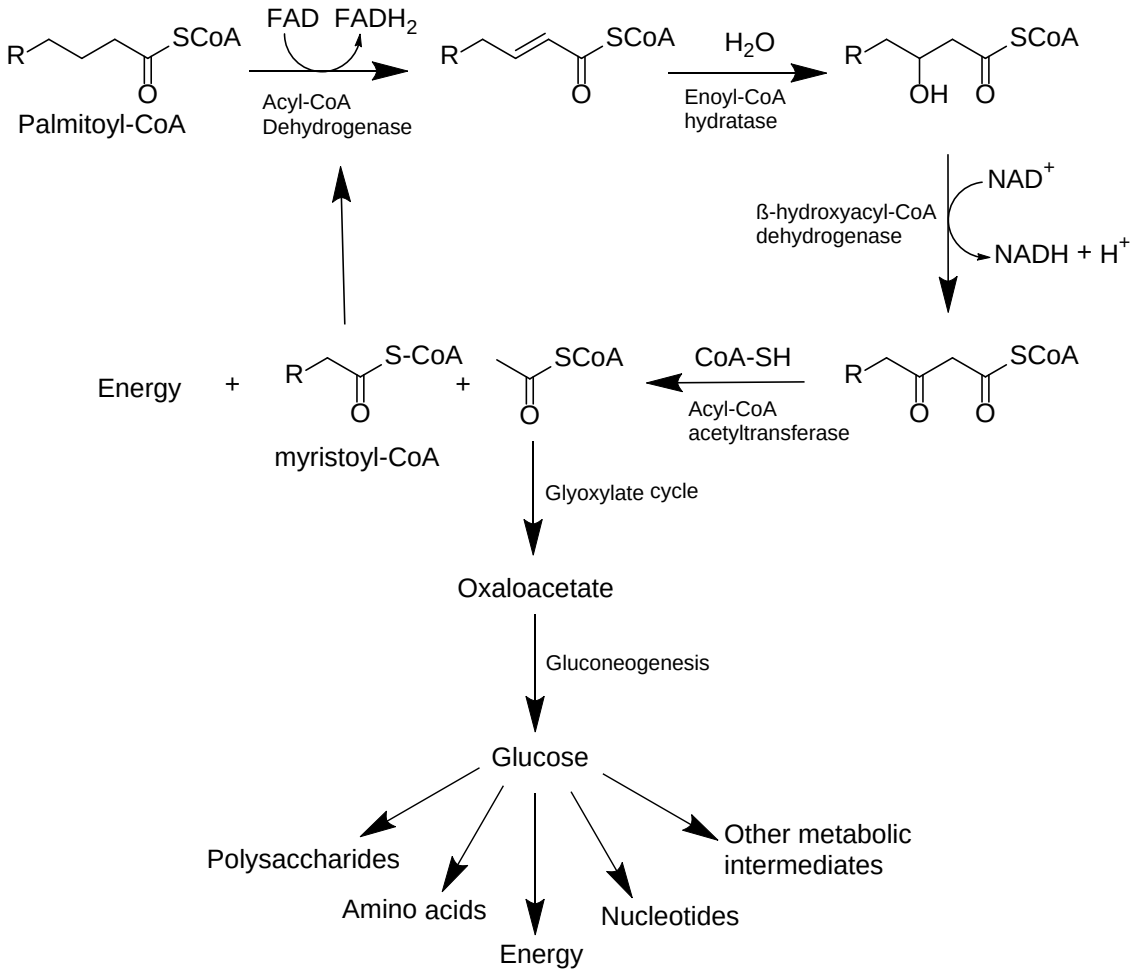


Figure 7-1. Catabolism cycle of saturated fatty acids via β-oxidation pathway and further derivatization of Acetyl-CoA to other primary metabolites in germinating seeds (Adapted from Lehninger's Principles of Biochemistry 5th Edition)

The tocopherols are a class of four naturally occurring lipophilic compounds with potent antioxidant properties. Collectively with tocotrienols, these compounds make up a class of nutrients commonly known as vitamin E **(Figure 7-2)** which have a vital role in human nutrition by preventing oxidative damage to cells**.** Oxidative damage to cells has been linked to several serious health conditions such as cancer, diabetes, and atherosclerosis (Machado-Sanchez et al., 2008). Tocopherols are present in most plants with the highest concentration occurring in seed oils (Zingg, 2007). Upon ingestion, tocopherols and tocotrienols are mostly deposited into cell

membranes where they guard the unsaturated fatty acids the cell membrane is composed from destruction by free radicals (Nelson and Cox 2008). The ability of vitamin E to neutralize free radicals without itself becoming a damaging radical stems from its aromatic ring moiety; after the hydroxyl substituent on the aromatic ring donates a hydrogen atom (one proton and electron) to the free radical, the electronic vacancy is easily stabilized by resonance by the adjacent aromatic system. The tocopherol radical will then combine with another free radical to form non-radical products (**Figure 7-3**) (Nagaoka et al., 1992). The activities of γ-tocopherol and δ-tocopherol are believed to be only 10% and 1%, respectively as active as α-tocopherol (Wyatt et al., 1998) due presumably due to their differential bioavailability. α-Tocopherol is more widely distributed throughout the body than other tocopherols because the carrier protein that transports tocopherols throughout the body known as α-tocopherol transport protein (α-TTP) has a stronger binding affinity for this form of vitamin E (Zingg 2007; Rigotti 2007). α-TTP not only discriminates tocopherols based on their substitution pattern around the aromatic ring, but also their stereochemistry; α-TTP is known to selectively bind to the natural diastereomer RRR-α-tocopherol and has a poor affinity for synthetic diastereomers (Brigelius-Flohe and Traber, 1999). α-TTP also has a lower binding affinity for tocotrienols as well (Sen et al., 2005). Although vitamin E can only be obtained through dietary sources, deficiency of this nutrient is very rare. Vitamin E deficiency is usually a result of defective α-TTP caused by genetic abnormalities, fat absorption issues, and low dietary protein (Ye and Eitenmiller, 2004).

They fatty acid profile of griffonia seeds have been previously reported to consist of palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and arachidic acid (20:0) (Ramazanov and Petkov, 2003). While their relative ratios have been reported, the absolute concentrations of fatty acids per gram of seeds have not been reported. One of the objectives of this study was to determine the absolute concentration of each fatty acid per gram of griffonia seed and to ask whether the fatty acid profile can vary by griffonia population. After 5-HTP is

extracted from griffonia seeds, the left over seed material is usually discarded. Determining the absolute content of fatty acids per gram of seed may encourage 5-HTP suppliers to attempt to recover some of these fatty acids as some of these compounds can be used in the formulation of nutraceuticals, drug vehicles, emulsifying agents, and soaps (Heinrich et al., 2012). To date, the tocopherol and tocotrienol composition of griffonia seeds has never been reported. As is the case with fatty acids, the development of an appropriate processing technology to extract tocopherols and tocotrienols from the seed material that is discarded after 5-HTP extraction could be of interest, should the tocopherol and tocotrienol content of griffonia seed warrant a by-product commercialization.

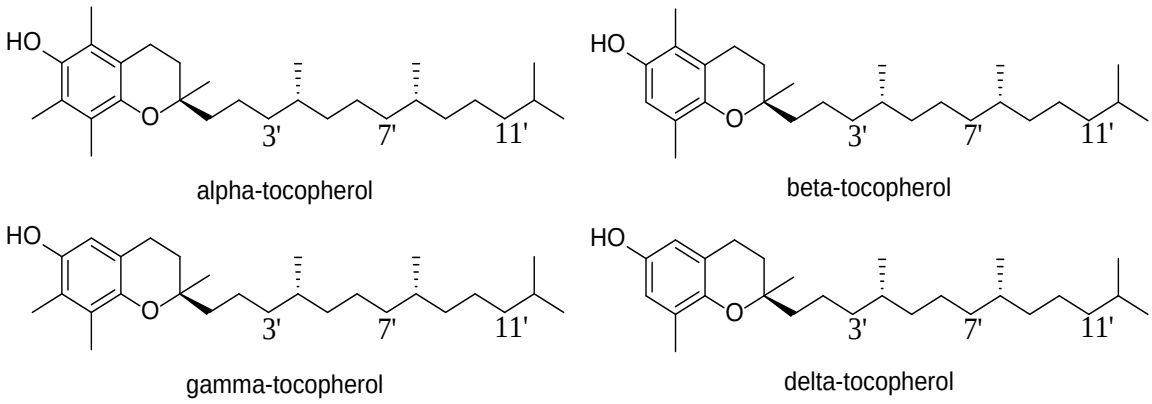


Figure 7-2. Chemical structures of alpha, beta, gamma, and delta tocopherol. Tocotrienols have the same substitution patterns around the aromatic system but have olefins at the 3', 7', and 11' positions

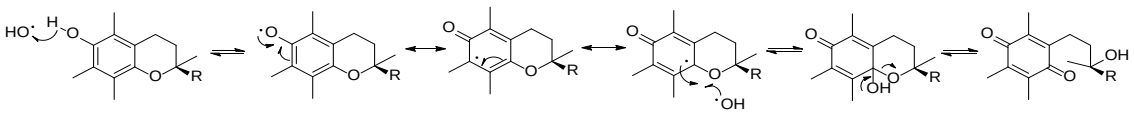


Figure 7-3. Example reaction between α-tocopherol and the hydroxyl radical. Other mechanisms and products are possible. Adapted from Brigelius-Flohe and Traber, 1999

7.2 Experimental

7.2.1 Materials

Hexane, toluene, methanol, water, acetonitrile, ethanol, from Fischer Scientific (Fair Lawn, NJ) sulfuric acid from Acros Organics (Geel, Belgium), Cellulose thimble (Whatman Ltd. England), griffonia seeds from various regions of Ghana and Liberia (see **Table 5-1**).

7.2.2 Equipment

Fatty acids were analyzed using an Agilent 6890 GC equipped with an FID detector in conjunction with an Agilent 5973 mass spectrometer. The column used was an Econo-cap EC-Wax 30 m x 0.25 mm, ID x 0.25 µM from Altech (State College, PA).

Tocopherols were quantitated using a Waters 2695 separation module equipped with a Waters 2996 photo diode array detector. Column used for separation was a Phenomenex Prodigy ODS3 (250 x 4.6 mm, 5 µM, 100Å).

7.2.3 GC Conditions for Fatty Acid Analysis

The Agilent GC was set with an initial temperature of 60 °C which was held for one minute. After the first minute, temperature was increased by 10 °C per minute until oven reached 220 °C and was held constant at this temperature for an additional 23 minutes. Injection was split (25:1), flow rate was 0.8 mL/min and injection volume was 2 µL.

7.2.4 HPLC Conditions for Tocopherol Analysis

The mobile phase consisted of solvent A (100% Acetonitrile) and solvent B (100% Methanol). Gradient consisted of 50% to 60% B over 15 minutes then held constant at 60% for an additional 7 minutes. The flow rate was set at 1.0 mL/min with UV detection set at 292 nm. The injection

volume was 10 µL and analysis occurred at room temperature. Analysis was conducted in triplicate.

7.2.5 Standards

Palmitic acid methyl ester (99% purity), heptadecanoic acid methyl ester (≥99% purity), stearic acid methyl ester (99% purity), oleic acid methyl ester (99% purity), arachidic acid methyl ester (≥99% purity), α-tocopherol (≥96% purity), β-tocopherol (racemic mixture), γ-tocopherol (≥96% purity), δ-tocopherol (≥96% purity) were purchased from Sigma Aldrich (St Louis, MO). Linoleic acid methyl ester from ICN biomedical Inc. (Aurora, OH) and heptadecanoic acid (95% purity) were purchased from Acros Organics (Geel, Belgium).

7.2.6 Soxhlet Extraction of Fatty Acids

Approximately 7 grams of ground seed material was placed in a cellulose thimble and was subjected to Soxhlet extraction for 20 hours using 300 mL of hexane. After extraction, liquid was dried with Na_2SO_4. Solvent was then removed by rotary evaporation to yield approximately 2.3 grams of a greenish yellow oil that would solidify upon refrigeration.

7.2.7 Methylation of Fatty Acids

Approximately 40 mg of seed oil was placed in a glass vial and to it was added one milliliter of toluene spiked with 15.3 mg of heptadecanoic acid to be used as an internal standard. 2 mL of 2% H_2SO_4/MeOH was then added and this mixture was then allowed to heat at 65 ºC for 3 hours and then was left to stand at room temperature overnight. The next morning, the mixture was diluted with 2 mL of water and then extracted with 30 mL of hexane 3 times. Hexane layers were then combined and washed with 3 mL of 3% $NaHCO_3$. Hexane was then removed by rotary evaporation.

7.2.8 Preparation of Fatty Acids for GC Analysis

Fatty acid methyl ester residue was diluted with 10 mL of toluene. Anhydrous Na_2SO_4 was then added and mixture was centrifuged for 5 minutes. After centrifugation, mixture was placed in an amber vial for GC analysis. Each sample was analyzed in triplicate in order to insure instrument reliability.

7.2.9 Preparation of Tocopherols for Analysis

Approximately 200 mg of oil from Soxhlet extraction was diluted with 3 mL of toluene and then placed in an amber vial for HPLC analysis. For each population, samples were prepared and analyzed in triplicate.

7.2.10 Calibration Curves for Fatty Acid Analysis

Each fatty acid was quantitated by construction of a calibration curve using fatty acid methyl ester standards supplied from Sigma Aldrich (St. Louis, MO) (**Table 7-1, Figures 7-4 to 7-9**). Calibration curve for palmitic acid methyl ester covered a range of 11.09 µg/mL to 1420 µg/mL and R^2 value was 0.9995 indicating excellent linearity. Calibration curve for heptadecanoic acid methyl ester covered a range of to 12.88 µg/mL to 1648 µg/mL and R^2 value was 0.9996 indicating excellent linearity. Calibration curve for stearic acid methyl ester covered a range of 11.84 µg/mL to 1516 µg/mL and R^2 value was 0.9995 indicating excellent linearity. Calibration curve for oleic acid methyl ester covered a range of 11.53 µg/mL to 1476 µg/mL and R^2 value was 0.9993 indicating excellent linearity. Calibration curve for linoleic acid methyl ester covered a range of 11.91 µg/mL to 1524 µg/mL and R^2 value was 0.9994 indicating excellent linearity. Calibration curve for arachidic acid methyl ester covered a range of 4.63 µg/mL to 592 µg/mL and R^2 value was 0.9989 indicating excellent linearity.

Table 7-1. Equations and R^2 values for standard curves used to measure fatty acids and tocopherols in griffonia seeds

	Equation	R^2 value
Palmitic acid methyl ester	y = 49293x - 539264	0.9995
Heptadecanoic acid methyl ester	y = 47654x - 523020	0.9996
Stearic acid methyl ester	y = 46890x - 506486	0.9995
Oleic acid methyl ester	y = 51216x - 669833	0.9993
Linoleic acid methyl ester	y = 40173x - 570674	0.9994
Arachidic acid methyl ester	y = 46233x - 193465	0.9989
α-Tocopherol	y = 4068.2x - 9869.7	1.0000
γ-Tocopherol	y = 4209.6x - 9049.4	0.9999

7.2.11 Calibration Curves for Tocopherol Analysis

Calibration curves for α and γ tocopherol covered ranges 1.88 µg/mL to of 964 µg/mL and 1.95 µg/mL to 1000 µg/mL, respectively. R^2 value for α-tocopherol was 1 (**Figure 7-10**) while R^2 value for γ-tocopherol was 0.9999 indicating excellent linearity (**Figure 7-11**).

