5-2012

Sassafras Tea: Using a Traditional Method of Preparation to Reduce the Carcinogenic Compound Safrole

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SASSAFRAS TEA: USING A TRADITIONAL METHOD OF PREPARATION TO REDUCE THE CARCINOGENIC COMPOUND SAFROLE

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Forest Resources

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by
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May 2012

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ABSTRACT

The purpose of this research is to quantify the carcinogenic compound safrole in the traditional preparation method of making sassafras tea from the root of *Sassafras albidum*. The traditional method investigated was typical of preparation by members of the Eastern Band of Cherokee Indians and other Appalachian peoples. Sassafras is a tree common to the eastern coast of the United States, especially in the mountainous regions. Historically and continuing until today, roots of the tree are used to prepare fragrant teas and syrups. These traditional uses can be found across cultures throughout its range. Products made from sassafras are banned from the market by the US Food and Drug Administration (FDA) due to a carcinogenic compound, safrole, found in the unprocessed root. Low levels of safrole are permitted in Europe due to the small concentration found in common spices, including nutmeg and cinnamon. However, in sufficient doses, safrole causes genotoxicity and cell toxicity, oxidative stress, and liver cancer in laboratory rats after ingestion. In this study, traditionally prepared tea and the FDA method of eliminating safrole were analyzed using high-performance liquid chromatography. These two methods were compared for effectiveness of eliminating or reducing safrole levels. The FDA method resulted in little to no safrole content. A smaller amount of safrole was present in the traditionally prepared tea compared to agitate samples, used to gauge a baseline concentration of safrole present in the root. Collaborations with the Center for Cherokee Plants within the Cherokee community will help us to return the results of the research and contribute resources emphasizing the cultural and historical importance of *Sassafras albidum*.

DEDICATION

This work is dedicated to my mother, the strongest and most loving person I know. Also for my father, for his generosity and humor.

ACKNOWLEDGMENTS

I would like to thank my major advisor, Dr. Karen Hall, first and foremost. Thank you for your unending support and dedication to not only this project, but also for helping me become a well-rounded individual. This project would not have been possible without your patience, wisdom, and guidance through every step. Dr. Feng Chen, thank you for your advice and support, as well as the generous use of your laboratory to complete the chemical analysis. Thank you Dr. Christina Wells, for helping with the propagation trials and planting of sassafras in North Carolina. Special thanks to Sarah McClellan-Welsh and Kevin Welch for everything you have done, including hosting and assisting with the tree planting project, GIS project, and sassafras tea conversations. Thank you for your kindness, hospitality, and understanding. To Dr. Chen's lab, especially Nina Huang and Greg Jones, thank you for teaching me everything you knew about chemical analysis and very basic chemistry. Thank you to Alyssa Palmer-Keriazakos, my summer intern, for being such a cheerful, independent, and patient student of GIS and allowing me to bring the SPRI experience full circle. I would like to acknowledge the Highlands Biological Station for the grant which funded my summer field research, and to the Highlands-Cashiers Land Trust, especially Kyle Pursel. Last but not least, thank you to the people who took time out of their day to talk to me about sassafras tea.

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CHAPTER ONE

INTRODUCTION

Sassafras albidum is a North American species primarily known for the tea made from its roots and root bark. Sassafras has been in use for centuries by the Cherokee, Chippewa, Creek, Delaware, Iroquois, Seminole, and other Native American tribes within the East Coast range of the tree (Moerman 2011; Hamel & Chiltoskey 1975). Many people who were raised in the Southeast remember drinking sassafras tea as a child, and families in the mountainous regions still make root tea and syrup today. The Eastern Band of Cherokee Indians in Western North Carolina is one such community that have preserved and passed down the traditional knowledge needed to utilize sassafras.

The essential oil extracted from the root of sassafras contains 80-90% safrole (Carlson & Thompson 1997). Safrole is a phenylpropanoid, a type of aromatic compound that most likely accounts for the strong smell associated with the root (Kamdem & Gage 1995; Rasch 1998). Safrole is found in small quantities in a number of species consumed on a daily basis, including cinnamon, ginger, nutmeg, and cocoa (Heikes 1994). On the commercial market, sassafras extract was a common ingredient in foodstuffs, cleaning products, and cosmetics as a flavoring agent and fragrance. Until 1960, the sale of food containing safrole was legal up to 20 ppm (Carlson & Thompson 1997). In 1960, the United States Food and Drug Administration (FDA) banned the presence of the compound safrole in foodstuff. Experiments had demonstrated the hepatocarcinogenetic (liver cancerous) effect of large doses of safrole on laboratory rats (CFR, Sec. 189). It is

possible to buy safrole-free extracts of sassafras tea concentrate where the safrole has been removed according to processes set by the FDA (CFR, Sec. 172).

Many studies have demonstrated the carcinogenic effects of high doses of safrole on laboratory rats, but all these studies have used pure safrole standard (Jin et al., 2011; Liu et al., 1999; Ueng et al., 2005). As of yet, no research has tested the traditionally prepared sassafras tea for carcinogenic properties. In fact, no research has investigated the potential concentration of safrole in traditionally prepared sassafras tea. This research is important because many people, particularly within southeastern mountain communities, continue to prepare and consume products made from sassafras.

The purpose of this study is twofold: first, to quantify the amount of safrole present in traditionally prepared sassafras tea. We will do this by running chemical analysis of tea samples and samples prepared following FDA guidelines through high performance –liquid chromatography (HPLC). The purpose of running both the FDA and traditional samples is to quantify the effectiveness of both methods at reducing safrole content. The second objective will focus on education and outreach projects, to return the results of the chemical analysis to the community, with which we collaborated and complete projects that highlight the cultural and historical importance of sassafras.

CHAPTER TWO

LITERATURE REVIEW

Project Proposal

The topic of this research project was suggested during a meeting with community and tribal members connected to traditional agriculture, history, and granting agencies of the Eastern Band. They believed researching the toxicity associated with sassafras products would be relevant and interesting to members of the tribe, as tea and syrup are commonly made and exchanged between Cherokee members. The products could eventually contribute to the economy of the Cherokee if the sale of products was achieved or desired. The banning of sassafras products on the market has raised concern among those that consume sassafras tea, syrup, and other foodstuffs made from *Sassafras albidum*. Although many continue to consume these products, there is a feeling of hesitancy and uncertainty in conversation about the use of sassafras. For the present, quantifying the concentration of the main carcinogenic compound in sassafras products would be meaningful for many who have consumed sassafras their entire lives. With the information from these results, consumers could make informed decisions on the personal use of sassafras. Though already a culturally important plant, a study on this species would support the continued emphasis of sassafras in the culture and honor the traditional knowledge associated with its use.

Eastern Band of Cherokee Indians

The Eastern Band of Cherokee Indians (EBCI) of Western North Carolina own more than 56,600 acres of land in five counties slightly south and adjacent to the Great Smoky Mountains National Park (Lambert 2007). There are approximately 13,000 enrolled members, about 8,200 of which live on EBCI-owned land called the Qualla Boundary (Hall 2006; Lambert 2007). Historically, the Cherokee territory covered about 25.6 million acres in the mountains of the southeast, primarily on the Savannah, Hiwassee, and Tuckasegee rivers, with their principle town, Echota, on the bank of the Little Tennessee. The Kituwah settlement, now known as the Kituwah mound, is located along the Tuckasegee River near Bryson City, North Carolina and was possibly the original capital of the Cherokee (Duncan and Riggs 2003; Hudson 1979).

Written history about the Cherokee began in 1540 with the exploration of De Soto and other Spanish explorers. As the French and Spanish settled along the coast and slightly inland in the 1500's and 1600's, there was only occasional contact between the groups. Some scholars estimate these early settlers brought diseases that killed 95% of Native Americans within the first century and a half of European contact (Duncan and Riggs 2003). Being on the edge of the territory claimed by early European settlers, the Cherokee were caught up in the politics and bloodshed of the Colonial Period, the French and Indian War, and the Revolutionary War, as well as the slave trade of several ethnic groups. Exploring settlers slowly absorbed the piedmont regions of Cherokee land and the tribe became concentrated in the southern Appalachian Mountains (Finger 1984).

The "Civilization Policy" of 1789 by the new American government was a crossroads for the Cherokee in terms of adapting to European-style schools, government, churches and economy. The written language developed by Sequoyah in 1819, which may have been a response to these pressures. The Treaty of New Echota of 1835, signed without permission of the Cherokee people as a whole, gave all Cherokee land east of the Mississippi to the United States. In 1838, the US government forced 17,000 Cherokee to territory in Arkansas and Oklahoma on a march now known as the Trail of Tears. About one-quarter to one-half of the ill-equipped travelers perished on the march (Finger 1984). A group of three to four hundred Cherokee hid in the mountains of Western North Carolina to evade capture by US soldiers. They, along with Cherokee that returned to the territory, were the foundation for the present day Eastern Band of Cherokee Indians (Duncan and Riggs 2003).

It is important to know this brief history of the Eastern Band when considering the contemporary Cherokee Indians and the efforts they have made to reclaim their rights and traditional knowledge. Many historians, ethnobotanists, and anthropologists have studied and written about the Cherokee, including James Mooney in the 19th century (Mooney 1992). He was followed by Frans Olbrechts, who worked with the healer Swimmer to publish a manuscript in 1932, and then by John Witthoft, who published articles from the 1940s to 1970s. There have been a number of books written on the ethnobotany of the Cherokee in latter half of twentieth century, including authors William Banks (1953), Myra Jean Perry (1974), David Cozzo (2004), and Karen C. Hall (2006). The Museum of the Cherokee Indian Press publishes the Journal of Cherokee Studies, as well as many

books on the history and culture of the tribe with authors like Barbara Duncan, Vernon Crowe, and Duane H. King (MCI 2012).

Today, tribal members continue their cultural traditions including the lacrosse-like stickball game, the Cherokee language, and traditional Cherokee arts and crafts, all of which are integrated with contemporary festivals. They cater to tourists through managing a casino, interpretive performances of the Trail of Tears, and selling traditional arts and crafts. The Cherokee worldview is represented throughout the landscape where they live, including street signs written in Cherokee language and the preservation of culturally and spiritually important locations like the Kituwah mound. The Cherokee language is reinforced through language immersion schools for members. Many families continue to pass traditional ecological knowledge of plant uses through generations and between members.

Ecology of *Sassafras albidum*

Sassafras is a native deciduous tree species of Eastern North America known for its brilliant fall color and a distinct fragrance. Leaves are bright to deep green in the summer and change to scarlet, orange, or yellow in the autumn. The tree has heterophyllic foliage with three leaf shapes: entire, mitten (both right- and left-handed), and trilobed, as seen in Figure 2.1. On rich sites in the Smoky Mountains, sassafras is typically a medium-sized tree with a straight bole reaching heights of 9 to 18 m (30 to 60 ft). On the edges of the habitat range sassafras tends to be shrubby. Sassafras is a dioecious species whose female plants produce small racemes of yellow flowers in March

or April (Griggs 1990). The odor of the root is very distinct; the scent has been described as ‘sweetshop’ or spicy (Dugan 2011).

Sassafras is found along the Atlantic coast (zones 4 to 9) of the United States from northern Florida to Canada, and west to Michigan, Illinois, and Arkansas (Cullina 2002; Rasch 1998). Sassafras is a generalist species, occupying both full sun and partial shade sites with a variety of soil types and moistures. The tree can be found in a wide range of ecosystems, including many species of pine dominated forests and oak-hickory, maple-beech-birch, and aspen-birch forests. The rapid growth is achieved through a deep taproot as well as shallow, laterally growing root system that send up root suckers (Griggs 1990). White-tailed deer, woodchucks, black bears, and rabbits all browse the leaves during the summer and winter. Many types of birds (wild turkeys, pileated woodpeckers, and northern bobwhites) and some small mammals eat the ripe fruit, a single-seeded drupe with a high energy value (Sullivan 1993). Those who wish to propagate sassafras usually need to stake out the tree for the highly favored drupe (See Appendix A for Propagation techniques). Sassafras is an important host plant for many species of moths and butterflies, particularly the host-specific spicebush swallowtail butterfly (Carter et al., 1999; Sternberg 2004).

Sassafras is typically considered shade intolerant, though it can adapt to low light intensities of an understory with soil pH of around 5 (Bazzaz et al., 1971). *S. albidum* is typically subdominant but can attain dominance by forming thickets on poor soils through allelopathic activity, which includes the release of terpenes and phenylpropanoids as well as other secondary defense compounds that deter pathogens

and herbivores (Bisset 1994; Gant et al., 1975; Sullivan 1993). These adaptations are important when considering the chemistry of the allelopathy: the strong presence of allelopathic compounds are found throughout the tree but mainly concentrated in the roots. Root wood is porous with characteristic pits and oil cells (Bisset 1994). These allelopaths include: 2-pinene, 3-phellandrene, eugenol, safrole, citrol, and s-camphor (Griggs 1990). The sassafras root cortex contains 6-9% essential oil, which consists of the compounds safrole (80-85% of the total oil), safrole camphor (3.25%), methoyleugenol (1.1%), tannins (sassafrid), resin, wax, mucilage, sugar, and sitosterol. Another one percent of the oil includes pinene, eugenol, apoil, 5-methoxyeugenol, elemincine, estragol, and myristicin (Rasch 1998).

Figure 2.1: Heterophyllic leaves of *Sassafras albidum*

Safrole

Safrole (5-(2-propenyl)-1,3-benzodioxole) is a phenylpropene, part of the aromatic phenylpropanoid family. Safrole has a benzene ring flanked on either side by a dioxolane ring and a terminal, highly reactive methylene group, as seen in Figure 2.2 (Wink et al., 2008). Safrole is insoluble in water but mixes readily with chloroform, ether, and other non-polar organic solvents (Budavaris 1989; Burdock 1997). Many of the aromatic rings that produce smell and taste of plants are derived from the phenylpropanoid metabolism through the shikimic acid pathway (Dewick 1997; Wink 2010). Phenylpropanoids secondary compounds associated with the taste and smell of a plant and therefore are used as deterrents against herbivores that shy away from strong secondary metabolites. The compounds also protect against ultraviolet radiation, fungi, and bacteria, and can act as pollinator attractants (Hahlbrock 1989). Secondary compound activity increases in nutrient-poor soils, which is why sassafras, known for its ability to inhabit roadsides and abandoned fields, would have high allelopathic and defense tendencies in these locations and therefore higher concentrations of associated secondary compounds (Ibrahim 2001).

Due to its pleasant smell, sassafras oil used to be a common ingredient (up to 20 ppm) in foodstuffs, cleaning products, and cosmetics as a flavoring agent and fragrance (Carlson and Thompson 1997). Then on December 3, 1960, the US FDA banned the presence of safrole, oil of sassafras, isosafrole, and dihydrosafrole in food after experiments showed a hepatocarcinogenic effect on laboratory rats (CFR, Sec. 189). Many common spices, including black pepper, cocoa, mace, nutmeg, cinnamon, tarragon,

star anise, fennel, parsley, basil, bay laurel, dill, pimento (allspice), and cloves, contain trace amounts of safrole (Heikes 1994; Zhou et al., 2007). Safrole is also detectable in the range of 3-5 mg/L (ppm) in soft drinks that use the spices listed above, including the well-known beverages Coca-Cola and Pepsi (Choong and Lin 2001). Alcoholic beverages contain between 0.15 and 3 mg/L (ppm) (Curró et al., 1987). In a 2001 document, the European Commission allowed, "1 mg/kg in foodstuffs and beverages, 5 mg/kg for alcoholic beverages with more than 25% alcohol by volume and 15 mg/kg for foods containing mace or nutmeg" for both safrole and isosafrole (Carlson & Thompson 1997; SCF 2002; SCF 2003). In 2005 the Council of Europe listed safrole as an Active Principle 1, or suspected weakly carcinogenic substance; no maximum daily limit can be set for these compounds, but they should be set as low as possible. Safrole is currently under evaluation by the Council of Europe (CD-P-SP 2005). Sassafras was most likely targeted because of its high concentration of safrole compared to other spices, including the often cited nutmeg, which has 2.46 % (w/w) compared to sassafras root (89% w/w) (Curró et al., 1987).

Pure safrole oil is still imported to Europe, Japan, and the United States from Brazil and countries in the South Pacific. Safrole-rich species in the *Cinnamomum* and *Ocotea* genera are harvested to be synthesized into piperonyl butoxide, used in pesticides, and piperonal, a flavoring and perfume ingredient (Miglierini 2008; Oltramari et al., 2004). Safrole is also an essential ingredient in the production of the illegal drug MDMA, also known as Ecstasy (Rasch 1998). Safrole is designated as a List I Chemical by the U.S. Drug Enforcement Administration, implying that the chemical is used in the

manufacture of controlled substances as well as having legitimate uses (CFR, Sec. 1310.02).

