YIELD AND ESSENTIAL OILS OF HOLY BASIL (*OCIMUM TENUIFLORUM* L., *O. GRATISSIMUM* L.): VARIETAL COMPARISON AND ANALYTICAL METHODS

by

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(Under the Direction of David Berle)

ABSTRACT

Holy basil (*Ocimum tenuiflorum, O. gratissimum)* is a medicinal herb native to India that has been used to promote longevity, reduce stress and restore balance to the body. Because it is easy to grow and adapted to a wide range of growing conditions, there is potential for domestic holy basil production. The purpose of this project was to evaluate holy basil varieties for production suitability and identify any differences in essential oil composition. Fourteen varieties of holy basil were grown during the 2015 and 2016 growing seasons and compared for harvestable weight and essential oil yield. Essential oils contribute to the therapeutic properties of holy basil, and therefore the composition of the essential oils was evaluated. Four unique essential oil profiles were identified, and differences in harvestable weight and essential oil yield were observed, indicating there are many factors to consider when choosing a variety to grow for commercial production.

INDEX WORDS: *Ocimum sanctum*, *Ocimum tenuiflorum*, *Ocimum gratissimum*, holy basil, tulsi, hydrodistillation, eugenol, essential oils, GC-MS, GC-FID, predicted relative response factors

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DEDICATION

To Chris Fuller, my husband, who supported me from the moment I told him I wanted to go back to school. You taught me about loyalty and community, and encouraged me every step of the way as my love for science and plants blossomed. To David Berle, for seeing my potential and talking me into grad school, as it turned out to be one of the best things that has ever happened to me.

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INTRODUCTION

Medicinal Herbs

Medicinal and aromatic Plants (MAPs) have been used for millennia to enhance human health and provide drug and fragrance materials. MAPs are sold for their therapeutic secondary metabolites that have biological action in the body. While the use of herbs in the US has been overshadowed by dependence on modern medications for the last 100 years, the World Health Organization estimates that 75% of the world's population still relies primarily on traditional healing practices, much of which is herbal medicine (2005). In these countries, the demand and economic value of medicinal herbs is enormous. Modern science and Western medicine are becoming interested in the healing herbs once more. This is due, in part, to the demand in Europe and North America for products that are "all-natural" as well as aggressive marketing of herbal remedies (Srivastava, et. al., 1996). Medicinal plants are returning to a place of esteem and popularity because they are generally considered safe, they are economical, and they can be obtained easily from many sources, including a home garden (Prakash & Gupta, 2005).

However, with growing concern about food safety, there have been barriers to the widespread use of herbs in the US. Quality control of herbal medicines is exceptionally difficult as the quality of source material is dependent upon genetics, growing conditions, harvesting protocols, post-harvest practices, transport, storage, and extraction methods for active compounds. According to Roy Upton, Executive Director of the American Herbal Pharmacopeia, "with Good Manufacturing Practices (GMPs) in full force, many companies are realizing that the

supply chain for ingredients that pass identity and quality requirements has shrunk dramatically" (Smith, 2011). A potential solution to sourcing quality materials is encouraging domestic cultivation of medicinal herbs where GMP's can be enforced and materials are more easily traced.

Some of the doubt surrounding the safety and efficacy of herbal medicines is a result of contradicting clinical trials. The model has been to isolate active compounds and determine their biological activity, however, in the context of herbal medicine, there are many examples in the literature where whole plant extracts are more effective than isolated compounds (Spinella, 2002). This phytochemical matrix of plant constituents, acting synergistically, likely contributes to the safety and health promoting effect of herbs compared to single-substance pharmaceuticals. This synergy, however, represents a challenge for the food scientist evaluating active compounds and developing quality control standards to enable consistency, safety and efficacy of herbal products (Junio et al., 2011).

Recognizing the importance of broadening the Western medical perspective, the World Health Organization has recommended the integration of traditional health and folk medicine systems with modern medical therapies to more effectively address health problems worldwide (Pattanayak, et. al., 2010). Bringing people together from different disciplines is required to overcome these challenges and create a rich tapestry of multicultural healing wisdom and scientific evidence that work together to improve and create a sustainable system of healthcare in the United States.

Holy Basil: Description, History and Use

Ocimum tenuiflorum syn. *O. sanctum,* known as "Tulsi" or "Holy Basil" is a medicinal herb native to India with a wide distribution over the subcontinent. It has been used in Ayurveda, the traditional healing system of India, for over 5,000 years. It is an aromatic plant in the Lamiaceae family, and grows as a small erect shrub with hairy stems. It has oval leaves with toothed edges and, depending on the variety, stems range from herbaceous to woody, leaf coloring ranges from purple to green, and bloom color ranges from white to purple.

Holy basil has been widely cultivated in gardens in India and grows as an escaped weed. It is a sacred plant to the Hindu people and many people grow it around their home in belief that it will purify its surroundings and protect the family from negative influences. It is one of their most cherished herbs and has been termed the "incomparable one" "queen of the herbs", and "elixir of life" (Pattanayak et al., 2010). It is taken as an extract, herbal tea, dried powder or fresh leaf. It has been used traditionally as an herb that rejuvenates and promotes longevity, and as a remedy for common colds, bronchitis, diarrhea, nausea, headaches, stomach disorders, inflammation, poor memory, ulcers, heart disease and various forms of poisoning and malaria (Kuhn & Winston, 2000; Pattanayak et al., 2010; Singh, et. al., 2012).