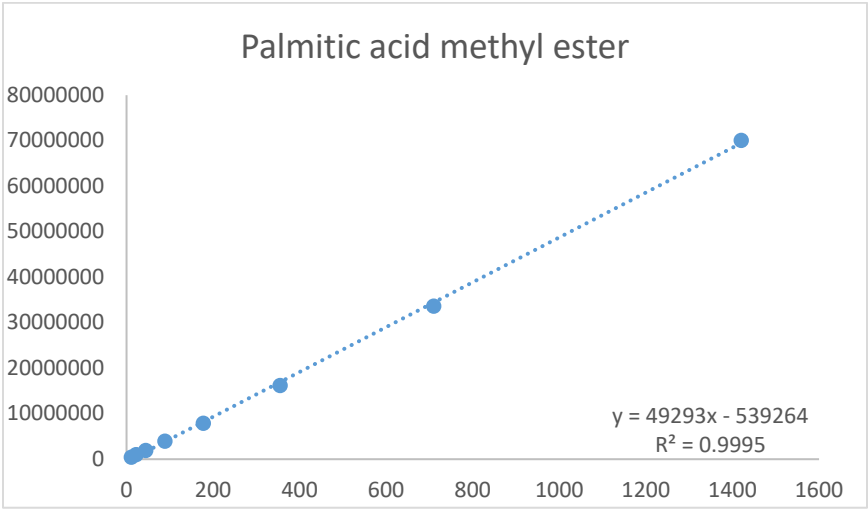


Figure 7-4. Calibration curve used to quantitate palmitic acid methyl ester

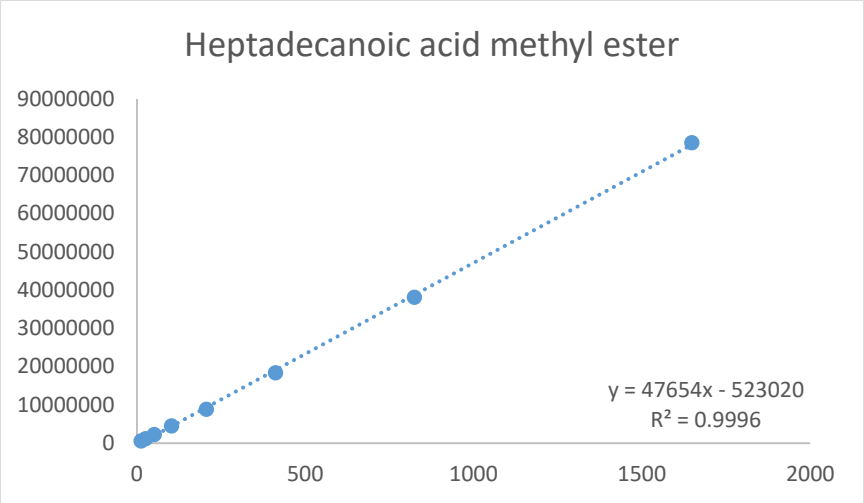


Figure 7-5. Calibration curve used to quantitate heptadecanoic acid methyl ester (Internal standard)

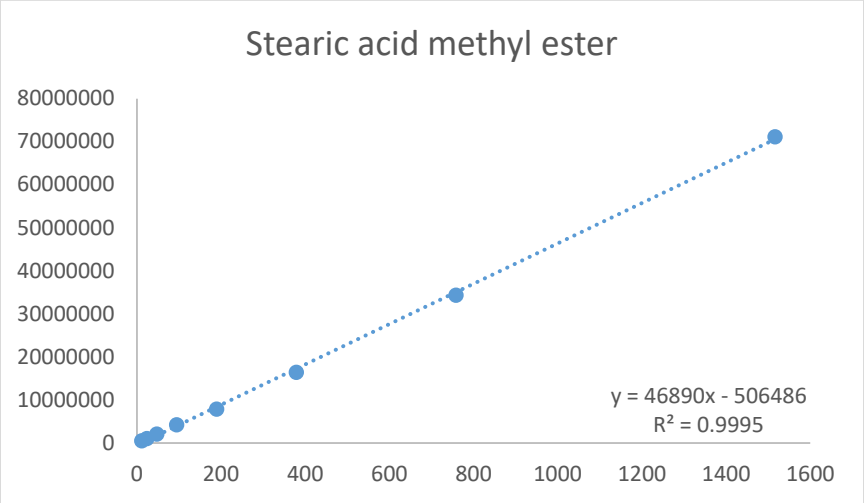


Figure 7-6. Calibration curve used to quantitate stearic acid methyl ester

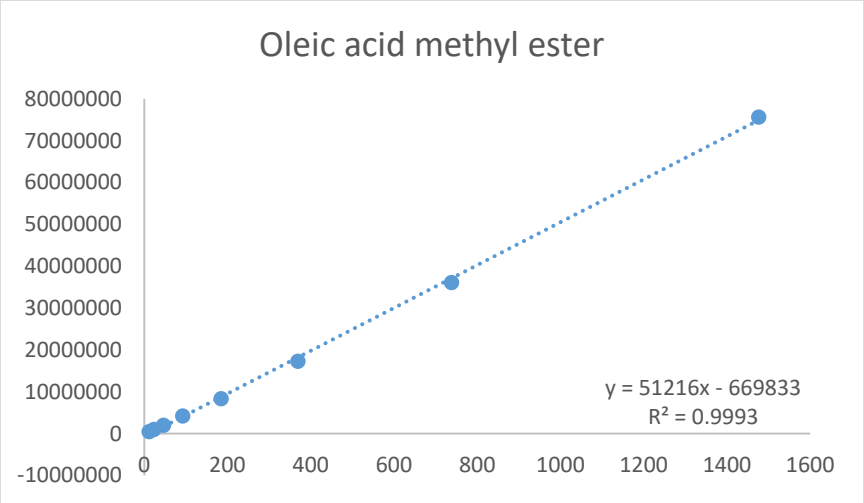


Figure 7-7. Calibration curve used to quantitate oleic acid methyl ester

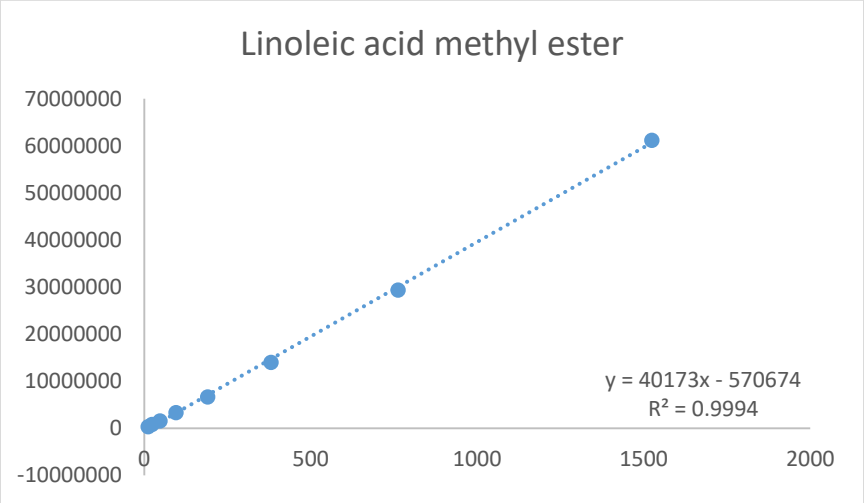


Figure 7-8. Calibration curve used to quantitate linoleic acid methyl ester

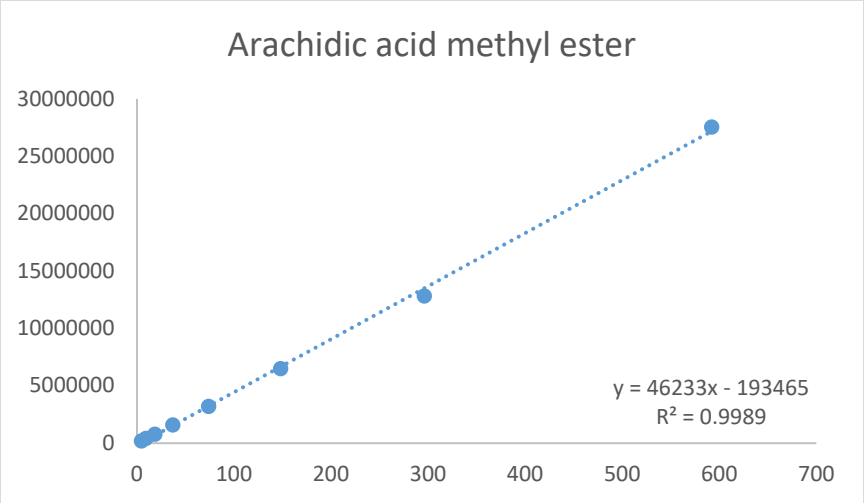


Figure 7-9. Calibration curve used to quantitate arachidic acid methyl ester

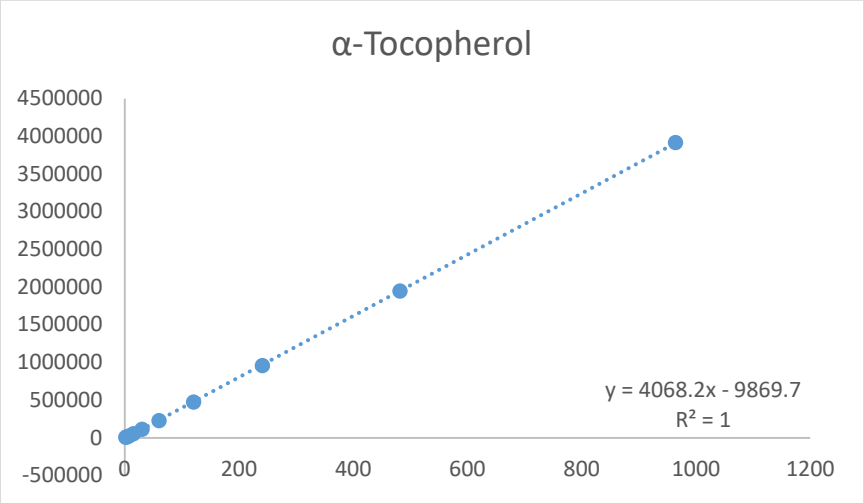


Figure 7-10. Calibration curve used to quantitate α-tocopherol

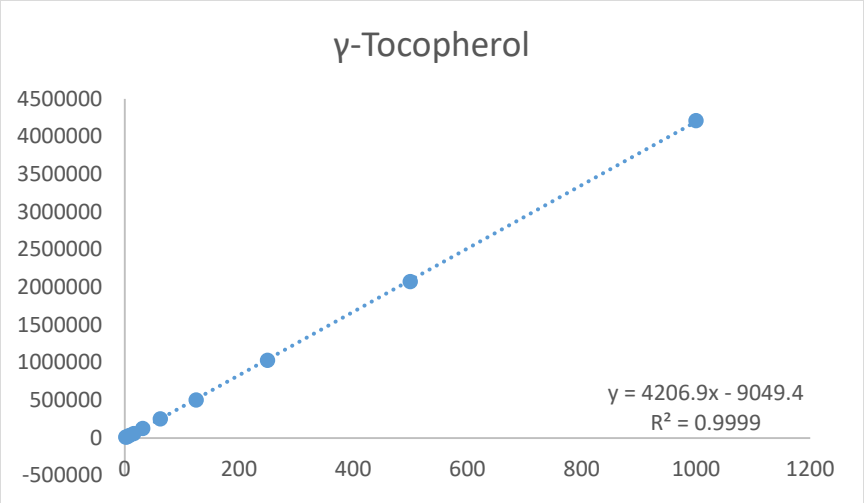


Figure 7-11. Calibration curve used to quantitate γ-tocopherol

7.3 Results and Discussion

The purpose of this study was to quantitate the free fatty acids (**Tables 7-3**, **7-4 and Graphs 7-1 to 7-10**) and tocopherol content (**Table 7-5, Graphs 7-11 and 7-12**) in griffonia seeds from various regions of Ghana and Liberia. This analysis did not seek to identify or quantify tocotrienol character of griffonia seeds. Since vitamin E refers collectively to both tocopherols and tocotrienols, overall content of this class of vitamins may therefore be underestimated. To extract these compounds from the raw seed material, a Soxhlet apparatus was used and each extraction yielded a greenish yellow oil that solidified upon standing. Yield of fixed oil obtained from all but one seed population ranged from 27% to 32% of original seed mass (**Table 7-2**). The yield of oil obtained from one Liberian population was only 21% of the original seed mass. Each of the four Ghanaian populations contained a higher percentage of fixed oils than all six Liberian griffonia populations. This could be due in part to better post-harvest practices among Ghanaian farmers or simply the be due to genetic differences although no information is available about the genetic relationships among and between the griffonia in each area in each country. Soil nutrition,

genetics, access to sunlight, biotic and abiotic stressors could have had a role in these outcomes as well.

GC method provided excellent separation of all fatty acids (**Figure 7-12**). Palmitic, stearic, oleic, linoleic, and arachidic acids were all detected and the concentrations of these fatty acids per gram of seed oil and per gram of seed were calculated (**Tables 7-3 and 7-4** respectively). Each sample was analyzed in triplicate in order to insure instrument reliability. In most cases, error was relatively low indicating instrument was very reliable. Also internal standard data indicated that 99% of free fatty acids were successfully converted to methyl esters using this methylation procedure. Fatty acid profile of all eight griffonia populations were both qualitatively and relatively proportionally similar to that which was previously reported in 2003 by Ramazanov and Petkov (**Table 7-6**).

Overall, free fatty acids comprised approximately 89% of the total seed oil composition and linoleic acid composed of approximately 53% of this oil. The high content of linoleic acid in griffonia seeds is not surprising as relatively high amounts of this acid is strongly characteristic of species within the Caesalpinoideae subfamily (Bagci and Sahin, 2004). In the Leguminosae family, linoleic acid generally accounts for 21-53% of free fatty acids (Grela and Gunter, 1995; Ryan et al., 2007). The other 11% is likely composed of unsaponifiables such as sterols, sterol esters, and pigments such as xanthophylls. In the seed oil of *Cercis siliquastrum* (Judas tree), which is one of the closest living relatives of griffonia, linoleic acid has been reported to be 66.4% of fatty acids in the fixed oils (Khatiashvili and Kemertelidze, 2007). This low proportion of unsaponifiable material to fatty acids may be unusual for members of the Caesalpinoideae subfamily (Grindley, 1946). The composition of palmitic and stearic acids was larger in griffonia seed oil than in sesame, soybean, peanut (Li et al., 2016), and sunflower (Jabeur et al., 2014) oils (**Table 7-6**). Linoleic acid composition of griffonia seed oil was similar to that of sunflower oil

(Jabeur et al., 2014). Oleic acid content of was significantly lower in griffonia seed oil than in palm (Wirasnita et al., 2013), sesame, soybean, peanut, and sunflower oils. Cooking oils with lower proportions of saturated fatty acids to unsaturated fatty acids are usually regarded as being healthier alternatives to oils with larger proportions of saturated fatty acids (Kabagambe et al., 2005). While the saturated fatty acid content of griffonia seed oil is slightly less than that of palm oil, other oils of sesame, soybean, peanut, and sunflower contain significantly lower proportions of saturated fatty acids thus there is little incentive to use griffonia seed oil as an alternative cooking oil.