Laboratory Studies

Safrole is classified as a weak carcinogen in laboratory rodents due to the creation of safrole intermediates that form hepatic DNA adducts (Liu et al., 1999). Conventional genotoxicity tests, including sister chromatid exchange and micronucleus tests, tested positive for *in vitro* toxicity of safrole, and many *in vivo* tests of safrole have established carcinogenic doses of safrole, both through incorporating safrole into the diet and injection (Jin et al., 2011; SCF 2002). Safrole is absorbed passively from the gastrointestinal tract, but it is thought that safrole is non-toxic in its unaltered form. Rather, safrole must be metabolically activated to be toxic to humans, a fact confirmed by neutral or negative results of an Ames test of safrole (Frohne & Pfander 2004; Swanson et al., 1979; Wink 2008). Some research has investigated utilizing the toxicity of safrole for human advantages, including anticancer drugs, insect fumigants, contact pesticides, and anti-fungicides (Casida et al., 1966; Catalán et al., 2010; Huang et al., 1999; Khayyat 2011; Khayyat & Al-Zahrani 2011; Kim & Park, 2008; Zhao et al., 2005).

Metabolites of safrole formed *in vivo* include but are not limited to 1'-hydroxysafrole (considered the most toxic), dihydrosafrole (p-*n*-propyl-methylenedioxybenzene), isosafrole (1-propenyl-3,4-methylenedioxybenzene), and eugenol (4-allyl-2-methoxyphenol) (Heikes 1994). As the body attempts to rid itself of the non-nutritive lipophilic molecule through the liver, the defense system undergoes a phase I reaction to convert the molecule to a nucleophilic substance (Shibamoto et al.,

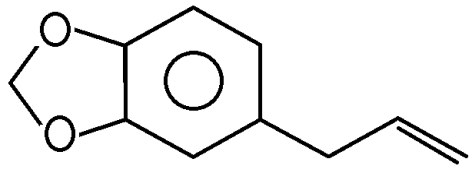

Figure 2.2: Chemical Structure of Safrole

2009). Metabolic activation of safrole to its carcinogenic derivatives can be simplified into four [4] different transformations. The first [1] transformation involves the oxidation of the allyl side chain in cytochrome P450 by the enzyme CYP2A6 to form 1'-hydroxysafrole. This compound can undergo sulfation to form 1'-hydroxysafrole sulfate (Daimon et al., 1997/8; de Vries 1997; Jeurissen et al., 2004; Zhou et al., 2007). These electrophilic, sulfuric acid esters form safrole-DNA adducts in human hepatoma (HepG2) cells and induce cancerous formations (Liu et al., 1999; Miller et al., 1983; Zhou et al., 2007). Safrole-DNA adducts lead to the induction of sister chromatid exchanges and chromosomal aberrations, which lead to mistakes in DNA replications and mutations that has the possibility of carcinogenesis, as well as cytotoxicity (Daimon et al., 1997). Adducts concentrate in hepatic DNA as the liver is the detoxifier and metabolizer of drugs for the body (Nakagawa et al., 2009).

A second [2] transformation falls within a different pathway to chemical carcinogenesis: oxidative stress, which causes faulty incorporation during DNA replication. Safrole can undergo cleavage of the dioxolane ring to form hydroxychavicol (4-allyl-1,2-dihydroxybenzene), shown in the pivotal Benedetti study to be the major urinary metabolite of rodents and humans. The Benedetti et al., study is the only research as of yet to test the results of human subjects ingesting safrole (Benedetti et al., 1977; Liu et al., 1999). Hydroxychavicol, best known in connection to betel quid chewing, has the potential to transform to the reactive elecrophiles *ortho*-quinone or *para*-quinone methide. These metabolites can further transform to reactive oxygen species that can induce oxidative damage. Hydroxychavicol is more toxic than safrole and has been

linked to mitochondrial dysfunction (Bolton et al., 1994; Nakagawa et al., 2009). Though, unlike DNA-safrole adducts, oxidative stress has been shown to be reversible. The damage was repaired within 15 days after safrole was administered, compared to stable DNA adducts that have been detected 30 to 140 days after ingestion (Gupta et al., 1993; Liu et al., 1999). The damage initiated by hydroxychavicol can also be prevented *in vivo* by antioxidants like vitamin E (Liu et al., 1999).

The first two transformations are the main metabolic pathways in animals. A third [3] transformation involves epoxidation of safrole by the double bond of the propenyl group to form safrole-2',3'-epoxide (de Vries 1997). The fourth [4] transformation is the gamma oxidation of the allylic side chain leading to a carboxylic acid, which can conjugate with glycine (CSF 2002). The two major safrole-DNA adducts for this transformation are N^2-(trans-isosafrol-3'-yl)2'-deoxyguanosine and N^2-(safrole-1'-yl)2'-deoxyguanosine (Gupta et al., 1993).

The effect of safrole on laboratory rats and mice is well documented. Rodents given large doses of pure safrole in their diet (via oral gavage, subcutaneous injection, or mixed into food) suffered from mortality and a range of liver and kidney damages. Physiological aliments included slight impairment such as weight loss, mild anemia, and growth retardation. Aliments also included moderate and severe damage, including liver tumor masses and nodules, liver enlargement, focal to chronic nephritis in the kidney and liver, bile-duct proliferation, fatty metamorphosis, and mortality (Abbott et al., 1961; Daimon 1998; Epstein et al., 1970). Genotoxicity assays were positive for sister chromatid exchange (SCE), unscheduled DNA synthesis (in cultured rat hepatocytes),

chromosomal aberrations, gene mutation, and cell transformation (Howes et al., 1990; Ishidate & Sufuni 1985; Mihr et al., 1985; Purchase et al., 1978). Safrole did test neutral or negative in an Ames test (Salmonella reverse mutation assay) as mentioned before, as well as mouse dominant lethal assay, bone-marrow micronucleus assay, and unscheduled DNA synthesis in HeLa cells (Baker & Bonin 1985; Epstein et al., 1972; Gocke et al., 1981; Martin et al., 1978; Mirsalis et al., 1982). Ingestion of safrole at high doses can cause symptoms such as nephritis, unconsciousness, weakness, liver disturbance, CNS stimulation, and skin irritation (Wink 2008). Also see Appendix C for complete chart of laboratory studies.

Toxicology and Dosage

Toxicology research utilizes two models to determine the potency of compounds, including qualitative and quantitative methods. Qualitative methods can include observations of lesions and clinical signs, while the quantitative model examines the dose and exposure levels. The ultimate goal is to determine a structure-activity relationship that outlines the biological effects of a substance at physiological and biochemical levels. To evaluate a compound for human safety, toxicology studies examine several species of laboratory animals (rats, mice, dogs, rabbits, primates) that have similar physiology and genetic structure to humans (Reagan-Shaw 2007). Researchers use several dose levels, where the higher dose levels are to understand the biochemical mechanisms and a maximum amount is established for safe consumption. The mode of ingestion or exposure should be considered in clinical tests; for example, feeding laboratory rats the

food additive compounds through their diet as opposed to subcutaneous injection or oral absorption (Dixon 1976).

As the 16th century Swiss-German chemist/physician Paracelsus held, 'Poison is in everything, and no thing is without poison. The dosage makes it either a poison or a remedy' ("Paracelsus" 2012). In estimating toxicity and the possibility of carcinogenicity, the dose is critical as is the perceived risk. For risk in using food additives in particular, no amount of carcinogen is accepted as additions to food are risks than can be avoided (Extoxnet 2012). In terms of dose, dose translation is necessary to compare doses between different species, particularly between human and laboratory animals. The use of body weight to compare doses between studies has been criticized as it may not accurately correlate to volumes of blood and plasma proteins (Dixon 1976). Body surface area (BSA) has been shown to associate these parameters, or total blood and plasma protein volume, in addition to basal metabolism, renal functions, and oxygen and calorie utilization. The equation used by Reagan-Shaw et al. (2007) to translate doses between animal species and human:

Human equivalent dose (mg/kg) = Animal dose (mg/kg) * Animal K_m/Human K_m (Reagan-Shaw et al., 2007). K_m values (mg/m^2) are listed in the paper for several species and are based on height-weight measurements. In this thesis, mg/kg bw (body weight) refers to mg of compound by body weight of the subject, either human or animal. The unit listed as mg/kg refers to mg of a compound in kg of material.

Another aspect of dosage to consider is the difference in employing high versus low dosage. The 'Virtual Safe Dose' for human consumption is calculated with a linear

model, "which assumes that cancer causation is directly proportional to dose and that there are no unique effects of high doses" (Ames & Gold 2000). A dosage margin of safety for humans is typically calculated by dividing a lethal dose of the compound for laboratory rodents by a factor of 100 (Segelman et al., 1976). An exaggerated high dose of a compound, as in experiments to understand the mechanisms of carcinogenicity, can cause wounding and death of tissue and cells and chronic cell division of neighboring cells. It is possible the resulting cellular necrosis and carcinogenesis is inflated in high doses, a point discussed in literature on safrole.

The Benedetti et al. (1977) study examined the response of rats and mice to consumption of safrole in addition to human subjects. Even at the smallest dose of 0.63 mg/kg bw (body weight), 88% of the safrole was eliminated by the rats within 24 hours. The authors point out that when the dose of safrole for the rodents was increased to levels common in carcinogen studies (between 500 to 1000 mg/kg bw), safrole rapidly accumulated in the liver and kidney, and safrole was not metabolized as quickly and therefore eliminated more slowly than rats fed smaller doses. They argue that the toxic accumulation associated with these carcinogenic studies was due to this slow metabolism of safrole and subsequent tissue absorption was a result of the unrealistic doses. Lower doses (or accurate amounts of safrole in food and drinks) would not lead to safrole accumulation in tissue muscle (Benedetti et al., 1977). Gupta (1993) found a dose of400 mg safrole/kg bw for mice to be the point of saturation of detoxification enzymes.

Safrole in Food and Spices

The HERP Index, or Human Exposure/Rodent Potency index lists a daily level of safrole ingestion at 1.4 mg/kg bw per day for a 60 kg person from the various safrole-containing spices like black pepper, star anise, cumin, cinnamon, and ginger root (HERP 2011). The European Commission estimated the daily intake of consumers (by the quantity of safrole added to food by industry) to be 0.3 mg/day, or 0.005 mg/kg bw for a 60 kg person (SCF 2002). The WHO estimates a daily range of safrole intake as 4 to 569 μg for Americans and between 0.6 and 879 μg for those in the European Union, based on spice content and annual volume consumed by country (WHO 2009). The median toxic dose (TD_{50}) of safrole, according to the HERP Index, for mice is 51 mg/kg bw per day (HERP 2011). Segelman et al. estimate a dose of 0.66 mg/kg bw may be toxic to man, based on a study by Epstein et al. (1970) that found 66 mg/kg bw of safrole administered over a 21 day period to be carcinogenic to infant male mice. Studies commonly use a margin-of-safety factor of 100 to estimate toxicity, hence the 0.66 mg/kg bw (Segelman et al., 1976).

In the only study found with human subjects, Benedetti et al. (1977) observed the effects of human consumption of safrole with two doses (0.163 mg and 1.655 mg). They concluded both amounts of safrole were completely eliminated in 24 hours with little possibility of safrole accumulating in tissue muscles. In the human subjects, safrole was eliminated as small amounts of unchanged safrole and safrole metabolites, specifically 1,2-dihydroxy-4-allylbenzene, eugenol, and an isomer of eugenol. The metabolites 3'-hydroxylisosafrole and 1'-hydroxysafrole (the most toxic metabolite) were not detected.

The authors suggest the absence of the latter metabolites could also be due to the dose or to the differences in metabolism between man and rodent (Benedetti et al., 1977).

Safrole is present in the flowers of *Piper betle*, used in the betel quid chewing tradition (similar to tobacco dip in the United States) prevalent among the male population of Taiwan. Betel quid has been linked to the high rates of oral cancer due to the high level (15,000 mg/kg plant material) of safrole present in the flowers, which are used in the betel quid mixture. Exposure to safrole in the saliva during chewing is estimated between 70 mg/L to 68120 mg/L (or 420 μmol/L) (Chen et al., 1999; Liu et al., 2000). Research on betel quid chewing in India and Southeast Asia suggested the presence of safrole could be causing the high rates of oral cancer and other diseases by inhibiting the bactericidal activity and releasing reactive oxygen species (Hung et al., 2003). A study examining betel quid chewing among pregnant women found that adverse pregnancy outcomes, including lower birth weight, stillbirth, fetal malformation, and premature delivery, were 2.8 times higher than women who did not chew betel quid (Yang et al., 2001). Overall, it is difficult to compare betel quid chewing to consuming sassafras tea due to the different paths of ingesting safrole (absorbed through the mouth and saliva versus gastrointestinal absorption) and the range of doses.

Previous Studies on Foods Containing Safrole

A handful of studies have examined sassafras tea. Carlson & Thompson (1997) examined several types of herbal products derived from sassafras, including leaves, tea concentrate, herbal powder capsules, and tinctures, as well as a ‘sassafras tea’ prepared

from instructions from a modern herbal guide. The 'tea' made in this study was infused, or the tea bag steeped in hot water for thirty minutes as opposed to the traditional method of decocting, or boiling, the whole or roughly chopped root. They demonstrated that herbal products of sassafras ranged from zero to a miniscule amount of safrole in tea made from the leaves, to containing 92.4% safrole in sassafras oils. The 'tea' they prepared had safrole content ranging from 0.03 to 1.37 mg per gram of powdered root material, or only 6.9% to 17.2% of total safrole present in the root (Carlson & Thompson 1997). There were several differences between the Carlson & Thompson study and this research, including the use of root bark powder instead of traditionally harvested and processed root and root bark. The researchers also infused the root bark powder in hot water instead of the decoction method that is used by all the consultants in this study.

Another study by Heikes (1994) quantified the amounts of known carcinogenic compounds in commercial, un-brewed tea mixes, without actually heating the material. Both of these studies (Carlson & Thompson and Heikes) focused on finding a more efficient, cost-effective method of analysis than the official method adopted by the FDA in the 1960's. Kapadia et al. (1978) did not prepare tea per se, but did extract in an aqueous solution using sassafras root bark. The researchers used petroleum ether, methylene chloride, and ethanol to extract the oil (which would include the safrole), and then injected 15 mg of the safrole-free ethanol extract in rats (Kapadia et al., 1978).

Traditional Knowledge & Implications for Chemical Analysis

Traditional knowledge, especially in Native American communities, comes from a cumulative, inter-generational custom of passing along practices, beliefs, ideas, and wisdom. It is transmitted through cultural behaviors and generated by close contact with the natural environment. It encompasses every part of a society: politics, history, anthropology, and philosophy (Huntington 2005). The inherent complexity in natural and human social systems is recognized and categorized with the assumption that humans are a part of the web. Balance in natural systems requires reciprocity and respect. Knowledge is indigenous or traditional when "the meanings as well as the categories of sense making are generated internally within a cultural community", as well as a physical environment (Gupta 2010; Viergever 1999).

Within the system of traditional knowledge, traditional medicine is less a remedy or cure, and more of a way of balanced well-being. The World Health Organization defines it as, "the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses" (WHO 2008). When traditional medicine is taken out of context of its originating culture, it can be termed alternative or complementary medicine. Without the history or tradition to sustain them, medicines can be prepared or used differently from their original intention, sometimes leading to harmful results. This is not to say that traditional knowledge regarding plant preparation is invalid, only that if the product is of poor quality, taken inappropriately, prepared differently, or used at the same time as other medicines, the

product can be ineffective or malignant (WHO 2008). The traditional knowledge and particular species are often targeted, as in the case of sassafras tea, rather than if and how the knowledge was taken out of cultural context.

People that use sassafras today look at the extended use of sassafras through history as proof of its safety. Steve Brill, a wild food forager in New York, states "People have enjoyed sassafras in moderation for thousands of years with no ill effects and consumed it in root beer before it was replaced by artificial chemicals. I think sassafras is safe" (Brill 1994). A home remedy book, or do-it-yourself herbal, says, "Everyone knows that sassafras has been used for centuries as a spring tonic. Try it, it's delicious" (Williams 1998). Unfortunately, there are instances of injury from traditional methods prepared incorrectly. For example, a case study published in the Journal of Postgraduate Medicine describes an elderly woman who complained of diaphoresis. The doctor traced the excess sweating and hot flashes to her recent habitat of consuming ten cups of sassafras tea a day, as instructed by a family member (Haines 1991). The article does not indicate how strongly she prepared the tea or exactly how many ounces she consumed from ten cups. Another case study from 1987 reports a 47-year-old woman ingesting a teaspoon, or 5mL, of sassafras oil and immediately vomiting, followed by trembling (Grande & Dannewitz 1987).

There have been many studies highlighting the importance of traditional methods of preparation, including several on safrole-containing species. This research closely followed the study of Reynertson et al. on a bark tea made from *Cinnamomum carolinense*, a study that specifically examined the differences in extracting the bark

using alcohol versus water (2005). Research in Egypt studied safrole-containing spices collected in local markets for the degradation of safrole during common cooking methods (Farag & Abo-Zeid 1997). A wild ginger species (*Asarum spp.*) used in traditional Chinese medicine was analyzed before and after a one-hour decoction (Chen et al., 2009). The kava-kava controversy is well known in the health food circle for multiple cases of severe liver damage when the roots are extracted with ethanol or acetone rather than the traditional method of maceration in water and coconut milk (Singh & Devkota 2003; Whitton 2003).