HPLC method provided excellent separation of α and γ-tocopherols (**Figure 7-13**). To date, the tocopherol profile of griffonia has not been reported. All populations of griffonia seeds were found to contain γ-tocopherol and all but one population studied was found to contain α-tocopherol (**Table 7-5, Graphs 7-11 and 7-12**). α-Tocopherol content ranged from 0 µg/gram to 121.21 ± 2.88 µg/gram of oil and 0 µg/gram to 41.30 ± 3.59 µg/gram of seed. γ-Tocopherol content ranged from 120.20 ± 13.06 µg/gram to 362.79 ± 13.62 µg/gram of oil and 36.06 ± 3.92 µg/gram to 127.74 ± 4.79 µg/gram of seed. Tocopherol content varied significantly among the various populations and in all populations, γ-tocopherol was found in higher concentrations than α-tocopherol. In general, the populations with the highest γ-tocopherol content were of Ghanaian origin while populations with the highest α-tocopherol content of Liberian origin. The average α-tocopherol content of griffonia seed oil is slightly lower than that of its close relative *Tylosema esculentum* while the average γ-tocopherol content of griffonia is slightly less than half that of *Tylosema esculentum* (Mitei et al., 2009). In comparison to other oils (**Table 7-7**), α-tocopherol content of griffonia seed oil was found to be slightly less than half that of soybean and olive oil, and about 1/8th that of sunflower oil (Zhang et al., 2016). γ-Tocopherol content of griffonia oil was less than 1/3 that of soybean oil and about 20 times and 37 times greater than olive oil and sunflower oil, respectively (Zhang et al., 2016). Griffonia seeds appear to be a much lower source

of α-tocopherol than other sources known to be rich in this micronutrient (National Institutes of Health, Office of Dietary Supplements) such as peanuts, almonds (Piironen et al., 1986), and raw sunflower seeds (Delgado-Zamarreno et al., 2001). Griffonia seeds appear to contain twice as much α-tocopherol than fresh spinach and four times as much α-tocopherol than lettuce (Piironen et al., 1986). Griffonia seeds appear to be much greater in γ-tocopherol content than almonds (Piironen et al., 1986) and raw sunflower seeds (Delgado-Zamarreno et al., 2001) and similar in γ-tocopherol content to peanuts (Piironen et al., 1986). It should be noted that during the Soxhlet extraction process, the tocopherols were subjected to prolonged exposure to light and elevated temperatures therefore it is possible that some of the tocopherol content may have been degraded by this process. It should also be noted that β-tocopherol and γ-tocopherol do not resolve well under reverse phase HPLC conditions (Xu, 2002) therefore β-tocopherol could also be present.

Table 7-2. Oil masses obtained from griffonia seeds of various origins

Ghana

Collection Site	**Seed material (grams)**	**Oil mass (grams)**	**Yield (%)**
Ashanti Region	7.1	2.2	31.99
Brong Ahafo Region	8.2	2.6	31.71
Eastern Region	8.2	2.6	31.71
Central Region	8.7	2.8	32.18

Liberia

Collection Site	**Seed material (grams)**	**Oil mass (grams)**	**Yield (%)**
Bargblor Town, Grand Gedeh County	7.0	2.2	31.43
Gaye Town, Grand Gedeh County	7.6	1.6	21.05
Tapita, Lower Nimba County	7.3	2.2	30.14
Unspecified Site 2, Nimba County	9.1	2.5	27.47
Unspecified Site 1, Nimba County	7.7	2.1	27.27
Upper Nimba County	7.0	2.1	30.00

Table 7-3. Griffonia seed fatty acid content per gram of seed oil for each Ghanaian and Liberian population

Ghana

Collection Site	Palmitic acid (mg/g)	Stearic acid (mg/g)	Oleic acid (mg/g)	Linoleic acid (mg/g)	Arachidic acid (mg/g)	Total FA's (mg/g)
Ashanti Region	79.54 ± 0.54	162.99 ± 1.86	82.72 ± 0.95	532.24 ± 0.40	48.28 ± 2.37	905.78 ± 3.23
Brong Ahafo Region	77.14 ± 0.09	158.84 ± 0.43	82.97 ± 0.19	528.45 ± 0.61	47.25 ± 0.39	894.65 ± 0.87
Central Region	72.13 ± 0.06	155.54 ± 0.17	116.14 ± 0.05	472.37 ± 0.49	45.87 ± 0.10	862.06 ± 0.53
Eastern Region	76.68 ± 0.15	164.21 ± 0.37	78.42 ± 0.35	538.11 ± 1.93	46.71 ± 0.59	904.12 ± 2.09
Average, all Ghanaian populations	**76.35 ± 0.28**	**160.39 ± 0.97**	**90.06 ± 0.51**	**517.79 ± 1.06**	**47.03 ± 1.24**	**891.65 ± 1.99**
Average, all griffonia seed populations	**78.50 ± 2.25**	**150.96 ± 6.49**	**87.23 ± 2.87**	**533.91 ± 9.33**	**44.13 ± 2.33**	**894.33 ± 12.16**

Liberia

Collection Site	Palmitic acid (mg/g)	Stearic acid (mg/g)	Oleic acid (mg/g)	Linoleic acid (mg/g)	Arachidic acid (mg/g)	Total FA's (mg/g)
Bargblor Town, Grand Gedeh County	78.26 ± 0.13	150.13 ± 0.18	89.50 ± 0.08	575.90 ± 0.83	46.12 ± 0.79	939.89 ± 1.17
Gaye Town, Grand Gedeh County	82.91 ± 6.80	148.04 ± 19.94	90.35 ± 8.68	587.34 ± 28.09	44.82 ± 5.02	953.46 ± 36.52
Tapita, Lower Nimba County	72.05 ± 0.08	135.79 ± 0.09	79.63 ± 0.18	492.08 ± 0.14	40.20 ± 0.08	819.75 ± 0.27
Unspecified Site 1, Nimba County	78.05 ± 0.003	149.64 ± 0.03	84.37 ± 0.07	559.28 ± 0.22	44.43 ± 0.27	915.78 ± 0.36
Unspecified Site 2, Nimba County	80.94 ± 1.96	149.16 ± 4.46	94.24 ± 2.41	587.63 ± 8.69	41.07 ± 4.52	953.04 ± 11.21
Upper Nimba County	87.29 ± 0.08	135.26 ± 0.06	74.00 ± 0.19	461.69 ± 0.32	36.54 ± 1.34	794.79 ± 1.40
Average, all Liberian populations	**79.92 ± 2.89**	**144.67 ± 8.34**	**85.35 ± 3.68**	**543.99 ± 12.01**	**42.20 ± 2.83**	**896.12 ± 15.61**
Average, all griffonia seed populations	**78.50 ± 2.25**	**150.96 ± 6.49**	**87.23 ± 2.87**	**533.91 ± 9.33**	**44.13 ± 2.33**	**894.33 ± 12.16**

Table 7-4. Griffonia seed fatty acid content per gram of seed for each Ghanaian and Liberian population

Ghana

Collection site	palmitic acid (mg/g)	stearic acid (mg/g)	oleic acid (mg/g)	linoleic acid (mg/g)	arachidic acid (mg/g)	Total FA's (mg/g)
Ashanti Region	24.65 ± 0.17	50.50 ± 0.58	25.63 ± 0.29	164.92 ± 0.13	14.96 ± 0.73	280.66 ± 1.00
Brong Ahafo	24.46 ± 0.03	50.36 ± 0.14	26.31 ± 0.06	167.56 ± 0.19	14.98 ± 0.13	283.67 ± 0.27
Central Region	23.22 ± 0.02	50.06 ± 0.05	37.38 ± 0.02	152.03 ± 0.16	14.76 ± 0.03	277.44 ± 0.17
Eastern Region	24.31 ± 0.05	52.07 ± 0.12	24.86 ± 0.11	170.62 ± 0.61	14.81 ± 0.19	286.67 ± 0.66
Average for Ghanaian seed populations	**24.16 ± 0.09**	**50.75 ± 0.31**	**28.54 ± 0.16**	**163.78 ± 0.34**	**14.88 ± 0.38**	**282.11 ± 0.62**
Average for griffonia seed populations	**23.64 ± 0.51**	**45.62 ± 1.43**	**26.37 ± 0.64**	**160.60 ± 2.12**	**13.31 ± 0.67**	**269.54 ± 2.77**

Liberia

Collection site	palmitic acid (mg/g)	stearic acid (mg/g)	oleic acid (mg/g)	linoleic acid (mg/g)	arachidic acid (mg/g)	Total FA's (mg/g)
Bargblor Town, Grand Gedeh County	24.59 ± 0.04	47.18 ± 0.06	28.13 ± 0.02	181.00 ± 0.26	14.49 ± 0.25	295.39 ± 0.37
Gaye Town, Grand Gedeh County	17.46 ± 1.43	31.17 ± 4.20	19.02 ± 1.83	123.65 ± 5.91	9.44 ± 1.06	200.73 ± 7.69
Tapita, Lower Nimba County	21.7 1± 0.02	40.92 ± 0.03	24.00 ± 0.05	148.30 ± 0.04	12.11 ± 0.02	247.05 ± 0.08
Unspecified Site 1, Nimba County	21.29 ±<0.01	40.81 ± 0.01	23.01 ± 0.02	152.53 ± 0.06	12.12 ± 0.07	249.76 ± 0.10
Unspecified Site 2, Nimba County	28.50 ± 0.69	52.52 ± 1.57	33.18 ± 0.85	206.91 ± 3.06	14.46 ± 1.59	335.58 ± 3.94
Upper Nimba County	26.19 ± 0.02	40.58 ± 0.02	22.20 ± 0.06	138.51 ± 0.10	10.96 ±0.40	238.44 ± 0.42
Average for Liberian seed populations	**23.29 ± 0.65**	**42.20 ± 1.83**	**24.92 ± 0.82**	**158.48 ± 2.72**	**12.26 ± 0.80**	**261.16 ± 3.53**
Average for griffonia seed populations	**23.64 ± 0.51**	**45.62 ± 1.43**	**26.37 ± 0.64**	**160.60 ± 2.12**	**13.31 ± 0.67**	**269.54 ± 2.77**

Table 7-5. Griffonia seed tocopherol content per gram of oil and per gram of seed for each Ghanaian and Liberian population

Ghana

Collection site	α-tocopherol content of oil (µg/g)	α-tocopherol content of seeds (µg/g)	γ-tocopherol content of oil (µg/g)	γ-tocopherol content of seeds (µg/g)
Ashanti Region	60.29 ± 2.96	18.68 ± 0.92	291.92 ± 10.14	90.45 ± 3.14
Brong Ahafo Region	61.44 ± 0.73	19.48 ± 0.23	335.97 ± 2.29	106.53 ± 0.72
Central Region	68.70 ± 1.80	22.11 ± 0.58	350.11 ± 3.30	112.68 ± 1.06
Eastern Region	57.11 ± 2.93	18.11 ± 0.93	300.89 ± 6.86	95.40 ± 2.18
Average of all Ghanaian populations	**61.88 ± 2.30**	**19.60 ± 0.72**	**319.72 ± 6.44**	**101.26 ± 2.02**
Average of all griffonia populations	**75.61 ± 4.77**	**22.62 ± 1.54**	**285.14 ± 13.31**	**86.58 ± 4.01**

Liberia

Collection site	α-tocopherol content of oil (µg/g)	α-tocopherol content of seeds (µg/g)	γ-tocopherol content of oil (µg/g)	γ-tocopherol content of seeds (µg/g)
Bargblor Town, Grand Geddeh County	102.00 ± 4.29	32.06 ± 1.35	252.02 ± 2.31	79.21 ± 0.72
Gayetown, Grand Gedeh County	121.21 ± 2.88	25.52 ± 0.61	299.77 ± 12.30	63.11 ± 2.59
Tapita, Lower Nimba County	107.43 ± 7.83	32.38 ± 2.36	279.03 ± 32.89	84.09 ± 9.91
Unspecified Site 1, Nimba County	60.62 ± 3.82	16.53 ± 1.04	258.66 ± 3.24	70.54 ± 0.88
Unspecified Site 2, Nimba County	117.31 ± 10.19	41.30 ± 3.59	362.79 ± 13.62	127.74 ± 4.79
Upper Nimba County	0	0	120.20 ± 13.06	36.06 ± 3.92
Average of all Liberian populations	**84.76 ± 5.87**	**24.63 ± 1.90**	**262.08 ± 16.35**	**76.79 ± 4.91**
Average of all griffonia populations	**75.61 ± 4.77**	**22.62 ± 1.54**	**285.14 ± 13.31**	**86.58 ± 4.01**

Table 7-6. Griffonia seed fatty acid content compared to the seeds of several of its closest relatives and other oils used for cooking

	Palmitic acid (%)	Stearic acid (%)	Oleic Acid (%)	Linoleic acid (%)	Arachidic acid (%)
Griffonia seed oil (as determined by this study)	**8.8**	**16.9**	**9.8**	**59.6**	**4.9**
Griffonia seed oil (as reported by Petkow and Ramazanov, 2003)	**9-11**	**16-18**	**10-11**	**53-60**	**3-4**
Bauhinia petersiana seed oil (Ketshajwang et al., 1998)	16.35	6.80	26.28	44.82	0.52
Bauhinia purpurea seed oil (Ramadan et al., 2006)	22.1 ± 0.88	13.6 ± 0.36	16.3 ± 0.39	45.9 ± 1.25	0.26 ± 0.07
Cercis siliquastrum seed oil (Khatiashvili and Kemertelidze, 2007)	5.8	1.7	21.8	66.4	Trace
Tylosema esculentum seed oil (Mitei et al., 2008)	12.93 ± 0.06	8.82 ± 0.12	47.27 ± 0.43	23.40 ± 0.42	3.31 ± 0.03
Palm oil (Wirasnita et al., 2013)	36.85 ± 0.24	2.97 ± 0.06	41.90 ± 0.13	16.09 ± 0.23	N/D
Soybean oil (Li et al., 2016)	5.80 ± 1.01	8.28 ± 0.24	31.55 ± 0.77	45.00 ± 0.31	N./D
Peanut oil (Li et al., 2016)	6.23 ± 0.30	2.95 ± 0.11	68.49 ± 2.06	18.28 ± 0.79	N./D
Sesame oil (Li et al., 2016)	5.14 ± 0.78	5.14 ± 1.09	43.44 ± 1.05	43.81 ± 2.06	N./D
Sunflower oil (Jabeur et al., 2014)	6.53 ± 0.08	3.64 ± 0.04	27.58 ± 0.33	61.54 ± 0.75	0.26 ± 0.00

Table 7-7. Griffonia seed tocopherol content per gram of seed oil and seed compared to other oils and tocopherol rich seeds, lettuce and spinach

	α-tocopherol content of oil (µg/g)	α-tocopherol content of seeds (µg/g)	γ-tocopherol content of oil (µg/g)	γ-tocopherol content of seeds (µg/g)
Griffonia seeds (as determined by this study)	**75.61 ± 4.77**	**22.62 ± 1.54**	**285.14 ± 13.31**	**86.58 ± 4.01**
Tylosema esculentum seeds (Mitei et al., 2009)	81.43 ± 0.74	N/D	117.27 ± 0.50	N/D
Soybean oil (Zhang et al., 2016)	179.6 ± 17.8	N/D	747.3 ± 70.9	N/D
Virgin olive oil (Zhang et al., 2016)	156.8 ± 19.7	N/D	13.4 ± 4.4	N/D
Sunflower oil (Zhang et al., 2016)	546.7 ± 48.9	N/D	7.7 ± 2.2	N/D
Sunflower seeds, raw (Delgado-Zamarreno et al., 2001)	N/D	227.0 ± 6.0	N/D	7.1 ± 1.1
Almond (Piironen et al., 1986)	N/D	264.4	N/D	7.6
Peanut (Piironen et al., 1986)	N/D	108.9	N/D	83.9
Spinach (Piironen et al., 1986)	N/D	12.2	N/D	N/D
Lettuce (Piironen et al., 1986)	N/D	6.3	N/D	3.4