It is important to remember as well that plant medicine is most likely derived from secondary compounds produced for protection against herbivores, pathogens, fungi, and other modes of attack. Plants have evolved a myriad of defense mechanism, from neurotoxin effects that can cause confusion or sedation to physiological effecting, including growth suppression or organ damage. These secondary compounds, often very toxic to predators, can be harnessed by humans for beneficial effects (Briskin 2000).

The purpose of this research was not to discredit or devalue the traditional knowledge that informed this study. Native American cultures have been using sassafras for centuries, along with other plant species with deadly and toxic compounds. As of the conclusion of this study, there have been no confirmed, direct linkages between drinking sassafras tea and harm reported in any CDC Morbidity and Mortality Report or the FDA MedWatch program (CDC 2012; MedWatch 2012). These programs are the monitoring methods used by the FDA to follow any adverse, unexpected, or unusual results of consuming FDA-regulated products (Love 1999).

CHAPTER THREE

TRADITIONAL KNOWLEDGE OF PREPARING SASSAFRAS TEA

Introduction

The methods and materials used in this study of harvesting, processing, and preparing sassafras root are based on discussions with enrolled members of the EBCI and non-Cherokee persons. These people have made sassafras tea for much of their lives and many were taught as children by their parents or grandparents. They were consulted on their methods of making tea in order to create a specific formula for tea-making to be used as the 'standard' in the laboratory, a process that is replicable and uses specific quantities. The 'standard' method represents a typical way to prepare tea and was a compromise between slight differences among the processes. The harvesting, processing, and preparing techniques varied with the consultants, including the age and habitat type of the sassafras tree, how the root was processed for tea and storage, the proportion of root to water in making the tea, and the length of time the root was boiled and steeped.

Traditional Harvesting, Processing, and Preparation

The processes included on harvesting, processing, and preparing sassafras root represent one way of demonstrating how decisions were made for this research and do not imply the totality of processes or people who use sassafras. Many consultants harvested saplings because they are abundant, easier to dig up, and are considered weedy by some homeowners. Some consultants dug from both young and mature trees. A few

indicated they dug roots in certain seasons, including only in late fall, winter, or early spring, as these were when the plant energy was concentrated in the root. The processing step was the most variable among root harvesters: some consultants dried the root spread on newspaper, while others hung them in bags. The consultants either chopped the root into chunks of various sizes and weights, whittled the root lengthwise into strips, or kept the smaller, thinner roots whole. In making the tea, the root was boiled from 10 minutes up to an hour, removed from the heat source, then let steep for a few hours, overnight, or up to 24 hours.

There were several points of general agreement among the consultants. They all emphasized the sustainable harvest of the root as to not to harm the larger trees, or only harvesting saplings if they are considered weedy or in a site about to be bulldozed. Every consultant indicated that they left the root bark on the roots, washed the root very thoroughly after harvesting, and hung the root in breathable containers, most commonly a mesh onion bag. Breathable containers are essential to keep the dried root from molding. The consultants agreed that dried root would last many years, though typically it was used in one-year period due to steady use.

All the consultants agreed on the proper color of the tea, though many found it difficult to describe: light brown, almost see-through, yellowish-brown, medium honey-colored, or the color of amber beer (See Figure 3.1). Any lighter and it would be too weak tasting, and any darker it would overpower the senses. Many described the amount of root to be used in a pot or single cup of tea in terms of handfuls, as in a double handful of root per kettle of water or one chunk of root for a mug of water. The art of making

sassafras tea is in the experience and practice of knowing how strong the root smells as to how strong the tea will be, and checking on the steeped tea for the color and taste. The amount of root and water and how long it is boils and steeps is secondary to the color, smell, and taste. The more root you use, the faster it will steep, while the less root used, the longer you steep it.

Tea is made with either fresh root or dried root, and there seemed to be no preference for either type. Dried root needs to be rehydrated in order to release the flavor, which can be done by boiling the root once, letting it steep overnight, and boiling again the next morning and let steep again, or by letting boil and steep for a longer period of time. For the amount of tea to drink, the consultants were all in agreement. If a person was sick, they should have about three cups of tea, otherwise one to two cups a day is enough. Tea is typically consumed in the fall, winter, and spring, especially in the spring months when people are liable to get sick or prepare for the changing season. Fresh tea is best, but if a big pot is made it can be kept in the refrigerator for up to a week.

Field Methods: Harvesting

All roots were dug within two weeks during July 2011 on property managed by either the Highlands Biological Station or the Highlands-Cashiers Land Trust. All the land was within Highlands, North Carolina, a small town in the southern plateaus of the Blue Ridge Mountains. Many of the harvesting sites were around 3,800 feet in elevation, with one site at 4,100 feet. Sassafras trees grow prolifically at this elevation and habitat, which is classified as temperate rainforest (Chamber of Commerce 2011).

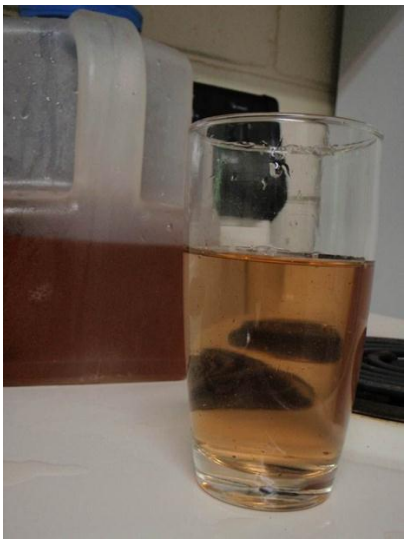

Figure 3.1: Traditionally prepared sassafras tea.

A total of nine trees were harvested for an average of 40 grams of root material per tree. The terrain shape index, landform index, and diameter at breast height (dbh) were measured for each of the trees, and the habitat type was described. The roots were removed with hand shears, and the soil samples were collected from around the excavated roots, to a depth of six inches. Soil samples were analyzed by the Clemson University Agricultural Services Lab for general soil properties and nutrients. With the exception of one site (Site 7), all the primary nutrients (nitrogen, potassium, and phosphorus) were in the low medium to low range recommended for adequate plant growth. The soils in all the sites were moderately to extremely acidic, with an average soil pH of 4.23 ± 0.6 (optimum soil pH for plants is 5.8 to 6.5). See Table 3.1 for complete descriptions of harvest trees and sites.

Positive root identification was achieved by following a large surface root three to four feet from the tree bole. The surface root sometimes dipped into the soil, where it was followed by tunneling along the root. Identification was also attained by olfactory identification of the excavated root, which smells characteristically of 'sweetshop', candy store, or 'spicy' when fresh. The two young saplings that are included in the sampling were dug up completely and the entire root system up to the main root of the colony was collected. Only trees approved by the Highlands-Cashiers Land Trust or the Highlands Biological Station were harvested.

The size and shape of the roots is most likely dependent on the age of the tree, site conditions like soil type and slope, and size of the colony, if part of one. Observations were made that the older trees had much thicker, darker roots, almost a deep reddish-

brown color, with potent smelling roots that smelled characteristically like a sweet-shop and earthy. Soil surrounding older roots had a very strong sassafras smell. Young saplings, three or four years old, had roots that were much lighter in color with a sharper, sweeter smell then the older trees without the associated smell in the soil. Surface roots, or roots typically acting as suckers in a colony, were very pliable and thinner compared to non-surface roots.

Field Methods: Processing

The roots were washed thoroughly (carefully keeping the root bark) in tap water and weighed on a Mettler H51 balance scale with 0.01 g precision. The length and diameter were measured with a digital caliper. The roots were dried on paper for a minimum of 48 hours to prevent mold growth, and then placed in separate brown paper bags until prepared for samples. The bags kept in an open box in a well-ventilated room until they were boiled for tea.

When the roots were dry, they were weighed and measured again. The roots had an average moisture loss of 61% when air-dried, or ranging between 51% to 67% moisture loss. One 4 g sample was dried in an oven for 6 hours, or until the dried weight did not fluctuate, where it lost a further 9% moisture, for a total of approximately 70% moisture difference between dried and fresh root.

The air-dried dried roots were cut into segments that each weighed from 0.1 g to 1 g approximately (See Figure 3.2). The segments were mixed together in equal parts, 10 g from each tree, so each tree root represented one-ninth of the total weight. When root

Site #	Height (feet)	DBH (inches)	Habitat	Slope	Soil pH
1	17	3.6	Acidic upland with *Rhododendron* and *Vaccinium*, part of a colony, sandy loam soil, understory	North facing, slight slope	3.4
2	12	1.8	Acidic upland with *Rhododendron* and *Vaccinium*, part of a colony, sandy loam soil, understory	Southwest facing, slight slope	3.9
3	15.5	2.2	Acidic upland with *Rhododendron* and *Vaccinium*, part of a colony, sandy loam soil, understory	Southwest facing, slight slope	4.5
4	<6	<1	Acidic upland with *Kalmia*, *Rhododendron*, and *Vaccinium*, Rocky, sandy soil, understory in a windy, exposed mountainside	Southwest facing, very steep	4.1
5	17	2.2	Oak-hickory forest, understory and part of a colony	Southwest facing, slight slope	4.3
6	57.5	28.8	Acidic soil with dense covering of *Rhododendron* and *Kalmia*, roadside	South facing, steep slope.	4.1
7	<6	<1	Sapling on disturbed site, roadside	South facing, slight slope	4.9
8	49	12.5	Old-growth, acidic upland forest with *Rhododendron.* Is dominant in the canopy with no colony	West facing, some slope	3.6
9	<6	<1	Sapling in open, disturbed site with full sun. Part of a colony of saplings	South facing, no slope	5.3

Table 3.1: Site and tree characteristics for *Sassafras albidum* trees harvested in Highlands, North Carolina.

segments were selected for preparing the tea, the bag was shaken thoroughly and root segments were chosen blindly and at random. Oven-dried root segments were not used in tea preparation as someone would not normally dried the root in the oven before making tea.

Field Methods: Preparing

The traditional method of preparing tea was standardized to consistently produce a cup of sassafras tea with approximately the same color and smell each time. This was a subjective observation, but acceptable for this study as tea is consumed with a wide range of color and smell intensities. Just as a variety of tree age classes and habitats are harvested, a variety of preferences exist within tea preparation. As the consultants drank tea within this spectrum of tea intensities, we intended to cover the range of tea consumed to reflect any differences in safrole content. Once a standard time of boiling, steeping, and re-boiling was established, the standard method did not change to accommodate changes in tea color.

The roots were boiled twice: once to rehydrate the roots, which were then left to steep ‘overnight’, then boiled again to make the tea. The water and root are heated together to boiling (when the water started visibly bubbling), which marked the beginning of the boil time. The heat source is turned down to ‘medium’ to simmer for 15 minutes (See Figure 3.3). The tea was removed from the heat source and left to steep ‘overnight’ for 12 hours without refrigeration. The second boil was performed as the first, or the root and water brought to a roiling boil together, with the time beginning at first

Figure 3.2: Segments of dried sassafras root chopped into small pieces.

Figure 3.3: Sassafras root being actively boiled while preparing the traditional method samples.

signs of boiling. The tea was allowed to actively boil for 20 minutes and removed from the heat source. The root segments were used once for each replicate of each sample.

Results and Discussion

The standardized method described in this section was based on conversations with people knowledgeable in the use of the species. This standardized method, particularly in the preparation steps, is as similar to the traditional method as could be replicated in a laboratory setting (using distilled water, hot plates, and laboratory glassware instead of kitchenware). Future studies might look closely at the traditional knowledge of sassafras, including the range of harvesting, processing, and preparation methods.

CHAPTER FOUR

CHEMICAL ANALYSIS USING HPLC

Introduction

The objective of this chemical analysis was to quantify the amount of safrole found in both the method outlined in the FDA regulation and the traditional method of preparing sassafras tea. A third sample set, the agitate samples, was added to measure the concentration of safrole present in the root without degrading the safrole with heat extraction. The samples were run through a high-performance liquid chromatography (HPLC) system using a reverse-phase C18 column method. The FDA used gas chromatography in the 1960's to detect and quantify safrole, but the developments in sensitivity and reliability of HPLC lead us to use this method (CFR Sec. 189; Larry 1970). Also, many recent papers have used HPLC to quantify safrole, including a paper by Reynertson et al., 2005 from which we adapted the methodology for the chemical analysis. This paper was used as a model because the study was recent, so it utilized modern analytical techniques, and because the research closely paralleled the ethnobotanical objectives of this project.

Plant material is inherently variable, particularly in the concentration of secondary compounds. The time of day or season of the year, soil and microclimate, herbivory and pathogen activity, and age and history of a particular tree can determine specific chemical quantities. The experiment was designed to encompass the spectrum of harvest and

preparation styles, as well as variation among young or mature trees and different habitats and elevations.

Materials and Methods

Laboratory materials

- Safrole standard (98%) – Chem Service Inc. in West Chester, Pennsylvania.
- ACS-grade MeOH and HPLC-grade ACN – Fisher Science in Georgia.
- Water – deionized
- YMC ODS-AQ S-8 4.6 x 250mm, 5μm, 120Å HPLC column

Traditional Method

Samples 1a, 2a, and 3a (see Figure 4.1 for chart of the samples) were prepared by boiling 2 grams of air-dried root segments in a 500 mL glass Erlenmeyer flask with 300 mL distilled water, using two hot plate/stirrers (VWR Scientific Products 370 and Fisher Science). A small piece of aluminum foil was loosely placed over the top of the flask to reduce water evaporation. The samples were prepared as described in Chapter 3, or boiled for 15 minutes, let steep for 12 hours, and re-boiled for 20 minutes.

Fifty micrograms (50 μg) of safrole standard was added to the samples 2a, 3a, 2b, and 3b, following the methods in Reynertson et al. 2005. These spiked samples would show whether safrole is degraded without complication of root material.

The traditional method samples were run twice due to the inconsistency of the results in the first run. Each sample set of the traditional methods has six samples whereas the FDA method and agitate sample set contains three samples each.

FDA Method

The FDA samples were prepared based on the generalized methods defined by regulations. Sassafras extract is the aqueous solution "obtained by extracting the bark with dilute alcohol, first concentrating the alcoholic solution by vacuum distillation, then diluting the concentration with water and discarding the oily fraction" (CFR, Sec. 172).

Samples 1b, 2b, and 3b were prepared using ACS grade methanol diluted to 80% methanol in 500 mL glass Erlenmeyer flasks. Each of the FDA samples were stirred using a stir plate and stir rod for 35 minutes, or the same boil time of the traditional method samples. Following the methodology outlined by the FDA, vacuum distillation was done using a Büchi Rotovap. The heating bath was set at 65°C, the evaporation point of methanol. The solution was diluted back to 300 mL using distilled water. The solution was poured into the funnel separator, allowed to settle for 10 minutes, and then run through the separator until 5 mL remained. The remaining liquid in the funnel separator represented any sassafras oil found in the root and was not included in the analyzed sample. The remaining aqueous solution (after removal of the oil fraction) had no detectable oil, characteristic sassafras scent, or color.

Agitate Samples

The agitate samples were prepared with 10 g of root and 300mL of cool distilled water. The samples were stirred with a stir plate and stir rod for 50 minutes, let sit for 20 hours, and filtered. The agitate sample went through a longer period of agitation and steeping until the color of the resulting samples was close to color of the traditional tea

samples. The color was not standardized but based on the descriptions provided by the consultants. A larger quantity of root was added for the same reason. No heat was applied during any part of the process and the stirring was kept at a low, steady speed as to not cause excess heat. The resulting concentration was divided by five to compensate for the increased amount of root but no correction was taken for the increased time of agitation.

HPLC Analysis

All samples were filtered through 0.2 μm syringe filters prior to analysis. Analysis was performed on a Shimadzu Prominence UFLC (Ultra-Fast Liquid Chromatography) system using a reverse-phase C_{18} column (YMC ODS-AQ S-8 4.6 x 250mm, 5μm, 120Å). Results were monitored at 235 and 254 nm using UV detection. The mobile phase consisted of two solutions, (solvent A) distilled water and (solvent B) acetonitrile (ACN). The HPLC conditions were as follows: linear gradient of 5% (B) at 5 minutes, 15% (B) at 10 minutes, and 100% (B) at 45 minutes for 10 minutes, with a stop time of 55 minutes. The injection volume was 10 μL. Retention time for safrole standard was 37.77 ± 0.23 minutes (See Figure 4.1).

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated based on the blank determination method cited in Sanagi et al., 2009. LOD is the lowest concentration of a compound that can be detected, but without the need to specifically quantify the concentration. LOQ is the lowest quantifiable concentration identified by the analytical procedure. LOD and LOQ are important analytical validations that define the limits of the analytical laboratory machine (Sanagi et al., 2009). There are many accepted

methods for quantifying LOD and LOQ, including the blank determination method outlined in Sanagi et al. (2009). The blank determination method was chosen for this study as the method is applicable when a blank sample returns results with non-standard deviations. LOD and LOQ is calculated by running at least three blank samples with no standards or compounds added. The mean concentration and mean standard deviation of the blank are found. LOD is the concentration of the blank sample plus three standard deviations ($x_m + 3S_d$) while the LOQ is the analyte concentration of the blank sample plus ten standard deviations ($x_m + 10S_d$), where (x_m) is the mean concentration and (S_d) is the standard deviation (Sanagi et al., 2009).

LOD and LOQ measured 1.514 ppm and 2.049 ppm for 235nm, respectively. Triplicate samples of diluted safrole standard of 10, 50, 100, 200, and 1000 ppm were run to configure a standard curve. Standard curves were calculated for 235 nm absorption, yielding the following equation which with to calculate the amounts of safrole present in the samples. For 235nm absorption: the equation was [Area = 628.2326*Concentration], as seen in Figure 4.2. For the 235nm absorption, the p-values for the intercept variable (or 'b' in the equation y = mx + b) were greater than the level of significance, meaning it is not included in calculating the sample concentrations. The R-squared value for 235nm absorption was 0.9579. $P<0.05$ was considered statistically significant. Based on the absorption spectrum of safrole, the 235 nm wavelength was determined to be a more stable absorption time and therefore was the only wavelength reported in these results.

Number	Sample	Extraction Method	Purpose
1a	Root	Traditional	Quantify the presence or absence of safrole
2a	Safrole Standard	Traditional	No root, to ensure traditional method eliminates safrole
3a	Safrole spiked Root	Traditional	Quantify amount of degradation
1b	Root	FDA	Quantify safrole to compare to #1a
2b	Safrole Standard	FDA	No root, to compare to #2a
3b	Safrole spiked Root	FDA	Quantify amount of degradation
Agitate	Root	Cold water	To extract unaltered safrole using water

Table 4.1: Table of samples, extraction methods, and purpose of sample type in preparation of HPLC analysis. Traditional method samples are signified with the letter ‘a’ after the sample number while FDA method samples have the letter ‘b’ after the sample number.

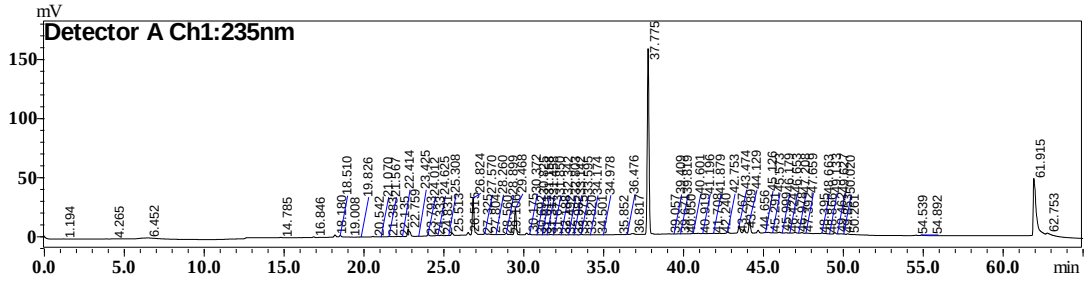

Figure 4.1: Safrole standard (5,000 ppm), detected at 235 nm with an elution time of 37.775 minutes.

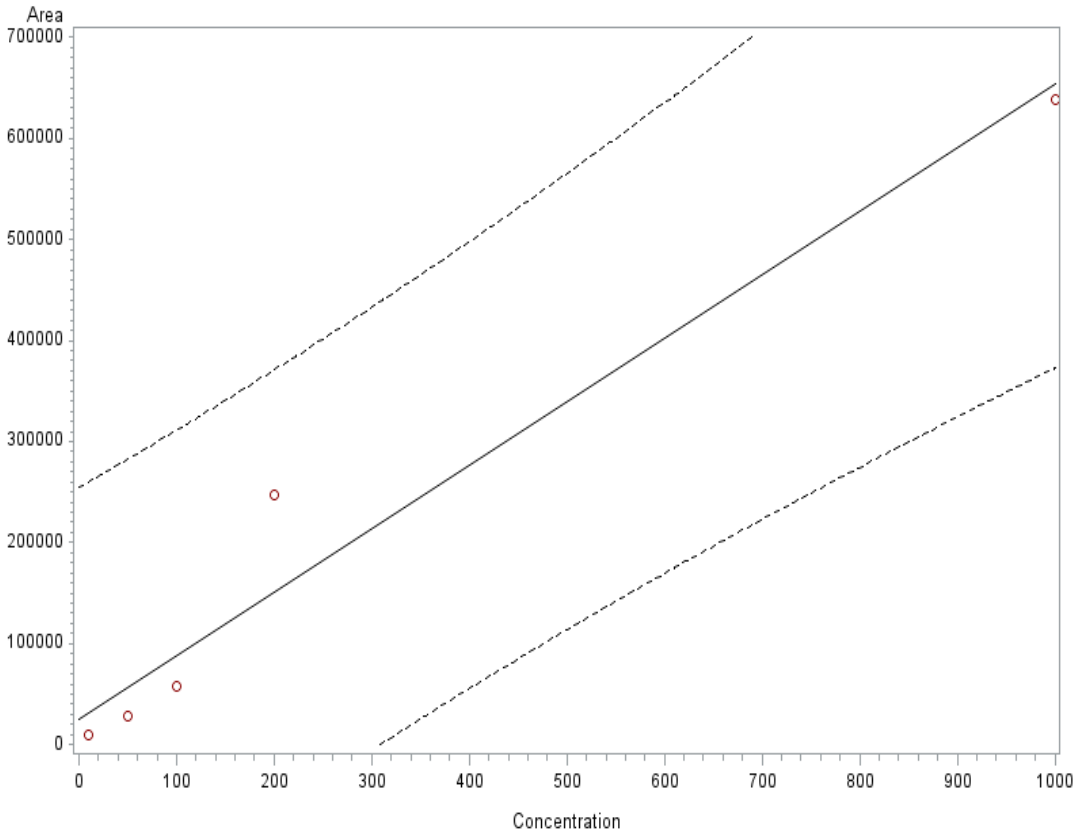

Figure 4.2: Standard Curve for 235 nm absorption. The equation generated by the linear regression: Area = 610.03*Concentration. Triplicate runs of 10, 50, 100, 200, and 1000 ppm of safrole standard are indicated by red open circles. Upper and lower 95% confidence intervals represented by dotted lines.

Sample	PPM	Mean (PPM)	Std Dev
1a-1	7.206		
1a-2	0		
1a-3	9.055		
1a-4	8.917		
1a-5	6.198		
1a-6*	35.36	6.275[1]	3.707
2a-1	0		
2a-2	1.854		
2a-3	0		
2a-4	0		
2a-5	0		
2a-6	0	0.309	0.757
3a-1	26.09		
3a-2	13.80		
3a-3	18.36		
3a-4	5.880		
3a-5	9.653		
3a-6	0	12.30	9.258
1b-1	0		
1b-2	0		
1b-3	0	0[1]	0
2b-1	0		
2b-2	0		
2b-3	0	0	0
3b-1	0		
3b-2	1.93		
3b-3	0	0.644	1.12
Agitate-1	21.25		
Agitate-2	24.12		
Agitate-3*	n/a*	22.69[1]	2.033

Table 4.2: Results of HPLC analysis in parts per million (PPM) of safrole at 235nm. The mean and standard deviation of each of the sample sets is listed.
*Samples 1a-6 and Agitate-3, were removed from the sample sets as the results were outliers or due to a contaminated column. The concentration was determined by the calibration curve.
[1]Samples 1a are significantly greater than the samples 1b and significantly less than Agitate 1 & 2 samples based on student t-tests at α=0.05

Sample	Average Safrole in mg/ 300 mL	Std. Dev.	Average Safrole in ppm or mg/L	Std. Dev.
1a	188.2	111.2	627.5	370.7
2a	9.27	22.7	30.9	75.7
3a	368.9	277.7	1229.7	925.8
1b	0	0	0	0
2b	0	0	0	0
3b	19.3	33.5	64.48	11.67
Agitate	680.6	61.0	2268.7	203.3

Table 4.3: Milligrams and ppm of safrole per 300 mL of aqueous solution. Concentrations calculated from Table 4.2.

Sample	Safrole in mg/ kg	Std. Dev.
1a	3.14	1.85
2a	0.15	0.38
3a	6.15	4.63
1b	0	0
2b	0	0
3b	0.32	0.56
Agitate	11.34	1.02

Table 4.4: Milligram of safrole hypothetically consumed by a 60 kg person. Concentrations calculated from Table 4.3.

pH of Traditionally Prepared Tea

The pH of three separately prepared, traditional tea samples was quantified using a pH probe. The probe was rinsed with distilled water, inserted into the tea sample, and rinsed again with distilled water. The traditionally prepared tea samples were prepared following the methods listed above.

Results

Concentrations in ppm of the HPLC analysis of the traditional, FDA and agitate samples can be seen in Table 4.2. One sample prepared using the traditional method with root only (1a-6) was discarded as an outlier with a large Cook's distance. Sample labeled Agitate-3 in Table 4.2 was discarded due to contamination of the column.

The methods outlined in the paper by Reynertson et al., 2005 were followed in preparing comparative samples, namely the spiked samples (2a, 3a, 2b, & 3b). The samples 2a and 2b were prepared with 50 μg safrole standard while the samples 3a and 3b were prepared with root and 50 μg safrole standard, as outlined in Table 4.1. When calculating the concentration of safrole standard added to the samples, the amount suggested by Reynertson et al. (2005), it was discovered that the amount was too small to be detected by the HPLC system. In a 300 mL solution, 50 μg contributes a concentration of 0.67 mg/L, or 0.67 ppm. The LOD and LOQ for this system were 1.514 ppm and 2.049 ppm. Unfortunately, these samples could not be used in discussion of the results of the chemical analysis.

The agitate samples were intended to provide a baseline amount of safrole found in the root and extracted with water. We compared the agitate samples to the traditionally

prepared teas to observe how the quantity of safrole differs between samples being actively heated and boiled and those samples simply being agitated. Although the agitate samples were agitated longer than the traditional methods, the samples were prepared based on color just as a person making sassafras tea will judge the readiness of the tea on its color. The elevated amount of root in the agitate sample was compensated by dividing the concentration by five, as five times as much root was used. It was interesting to note that while the agitate samples did not have the characteristic sassafras smell, but they did have a woody, earthy smell that was not unpleasant.

Based on a student t-test, the concentrations of the traditional method samples were significantly greater than the FDA method samples ($P < 0.05$) and significantly less than the agitate samples ($P<0.05$) based on $\alpha=0.05$.

Observations and Discussion

We found three important points of discussion from the chemical analysis. First, the FDA method was extremely efficient at removing the safrole oil by agitation using organic solvents. There was one sample with a small amount (1.878 ppm) of safrole present, but this is probably due to imperfect laboratory procedures and not a reflection of the methodology. We observed that the resulting liquid from the FDA method was odorless and colorless. There was no characteristic sassafras smell associated with the liquid and therefore, it may be unappealing for a marketable product.

The second observation related to the results of concentrations of the agitate and traditional method samples, as listed in Table 4.3. The average safrole concentration of

the agitate samples was 2268.7 ppm ± 203.3, or 681 ± 61.0 mg of safrole per 300 mL of aqueous solution. The traditional method of preparing sassafras tea (Sample 1a) resulted in an average concentration of 627.5 ± 370.7 ppm or 188 ± 111.2 mg of safrole per 300 mL of aqueous solution. Based on these results, the traditional method samples have 3.6 times fewer grams of safrole per cup (300 mL) of tea as compared to agitate.

Since essential oil composes 6-10% of the weight of the root cortex of sassafras, and safrole composes 80-90% of the essential oil, by taking the highest estimates the root contains around 9% safrole (Carlson & Thompson 1997; Kamdem & Gage 1995). A cup of tea, or around 300 mL, is typically prepared with 2 grams of root. We would then expect the cup of tea to contain 180 mg of safrole oil. The traditional method samples contained 188 ± 111.2 mg of safrole per cup, or close to the expected concentration. On the other hand, roots extracted with water by agitation yielded 681 ± 61 mg of safrole per 300 mL of tea. It is difficult to compare this experiment to previous research as the methods to agitate the sample are going to differ with each study, but it was interesting to note the difference between the amount of safrole we expected to find and the amount of safrole extracted by aqueous agitation. The study by Carlson & Thompson (1997) showed an 88.9% reduction of safrole from the unbrewed teas compared to the brewed teas. The study found ranges of 0.18 – 16.0 mg/g product in unbrewed teas that were reduced to 0.09 – 4.12 mg/g product in the brewed teas.

Chemically, it seems unlikely that using a polar solvent (water) to extract a non-polar solute (safrole oil) should extract more oil than a non-polar solvent (methanol or another organic solvent). Safrole is miscible in alcohols, not water (Kamden & Gage

1995; Sethi et al., 1976). Though, it is difficult to compare the studies as many journal articles do not detail extraction processes. However, it is possible to say that boiling the root as in the traditional method aided in reducing the concentration of safrole in the resulting aqueous solution, as compared to the agitate samples.

The third and last observation relates to the literature on safrole content and regulations. Returning to the previous calculations, if we expect one cup of tea with 2 grams of root and root bark to have about 180 mg of safrole, a person weighing 60 kilograms would consume 3.0 mg of safrole per cup. This calculation is supported by a similar estimate by Segelman et al. (1976) who calculated 3.0 mg/kg safrole is found in one tea bag containing 2.5 g of sassafras bark. We found that sassafras tea has 188 ± 111.2 mg of safrole per cup (300 mL) of tea, as listed in Table 4.3. Therefore, a 60 kg person would consume 3.13 ± 1.85 mg/kg bw of safrole per 300 mL of tea (Table 4.4). A person consuming agitated, non-boiled tea would be consuming 11.34 ± 1.02 mg/kg bw of safrole per 300 mL. The United States FDA does not allow the presence or addition of safrole to any foods or beverages and the European Commission of the European Union allows the presence of safrole in foods containing nutmeg and mace only up to 15 ppm. Traditional tea, according to this study, has around 628 ± 370.7 ppm of safrole per 300 mL (Table 4.3).

Using the Body Surface Area (BSA) dose translation formula discussed earlier, a 60 kg person would consume 3.14 mg of safrole, which is equivalent to a dog consuming 5.8 mg/kg bw. A study by Hagan et al. (1965) conducted a six-year study that administered safrole to dogs in doses of 5, 20, 40, and 80 mg/kg bw. After six years, with

the dogs receiving a tablet six days a week, the dogs experienced microscopic, minimal focal necrosis, bile-duct proliferation, fatty metamorphosis, and hepatic cell atrophy (Hagan 1965). Another study with dogs that spanned seven years found liver injury early in the experiment, but hypothesized that the lack of damage found later in the study was due to adaptation to the continuous intake of safrole (Weinberg & Sternberg 1966).

The dose equivalent for rats would be 19.4 mg/kg bw. As stated before, many toxicity studies administer doses in the range of 100 to 1000 mg/kg bw, though Daimon et al. (1998) tested single doses of 1, 10, 100, 250, and 500 mg/kg bw. Two DNA-safrole adducts per 10^7 nucleotides were found after a dose of 10 mg/kg bw, while a 1 mg/kg bw dose did not produce adducts and a dose of 100 mg/kg bw produced two major and two minor adducts per 10^7 nucleotides. Friedman et al. (1971) found that a 10 mg/kg dose of safrole had no effect on microsomal enzyme activity, which aid in the detoxification process. Chang et al. (2002) found the metabolic saturation of safrole occurred between 150 and 300 mg/kg for rats, or much greater than the amount of safrole found in a cup of tea. Saturation of safrole means the body cannot eliminate the compound quickly and the safrole accumulates in tissue muscle, especially in the liver and kidneys, to increase the risk of DNA adducts and carcinogenesis (Benedetti et al.,1977).

Based on these few studies, there was no indication that rats or dogs fed safrole at the amounts listed above developed liver tumors or cancer. There are not enough studies that have tested these particular doses on laboratory animals to conclude with certainty that the amount of safrole found in sassafras tea will lead to carcinogenesis. These few studies are promising, but more research needs to be conducted.

Plant secondary compounds, used by the plants for defense against predators and pathogens, are utilized by humans for the same purpose, to rid the human body of disease by way of pharmaceuticals (Bourgaud et al., 2001). Secondary compounds must be used properly, as the same compounds that humans harness to starve off disease can harm the body. Safrole is a proven hepatocarcinogen to laboratory animals, causing macro- and microscopic liver lesions, liver tumors, inability to gain weight, and reduced feeding (Jin et al. 2011; Long et al., 1963; Miller et al. 1983; Taylor et al., 1964). These symptoms exhibited by laboratory animals make sense when we consider the traditional medicinal qualities of sassafras tea, including 'overfatness' and to thin the blood during the change of seasons, especially during the spring (Moerman 2011). Considering the laboratory tests use high doses of safrole compared to the amount of safrole found in traditional tea, we can hypothesize that the extreme symptoms observed in laboratory animals occur to some degree in those drinking sassafras tea, but possibly to the human advantage. While laboratory rats had difficulty gaining weight or had a reduced appetite while consuming safrole, humans perhaps used small amount of safrole in sassafras tea to help shed winter pounds. A seven year study of administering safrole to dogs found evidence of liver injury early in the research but not late in the study. The researchers suggested the dogs adapted to the continuous intake of safrole and regenerated the damaged liver tissue (Weinberg & Sternberg 1966).