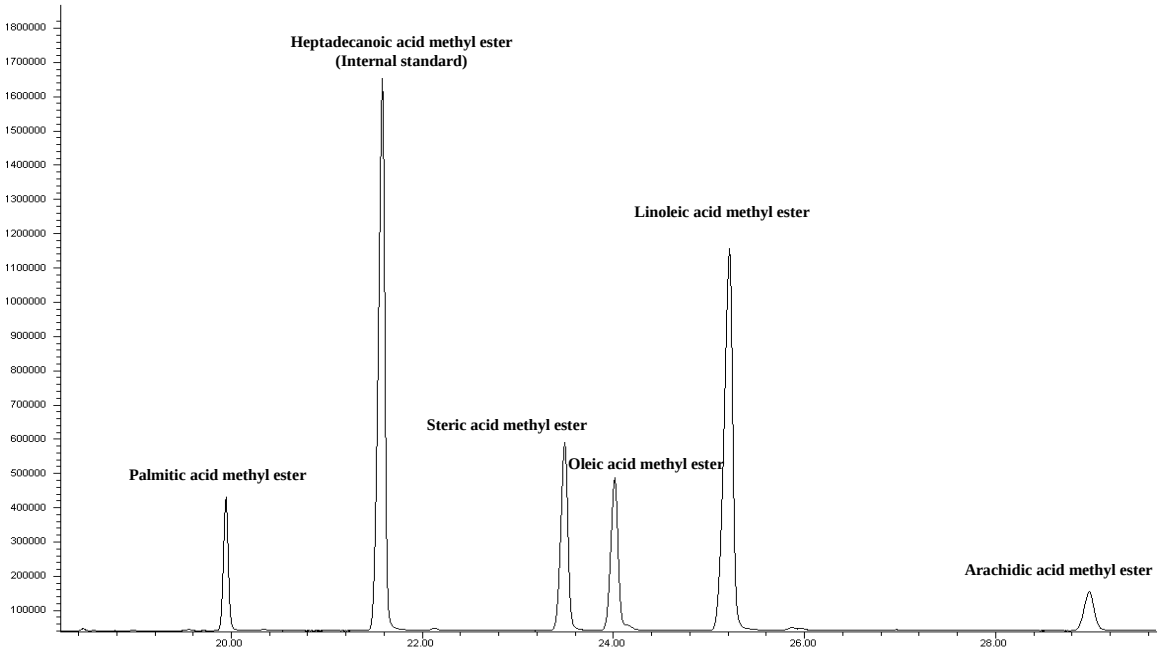


Figure 7-12. Representative GC Chromatogram depicting fatty acid methyl esters of griffonia seeds from Central Region, Ghana

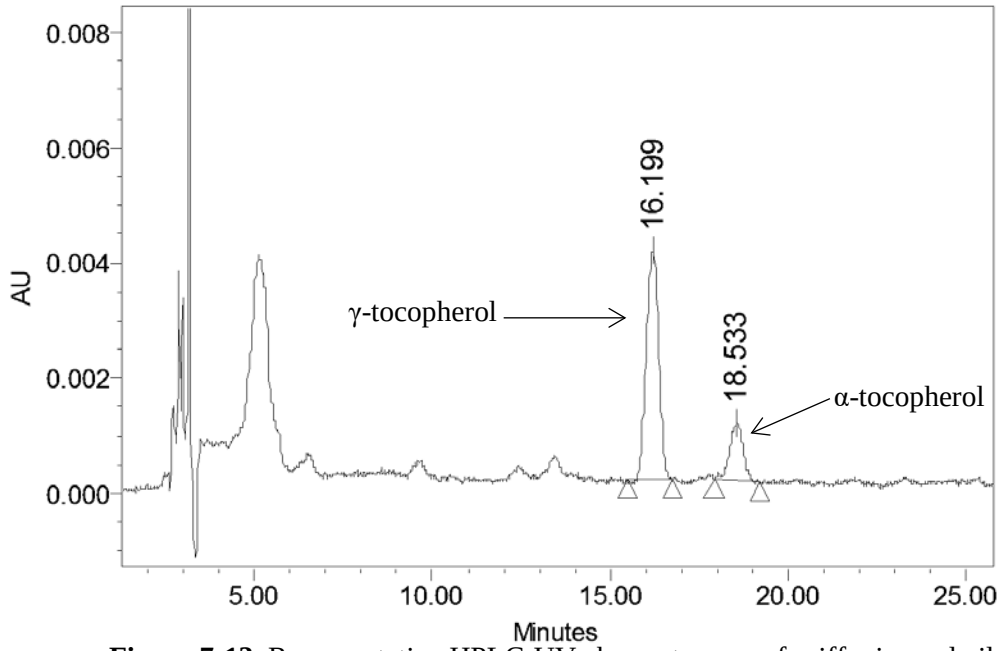
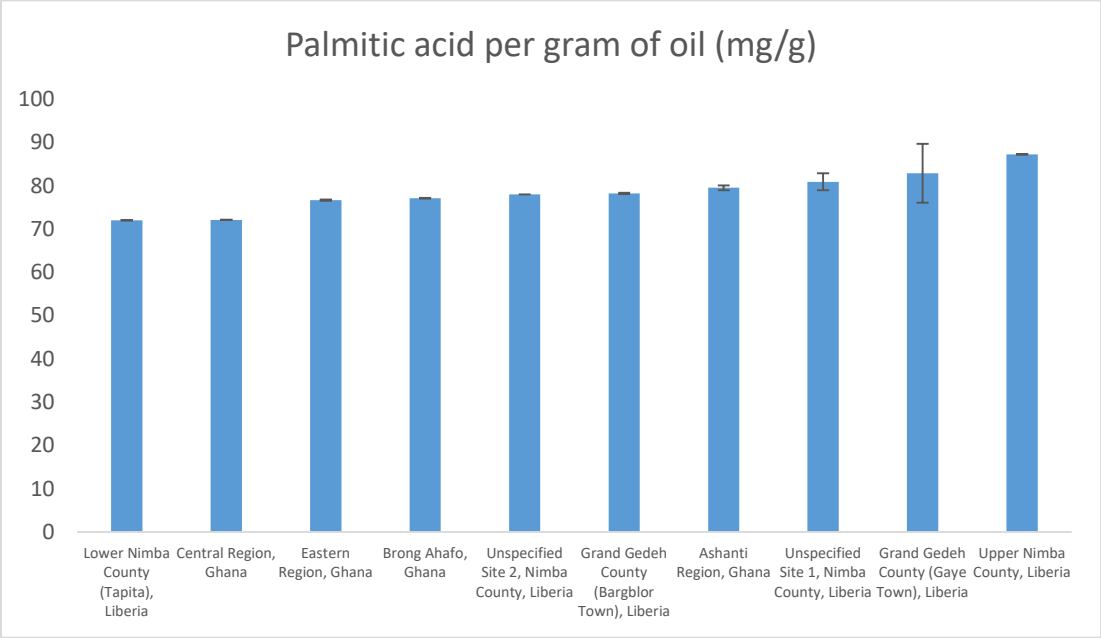
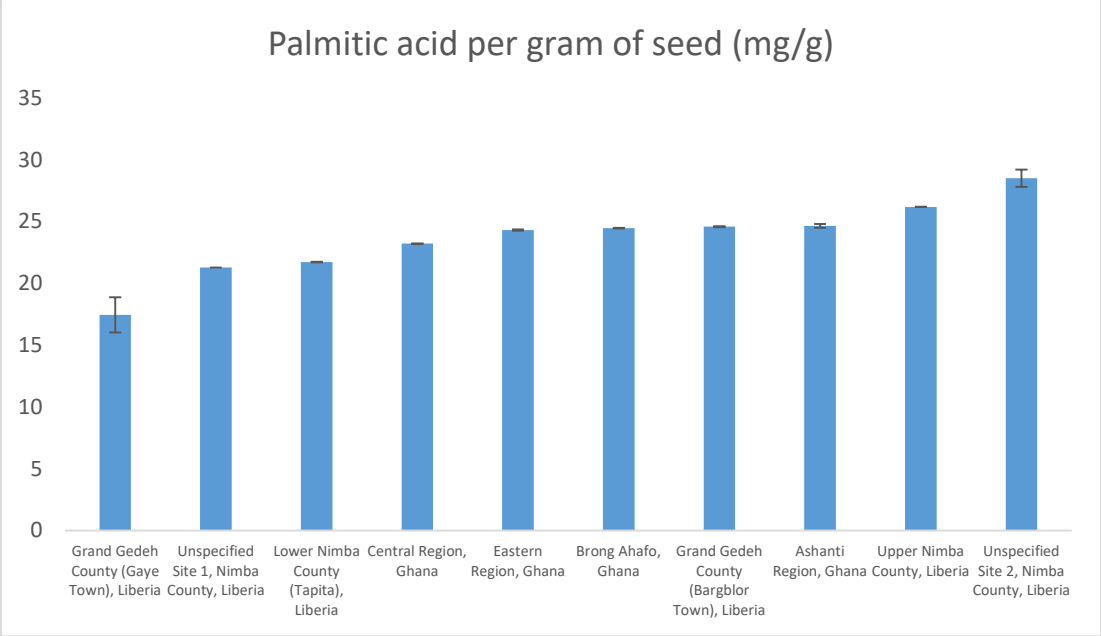


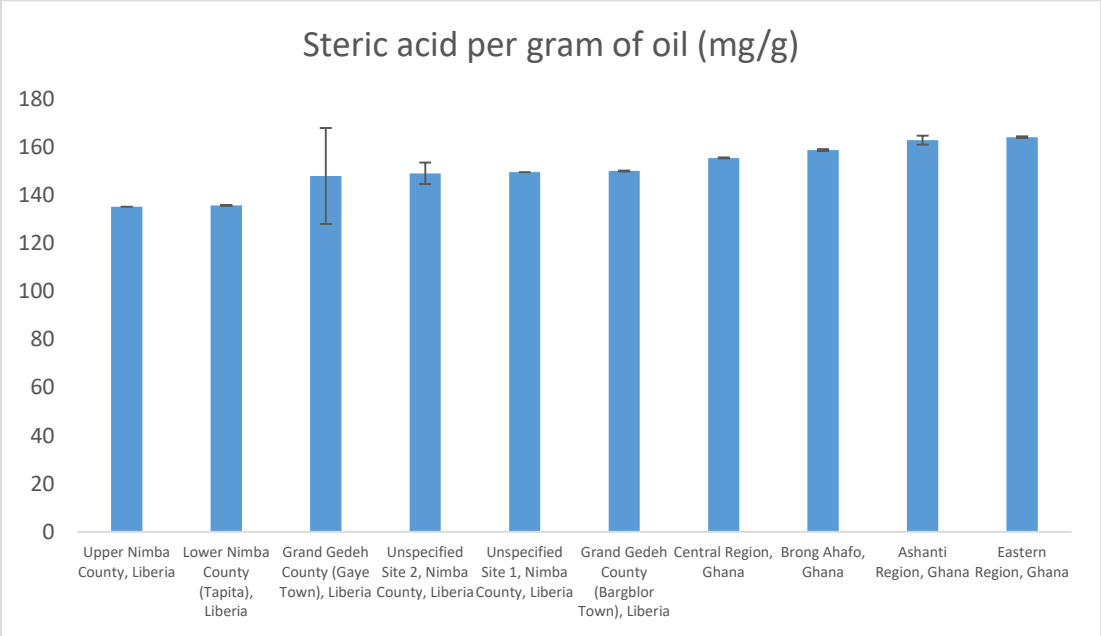
Figure 7-13. Representative HPLC-UV chromatogram of griffonia seed oil at 290nm depicting α-tocopherol and γ-tocopherol



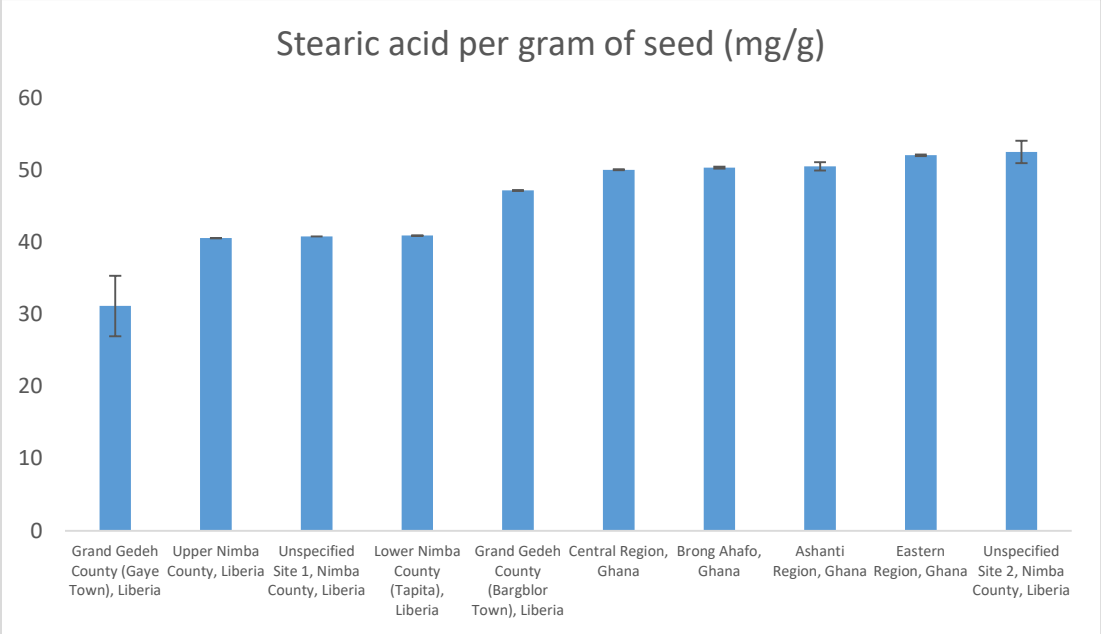
Graph 7-1. Palmitic acid content per gram of seed oil



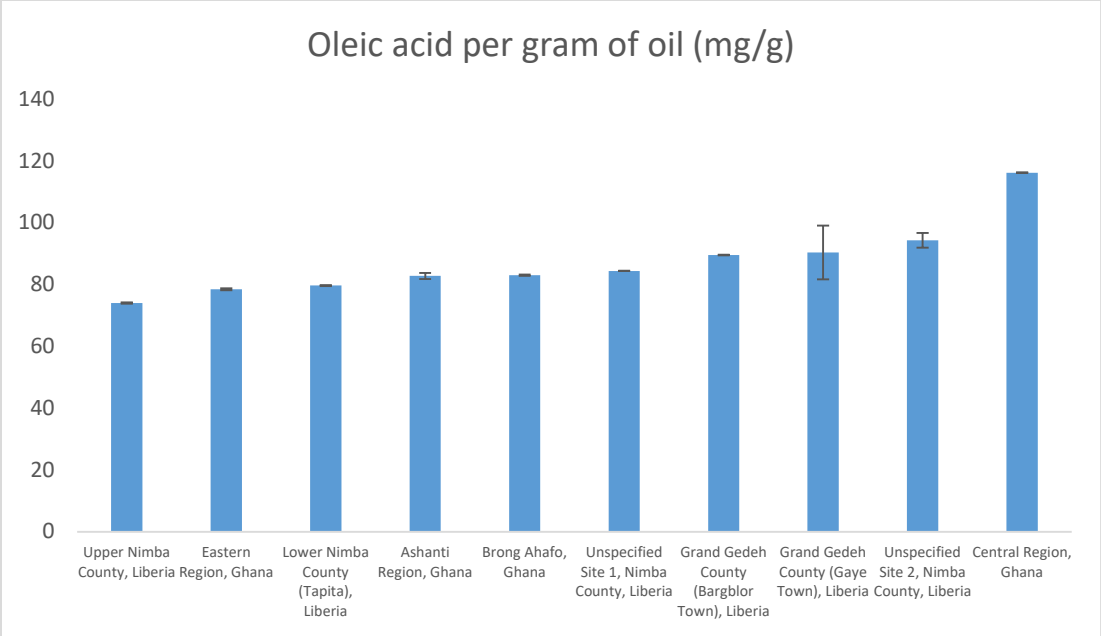
Graph 7-2. Palmitic acid content per gram of seed