Reduction of Safrole during Boiling

A handful of studies have attempted to explain the process that occurs when safrole-containing substances are subjected to heat. One study examined the spices star anise, cumin, black pepper, and common cooking ginger, which contain safrole in the essential oil, and found that washing, drying, and cooking the spices all reduced safrole to safe levels. Boiling the spices reduced the safrole content from 955 mg safrole per kg material in untreated seeds to 375 mg/kg in whole seeds boiled for one minute all the way to 9 mg/kg in powdered seeds boiled for five minutes (Farag & Abo-Zeid 1997). The study on a Chinese wild ginger reduced the 1.57 to 2.76 mg safrole per g material to 0.2 mg/g or undetected in most samples after a one-hour decoction of the root (Chen et al., 2009). Carlson & Thompson (1997) hypothesized that the lower safrole content in their sassafras 'tea' (the tea was infused not decocted) could have been due to either the decreased solubility of sassafras oil in water or volatilization of the compounds.

Three chemical reactions could be occurring to reduce the safrole content in a cup of boiled tea. First, the essential oil, including the safrole oil, could be still in the root. Carlson & Thompson (1997) hypothesized that safrole content was reduced due to the insolubility of the essential oil in the root, which agrees with their finding of high recovery of safrole from alcohol- based sassafras oil tinctures. The second situation could involve volatilization of safrole as it is being heated. The compound has a low vapor pressure (0.0706 mm Hg at 25°C or 1 mmHg at 63.8°C), making safrole primarily a vapor at room temperature and pressure (Sigma-Aldrich MDMS S9652). If the compound was boiled at high temperatures for an extended period of time safrole would have some

volatility. Carlson & Thompson (1997) cited volatilization as well as reduced solubility of safrole in water for the reason safrole content was reduced an average of 88.9% from unbrewed to brewed tea. Many laboratory studies mention the volatilization of safrole from safrole-spiked rodent diet, which was left in open in room temperature. One study includes Homburger et al. (1965) that measured the evaporation rate of safrole to be 11.3% per three day period (Crampton et al., 1997; Huang et al., 1999; Long et al., 1963).

The last possibility is that safrole is being altered or degraded during the boiling process, as described in the Reynertson et al. (2005) paper. Reynertson et al. hypothesized that even though safrole is insoluble in water, insolubility is not the issue as samples spiked with safrole still resulted in no detected safrole. Since there were a number of degraded by-products after samples were boiled, it is probable that the safrole was not volatilized. Rather, the safrole was probably degraded during the boiling process by hydroxylation of the dioxolane ring. The hydroxylation could be due to the slightly acidic nature of the tea, which they measured at pH 4.4 (Reynertson et al., 2005). For this research, the average pH for the traditional method tea sample was measured at pH 5.1 ± 0.1, which is considered acidic.

CHAPTER FIVE

WORKING WITH NATIVE COMMUNITIES: EDUCATION AND OUTREACH

Introduction

During the research, we have collaborated with the Cherokee community, who continue to drink sassafras tea according to their traditional method. In addition, there are many non-Native people that grew up harvesting, processing, preparing sassafras, and drinking sassafras tea. The Cherokee best represents a cohesive community of people that maintain the knowledge and tradition of preparing sassafras tea. Since the Center for Cherokee Plants has worked to maintain and continue traditional knowledge of native, culturally signification plants, this work might not have been possible.

Working with Native Communities

This research, although not strictly participatory, is based on a model of working with Native communities through participatory research used by many researchers. In this model, research, education, and action are combined and research is based on the circumstances of the community. The researchers work to connect communities with resources, data, and scientific knowledge of benefit to them. There is an underlying assumption that communities will benefit from the research as resources are based on an "ecological give-and-take approach", where knowledge, time, or mentoring by a community is returned to them through conscience acts of reciprocity (Davis & Reid 1999; Cotton 1996).

Linda Tuhiwai Smith said, “The term ‘research’ is inextricably linked to European imperialism and colonialism. The word itself, ‘research’ is probably one of the dirtiest words in the indigenous world’s vocabulary” (1999). Unfortunately, many Native communities around the world have been exploited by researchers. Some research has even been harmful, especially research related to data collection and reporting on negative issues like alcoholism or drug addiction. Such research can lead to communities being ostracized or stigmatized, with participants feeling betrayed. In the worst cases, participatory research has left participants feeling invaded, patronized, or inferior, which is why many Native communities today have created strict rules to how and with whom research can be conducted on their land (Davis & Reid 1999).

Anthropological and botanical studies in particular are in danger of being of little use to Native communities, and unfortunately some research on traditional medicines has been unconcerned with collaboration, effects of publication, or reciprocity. Given the history of medical, anthropological and archeological research, contemporary research projects with Native communities absolutely should be a collaborative effort between the community and the researchers. Christopher (2005) outlines several recommendations for non-Indian researchers, including involving the community from the formation of the question to the conclusion, as well as ensuring that the community has access to the research and results. The research should be based on values, concerns, and interests of the community and contain culturally-appropriate methodologies (Weaver 1997). The project should also have clear and immediate benefits for the community. Knowledge for

the sake of knowledge, according to activist and historian Vine Deloria Jr., should not be tolerated by Native Americans (1969).

Collaborating with the Cherokee

Although this project was concerned with the perceived toxicity of products made from *Sassafras albidum*, this research did not draw the official support of the tribe due to the historical use of sassafras for spiritual and medicinal purposes. However, we were able to work with the Eastern Band of Cherokee Indians Cooperative Extension to provide a means for education and outreach about the species. The Cooperative Extension acted as a 'cultural consultant' to advise us on the appropriate channels to distribute the research results (Weaver 1997). We planted sassafras trees and created a GIS map and pamphlets for the Center for Cherokee Plants (CCP), a project under the Extension office. The Center for Cherokee Plants, located between Cherokee and Bryson City, North Carolina and near the Kituwah mound, is a nursery that cultivates and experiments with Cherokee heirloom varieties of edible crops. They collect, save, and distribute seed within the tribe to encourage gardening with old varieties and healthy eating. The Center emphasizes youth and elder participation by establishing community and school gardens around the Qualla Boundary and projects including the Backyard Ramp Patch, Native Plant Study, Cherokee Community Greens Patch, and the Cherokee Farmers Market. They are the hub of agriculture and gardening in Cherokee as they work with community civic clubs and are connected with youth and elders alike. The Center is

a medium to encourage interactions between elders, adults, and youth and the exchange of traditional and indigenous knowledge (CCP 2011).

By working with the CCP, we were able to hold discussions about sassafras tea and most importantly, give back to the community. Education and outreach comprise a significant part of this project for several reasons, including to disseminate scientific knowledge about the properties and safety of sassafras products and to highlight the cultural and historical importance of sassafras products. We promoted the use of sassafras in several ways: first, by planting a stand of twenty trees of sassafras on the property of the CCP, as seen in Figure 5.1. The CCP can decide in what ways they will use trees, either by harvesting material for demonstration purposes, allowing tribal members to harvest material for their own use, or as a physical reminder of how sassafras is a culturally significant species for the Cherokee. The trees also provide a basis for future research, ranging from topics on forestry to food chemistry. We also are in the process of creating pamphlets to be displayed with other educational material at the Cooperative Extension office in the city of Cherokee as well as at the Center for Cherokee Plants. Appendix A, focused on simple means of propagating sassafras, will be modified for these pamphlets. A copy of the thesis will be given to the Museum of the Cherokee Indian, which is also required for research under the auspices of the Tribal Research Committee, though we did not submit this research for their approval per their request (EBCI C.O., Sec. 70-3).

We hosted a high school intern during Summer 2011 to complete a Geographic Information System (GIS) project to map the nursery grounds of the Center for Cherokee

Plants. The GIS map was made into a 3’ x 4’ laminated wall display and also into printale sheets for record-keeping. The Center asked not for the maps to be either replicated in print form, other than their own copies, or available on the internet, which we have agreed not to do. This is an example of how researchers need to be flexible to the wishes of the Native community which with they work.

The continued use of sassafras trees for traditional foods and beverages could bring attention to land that is under development on the Qualla Boundary. Many current stands of sassafras in use by tribal members are open fields or sites slated for bulldozing. In the future, a non-timber forest product or products could be developed to sell if tribal members so desired. There could be potential for members of the tribe to petition the Food and Drug Administration based on this research, but more research and chemical analysis would most likely be needed before individuals could market and sell sassafras products. Appendix B contains more information on how to submit a petition to the FDA.

Figure 5.1: Stand of twenty sassafras trees planted at the Center for Cherokee Plants in Bryson City, North Carolina. The three-year-old trees were planted in Spring 2011.

CHAPTER SIX

SUMMARY

Sassafras tea is consumed today by many communities in the Southeastern United States, particularly in the Appalachian Mountains. Products made from the roots of *Sassafras albidum* have been banned from the market by the FDA due to the presence of the confirmed carcinogenic compound, safrole, based on tests using pure safrole standard. Toxicological studies typically test compounds by administering large doses of the compound to laboratory animals in order to understand the mechanisms of carcinogenesis. These studies are needed to understand the mechanisms but should be carefully analyzed when attempting to discuss the effects of ingesting safrole at daily levels. Before this project, no research had quantified the concentration of safrole in traditionally prepared sassafras tea. Additionally, no studied have used traditionally prepared teas in laboratory rodent cancer potency tests. Even though the number of people that consume traditional sassafras products is unknown, we can assume that many people grew up drinking the tea continue to consume it, as well as people that recently learned through herbal medicine guides. Due to the regular use of sassafras tea, it is important to understand any potential dangers associated with consuming products derived from the tree.

This study was informed by traditional knowledge of harvesting, processing, and preparing sassafras tea, as understood from conversations with members of the Eastern Band of Cherokee Indians (EBCI) as well as non-Cherokee well-versed in making

sassafras tea. The first objective of this study was to conduct chemical analysis using HPLC. The intention was to compare the FDA method of extracting sassafras oil with a standardized process of preparing sassafras tea to quantify the amount of safrole present. An agitate sample was analyzed to compare the influence of heat on the quantity of safrole. The second objective was to return the results of this study to communities that utilize sassafras tea, namely the EBCI. We also emphasize the importance of reciprocity for the Cherokee community, who has dedicated their own resources to this project.

The results of the chemical analysis demonstrated that the FDA method was very effective at eliminating safrole entirely, as only one sample showed a miniscule amount of safrole. Additionally, analysis revealed that traditionally-prepared sassafras tea contains 188 ± 111.2 mg of safrole per 300 mL, or 0.63 ± 0.37 mg safrole per mL of solution. This is a safrole concentration of 628 ppm in traditionally prepared sassafras tea. Previous research examining the safrole content in root and root bark predicted a similar quantity of safrole, or 180 mg per 300 mL. On the other hand, the agitate samples from this research contained a higher concentration of safrole, or 680 ± 0.061 mg per 300 mL, or 2.3 ± 0.2 mg/mL of solution. This is a concentration of 2269 ± 203.3 ppm, or more than predicted from previous estimates of safrole concentration in essential oil. As the agitate samples in this research used water as the extraction solvent (versus organic solvents used previously), it is difficult to conclude if safrole is reduced in the boiling process. If we assume the agitate samples in this study are the reference point of safrole content, then boiling does reduce the amount of safrole found in traditionally prepared tea.

These three methods of extracting sassafras root resulted in important observations. First, the traditionally made sassafras tea had a pleasant smell and color - properties that many cultures associate with the tea. Second, the agitate samples did not elicit the characteristic sassafras smell and it took more root and time to achieve the desired color. Lastly, the FDA method resulted in a clear, odorless product with undetectable sassafras odor. In hindsight, the concentration of safrole found in sassafras tea may be serving some purpose, including simply contributing taste and scent properties to the tea or functioning medicinally.

In conclusion, this research contributes to a growing body of knowledge, both for those in the academic world as well as practitioners and caretakers of traditional knowledge. This research will serve at least two purposes: first, the results of this research will help people make informed decisions about their personal consumption of sassafras products. Second and more broadly, this will highlight the importance of traditional preparation methods when analyzing foods and beverages, especially those historically used by Native communities.

APPENDICES

Appendix A

Propagating *Sassafras albidum*

Sassafras is notoriously challenging to propagate or even find in nurseries. Not only is it problematic to transplant (due to its deep taproot) but ripe seeds are difficult to find in the wild (Dirr 1998). The species is dioecious so only female plants produce seed, which are often eaten quickly by birds and wildlife. Greenish-yellow flowers produce single-seed drupes on red pedicles that turn a dark blue-purple when mature (Bonner 2012). The species can be propagated from fresh, ripe seeds, dried seeds, cuttings, or root scions (Rasch 1998).

Transplanting

Sassafras can be grown in a container from seed and the tree transplanted any time of the year. A tree taken from the wild or from field plantings should be true seedlings (not root sprouts) that are transplanted in the late winter or early spring. The trees started by seed and grown in large pots until planting seem to transplant better than those transplanted directly from a field (Sternberg 2004). The colonal root system of sassafras, along with its deep taproot, makes it difficult to transplant saplings or trees easily. Root pruning in the late fall or early winter can encourage a tree to develop a healthy root ball before it is transplanted in the spring.

When transplanting saplings, the tree should be balled and burlapped in moist, loamy, acidic, well-drained soil (Dirr 1998). About a third of the plant should be pruned to encourage root growth (See Figure A.1). If the main bole or branches break, as

Figure A.1: Transplanted and pruned sassafras sapling.

brittle wood tends to do, then the stem is cut to the ground and a new stem will sprout. A sassafras thicket is obtained by cutting the stem back to encourage root suckers, but if a single stemmed tree is desired, the suckers should be removed (Sternberg 2004).

Seeds

Sassafras trees produce seed around 10 years of age (minimum of 4 years old) and reach maximum production from ages 25 to 50 with seed crops every year or every other year. Seed viability is around 35% and the seed can remain viable for six years on the forest floor. A study on *Sassafras randaiense* (Hayata) Rehder cited inhibitors in the seed coat and cotyledon that cull germination, requiring removal or scarification of the seed coat and followed by two to three months of cold stratification (Chen & Wang 1985). Sassafras seeds do not store well as they are an oily seed (versus a more starchy seed), with 47% lipid content (Bonner 2012). To break a natural dormancy of the embryo, the seeds need a cold-wet stratification with a period of 120 days at 41°F (5°C) in moist sandy or mineral soil (Bonner 2012; Griggs 1990; Haywood 1994). Germination can then be tested in sand or a germination soil mix at 70° to 85° F. (21-29° C) for up to 120 days.

One method of germinating seed by the Woodlanders Nursery in Aiken, SC is to sow seed in a ‘community pot’, or a three-gallon container with well-draining potting soil. The seeds should be rehydrated in water for 24 hours, surface sterilized, and scattered in a sterilized three gallon pot of soil with a half inch of soil covering the seed. The community pot should be watered, covered with hardware cloth, and placed outside for the entire winter until the seeds germinate in the spring. The pot is dumped out and

seedlings separated to be potted individually (Bob McCartney, personal communication November 21, 2011).

Root Cuttings

A study by Sicuranza (2007) showed poor results for sassafras propagation from stem sprouts, but worthwhile results from root cutting propagation. It has been found that root cuttings yield plants with longer life spans, less root suckering, and better architecture than grafted plants (Orndorff 1977). Root cuttings are collected from October to December, when roots contain the highest percentage of carbohydrates, and placed in a 2:1:1 peat, loam, sand mixture can be planted out successfully (Del Tredici 1995, Dirr 1998). Some suggest taking root cuttings in February when the ground thaws and storing the roots in dry sand for three weeks before planted in sandy soil. If roots are harvested in the summer, Sicuranza (2007) showed July as the most successful month.

Evans & Blazich suggest harvesting root pieces that are 5 to 10 cm in length in early winter. Del Tredici (1995) recommends root pieces that are 10 to 15 cm long for roots being planted outdoors in late fall or early winter. A straight cut is made on the end closest to the parent plant while a slanted cut is made on the opposite end (Evans & Blazich 2011). The roots need to be washed and fibrous roots removed. The root pieces are placed vertically in a moist medium, preferably pure sand or 1:1 peat to perlite mix with 10-20% sand. Griggs (1990) recommends placing stem sprouts vertically or larger roots horizontally. The straight cut, or where the root was closest to the parent plant, should be covered by 2 to 3 inches of soil (Evans & Blazich 2011). The medium and

roots are stored in a cool, dark location for three weeks until put in a warm greenhouse. There is a danger of water stress as the sudden warmth will cause shoots to develop before roots (Sicuranza 2007).

Pests & Disease

Foliage disease is the most prominent problem among *S. albidum*, along with a strong susceptibility to fire damage. Overall, sassafras is usually free from pests and disease, probably due to the strong presence of secondary defense compounds, but it does suffer from the occasional cankers, leaf spots, mildew, wilt, root rot, Japanese beetle, and sassafras weevil (Dirr 1998).

Propagation Trials

Sassafras seeds were collected in August in Clemson, South Carolina and left to air dry on newspaper. For these trials, the fleshy seed coats were allowed to dry and then removed manually, but Bonner (2012) recommends removing the pulpy flesh before storage or propagation by rubbing the seed over hardware cloth. Two methods of processing and cold-wet stratifying the seeds were tested. The first method involved removing the dried seed coat entirely, which can be done by carefully cracking open with a small knife or fingernail and extracting the round, light brown seed. The seeds were surface sterilized in a 10% bleach solution for 30 seconds, rinsed, and placed in a sterilized plastic snap-lid container with damp perlite. The second method leached the seeds of any chemicals that would inhibit germination. The seeds were placed in

cheesecloth like fabric and placed in a back toilet bowl for fourteen days. The seeds were scraped with sandpaper, surface sterilized, and placed in damp perlite.

All containers were placed in the vegetable drawer of a refrigerator (at 41° F or 5° C) for four months. In March 2012 the seeds were sown in potting soil and placed outside to germinate. The seeds will be monitored for success rate of germination of either the leaching method or removal of seed coat.

A third method of stratifying and germinating the seeds was a method recommended by Woodlanders Nursery in Aiken, SC, termed 'community pot' (personal communication, Nov. 21, 2011). This method (see above description) was begun for the experiment but unfortunately had to be terminated. Future studies in sassafras propagation should strongly consider this method.

The propagations trials are still ongoing. In Fall 2011, a dozen sassafras saplings considered weedy by the landowner in Cherokee, NC were cut around with a sharp shovel to encourage a root ball. Six of the trees were transplanted in March 2012 and six more will be transplanted in Fall 2012 to the Center for Cherokee Plants. The saplings will be monitored to measure success rate of spring and fall transplanting.

Appendix B

Petitioning the Food and Drug Administration

According to the FDA website, the government agency receives about 200 petitions a year (webpage last updated in 2008). The evaluation of a petition can take a few weeks or up to a year. The majority of the petitions come from industry and consumer groups, though individuals are allowed to submit petitions. There is a specific format for citizen petitions that must be followed for submission, which can be found by searching for Title 21 of the Code of Federal Regulations, Sections 10.30. It contains six parts: action requested, statement of grounds, environmental impact, economic impact (if applicable), certification, and identifying information.

The code that specifically targets the use of sassafras bark to make sassafras tea is [Code of Federal Regulations: Title 21, Volume 3, Sec. 189.180 Safrole].
The full title: Title 21 – Food and Drugs, Chapter I -- Food and Drug Administration, Department of Health and Human Services, Subchapter B – Food for Human Consumption (Con't), Part 189 – Substances Prohibited from Use in Human Food, Subpart C – Substances Generally Prohibited from Direct Addition or Use as Human Food. Sec. 189. 180 Safrole.

The code states in part (b), "Food containing any added safrole, oil of sassafras, isosafrole, or dihydrosafrole, as such, or food containing any safrole, oil of sassafras, isosafrole, or dihydrosafrole, e.g., sassafras bark, which is intended solely or primarily as a vehicle for imparting such substances to another food, e.g., sassafras tea, is deemed to

be adulterated in violation of the act based upon an order published in the Federal Register of December 3, 1960 (25 FR 12412)" (CFR, Sec. 189).

Specifically, the petition should outline the confirmed carcinogenic properties of pure safrole standard and related metabolites in comparison to pivotal papers like Benedetti et al. (1977) that found a lack of 1'-hydroxysafrole (a carcinogenic derivative of safrole) in human subjects after ingestion of safrole. Next, the recent research on the non-toxicity of traditionally prepared foods, beverages, and medicines should be mentioned **(**Chen et al., 2009; Farag & Abo-Zeid 1997; Reynertson et al., 2005; Singh & Devkota 2003; Whitton 2003). The results of this research should be cited as well as any other future research on the toxicity or carcinogenicity of traditionally prepared sassafras products.

Summaries of all the studies can be found in the following reviews:

- Cropwatch.org/: Safrole
- SCF. 2002. Scientific Committee on Food, European Commission: Opinion of the Scientific Committee on Food on the safety of the presence of safrole (1-allyl-3,4-methylene dioxy benzene) in flavourings and other food ingredients with flavouring properties. (SCF/CS/FLAV/FLAVOUR/6 ADD3 Final).
- WHO 2009. World Health Organization: WHO Food Additives Series: 60. Safety evaluation of certain food additives. The 69th Meeting of Joint FAO/WHO Expert Committee on Food Additives.

Appendix C

Reference of Previous Laboratory Studies Using Safrole

Studies on betel quid chewing were included in this chart as they gave the unique opportunity to study the effects of safrole injection in humans. The inflorescence of *Piper betle* contains 15.35 mg safrole when fresh, which metabolize to dihydroxychavicol and eugenol (both mentioned in the chart) (Chang et al., 2002). Betel quid chewing is vastly different from consuming sassafras products, both in the complexity and composition of material and in the mode of safrole absorption. Both products contain safrole, so we can observe similarities in physiological effects from a range of concentrations.

Many studies quantify the presence of carcinogen- DNA adducts as a risk for cancer is influenced by the capability of a compound to form these covalent bonds. There is a correlation between the incidence of carcinogen-DNA adducts and the formation of carcinogenic tumors, but adducts do not predict the risk of cancer (Groopman & Skipper 1991).

Table C.1: Review of studies using traditional or modern methods of preparation.

Study	Plant Material	Extraction method	Original Safrole Level	Reduction method	Reduced Safrole Level
Reynertson et al., 2005	*Cinnamomum* bark	MeOH extraction	0.435 mg (in 1 g solution)	Aqueous decoction for 20 minutes	Not detected after boiling
Farag & Abo-Zeid 1997	Black pepper	Steam distillation	0.955 mg (in 1 g material)	Aqueous decoction of whole seeds for 1, 5, and 30 minutes and of powdered seed for 1 and 5 minutes.	0.375 to 0.009 mg (in 1 g material)
Chen et al., 2009	Wild ginger	MeOH extraction	0.14 to 2.76 mg (in 1 g material)	Aqueous decoction for 1 hour	Mainly undetected to 0.2 mg (in 1 g material). Also found in the traditional Chinese preparation no more than 0.02 mg
Carlson & Thompson 1997	*Sassafras albidum* - root bark powder		0.18 to 16 mg (in 1 g material)	Aqueous infusion for 30 minutes	0.03 to 1.37 mg (in 1 g material)
Current Research 2012	*Sassafras albidum* – root	Aqueous agitation	2.2 mg (in 1 mL solution)	Aqueous decoction for 35 minutes	0.609 mg (in 1 mL solution)

Table C.2: Review of studies using laboratory animals and bacterial studies to test the toxicity of safrole and derivatives.

Year	Article	Trial Model	Cmpd. source	Conc.	Results
1961	Abbott, D. D., E.W. Packman, B.M. Wagner and J.W.E. Harrisson. Chronic oral toxicity of oil of sassafras and safrole. Pharmacologist 3: 62.	Rodent	Natural oil of sassafras and safrole	390 ppm for 24 months and 1170 ppm for 22 months	No liver tumors, some sign of kidney congestion. At 24 months liver cancers was found, & cellular changes in the kidneys, adrenals, thyroid, pituitary, and reproductive organs. Requires prolonged exposure before cellular changes.
1985	Baker, R.S.U. and A.M. Bonin. Tests with *Salmonella* plate incorporation assay. In: Ashby and de Serres (eds.) Evaluation of Short-term Tests for Carcinogens. *Elsevier*, N.Y.	In vitro Salmonella Ames test			Safrole was neutral or weakly positive
1977	Benedetti, M. S., A. Malone, A. L. Broillet. Absorption, metabolism and excretion of safrole in the rat and man. Toxicology 7(1): 69-83.	Male Sprague-Dawley rats(100-200 g) and human (50-85 kg)	Safrole standard	Rats: One dose, from 0.63 to 750 mg/kg; Humans: 0.163 to 1.655 mg (1.655 mg is 0.024 mg/kg for a 68 kg person)	For rats, within 24 hours 88% of the lowest dose of 0.63 mg/kg was eliminated, 78% of the 60 mg/kg dose was eliminated, and 25% was eliminated at a dose of 745 mg/kg. For humans, the dose levels were absorbed and eliminated within 24 hours. Unable to detect metabolites in man.
1994	Bolton, J.L., et al. Evidence that 4-allyl-O-quinones spontaneously rearrange to their more electrophilic quinone methides: potential bioactivation mechanism for the hepatocarcinogen safrole. Chem Res. Toxicol. 7: 443-450	Male Sprague-Dawley rats (180-200 g)	Safrole standard		Examined a different metabolic pathway safrole could undergo, namely through oxidation to quinoid metabolites in liver microsomes in a non-enzymatic process.

1973	Borchert, P., et al. The metabolism of the naturally occurring hepatocarcinogen safrole to 1'-hydroxysafrole and the electrophilic reactivity of 1'-acetoxysafrole. Cancer Research 33: 575-589.	Rats (300 g), hamsters, and guinea pigs.	Safrole standard	90 mg was injected into rats or 30 mg/100 g body weight	Testing the theory that safrole undergoes *in vivo* conversion to electrophyllic allylic and benzylic esters that are the true carcinogens. 1'-hydroxysafrole is the primary metabolite of safrole and a proximate carcinogen, more so than safrole.
1973	Borchest, P., et al. 1'-hydroxysafrole, a proximate carcinogenic metabolite of safrole in the rat and mouse. Cancer Res. 33: 590- 600.	CD rats (230-260 g) and CD-1 mice (27-29 g)	Safrole standard	Rats and mice: fed via diet conc. of 0.5%, 0.4%, or 0.3% safrole, 0.55% or 0.44% 1'-hydroxysafrole for 8.5 to 11 months	Showed 1'-hydroxysafrole is more carcinogenic than safrole in adult male rats, almost half of rats given 1'-hydroxysafrole developed liver carcinomas by 8 months versus rats given safrole 0.04% developing liver carcinomas by 16 months. The rodents gained weight more slowly, but until they started developing tumors, survivorship was high.
1985	Bradley, M.O. Measurement of DNA single-strand breaks by alkaline elution in rat hepatocytes. In: Ashby and de Serres (eds). Evaluation of Short-term Tests for Carcinogens. Elsevier, N.Y., 353-357.	*In vivo* mammalian cell genotoxicity assay			Safrole tested positive for Sister Chromatid Exchange assay.
1966	Casida, J. E., et al. Methylene-C^{14}-dioxyphenyl compounds: Metabolism in relation to their synergistic action. Science 153(3740): 1130-1133	House fly and male albino mice	Standard		Showed that many of the compounds volatilized during the incubation period. Tested the recovery of the compounds from houseflies and mice.

2010	Catalán, L. E., et al. Synthesis of Nine Safrole Derivatives and Their Antiproliferative Activity Towards Human Cancer Cells. Journal of the Chilean Chemical Society 55: 2, 219-222.	Breast and colorectal cancer cell lines	Safrole standard		Safrole and six derivatives were tested- the derivatives proved to be more cytotoxic to human cancer cells than safrole.
2002	Chang, M.J.W., C.Y. Ko, R.F. Lin & L.L. Hsieh. Biological monitoring of environment exposure to safrole and the Taiwanese betel quid chewing. Arch. Environ. Contam. Toxicol. 43: 432–437.	Adult male Wistar rats and human urine	Safrole standard	Rats: 0, 30, 75, 150, 300 mg/kg. Tested urine of rats and human who chewed quid between 2 - 100 times a day	Rats were used to establish a linear dose-response relationship- found that there was a metabolic saturation between 150 and 300 mg/kg in rats. The safrole metabolites dihydroxychavicol and eugenol were found in the urine of non-betel quid chewers, at the levels of not detected to 5.4 micrograms/mg creatinine, probably due to spices in the diet.
1977	Crampton, R.F., et al. Long-term studies on chemically induced liver enlargement in the rat. II. Transient induction of microsomal enzymes leading to liver damage and nodularhyperplasia produced by safrole and Ponceau MX. Toxicology 7: 307-326.	Female Wistar albino rats (80-100 g)	Safrole standard	Diet containing 0.25% (w/w) safrole for 85 weeks, 10% more safrole was initially added due to volatility	Liver weight increased to 20% after week 1. Liver weight continued to increase to 60% more than the control group by week 8 with a decrease in drug metabolizing enzyme activity. Cytochrome P-450 was initially 80% higher, but lowered to 20-30% higher after 16 weeks. Noticed enlarged centrilobular hepatocytes, individual cell necrosis, and necrosis of hepatocytes.
1997	Daimon H., S. Sawada, S. Asakura & F. Sagami. Analysis of cytogenetic effects and DNA adduct formation induced by safrole in Chinese hamster lung cells. Teratogenesis Carcinogenesis Mutagenesis 17(1): 7-18.	Chinese hamster lung cells	Safrole standard	0.025 to 0.2 mg/ml	Used 32P-postlabeling assay that safrole forms DNA adducts, in turn induces sister chromatid exchanges and chromosomal aberrations assays.

1998	Daimon, H., S. Sawada, S. Asakura, and F. Sagani. *In vivo* genotoxicity and DNA adduct levels in the liver of rats treated with safrole. Carcinogenesis 19: 141-146.	Rat (F344) model	Safrole Standard	Five doses of 62.5, 125 or 250 mg/kg, single doses of 1, 10, 100, 250, or 500 mg/kg via stomach tube	No mortality. The single doses did not induce chromosome aberration, but the repeated doses increased aberrant cells in the liver. The single doses at 100, 250 and 500 mg/kg and the repeated doses induced sister chromatid exchanges. DNA adducts were found in the single doses of 10 (2 adducts) and 100, 250, & 500 (4 adducts) mg/kg while no adducts were observed in 1 mg/kg
1978	Dorange, J. L. et al. Comparative survey of microsomal activation systems for mutagenic studies of safrole, Mutation Research 53: 179-180.	Adult rats		42 mg/rat/day via i.p. injection for three days	Purpose of this study was to discover the activation or enhancement of safrole that tests positives for the Ames test. Safrole tested positive using strain TA 1535, activated by rat liver injected with safrole *in vivo*. Previous studies tested safrole without being modified and showed safrole to be negative in an Ames test.
1976	Drinkwater, N. R. et al. Hepatocarcinogenicity of estragole (1 allyl-4-methoxybenzene) and 1'-hydroxyestragole in the mouse and mutagenicity of 1'-acetoxyestragole in bacteria. J. Natl. Cancer Inst. 57: 1323-1331.	CD-1 mice, newborn (16 g) and 9-12 week old (35 g)	Safrole standard, synthesized to 1'-hydroxy safrole	Newborn mice: total of 4.43 micromoles; 9-12 week old mice: total of 5.19 micromoles, via se injection	The 1'-hydroxy metabolites of both estragole and safrole were found to be more carcinogenic than the parent compounds. 59% of the mice given 1'-hydroxysafrole developed liver tumors.

1975	Elcombe, C. R. et al. Studies on the interaction of safrole with rat hepatic microsomes. Biochem. Pharmacol. 24: 1427-1433.	Male Sprague-Dawley rats (200 g)	Safrole standard	2% (w/w) via diet for two weeks.	Safrole reacts to form a safrole metabolite- cytochrome P-450 complex.
1972	Epstein, S.S., et al. Detection of chemical mutagens by the dominant lethal assay in the mouse. Toxicology and Applied Pharmacology 23: 288- 325.	Swiss mice 8-10 weeks old			Dominant lethal assay: safrole tested negative
1967	Fishbein, L. et al. Thin-layer chromatography of rat bile and urine following intravenous administration of safrole, isosafrole, and dihydrosafrole. J. Chromatog. 29: 267-273.	Sprague-Dawley mice (350 g)	Safrole standard	0.04 ml via intravenous injection	The bile and urine samples showed safrole metabolized into isosafrole and dihydrosafrole. The safrole and metabolites were slowly eliminated in the bile.

1971	Friedman, M. A., E. Arnold, Y. Bishop, and S.S. Epstein. Additive and synergistic inhibition of mammalian microsomal enzyme functions by piperonyl butoxide, safrole and other methylenedioxyphenyl derivatives. Experientia 27: 1052-1054.	Male Swiss Albino mice (20-25 g)	Safrole standard	10 mg/kg one time dose of safrole via i.p. injection	The safrole had no effect on microsomal enzyme activity.
1981	Gocke, E., M.T. King, K. Eckardt, and D.Wild. Mutagenicity of cosmetics ingredients licensed by the European Community. Mutat. Res. 90: 91-109.	Bone-marrow micronucleus assay			Safrole tested negative
1978	Green, N.R. and Savage, J.R.,. Screening of safrole, eugenol, their ninhydrin positive metabolites and selected secondary amines for potential mutagenicity. Mutation Research, 57, 115- 121.	In vitro Salmonella reverse mutation assay (Ames test)			Safrole was neutral or weakly positive
1993	Gupta, K.P., K.L. van Golen, K.L. Putman & K. Randerath. Formation and persistence of safrole-DNA adducts over a 10,000-fold dose range in mouse liver. Carcinogenesis 14 (8): 1517–1521.	Female CD-1 mice (25 g)	Safrole standard	One dose of 0.001, 0.01, 0.1, 1.0, and 10.0 mg/mouse or 0.04, 0.4, 4, 40, or 400 mg/kg bw	Examined DNA using 32P-postlabeling assay at 0.5, 1, 2, 3, 7, 15, and 30 days. Showed a linear response of safrole-DNA adducts, reaching a threshold at the 10 mg dose, where the tissue could be saturated and the elimination of the compound would occur more slowly.