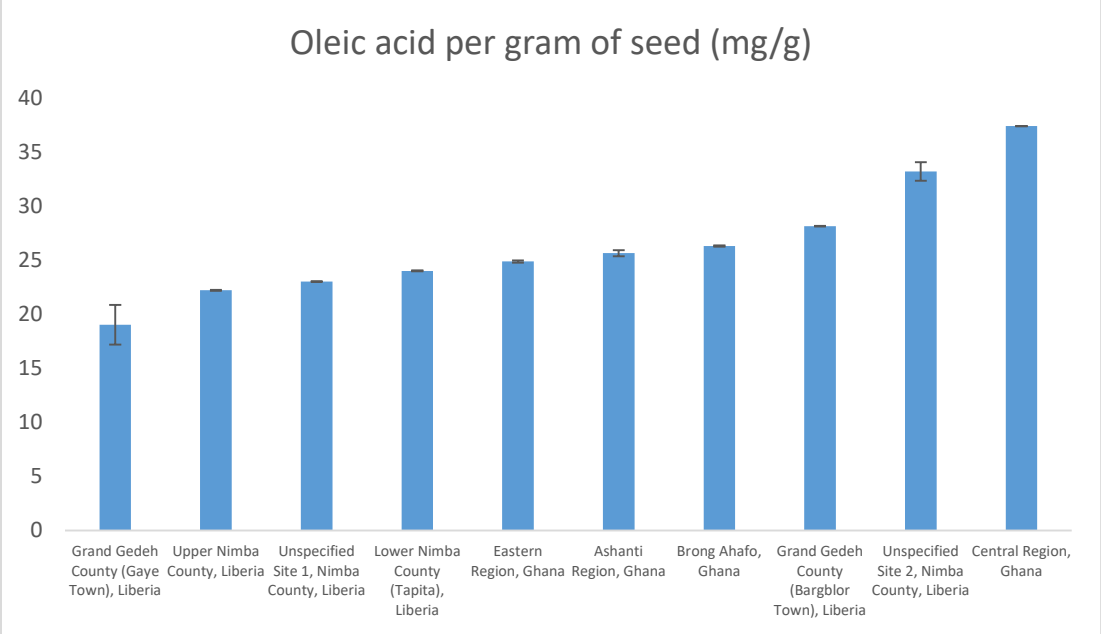
Graph 7-3. Stearic acid content per gram of seed oil



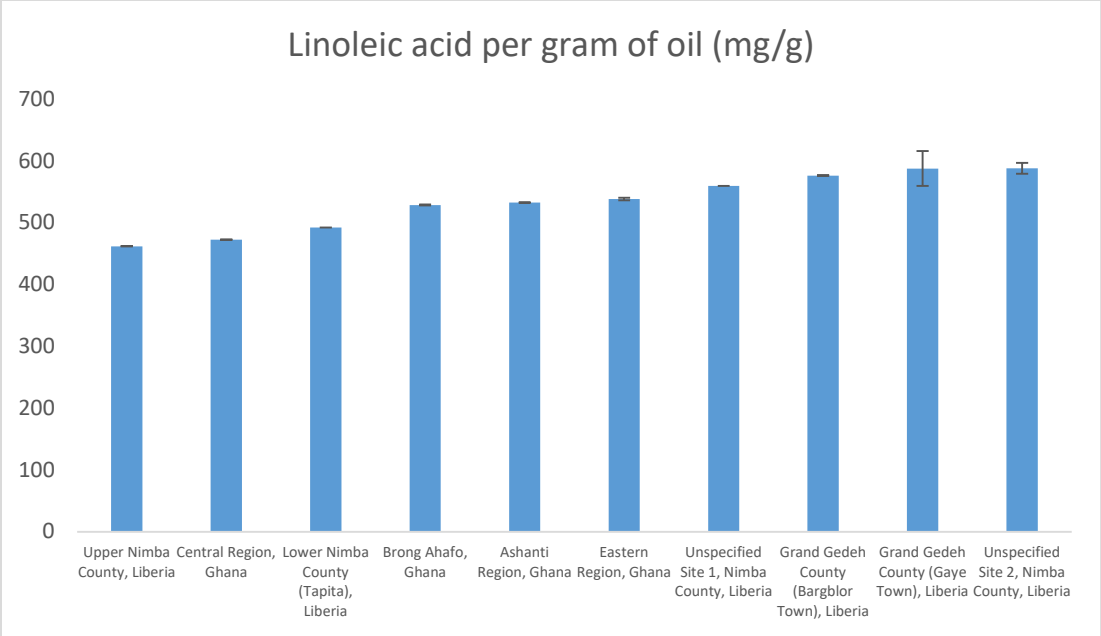
Graph 7-4. Stearic acid content per gram of seed



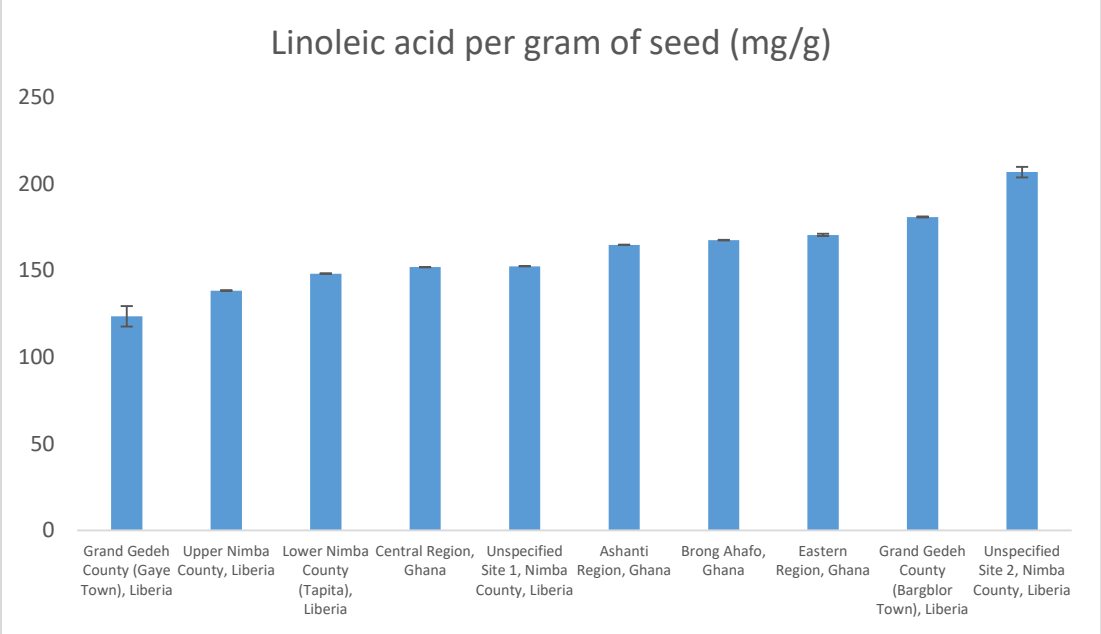
Graph 7-5. Oleic acid content per gram of seed oil



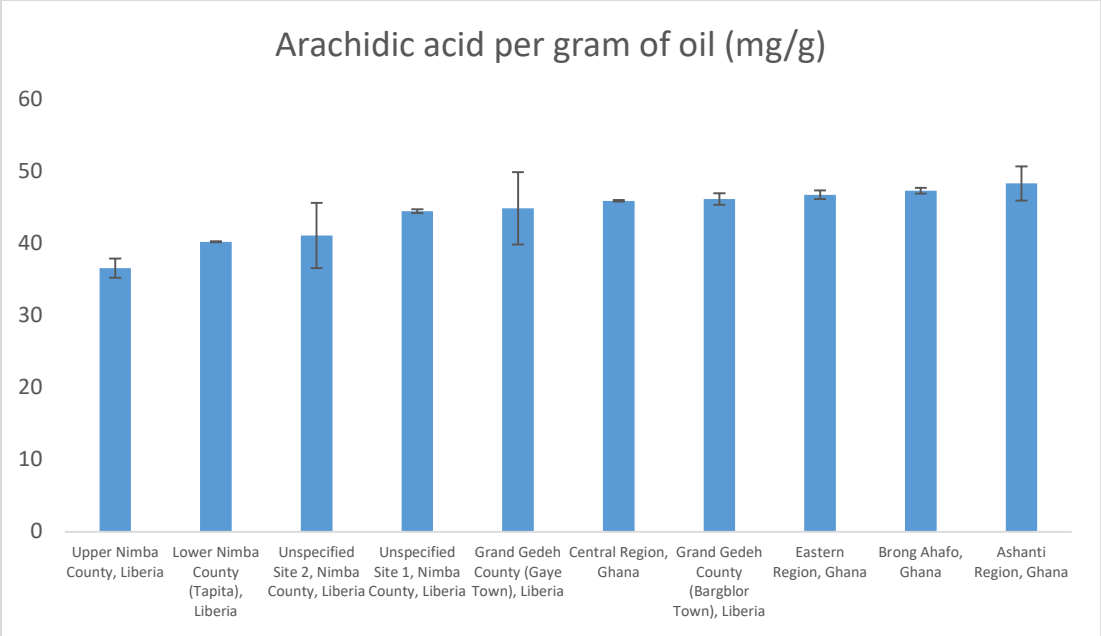
Graph 7-6. Oleic acid content per gram of seed



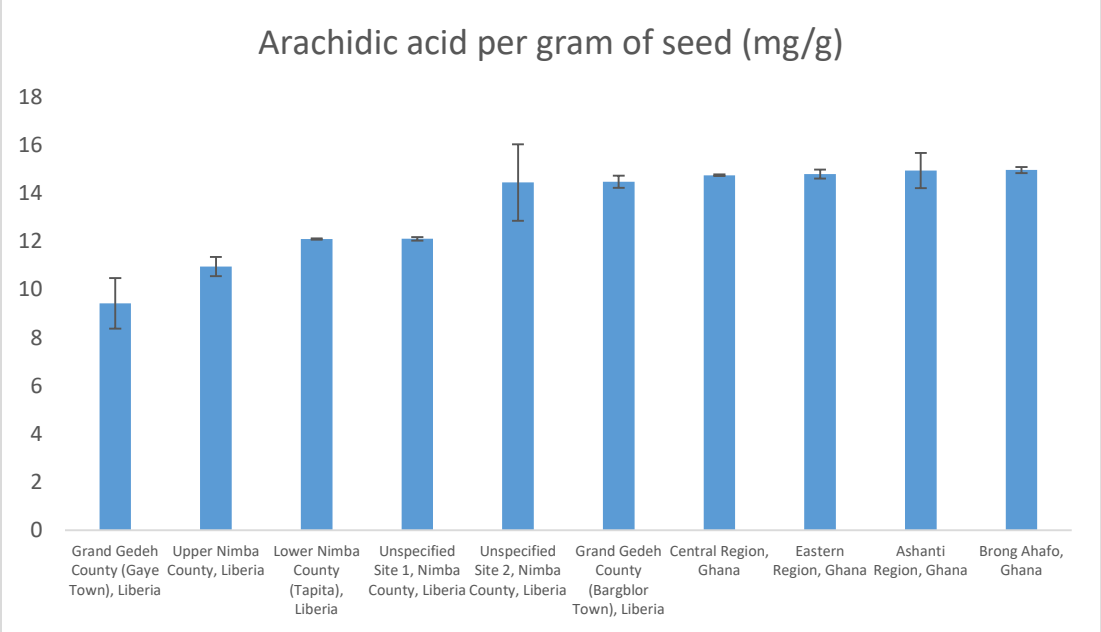
Graph 7-7. Linoleic acid content per gram of seed oil



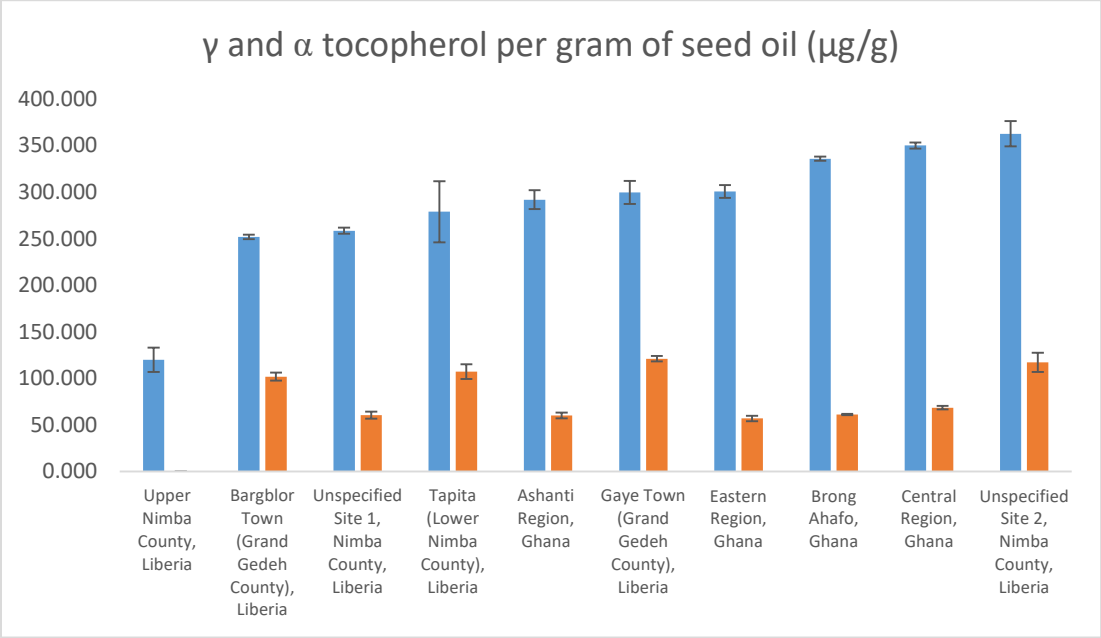
Graph 7-8. Linoleic acid content per gram of seed



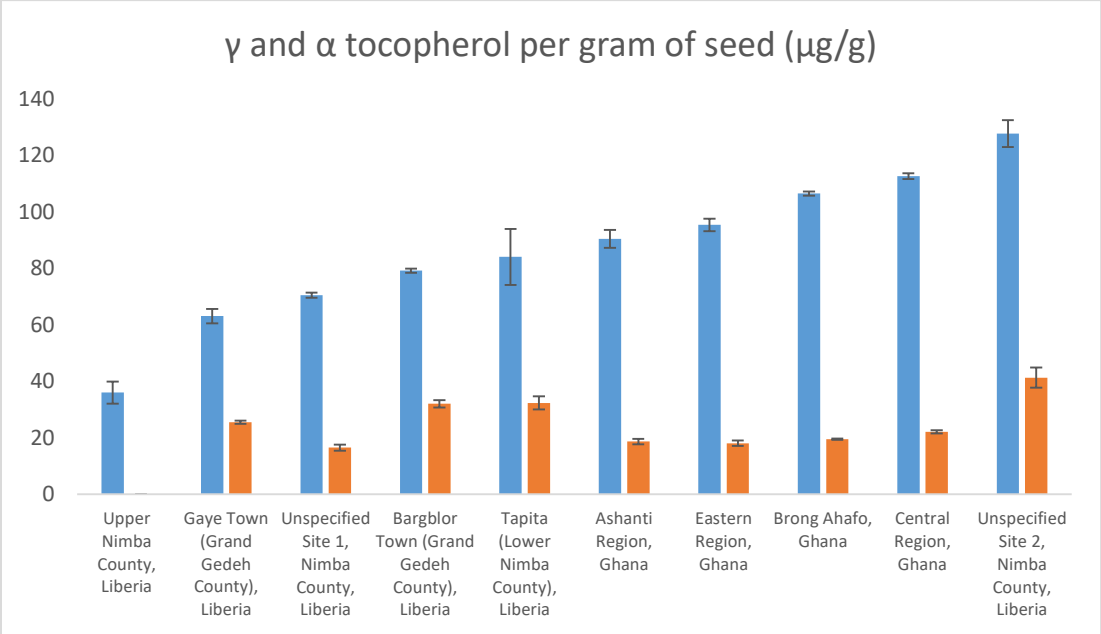
Graph 7-9. Arachidic acid content per gram of seed oil



Graph 7-10. Arachidic acid content per gram of seed



Graph 7-11. γ-Tocopherol (blue) and α-tocopherol (red) content per gram of seed oil (µg/g)



Graph 7-12. γ-Tocopherol (blue) and α-tocopherol (red) content per gram of seed

7.4 Conclusions

The purpose of this study was to quantify and compare fatty acid and tocopherol content of griffonia seeds from various regions of Ghana and Liberia. Fatty acid content did not vary significantly among the various populations and relative ratios of these fatty acids were consistent with that was reported for this species previously (Petkov and Ramazanov, 2003). In all cases, linoleic acid was the dominant lipid, accounting for 53% of griffonia seed oil mass and and 16% of the total mass of the seed. This is in agreement with what has been reported for other members of the Leguminosae family (Grela and Gunter, 1995; Ryan et al., 2007) especially in the Caesalpinoideae subfamily (Bagci and Sahin, 2004). According to Petkov and Ramazanov (2003) griffonia seed oil is a rich source of linoleic acid not only because it is high in content of this fatty acid, but also because the relatively lower content of oleic acid allows for seed oil extracts to be easily enriched with this essential fatty acid. Saturated fatty acids comprised of a larger proportion of griffonia oil than soybean, peanut, sesame, and sunflower oil. Since oils with lower saturated fatty acid content have been associated with lower cardiovascular risks (Kabagame et al., 2005), interest in using griffonia oil as an alternative cooking oil is unlikely to materialize.