1967	Hagan, E.C., W.H. Hansen, O.G. Fitzhugh, P.M. Jenner, W.I. Jones, J.M, Taylor, E.L. Long, A.A. Nelson, and J.B. Brouwer. Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. Food Cosm. Toxicol. 5: 141-157.	Osbourne-Mendel rats and pure-breed beagle dogs	Safrole standard	Rats, adult: 1000, 2500, 5000, and 10,000 ppm for two years; Dogs (2M, 2F): 5 and 20 mg/kg for 6 years	The rats feed 10,000 ppm died after 62 weeks, and the rest experienced growth retardation, increased mortality in the males (at 5000ppm), liver enlargement with tumor masses and nodules, mild hyperplasia of thyroid, and increase of chronic nephritis in the kidney. The dogs experienced liver enlargement and nodules at the 20 mg/kg dose and liver damage by focal necrosis, bile-duct proliferation, fatty metamorphosis, and other changes at the 5 mg/kg dose.
1965	Hagan, E.C., P. M. Jenner, W. I. Jones, O. G. Fitzhugh, E. L. Long, J. G. Brouwer, W. Welfare. Toxic properties of compounds related to safrole. Toxicology and Applied Pharmacology 7(1): 18-24.	Osbourne-Mendel rats and Swiss mice		Rats, adult: 250, 500, 750 mg/kg/day for 105 days; Mice: 250 and 500 mg/kg for 60 days	At 750 mg/kg, 9 out of 10 rats died at 19 days, at 500 mg/kg, 1 of 10 rats died after 46 days, and at 250 mg/kg, no rats died but they experienced liver enlargement and focal necrosis. No evidence of toxicity for isosafrole. Dihydrosafrole was found to be a esophageal carcinogen. Mice showed similar liver changes as found in the rats.
1965	Homburger, F., P.D. Bogdonoff and T.F. Kelley. Influence of diet on chronic oral toxicity of safrole and butter yellow in rats. Proceedings of the Society for Experimental Biology and Medicine 119 (4): 1106-1110.	Osbourne-Mendel male rats (114 g)	Safrole standard	0.5% safrole via diet for five diets with varying levels of protein, fat, carbohydrates, and salts.	The rats receiving high protein diets (30% protein), the liver appeared normal compared to the control while other diets with lower protein had smaller livers when fed with safrole. The safrole spiked diets resulted in livers with no fat present. Rats given safrole lived longer than the control, but therefore had a higher level of hepatomas.

1990	Howes, J.A., V.S.W. Chan, and J. Caldwell. Structure-specificity of the genotoxicity of some naturally occurring alkenyl-benzenes determined by the unscheduled DNA synthesis assays in rat hepatocytes. Food Chem. Toxicol. 28: 537-542.	Male Fischer 344 rats.	Safrole standard	10^-3 M safrole for a unscheduled DNA synthesis assays in cultured rat hepatocytes.	Safrole tested positive for genotoxicity activity, at a higher dose than previous studies (Probst et al. 1981) that tested small doses.
1999	Huang, Y. Ho SH, Kini RM. Bioactivities of safrole and isosafrole on *Sitophilus zeamais* (Coleoptera: Curculionidae) and *Tribolium castaneum* (Coleoptera: Tenebrionidae). Journal of Economic Entomology 92: 676-683.	*Sitophilus zeamais* adults and *Tribolium castaneum* adults and larvae	Safrole standard	Fumigant study at 0.9 mg/cm^3 and contact toxicity study at 2.04-16.2 mg/g of food.	Both insects showed susceptibility to the fumigant toxicity of safrole. There was slight feeding deterrence against *S. zeamais* due to reduced food consumption and no deterrence for *T. castaneum*.
2003	Hung, S.-L., Y.-L. Chen, and Y.-T. Chen. Effects of safrole on the defensive functions of human neutrophils. Journal of Periodontal Research 38: 130–134.	Oral pathogens including *Actinobacillus actinomycetem comitans, Streptococcus mutans,* and *Poryphyromon as gingivalis*	Safrole standard	5 mM and 10 mM	Safrole did not effect growth of *A. actinomycetemcomitans* or *S. mutans,* but did decrease the growth of *E. coli*. Safrole reduces the release of superoxide anion, but not through a direct killing of neutrophils, or a host defense mechanism. Safrole reduced antimicrobial activity but did not demonstrate cytotoxicity.

1969	Innes, J.R., B.M. Ulland, M.G. Valerio, L. Petrucelli, L. Fishbein, E.R. Hast, A.J. Pallota, R.R. Bates, H.L. Falk, L.L. Gart, M. Klein, I. Mitchell, and J. Peter. Bioassay of pesticides and industrial chemicals for tumorigenicity in mice: a preliminary note. J. Natl. Cancer Inst. 42: 1101-1114.	Hybrid mice from C57BL/6 (female) and C3H/Anf or AKR (male) strains	Safrole standard	Hybrid mice, 7 days old: 464 mg/kg over 21 days via stomach tube, then 1265 mg/kg over 82 weeks via diet	Liver-cell tumors found in 14 of 34 males and 32 of 33 females.
1985	Ishidate, M. and T. Sofuni. The in vitro chromosomal aberration test using Chinese hamster lung (CHL) fibroblast cells in culture. In: Ashby and de Serres (eds). Evaluation of Short-term Tests for carcinogens. Elsevier, N.Y.: 427-432.	*In vivo* mammalian cell genotoxicity assay: chromosomal aberrations			Safrole tested positive
1964	Jenner, P.M., E.C. Hagan, J.M. Taylor, E.L. Cook, and O.G. Fitzhugh. Food flavourings and compounds of related structures. I. Acute Oral Toxicity. Food Cosm. Toxicol. 2: 327-343.	Rat and mice		1950 mg/kg bw for rats and 2350 mg/kg for mice	Established acute toxicity at these concentrations.

2004	Jeurissen, S. M. F. et al. Human Cytochrome P450 Enzyme Specificity for Bioactivation of Safrole to the Proximate Carcinogen 1-Hydroxysafrole. Chemical Research in Toxicology. 17 (9): 1245-1250.	Human cytochrome P450 enzymes and microsomes from liver cells lines	Safrole standard		Found the formation of 1'-hydroxysafrole in the human liver microsomes. Various human cytochrome P450 enzymes metabolize safrole, including enzymes P450 2C9*1, P450 2A6, P450 2D6*1, and P450 2E1.
2011	Jin, M., A. Kijma, Y. Suzuki, D. Hibi, T. Inoue, Y. Ishii, T. Nohmi, A. Nishikawa, K. Ogawa, T. Umenura. Comprehensive toxicity study of safrole using a medium-term animal model with *gpt* delta rats. Toxicology 290: 312-321.	Male and Female F344 gpt delta rats	Safrole standard	Rats: 69.1 (0.1% in diet) and 275.6 (0.5%) mg/kg/day for 13 weeks	Both doses experienced decreased body weight gain, liver enlargement in the males and females (0.5% group only), and single cell necrosis. Male rats experienced tubular hyaline droplets, tubular regeneration, granular cast, pelvic calcification, and interstitial cell infiltration in the kidneys.
1970	Kamienski, F.X. and J.E. Casida. Importance of demethylenation in the metabolism *in vivo* and *in vitro* of methylene-dioxyphenyl synergists and related compounds in mammals. Biochemical Pharmacololgy 19: 91-112.	Male Swiss-Webster mice (18-20 g), male Sprague-Dawley rats (150-170 g), male hamster (180-200 g)	Safrole standard	Mice: 5 micromoles/kg bw; rats & hamsters: 10 micromoles/kg bw	The major metabolic pathway for safrole is cleavage of the methylenedioxyphenyl moiety as well as expiration of carbon dioxide from the methylene carbon. Metabolism of safrole, (which is volatile) resulted in less volatile, more polar, and ether-soluble metabolites. Found the metabolites of safrole were more volatile.
1978	Kapadia, G. J., E. B. Chung, B. Ghosh, Y. N. Shukla, S. P. Basak, J. F. Morton, S. N. Pradhan. Carcinogenicity of some folk medicinal herbs in rats. Journal of National Cancer Institute 60 (3): 683-686.	NIH Black rats, 1-2 month old	Sassafras root bark, extracted with petroleum ether, methylene chloride, and ethanol	Rats: 15 mg via s.c. injection for 78 weeks	The safrole-free sassafras extract produced local tumors in 66% of the rats, though study does not indicated if the samples were tested for safrole levels prior to injection.

2011	Khayyat, S. A and S. H. Al-Zahrani. Thermal, photosynthesis and antibacterial studies of bioactive safrole derivative as precursor for natural flavor and fragrance. Arabian Journal of Chemistry, doi:10.1016/j.arabjc.2011.09.014.	Gram-positive bacteria *Bacillus subtillis*, *Staphylococcus aureus*, and gram-negative bacteria *E. coli*	Safrole standard		*B. subtilis* was not inhibited by safrole or safrole epoxide, but it was inhibited by safrole hydroperoxide. *S. aureus* was also not inhibited by safrole, but it was by safrole epoxide and safrole hydroperoxide. *E. coli* was inhibited by all three compounds.
2011	Khayyat, S. A. Photosynthesis of dimeric cinnamaldehyde, eugenol, and safrole as antimicrobial agents. Journal of Saudi Chemical Society, doi: 10.1016/j.jscs.2011.07.014.	*Candida albicans*, fungal pathogen	Safrole standard, photosynthesized to compound		The photosynthesized compounds derived from safrole inhibited growth of *Candida albicans.*
2008	Kim, J. and I-K. Park. Fumigant toxicity of Korean medicinal plant essential oils and components from *Asiasarum sieboldi* root against *Sitophilus oryzae* L. Flavour and Fragrance Journal 23: 79-83.	Fumigation bioassay against the rice weevil *Sitophilus oryzae*	*Asiasarum sieboldi* essential oil	25 microgram/mL of essential oil on filter paper for the fumigant bioassay	Safrole was effective against the rice weevil via fumigation, likely through vapor action through the respiratory system. Found that the essential oil directly from the plant was more effective than purified compounds, as the compounds in the essential oil from the plant could be acting synergistically.

1972	Lake, B. G. & D. V. Parke. Induction of aryl hydrocarbon hydroxylase in various tissues of the rat by methylenedioxyphenyl compounds. Biochem. J. 130: 86.	Male Wistar albino rats	Safrole standard	Diet of 0.25% isosafrole for 14 days	Induction of benzopyrene hydroxylase, with maximum enzyme activity within 14 days. Increases where observed in hepatic biphenyl 2-hydroxylase and 4-hydroxylase activity, microsomal protein, liver weight, cytochrome P-450 content. Hepatic aniline 4-hydroxylase activity was inhibited.
1999	Liu, T. Y., C. C. Chen, C. L. Chen, and C. W. Chi. Safrole-induced Oxidative Damage in the Liver of Sprague-Dawley Rats. Food and Chemical Toxicology 37: 697-702.	Male Sprague-Dawley rats (200 g)	Safrole standard	Rats: single 0, 250, 500,1000 mg/kg via ip injection, with one group given 300 mg/kg Vitamin E	Safrole induces a dose-dependent increase of hepatic lipid hydroperoxides and 8-hydroxy-2'-deoxyguanosine. The higher the dose, the earlier the lipid hydroperodixe levels reached maximum. Oxidative damage is repaired within 15 days. Vitamin E prevented lipid peroxidation but not 8-hydroxy-2'-deoxyguanosine formation.
1963	Long, E.L., A.A. Nelson, O.G. Fitzhugh and W.H. Hansen. Liver tumours produced in rats by feeding safrole. Archives of Pathology 75: 595-604.	Osborne-Mendel rats	Safrole standard	Rats, adult: 100, 500, 1000, 2000 mg/kg over two years, or 5, 25, 50, and 100 mg/kg/day.	The highest dose -reduced body weight gain, mild anemia, and leukocytosis. Liver injury - moderate to severe at the highest dose, to very slight at the 100 mg/kg dose. Maglignant and benign tumors at highest dose, decreasing amounts of benign tumors.
1972	Lotlikar, P. D. & Wasserman, M. B. Effects of safrole and isosafrole pretreatment on N- and ring-hydroxylation of 2-acetamidofluorene by the rat and hamster. Biochem. J. 129: 937-943.	Male albino Wistar rats (60-120 g) and Syrian golden hamsters (100 g)	Safrole standard	Rats, in vivo study: 100 mg/kg bw, In vitro study: 100 mg/kg bw, and 300 mg/kg bw; Hamsters: 200 mg/kg bw	Animals were pretreated with safrole and isosafrole before injected with 2-acetamidofluorene increased the activity of liver microsomal material for hydroxylation of 2-acetamidofluorene, which can inhibit liver carcinogenesis.

1986	Lu, L.J., et al. Differences in the covalent binding of benzo(a)pyrene, safrole, 1'-hydroxysafrole and 4-aminobiphenyl to DNA of pregnant and non-pregnant mice. Cancer Letters 31: 43-52.	ICR mice	Safrole Standard	One time dose of 97 mg/kg to pregnant and non-pregnant mice	Pregnancy altered the binding of safrole to DNA by increasing the adduct binding to liver and kidney DNA by 2.3-3.5 times.
1986	Lu, L.J., et al. 32P-Post-labelling assay in mice of transplacental DNA damage induced by the environmental carcinogens safrole, 4-aminobiphenyl, and benzo(a)pyrene. Cancer Res. 46: 3046-3054.	Mice			2 major adducts formed in the N^2 position of guanine with safrole and 1'-hydroxysafrole
1978	Martin, C.N., A.C. Mc Dermid and R.L. Garner. Testing of known carcinogens and noncarcinogens for their ability to induce unscheduled DNA synthesis in HeLa cells. Cancer Research 38: 2621-2627.	Unscheduled DNA synthesis assays in HeLa cells	Safrole standard		Safrole tested negative for unscheduled DNA synthesis and is not mutagenetic in bacteria. 1'-hydroxysafrole was also nonmutagenetic.
1975	McCann, J. et al. Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proceedings of the National Academy of Science of the USA 72 (12): 5135-5139.	*Salmonella*/ microsome test			Safrole identified as a carcinogen, non-mutagenic, with less than 70 revertant colonies on a petri dish per 1000 micrograms of chemical incorporated in the plate and <0.01 revertants per nmol per plate. Used strains TA100, TA1535, TA1537, and TA98.

1985	Mihr, B., L. Bowers, and W.J. Caspary. Assays for the induction of gene mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells in culture. In: Ashby and de Serres (eds.). Evaluation of Short-term Tests for Carcinogens. Elsevier, N.Y.: 555-568.	*In vivo* mammalian cell genotoxicity assay: gene mutation			Safrole tested positive for gene mutation assay.
1983	Miller, C., A.B. Swanson, D.H. Phillips, T.L. Fletcher, A. Liem, and J.A. Miller. Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. Cancer Res. 43: 1124-1134.	CD-1 mice (1.4 g: Day 1; 3.5 g: Day 8; 7 g: Day 15; 13 g: Day 22)	Standard	Mice: safrole at 400 mg/kg ten times & 2300 or 4600 mg/kg; 1'hydroxysafrole at 81 mg/kg, 45 mg/kg, & 3900 mg/kg. Rats: 1000, 1100, 1200 mg/kg	Found liver cancer in 61% (v. control of 24%) in males and 13% in females. Liver cancer in 67% (v. 26% control), lung adenomas in 14% for both compounds. At both doses, liver cancer in 68% of the mice. Lung adenomas were found in 5% for safrole and 10% in 1'-hydroxysafrole. Liver cancer in 55% by 1'-hydroxysafrole.
1982	Mirsalis, J.C., K. Tyson, and B.E. Butterworth. Detection of genotoxic carcinogens in the in vivo – in vitro hepatocyte DNA repair assay. Environ. Mutagen 4: 553-562.	Male Fischer-344 rats	Safrole standard	A single dose of 200 or 1000 mg/kg	Safrole did not induce unscheduled DNA synthesis, maybe from the low metabolization of 1'-hydroxysafrole.
2009	Nakagawa Y., T. Suzuki, K. Nakajima, H. Ishii, A. Ogata. Biotransformation and cytotoxic effects of hydroxychavicol, an intermediate of safrole metabolism, in isolated rat hepatocytes. Chemico-Biological Interactions 180: 89-97.	Male F344/Jcl (200-240 g)-hepatocytes were isolated	Safrole standard	1 mM of safrole in 1 x 10^6 cells/mL	Hydroxychavicol is an intermediate metabolite of safrole and more toxic than safrole as it causes liver cellular mitochondria to dysfunction and causes generation of reactive oxygen species.

1969	Oswald, E. O., L. Fishbein & B.J. Corbett. Metabolism of naturally occurring propenylbenzene derivatives. I. Chromatographic separation of ninhydrin-positive materials of rat urine, Journal of Chromatography 45: 437-445.	Male Sprague Dawley rats (200-500 g)	Safrole standard	Single dose of 75-300 mg/kg bw	Ninhydrin-positive basic materials were excreted in the urine.
1970	Parke, D. V. & H. Rahman. The induction of hepatic microsomal enzymes by safrole. Biochem. J. 119: 53P.	Rat model			Safrole decreased the amount of glycogen in the liver. Adenylate cyclase and 3:5-cyclic AMP may contribute, but noradrenaline did not increase biphenyl 2- or 4-hydroxylases.
1971	Parke, D. V. & H. Rahman. Induction of a new hepatic microsomal haemoprotein by safrole and isosafrole. Biochem. J. 123: 9P.	Rat model		125 mg/kg per day	Increased cytochrome b5 and changed the absorption spectrum, which could result from biosynthesis of a hepatic microsomal haemoprotein. The new absorption spectrum was not observed when thioacetamine was added to the safrole induction.
1984	Phillips, D., M.V. Reddy and K. Randerath. 32P- Postlabelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturallyoccuring alkenylbenzenes. II. Newborn male B6C3F1 mice. Carcinogenesis 5: 1623-1628.	Mice model			2 major adducts formed in the N^2 position of guanine with safrole and 1'-hydroxysafrole

1981	Phillips, D.H., J.A. Miller, E.C. Miller, and B. Adams. N2 atom of guanine and N6 of adenine residues at sites for covalent binding of metabolically activated 1'- hydroxysafrole to mouse liver DNA *in vivo*. Cancer Res. 41: 2624-2671.	Female CD-1 mice, 8-10 weeks old (30 g)	Safrole standard	12 micromoles/ mouse of 1'-[2',3'-3H]-hydroxysafrole via i.p. injection.	2 minor adducts formed in the N6 position of adenine with safrole and 1'-hydroxysafrole
1979	Poirier, L.A. and F. J. de Serres. Initial National Cancer Institute studies on mutagenesis as a pre-screen for chemical carcinogens: an appraisal. J. Natl. Cancer Inst. 62: 919-926.	*E. coli* and *Saccharomyces cerevisiae* in vitro assays			Safrole tested positive in both
1978	Purchase, I. F. H. et al. An evaluation of 6 short-term tests for detecting organic chemical carcinogens, Br. J. Cancer 37: 873-903.	Ames test with *Salmonella typhimurium;* Rabin's test and others	Safrole standard		Safrole tested positive for the Ames test, cell transformation, degranulation, and sebaceous-gland suppression and tested negative for tetrazolium reduction and the implant tests.
1984	Randerath, K., R.E. Haglund, D.H. Phillips and M.V. Reddy. 32P-Postlabelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. Carcinogenesis 5: 1613-1622.	Female CD-1 mice (25 g)	Safrole standard	2 mg for DNA binding studies or 10 mg for adduct persistence studies. Mice: 80 mg/kg or 400 mg/kg.	2 major adducts formed in the N^2 position of guanine with safrole and 1'-hydroxysafrole. After a single dose of 10 mg of safrole, one adduct formed in 140,000 nucleotides. RAL x 10^7 values for safrole were 491 +/- 129 for 10 mg and 114 +/- 50 for the 2 mg dose.

1993	Randerath, K.P., K.L. Putman, and E. Randerath. Flavor constituents in cola drinks induce hepatic DNA adducts in adult and fetal mice. Biochemical Biophysical Research Communications 192 (1): 61-68.	Female ICR mice, 6-7 weeks	Safrole Standard	Mice :cola drinks instead of water (for 4 or 8 weeks), mice given a single 10 mg dose of myristicin, nutmeg, or mace via gastric intubation.	Mice that only drank cola-drinks developed 100-200 DNA adducts in 10^9 DNA nucleotides, with safrole 5-6% of the adducts and myristicin 50-80%. At 8 weeks, safrole represented 5-6% of the total adducts. For myristicin, nutmeg, and mace, safrole adducts represented 3.5-8.5% of the total adducts.
1979	Reuber, M. D. Neoplasms of the forestomach in mice ingesting dihydrosafrole, Digestion, 19, 42-47	Hybrid mice	Safrole standard	464 mg/kg of safrole & dihydrosafrole: 215 mg/kg of isosafrole; 1,112 ppm safrole, 1,400 ppm dihydrosafrole, & 517 ppm isosafrole.	Dihydrosafrole injestion resulted in neoplasms of the forestomach. Safrole and isosafrole did not have stomach tumors, but safrole did increase the incidences of liver carcinomas.
1969	Seto, T. A. & W. Keup. Effects of alkylmethoxybenzene and alkylmethylenedioxybenzene essential oils on pentobarbital and ethanol sleeping time. Arch Internde Pharmacodynamie et de therapie 180: 232-240.	Female white mice (22-40 g)	Safrole standard	10 or 20 mg/kg via i.p. injection along with sodium pentobarbital and 100 mg/kg via i.p. injection of ethanol	A dose of 20 mg/kg of safrole doubled the mean sleeping time compared to the control when added to the sleeping tablet compound sodium pentobarbital. Safrole did not significantly affect the sleeping time in association with ethanol.
1973	Stoner, G. D. et al. Test for carcinogenicity of food additives and chemotherapeutic agents by the pulmonary tumor response in strain A mice, Cancer Research 33: 3069-3085.	A/He mice, 6-8 weeks old (18-20 g)	Safrole standard	Over 24 weeks, mice received a total of 4.50 or 0.90 g/kg bw of safrole over 12 injections.	In the mice that received 4.50 g/kg bw, 10 out of 30 died and three mice had lung tumors. The mice the received 0.90 g/kg bw, 3 out of 30 died and 6 mice had lung tumors.

1979	Swanson, A.B., D.D. Chambliss, J.C. Blanquist, E.C. Miller and J.A. Miller. The mutagenicities of safrole, estragole, trans-anethole and some of their known or possible metabolites for Salmonella thyphimurium mutants. Mutat. Res. 60: 143-153.	In vitro Salmonella reverse mutation assay (Ames test)	Safrole standard	*Salmonella typhimuri um* strains TA100, TA1535, and TA98	Safrole was neutral or weakly positive in the Ames test. 1'-hydroxysafrole was mutagenic for strain TA100. Toxicity increased with addition of NADPH-fortified rat liver microsomes and cytosol. Previous studies with positive mutagenic properties used a preincubation period, which maybe have created metabolites.
1964	Taylor, J.M.,P.M. Jenner and W.I. Jones. A comparison of the toxicity of some allyl, propenyl and propyl compounds in the rats. Toxicology and Applied Pharmacology 6: 378-387.	Osborne-Mendel or Sherman rats (180-350 g)	Safrole standard	650 mg/kg bw via gavage, or 1/3 of the LD50 of safrole	No rats died out of six tested for safrole, but an average of 2.5 macroscopic liver lesions were observed. The rats lost weight and were in poor condition at the end of the experiment.
2005	Ueng, Y-F., et al. Inhibition of human cytochrome P450 enzymes by the natural hepatotoxin safrole. Food and Chemical Toxicology 43: 702-712.	*Escherich ia coli*-expressed human P450	Safrole standard		Safrole inhibits CYP1A2, CYP2A6, CYP2D6, CYP2E1, and CYP3A4
1979	Vesselinovitch, S.D., K.V. Rao, and N. Mihailovich. Transplacental and lactational carcinogenesis by safrole. Cancer Res. 39: 4378-4380.	Female C57BL/6J and male C3HeB/FeJ mice, 8 weeks		120 microgram per g of bw to pregnant mice, lactating mothers, and 4 week old offspring	Safrole and/or metabolites crossed the placenta and was delivered to infants via lactation, but young mice had had much higher survival rate (>90%) when in utero and nursing from mothers receiving safrole compared to weaned rats that received direct safrole doses.

1966	Weinberg, M. S. & S. S. Sternberg. Effect of chronic safrole administration on hepatic enzymes and functional activity in dogs. Toxicology and Applied Pharmacology 8: 2.	Dog model		Bromsulfalein excretion curve, soluble enzyme, the lipid and glycogen of the liver, and tnitro-reductase	Examined the over 7 years. Found some tissue injury in the beginning of the experiment, but concluded the dogs may have adapted to the continuous intake of safrole.
1985	Wiseman, R.W., et al. Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1'-hydroxysafrole and 1'-hydroxyestragole *in vitro* and in the mouse liver *in vivo*, including new adducts at C-8 and N-7 of guanine residues. Cancer Res. 45: 3096-3105.	Male hybrid mice B6C3F1	Synthesized from standards	0.1 micromoles of 1'-hydroxysafrole per g of body weight	2 major adducts formed in the N^2 position of guanine with safrole and 1'-hydroxysafrole.
1977	Wislocki, P.G., E.C. Miller, J.A. Miller, E.C. McCoy, and H.S. Rosenkranz. Carcinogenic and mutagenic activities of safrole, 1'-hydroxysafrole and some known or possible metabolites. Cancer Res. 37: 1883-1891.	Male CD rats; CD-1 mice, and male hamsters; Ames test	Synthesized from standards	10 or 2 mg per 100 g of body weight via i.p. injection; *Salmonella* strain TA100	20% had hepatomas. Ames test: 1'-acetoxysafrole, safrole-2',3'-oxide, 1'-acetoxysafrole, and 1'-oxysafrole are directly mutagenic in mutagenic in Salmonella strain TA1535.
2005	Zhao, J, J. Miao, B. Zhao, S. Zhang, D. Yin. Safrole oxide inhibits angiogenesis by inducing apoptosis. Vascular Pharmacology 43: 69-74.	Vascular endothelial cells, A549 lung cancer cells, rat aorta from male Sprague-Dawley rats	Safrole standard synthesized to safrole oxide		Safrole oxide inhibits angiogenesis induced by cancer cells both in vitro and in vivo.

Table C.3: Review of studies using chemical analysis of foods containing safrole.

Year	Article	Source	Chemical Analysis	Concentrations	Results
1994	Bolton, J.L., et a. Evidence that 4-allyl-O-quinones spontaneously rearrange to their more electrophilic quinone methides: potential bioactivation mechanism for the hepatocarcinogen safrole. Chem Res. Toxicol. **7:** 443-450.	Safrole standard	HPLC, UV spectrophotometer		Examined a different metabolic pathway safrole could undergo, namely through oxidation to quinoid metabolites in liver microsomes in a nonenzymatic process.
1997	Carlson, M. and R. D. Thompson. Liquid Chromatographic Determination of Safrole in Sassafras-Derived Herbal Products. Journal of AOAC International 80 (5): 1023-8.	Powdered leaves, gumbo file, root bark, root powder, herbal capsule material, unbrewed tea.	Liquid Chromatography		The 'tea' made in this study was infused or the tea bag steeped in hot water for thirty minutes as opposed to the traditional method of decocting, or boiling, the whole or roughly chopped root. They demonstrated that herbal preparations of sassafras ranged from harmless for tea made from the leaves, to containing 92.4% safrole in sassafras oils. The 'tea' they prepared had safrole content ranging from 0.09 to 4.12 mg per cup, or only 6.9 to 17.2% of total safrole present in root.
2002	Chang, M.J.W., C.Y. Ko, R.F. Lin & L.L. Hsieh. Biological monitoring of environment exposure to safrole and the Taiwanese betel quid chewing. Arch. Environ. Contam. Toxicol. 43: 432–437.	Safrole standard	HPLC	0, 30, 75, 150, 300 mg/kg one time. Tested the urine of the rats and of human subjects who chewed betel quid between 2 - 100 times a day.	The safrole metabolites dihydroxychavicol and eugenol were found in the urine of non-betel quid chewers, at the levels of not detected to 5.4 micrograms/mg creatinine, probably due to spices in the diet.

2009	Chen, C., D. Spriano, T. Lehmann, and B. Meier. Reduction of Safrole and Methyleugenol in Asari radix et rhizoma by Decoction. Forsch Komplementmed 16: 162-166.	Asari radix et rhizoma	HPLC	3 g of powdered herbal drug decocted in water or extracted with MeOH. A traditional Chinese medicine formula that contained 3 g of Asari radix was decocted.	Safrole content of the herbal drug extracted by MeOH ranged between 1.57 to 2.76 mg/g. After a one hour decoction, safrole content was between 0.20 mg/g and not detectable. The medicine formulas contained 0.06 mg/g of safrole to not detectable.
2001	Choong, Y.-M. & H.-J. Lin. A Rapid and Simple Gas Chromatographic Method for Direct Determination of Safrole in Soft Drinks." Journal of Food and Drug Analysis 9(1): 27-32.	Commercial soft drinks, apple cider, and root beer	Gas Chromatography	Soft drinks contained 3-5 mg/L (ppm) of safrole.	Developed simple method to analyze soft drinks; found 20 out of 25 soft drinks had levels of safrole and/or cis-isosafrole exceeded the limit of 1 microgram/mL set by regulations, though the safrole all came from 'natural seasoning extracts'.
1987	Curró, P., G. Micali, and F. Lanuzza. Determination of β-asarone, safrole, isosafrole, and anethole in alchoholic drinks by high-performance liquid chromatography. Journal of Chromotography 404: 273-278.	Commercial alcoholic beverages and essential oils of nutmeg, sassafras, cinnamon, and anise	HPLC with fluorimetric detection and GC-MS		Found safrole present in alcoholic drinks with levels from 6.6 mg/l to absent. Determined HPLC had a high recovery rate of essential oils- for safrole recovery ranged from 95.6 to 100%.

1997	Farag, S.E.A. & M. Abo-Zeid. Degredation of the natural mutagenic compound safrole in spices by cooking and irradiation. Nahrung 41 (6): 359-361.	Spices: Black pepper, ginger root, cumin, star anise.	Gas Chromatography	120 g of black pepper seeds were washed & dried, boiled, exposed to gamma irradiation, and microwave irradiation both in whole seed and powdered seed form.	Washing and drying reduced the safrole content by anywhere from 94 to 74%. Gamma irradiation reduced the safrole content by 97 to 86%, or only to safe levels of safrole at the high levels. Microwave radiation reduced the safrole content 92 to 57%, but only the longest time reduced to safe levels and the samples burned. Boiling the seeds found boiling whole seed for 5 minutes and boiling powdered seed for 1 minute was effective at reducing safrole to safe levels.
1994	Heikes, D.L. SFE with GC and MS determination of safrole and related allylbenzenes in sassafras teas. Journal of Chromatographic Science 32 (7): 253-258.	Unbrewed sassafras tea, compound standards	SFE with GC-MS		New method of determining safrole, eugenol, and 4-allyl-1,2-dimethoxybenzene. Tested unbrewed tea and found safrole levels between 299 and 17,400 mg/kg.
1970	Larry, D. Gas Chromatographic Determination of Safrole and Related Compounds in Nonalcoholic Beverages: Collaborative Study. Journal of the AOAC 54 (4): 900-902.	Safrole standard	Gas Chromatography		Tested the recovery rates of compounds using GC and recommends adopting the method for quantitative determination of safrole, which has been adopted by the FDA.

2005	Reynertson, K. A., M. J. Balick, R. Lee, W. Raynor, Y. Pelep, and E. J. Kennelly. A Traditional Method of *Cinnamomum carolinense* Preparation Eliminates Safrole from a Therapeutic Pohnpean Tea. Journal of Ethnopharmacology 102 (2): 269-274.	*Cinnamomum carolinense* trunk bark	HPLC	Extracted 2.01 g of bark with MeOH. Extracting 5.01 g of bark with water by boiling, including one sample set that was also extracted with MeOH. Another sample set was spiked with 50 micrograms of safrole standard and boiled in water	Extracts by MeOH showed safrole present in the bark by 0.435% (w/w). Safrole measured 0.0001% (w/w) in the tea made by boiling in water, which is about 300 times more safrole present in the methanol extracted samples.
1976	Sethi, M. L., G. Subba Rao, B. K. Chowdhury, J. F. Morton, & G. J. Kapadia. Identification of volatile constituents of *Sassafras albidum* root oil. Phytochemistry 15: 1773-1775.	Powdered root bark	GC-MS		Extracted sassafras oil using petrol, which was evaporated and extracted again with MeOH. Oily fraction was analyzed to find 19 components of sassafras oil.
1985	Wiseman, R.W., T.R Fennell, J.A Miller, and E.C. Miller. Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1'-hydroxysafrole and 1'-hydroxyestragole *in vitro* and in the mouse liver *in vivo*, including new adducts at C-8 and N-7 of guanine residues. Cancer Res. 45: 3096-3105.	Synthesized from standards	HPLC	0.1 micromoles of 1'-hydroxysafrole per g of body weight	2 major adducts formed in the N^2 position of guanine with safrole and 1'-hydroxysafrole.

Appendix D

Abbreviations

Abbreviation	Meaning
Bw	Body Weight
CCP	Center for Cherokee Plants
CDC	Center for Disease Control and Prevention
CFR	Code of Federal Regulations
EBCI	Eastern Band of Cherokee Indians
FDA	Food and Drug Administration
GIS	Geographic Information System
HPLC	High Performance-Liquid Chromatography
MCI	Museum of the Cherokee Indian
mg/kg bw	milligram/kilogram of body weight
ppm	Parts Per Million
SCF	Scientific Committee on Food (European Commission)
WHO	World Health Organization

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