Griffonia seeds appear to be much lower in α-tocopherol content than other sources known to be rich in this form of vitamin E such as sunflower seeds, almonds and peanuts. It should be noted however that the total vitamin E content of griffonia seeds may be underestimated in this study for two reasons. During the Soxhlet extraction process, fixed oils were subjected to prolonged exposure to heat and light which are known to degrade tocopherols and tocotrienols. Additionally, no attempt was made to identify or quantify tocotrienol content. Since the solubility properties of 5-HTP are significantly different than those of fatty acids and tocopherols, a modified extraction process employing Soxhlet extraction using hexane could effectively separate these lipophilic substances from 5-HTP without loss of this value commodity.

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Chapter 8. Inorganic Mineral Composition of *Griffonia simplicifolia* Seeds from Various Origins in Ghana and Liberia

8.1 Introduction

Minerals are important inorganic nutrients that the body requires for healthy growth and proper cellular and metabolic function. Calcium is of one of the most important dietary minerals as it has a role in assuring proper health. Along with sodium and potassium, calcium serves as an important electrolyte that has a crucial role in regulating osmotic pressure between intracellular and extracellular compartments as well as intercellular electrochemical potentials (Sawka and Montain, 2000). Calcium deficiency has been linked to impaired growth, delayed skeletal consolidation, rickets, vitamin D deficiency, and osteoporosis (Nordin, 1997). In the developed world, deficiency of calcium and other minerals is very rare. Yet, in many other parts of the world where undernutrition is common and quality health care is less accessible, this issue becomes more prevalent. Phosphorus deficiency has also been linked to osteoporosis as well as changes in energy metabolism (Shapiro and Heaney, 2003). Hypomagnesemia can result in arrhythmias, seizures, paralysis, and cardiac arrest (Topf and Murray, 2003) while in severe cases of low potassium, muscle necrosis, muscle paralysis, and respiratory function impairment can develop as the body breaks down tissue to release more potassium into the blood serum (Gennari, 1998). Individuals with zinc deficiency have an increased susceptibility to malaria and other infections as well as neurological issues (Hambidge, 2000) while iron deficiency has been linked to impaired neurological development in infants as well as low birthweight in newborns (Sandberg, 2002).

The purpose of this study was to determine the abundances of minerals in griffonia seeds from various Ghanaian and Liberian populations and to see if griffonia seeds could be used as an unconventional dietary source of mineral nutrients in those areas where the crop is wildcrafted and seeds collected from the forest floor. While griffonia seeds are not commonly consumed, a high mineral content may spur an interest in incorporating these seeds into the diet, especially because this species is present in many areas of Sub Saharan Africa where malnutrition is

prevalent. If griffonia seeds are indeed high source of minerals, this crop may also prove useful as a feed for livestock or to enrich soils with nutrients.

8.2 Experimental

8.2.1 Materials

HCl, water, and griffonia seeds from various regions of Ghana and Liberia as listed in **Table 5-1** were subjected to mineral elemental analysis.

8.2.2 Equipment

Muffle furnace and an Inductively Coupled Plasma Optical Emission Spectrometer was used for mineral elemental analysis. Samples were sent to the Agricultural Analytical Services Lab at Pennsylvania State University and analyzed for nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, manganese, ion, copper, boron, aluminum, and zinc by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP).

8.2.3 Method

Approximately 200 mg of seed material was placed in a crucible and heated to 500 ºC for two hours. After heating, ash was dissolved in 10 mL of 1N HCl and analyzed by ICP (Miller, 1998).

8.3 Results and discussion

The purpose of this study was to determine the mineral content of griffonia seeds (**Table 8-1** and **Graphs 8-1 to 8-13**) and to determine if contain high enough amounts of these minerals to be considered for use against malnutrition. Based on the *Codex Alimentarius* Guidelines on Nutritional Labeling CAC/GL 2-1985 and Guidelines for Use of Nutrition and Health Claims CAC/GL-23-1997 (Web addresses are listed in reference section) which defines the criteria for a food to be considered a source or high source of a mineral or other nutrient, griffonia seeds met

the criteria to be considered a high source of iron, zinc, magnesium, copper, and phosphorus (**Table 8-3**) Additionally, griffonia seeds met the criteria to be considered a source of manganese but did not meet the criteria to be a source of calcium.

Among the various seeds populations, the most significant differences in mineral content was their aluminum and sodium content which ranged from 12 to 68 ppm and 14 to 74 ppm, respectively with the Liberian populations being overall significantly higher in aluminum than the Ghanaian populations. Phosphorus, potassium, calcium, copper, and boron did not vary significantly among all Ghanaian and Liberian populations. Overall, the Liberian populations were higher in manganese, aluminum, and sodium than Ghanaian populations and Liberian populations overall tended to be higher in minerals than Ghanaian populations. Factors such as access to soil nutrients, genetics, and biotic and abiotic stressors could have had a role in these results. Griffonia seeds appear to contain a slightly higher amount of phosphorus than most plants where phosphorus averages approximately 0.2% dry weight (Schachtman et al., 1998). Boron which may have an important role in metabolic enzyme activity and in the metabolism of hormones and mineral nutrients (Devirian and Volpe, 2003) is within the general range for legumes (Devirian and Volpe, 2003). Aluminum, which does not appear to have any biological function in humans (Soni et al., 2001), was found in similar concentrations to many other commonly consumed foods (Sont et al., 2001; Stahl et al., 2011) and thus griffonia seeds are not believed to contain toxic amounts of this element. Sodium content of griffonia seeds was found to range among foods that are typically associated with being low in sodium such as lentils (USDA online: Lentils, Raw) and tomatoes (USDA online: Tomatoes, red, ripe). The copper content of griffonia seeds appears to be approximately three times greater than most others plant species (Yruela, 2005) although this is not surprising as legumes are often cited for having a high content of this mineral. The electronic properties of copper are such that this element readily catalyzes the formation of reactive oxygen species that can damage proteins, lipids, and DNA

thus excess consumption of copper can be a health risk. It is recommended that intake of copper does not exceed 5 mg per day (Xiong and Wang, 2005).

Legumes are often regarded as having a high content of iron, zinc, and magnesium however legumes are also known to contain large amounts of metal binding antinutrients such as phytate, lectins, protease inhibitors, polyphenols, ogliosaccharides, (Sandberg, 2002) and tannins (Beebe et al., 2000). These molecules interfere with the absorption of these minerals by chelating to them to form complexes that the body cannot absorb (Sandberg, 2002; Flyman and Afolayan, 2006). If interest was to arise in using griffonia as a new source of mineral nutrients, radiolabel studies could be used to assess the bioavailability of minerals from this source. If studies find that mineral absorption is significantly inhibited by the presence of these antinutrients, the addition of vitamin C and/or phytases may be useful in blocking the formation of these metal chelates (Sandberg, 2002).

Ghana

Table 8-1. Inorganic mineral content of griffonia seed populations from Liberia and Ghana

Collection site	**N %**	**P %**	**K %**	**Ca %**	**Mg %**	**S %**	**Mn ppm**	**Fe ppm**	**Cu ppm**	**B ppm**	**Al ppm**	**Zn ppm**	**Na ppm**
Ashanti Region	5.40	0.28	1.61	0.10	0.20	0.18	5	47	29	26	23	30	14
Brong Ahafo Region	5.70	0.29	1.65	0.09	0.19	0.19	5	51	30	25	18	34	14
Central Region	5.56	0.26	1.56	0.09	0.19	0.18	6	46	30	22	22	40	22
Eastern Region	5.62	0.29	1.65	0.10	0.20	0.19	6	44	31	23	14	34	18
Average of all Ghanaian populations	**5.57**	**0.28**	**1.6175**	**0.095**	**0.195**	**0.185**	**5.5**	**47**	**30**	**24**	**19.25**	**34.5**	**17**
Average, all *Griffonia simplicifolia* populations	**5.695**	**0.306**	**1.737**	**0.101**	**0.205**	**0.195**	**7.7**	**51.7**	**29**	**24.8**	**32.6**	**35.3**	**29.2**

Liberia

Collection site	**N %**	**P %**	**K %**	**Ca %**	**Mg %**	**S %**	**Mn ppm**	**Fe ppm**	**Cu ppm**	**B ppm**	**Al ppm**	**Zn ppm**	**Na ppm**
Bargblor Town, Grand Gedeh County	5.74	0.32	1.82	0.11	0.23	0.20	11	73	32	24	68	40	40
Gaye Town, Grand Gedeh County	5.60	0.28	1.75	0.10	0.20	0.18	7	33	22	26	12	26	30
Tapita, Lower Nimba County	5.72	0.34	1.93	0.10	0.22	0.21	9	51	29	26	31	36	30
Unspecified site 1 Nimba County	5.84	0.33	1.82	0.11	0.20	0.21	10	66	30	23	53	36	74
Unspecified Site 2 Nimba County	6.02	0.35	1.83	0.10	0.21	0.20	8	61	28	28	48	33	20
Upper Nimba County	5.75	0.32	1.75	0.11	0.21	0.21	10	45	29	25	37	44	30
Average of all Liberian populations	**5.778**	**0.323**	**1.817**	**0.105**	**0.212**	**0.202**	**9.17**	**54.8**	**28.33**	**25.33**	**41.50**	**35.83**	**37.33**
Average, all *Griffonia simplicifolia* populations	**5.695**	**0.306**	**1.737**	**0.101**	**0.205**	**0.195**	**7.7**	**51.7**	**29**	**24.8**	**32.6**	**35.3**	**29.2**

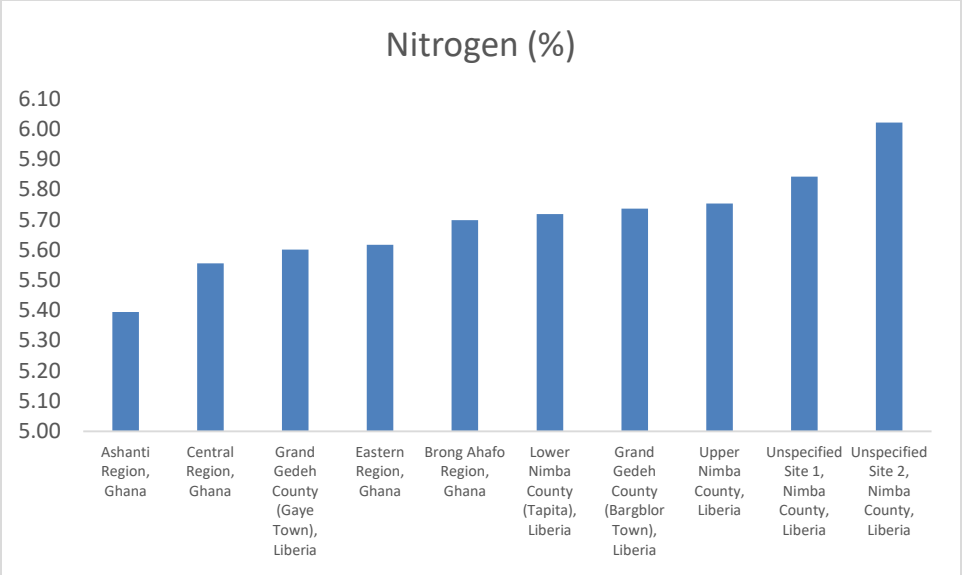
Table 8-2. Inorganic mineral content of Griffonia seeds compared to other species

	N %	**P %**	**K %**	**Ca %**	**Mg %**	**S %**	**Mn ppm**	**Fe ppm**	**Cu ppm**	**B ppm**	**Al ppm**	**Zn ppm**	**Na ppm**
Average, all *Griffonia simplicifolia* populations	**5.695**	**0.306**	**1.737**	**0.101**	**0.205**	**0.195**	**7.7**	**51.7**	**29**	**24.8**	**32.6**	**35.3**	**29.2**
Amaranthus tricolor (Shukla et al., 2006)	N/D	N/D	3.7	1.7	2.9	N/D	108.1	1233.8	N/D	N/D	N/D	791.7	N/D
Moringa oleifera leaves (Ogbe and Affiku, 2011)	N/D	0.003	0.97	1.91	0.38	N/D	81.65	107.48	6.10	N/D	N/D	60.06	192.95
Soybean (USDA online)	N/D	0.194	0.620	0.197	0.065	N/D	N/D	35.5	N/D	N/D	N/D	9.9	150

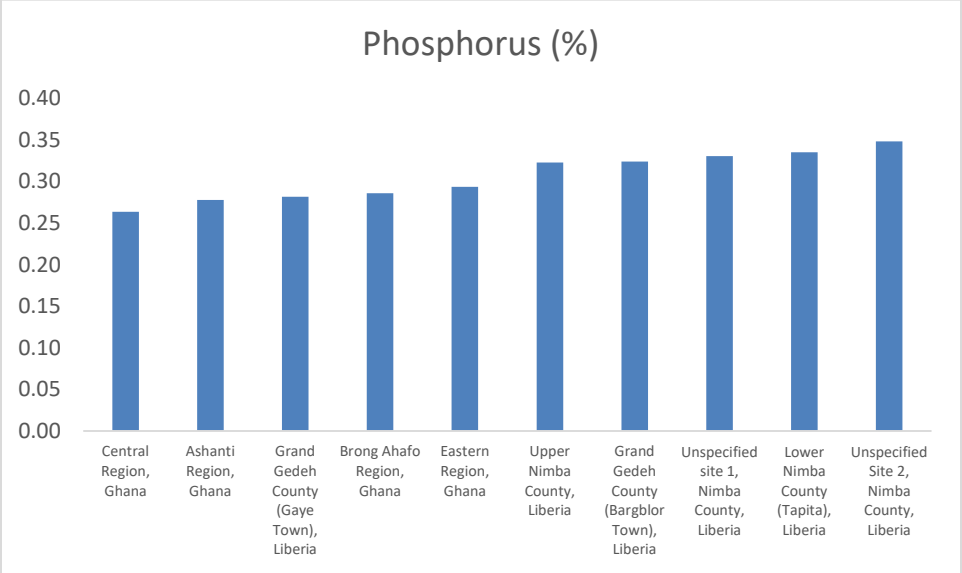
Table 8-3. Mineral content of griffonia seeds and content required to be a mineral source*

	Content of mineral in griffonia seeds (ppm)	Content of mineral in griffonia seeds (mg/100g)	Content to be considered a source of mineral* (mg/100g)	Content to be considered a high source of mineral* (mg/100g)
Fe	51.7	5.17	>2.1	>4.2
Zn	35.3	3.53	>1.65	>3.3
Ca	1010	101	>150	>300
Mg	2050	205	>46.5	>93
Mn	7.7	0.77	>0.45	>0.9
Cu	26	2.6	>0.135	>0.270
P	3060	306	>105	>210

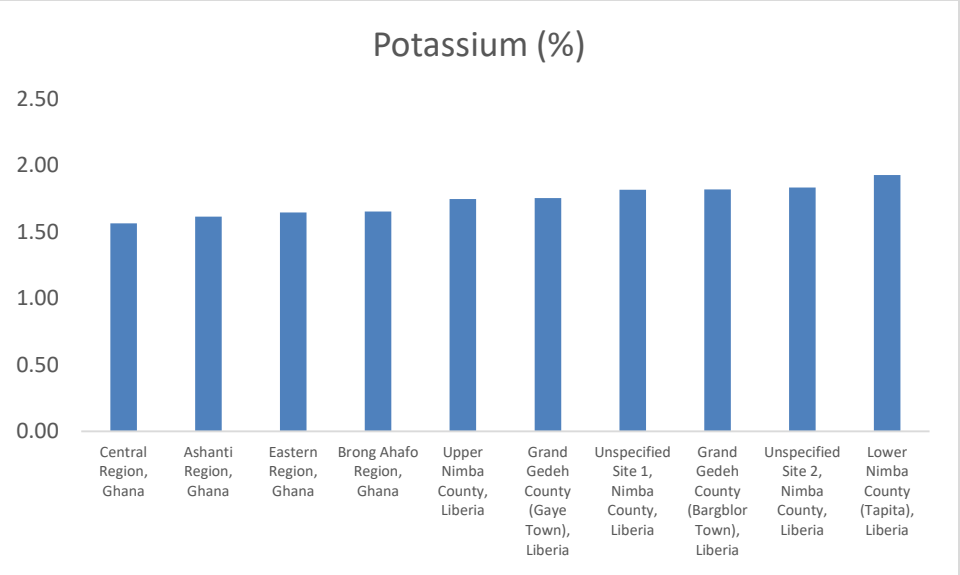
*Content required to be a source of a particular mineral was based on Based on the *Codex Alimentarius* Guidelines on Nutritional Labeling CAC/GL 2-1985 and Guidelines for Use of Nutrition and Health Claims CAC/GL-23-1997 (http://www.fao.org/fao-who-codexalimentarius/standards/list-of-standards/en/).



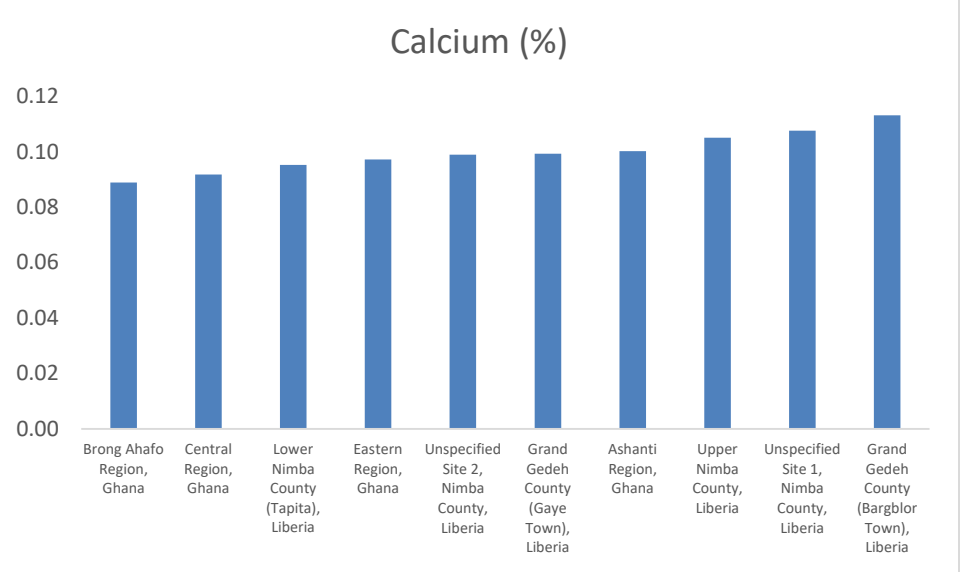
Graph 8-1. Nitrogen content of griffonia seed populations from Liberia and Ghana



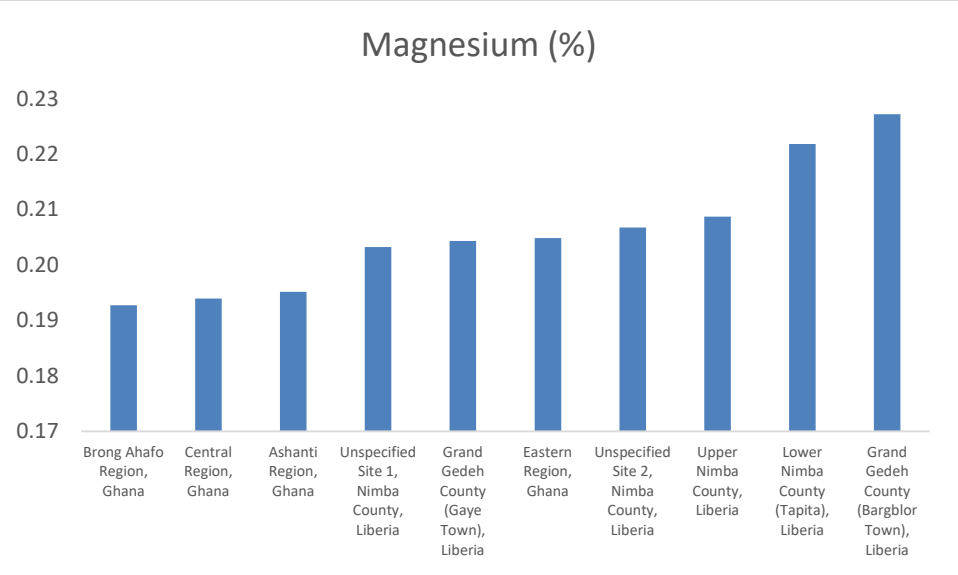
Graph 8-2. Phosphorus content of griffonia seed populations from Liberia and Ghana



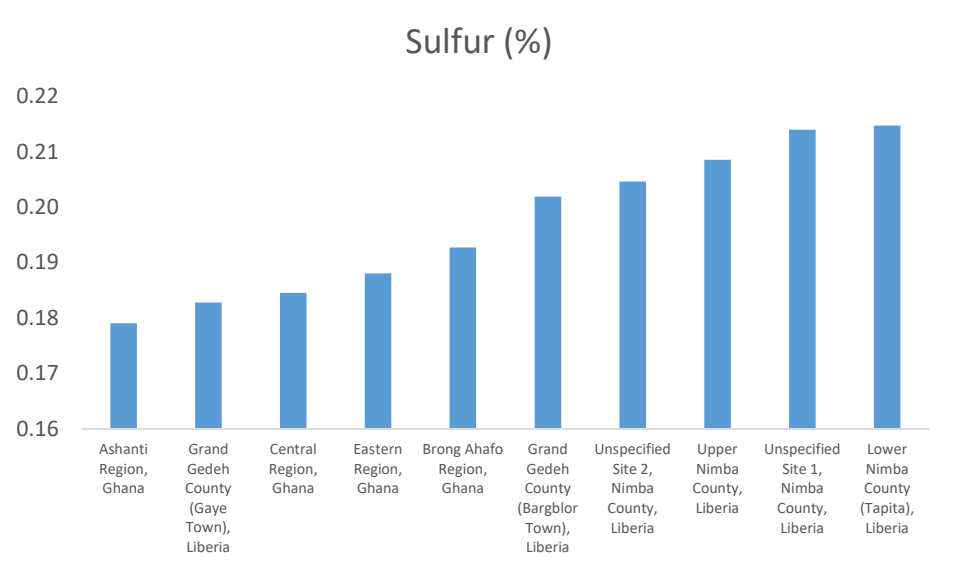
Graph 8-3. Potassium content of griffonia seed populations from Liberia and Ghana



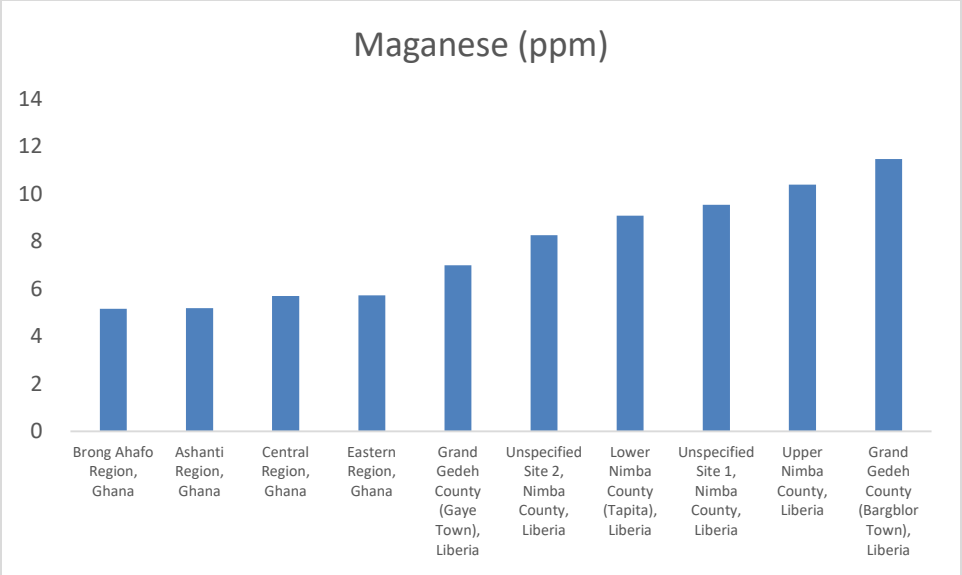
Graph 8-4. Calcium content of griffonia seed populations from Liberia and Ghana



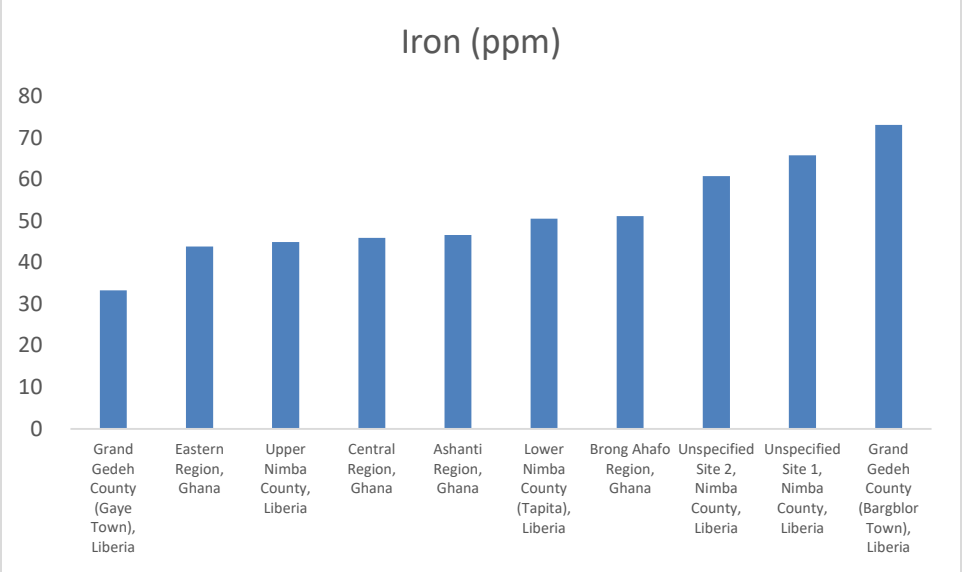
Graph 8-5. Magnesium content of griffonia seed populations from Liberia and Ghana



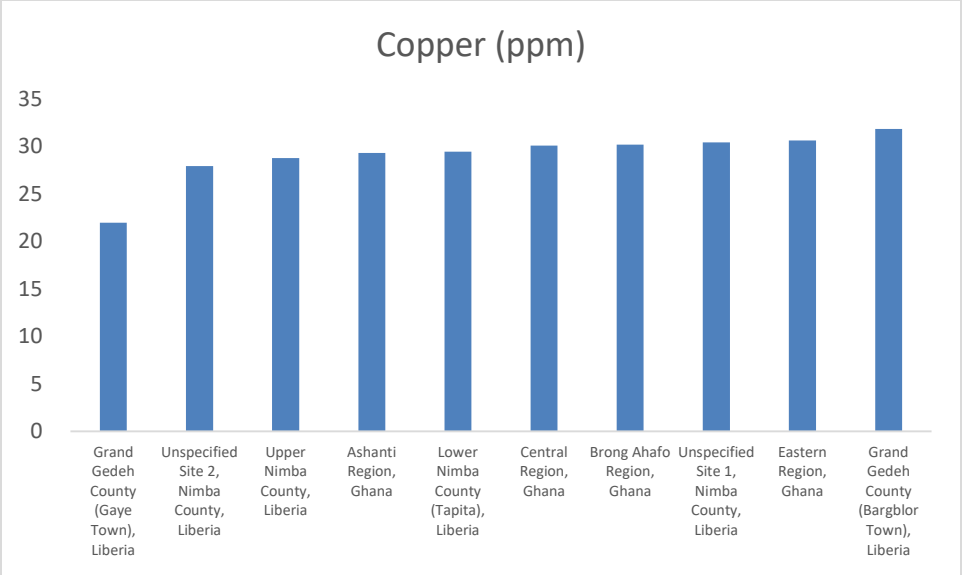
Graph 8-6. Sulfur content of griffonia seed populations from Liberia and Ghana



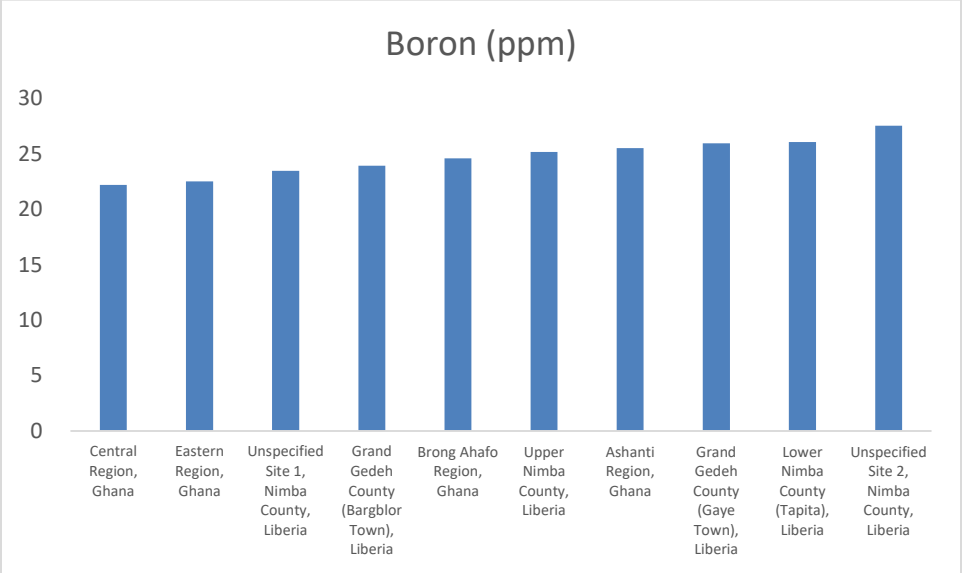
Graph 8-7. Manganese content of griffonia seed populations from Liberia and Ghana



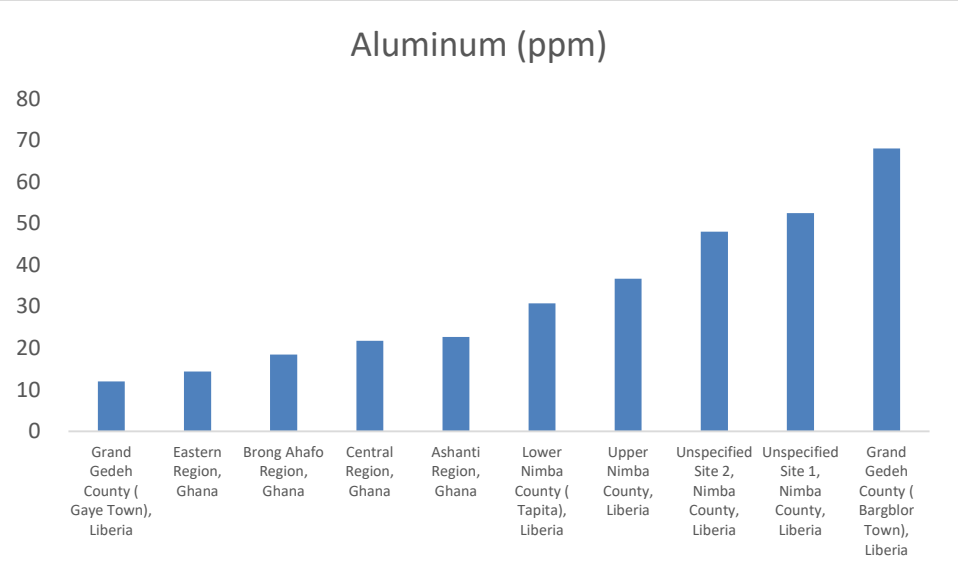
Graph 8-8. Iron content of griffonia seed populations from Liberia and Ghana



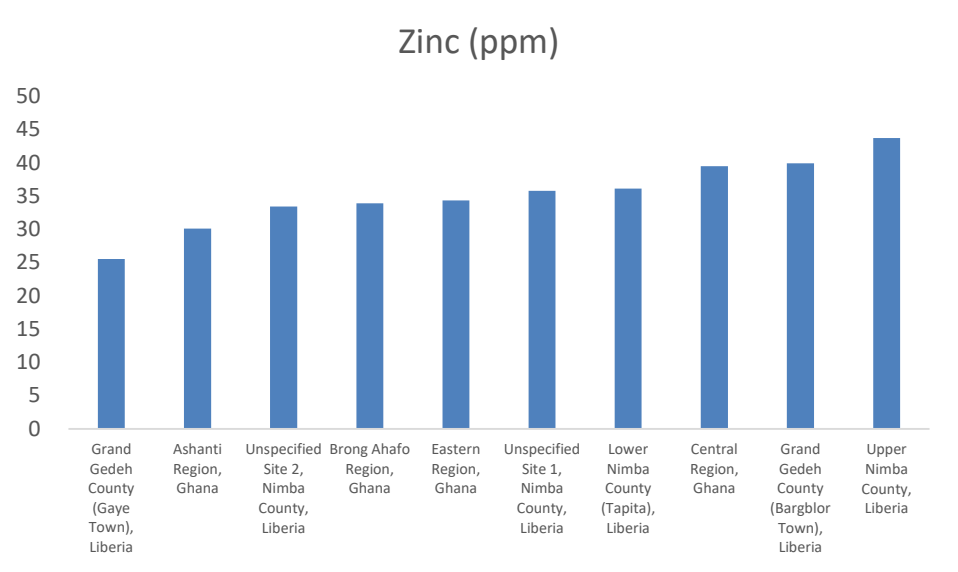
Graph 8-9. Copper content of griffonia seed populations from Liberia and Ghana



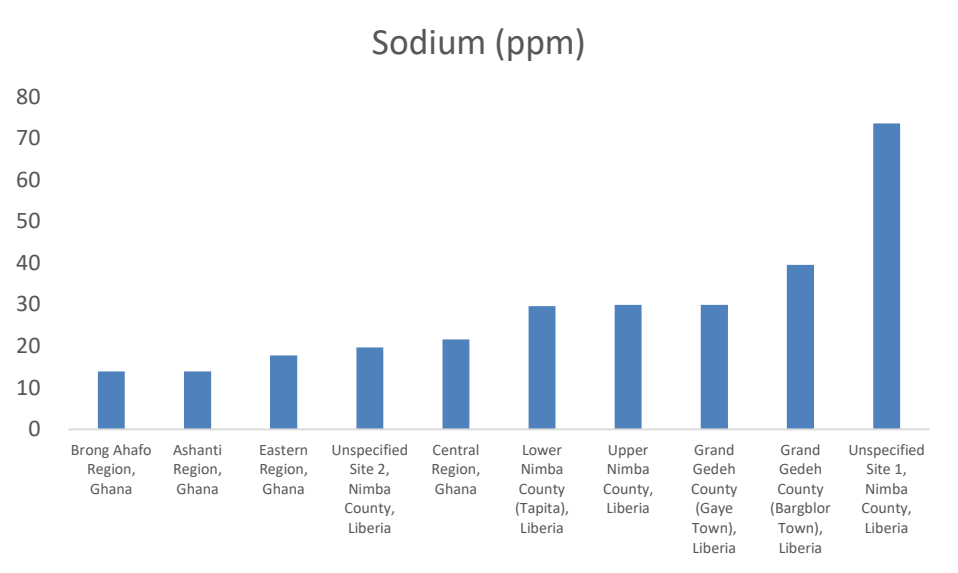
Graph 8-10. Boron content of griffonia seed populations from Liberia and Ghana



Graph 8-11. Aluminum content of griffonia seed populations from Liberia and Ghana



Graph 8-12. Zinc content of griffonia seed populations from Liberia and Ghana



Graph 8-13. Sodium content of griffonia seed populations from Liberia and Ghana

8.4 Conclusions

The purpose of this study was to determine the abundances of minerals found in griffonia seeds from various populations across Ghana and Liberia and to determine if griffonia seeds can be useful as a non-traditional source of these minerals. Malnourished populations in sub-Saharan Africa and southeast Asia are often deficient in micronutrients such as iron, iodine, zinc, (Muller and Krawinkel, 2005) and to a lesser extent, calcium, magnesium, copper, and selenium (White and Broadley, 2005) all of which can have severe health effects (Stein, 2010; Welch, 2002) when the body lacks sufficient amounts of these minerals. Based on the *Codex Alimentarius* Guidelines on Nutritional Labeling CAC/GL 2-1985 and Guidelines for Use of Nutrition and Health Claims CAC/GL-23-1997 (Web addresses are listed in reference section), griffonia seeds were found to be a source of manganese but not calcium, and a high source of iron, zinc, magnesium, copper, and phosphorus. Griffonia seeds were not found to be a source of calcium. On average, griffonia seeds contained approximately 1/2 as much calcium and iron, 1/4 as much zinc, and three times

as much magnesium as soybeans (USDA online: Soybeans, green raw). Copper content of griffonia seeds is similar to that of sesame seeds (Elleuch et al., 2007). Although griffonia seeds appear to contain useful amounts of manganese, iron, zinc, magnesium, copper, and phosphorus, antinutrients such as phytate, lectins, protease inhibitors, polyphenols, ogliosaccharides, (Sandberg, 2002) and tannins (Beebe et al., 2000) could be present that could hinder the absorption of vital minerals (Sandberg, 2002; Flyman and Afolayan, 2006). For this reason, future studies should focus on quantifying the amounts of antinutrients present in griffonia seeds in order to better evaluate the value of this legume in alleviating malnutrition.

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Chapter 9. Conclusions

9.1 Summary

While griffonia is already well known as the most abundant natural source of 5-HTP, a widely used and consumer promoted treatment for conditions involving serotonin imbalance, other aspects of this plant's phytochemical composition such as mineral, polyphenol, antioxidant, protein, flavonoid, absolute fatty acid, and tocopherol content have remained unexplored. The purpose of this study was to further characterize these phytochemical characteristics of *Griffonia simplicifolia* leaves and seeds as well as to identify any significant differences in phytochemistry between *Griffonia simplicifolia* populations collected from various regions of Ghana and Liberia. The most substantial differences in secondary metabolite accumulation was observed in polyphenol, antioxidant, and flavonoid content of leaves as well as cyanoglycoside and tocopherol content of seeds.

Total polyphenol content of griffonia leaves as determined by the Folin-Ciocalteu assay ranged from 7.83 ± 0.49 GAE/gram to 39.86 ± 0.60 GAE/gram with all populations averaging 21.16 ± 0.48 GAE/gram. Liberian leaf populations were on average greater in polyphenol content than Ghanaian populations. A 2007 study by Chan et al., found that green tea leaves contained 75.86 ± 19.9 GAE/gram, thus griffonia leaves appear to be significantly lower in polyphenol content than green tea leaves.

The total antioxidant capacities of griffonia leaves as determined by the ABTS assay ranged from 5.14 ± 0.19 TEAC/gram to 22.15 ± 0.64 TEAC/gram and averaged 12.13 ± 0.45 TEAC/gram. Ghanaian leaf populations were on average slightly greater in antioxidant capacity than Liberian populations. The antioxidant capacities of griffonia seed populations ranged from 163.65 ± 15.46 TEAC/gram to 257.35 ± 11.43 TEAC/gram and averaged 216.50 ± 13.88 TEAC/gram. No significant differences in antioxidant capacities were observed among seed populations from Ghana although Liberian seed populations varied significantly in antioxidant capacities. Liberian

seed populations were approximately 20% less concentrated in antioxidants than Ghanaian seed populations. A 2010 study by Ku et al., found that Korean grown green tea leaves contained approximately 196.48 ± 3.50 TEAC/gram thus griffonia leaves appear to be significantly lower in total antioxidants than Korean green tea leaves. Total antioxidant activity of griffonia seeds were similar to that of Korean grown green tea leaves.

Using the Bradford assay, total protein content of griffonia leaves were found to range from 2.12 ± 0.36 BSAE/gram to 10.73 ± 0.65 BSAE/gram and averaged 6.89 ± 0.47 BSAE/gram. All griffonia seed populations were similar in protein content and ranged from 28.36 ± 0.48 BSAE/gram to 36.51 ± 0.53 BSAE/gram and averaged 33.58 ± 1.52 BSAE/gram. Boyes et al. (1997) reported that the protein content of corn kernels was 7.2 to 8.4 BSAE per gram thus griffonia leaves appear to be similar in protein content to corn kernels while griffonia seeds are significantly greater in protein content than corn kernels. Future studies should focus on the content and quality of essential amino acids in griffonia seeds.

To the best of our knowledge, this is the first study on the flavonoids in griffonia leaves. In this study, eight flavonoids were detected in griffonia leaves of which six have been tentatively identified as apigenin-di-C-glycosides based on analyzing UV and MS data. The remaining two flavonoids have been identified as vitexin and isovitexin based on MS data and a retention time comparison with an authentic vitexin reference standard. The flavonoid profile of griffonia leaves is much different from its closest relatives although flavonoid data for many of griffonia's closest relatives have yet to be reported. There were significant differences in flavonoid content between populations from Ghana and Liberia. Ghanaian populations were significantly more abundant in flavonoid peaks A, B, E, and F than Liberian populations while Liberian populations were slightly more abundant in flavonoid peaks D, E, vitexin, and isovitexin than Ghanaian populations. The average flavonoid content of all griffonia leaf populations was determined to be

9182.02 ± 140.90 µg/g. Griffonia leaves were found not to be as rich in flavonoids content as dry spinach (Bergquist et al., 2005) and green tea leaves (Peterson et al. 2005) and griffonia leaves were similar in flavonoid content to dried celery (Lin et al., 2007). To fully assess the potential nutritional benefits of griffonia leaves, future studies should focus on the bioavailabilities of these apigenin-C-glycosides.

The relative percentages of fatty acids in griffonia seeds has been previously reported (Petkov and Ramazanov, 2003) and this study sought to elaborate by determining the content of fatty acids per gram of seed and seed oil. Palmitic acid ranged from 17.46 ± 1.43 mg/g to 28.50 ± 0.69 mg/g of seed. Stearic acid ranged from 31.17 ± 4.20 mg/g to 52.52 ± 1.57 mg/g of seed. Oleic acid ranged from 19.02 ± 1.83 mg/g to 37.38 ± 0.02 mg/g of seed. Linoleic acid ranged from 123.65 ± 5.91 mg/g to 206.91 ± 3.06 mg/g of seed. Arachidic acid ranged from 9.44 ± 1.06 mg/g to 14.98 ± 0.73 mg/g of seed. Free fatty acids comprised of approximately 89% of the total seed oil composition and linoleic acid composed of approximately 53% of this oil. The high content of linoleic acid in griffonia seeds is not surprising as relatively high amounts of this acid is strongly characteristic of species within the Caesalpinoideae subfamily and Leguminosae family (Bagci and Sahin, 2004). While griffonia oil would be expected to be edible, the relatively high ratio of saturated fatty acid content compared to other oils such as peanut, sesame, soybean and sunflower gives little incentive to use griffonia oil as an alternative cooking oil. The α-tocopherol content of griffonia seeds ranged from 0 µg/gram to 41.30 ± 3.59 µg/gram of seed and the γ-tocopherol content ranged from 36.06 ± 3.92 µg/gram to 127.74 ± 4.79 µg/gram of seed. While they do not appear to be as concentrated in α-tocopherol as sunflower seeds, almonds, and peanuts, griffonia seeds do appear to be a richer source of this nutrient than some commonly consumed leafy greens such as lettuce and spinach. Griffonia oil also appears to be less concentrated α-tocopherol than soybean, sunflower, and olive oil. It should be noted however that the total vitamin E content of griffonia

seeds may be underestimated because this study did not seek to identify any tocotrienols, and during the extraction process, oils were subjected to elevated temperatures and long exposure to light which could cause these compounds to degrade.

5-HTP content ranged from 110.23 ± 1.10 mg/g to 132.65 ± 17.80 mg/g and averaged 120.84 ± 9.00 mg/g. Ghanaian populations were slightly greater in 5-HTP content than Liberian populations. Endosperm color is usually correlated with the health of the seed and green to yellow coloring usually indicates seeds are healthy while black coloring is regarded as a sign that seeds have been subjected to decay either by microbial growth and/or improper drying. In this study, we found heavily discolored endosperms (i.e. nearly or completely black) contained significantly less 5-HTP than healthy seeds with yellow colored endosperms. In order to determine impact of pasteurizing and sterilizing temperatures on 5-HTP content, griffonia seeds were exposed to 90 ºC and 125 ºC for 180 and 60 minutes respectively. It was found exposure to these temperatures for these periods of time did not significantly affect the 5-HTP content.

The HPLC-UV chromatogram of Liberian griffonia seeds revealed the presence of two non-cyanogenic cyanoglycosides, which were then isolated and identified by MS and NMR as riachin and lithospermoside. Lithospermoside has been previously reported to occur in griffonia roots (Dwuma Badu et al., 1976) while riachin has only been isolated from the new world legume *Bauhinia pentandra* (Silva et al., 2013). This is the first report of these compounds occurring in griffonia seeds. In this study, riachin and lithospermoside were only detected in the endosperms of Liberian populations. Riachin content ranged from 1.41 ± 0.01 mg/g to 6.43 ± 0.63 mg/g and averaged 4.59 ± 0.95 mg/g. Lithospermoside content ranged from 7.39 ± 0.81 mg/g to 14.48 ± 0.32 mg/g and averaged 9.65 ± 1.35 mg/g. Riachin content was lower in populations from Grand Gedeh County while lithospermoside was more deficient in populations from Nimba County than

Grand Gedeh County. In all populations, riachin content was lower than lithospermoside content. Similar to 5-HTP content, seeds with endosperms that were nearly or completely discolored contained significantly less riachin and lithospermoside. Liberian griffonia seeds could be a useful source of riachin and lithospermoside for future bioactivity studies as these compounds are present in milligram per gram quantities.

Although griffonia seeds are not traditionally consumed, the mineral content of griffonia seeds was evaluated in order to determine if these seeds could be a useful source of vital minerals as this species thrives in tropical areas where malnutrition is prevalent. Based on the *Codex Alimentarius* Guidelines on Nutritional Labeling CAC/GL 2-1985 and Guidelines for Use of Nutrition and Health Claims CAC/GL-23-1997 (Web addresses are listed in reference section) griffonia seeds were found to be a high source of iron, zinc, magnesium, copper, and phosphorus. Legumes are known to contain large amounts of metal binding antinutrients such as phytate, lectins, protease inhibitors, polyphenols, ogliosaccharides, (Sandberg, 2002) and tannins (Beebe et al., 2000). thus the absorbance of any mineral nutrients supplied from griffonia seeds will likely be hindered.

The 5-HTP content of 18 commercial supplements were measured in order to verify that the declared amounts of this amino acid match within ±15% what is declared on the product label. The measured 5-HTP content of all supplements were within ±15% of the amounts declared on their labels thus all were in accordance with USP guidelines (Tunna and Patel, 2013).

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