Due to its large popularity in India, there have been many research studies on the medicinal action of holy basil. These studies have demonstrated that it can be used to promote healthy metabolism, support the immune system, function as an antioxidant and anti-inflammatory, act as a neuroprotective, as well as protect against the negative effects of ionizing radiation (Winston & Maimes, 2007). Holy basil can be used in all these capacities, but it is best known for its stress reducing adaptogenic properties, meaning it has the ability to help the body adapt to stress, normalize physiological function, and restore balance regardless of the origin of the stressor. As

an adaptogen, holy basil is popular in the herbal community because it is aromatic and provides a pleasing addition to teas and other herbal formulas.

It is known that stress can be a contributing variable to many chronic degenerative diseases such as cardiovascular disease, neurodegenerative diseases and immunological diseases (Esch, et. al., 2002). There is a growing body of evidence indicating the adaptogenic properties of holy basil have a wide range of therapeutic applications to address these common diseases, suggesting this herb is likely to increase in popularity and demand in the coming years (Pattanayak et al., 2010).

Holy Basil: Bioactive compounds

The flowers and leaves of holy basil contain a variety of phytochemicals believed to have therapeutic activity. These include saponins, flavonoids, alkaloids, glycosides, tannins, and ascorbic acid (vitamin C). However, the bulk of the therapeutic potential of holy basil is commonly attributed to the essential oil fraction (Prakash & Gupta, 2005). For instance, eugenol (2-methoxy-4-(prop-2-en-1-yl)phenol; CAS No. 97-53-0) is a notable phenolic found in holy basil's essential oil fraction. Other notable compounds found in the essential oil of holy basil include methyl eugenol, β-caryophyllene, β-elemene, estragole, eucalyptol, and β-bisabolene. Studies on the essential oils of holy basil have shown they can be effective in reducing blood sugar, improve blood lipid profiles, with antidiabetic, cardioprotective, hepatoprotective, antimicrobial, antifungal and antiviral properties (Prakash & Gupta, 2005). Eugenol, specifically, has been shown to be an effective vasodilator, and has been used therapeutically for neurological, inflammatory, allergic, and immunological disorders (Sen, 1993).

There are many challenges associated with the study of holy basil. Currently, there are no published standard reference ranges for known biologically active compounds, and the mechanism of action of the holy basil plant is not well understood (Pattanayak et al., 2010). There is inherent botanical and biochemical complexity and synergistic interaction of all the phytochemicals present in the whole plant that can't be reproduced with isolated extracts or pure chemicals. Additionally, holy basil has different chemotypes, defined as morphologically indistinguishable plants differing in their chemical constituents (Raina, et. al., 2013). Unfortunately, the chemotype is usually not specified in studies investigating the health benefits of holy basil (Tucker, et. al., 2009). To truly standardize these studies and be able to better understand the activity of this plant, more research is needed on the essential oil composition and applied to controlled and replicated studies. This work is important for building a body of knowledge around the range and efficacy of this sacred herb.

Essential Oils: Biosynthesis

Essential oils are volatile liquids of a plant, containing complex mixtures of many different classes of chemical constituents, usually of low molecular weight. Between 40 to 60,000 tons of essential oils are produced annually with a market value of $700 million USD. They are commonly used as flavors and additives in the food industry, as well as in the cosmetic, health and various agricultural industries (Raut & Karuppayil, 2014).

Essential oils are produced in the plant in response to stress and to help the plant deter pests, prevent disease, attract pollinators, and may function as antioxidants to protect the plant from oxidative damage (Preedy, 2015). In the genus *Ocimum*, essential oils are produced and stored in trichomes (surface glands) that are in highest concentration on the leaves and

inflorescences (Iijima et al., 2004). *Ocimum* essential oils are predominantly comprised of terpenoids and phenylpropanoids. The terpenoids are synthesized from the five carbon units of isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAPP). Condensation of IPP and DMAPP by prenyl pyrophosphate synthases form prenyl diphosphate molecules of set chain length (C10, C15, C20,... C5n) and form the starting material for all the different mono, di, tri and sesquiterpenes. After condensation, a variety of secondary metabolic processes such as isomerization, oxidation, reduction, derivitization and conjugation produce the different variants that form the different families of terpenoids and their unique individual compounds.

Two different metabolic pathways form the isoprene units IPP and DMAPP. The Mevalonate (MVA) pathway is localized in the cytosol and uses Acetyl-CoA as the precursor to form the sesquiterpenes (Kalkan, 2012). The Methylerythriol 4-phosphate (MEP) pathway, also known as the Deoxyxylulose 5-Phosphate (DXP) pathway, is localized in the plastid and uses glyceraldehyde phosphate and pyruvate to form IPP and eventually go on to form the mono- and di-terpenes.

Phenylalanine, produced through the shikimate pathway, is the precursor to the phenylpropene molecules, the second major class of compounds (Baser & Buchbauer, 2015). The phenylpropanoids, while not found in all aromatic plants are in abundance in the *Ocimum* genus and they usually contribute meaningful flavor and odor to the oil.

Essential oil: Extraction and Analysis

The International Organization for Standardization (ISO) defines an essential oil as a product recovered by distillation with either water or steam or by mechanical processing of citrus

rinds or by dry distillation of natural materials (2013). Currently, other methods of extraction such as solvent extraction and supercritical fluid extraction are being explored. However, of all the different types of extraction, hydrodistillation is the standard method for quality control of essential oils, and has been shown to produce good yield and good recovery of the essential oil fraction, even with small amounts of plant material (Charles & Simon, 1990; Preedy, 2015).

Additionally, hydrodistillation has been shown to produce the greatest quantity of essential oil compared to steam distillation (Charles & Simon, 1990). To perform the extraction, ground plant material is mixed with water and boiled. The steam releases the volatiles from the plant material and after it is condensed, the oil and water separate as two layers in a special piece of glassware called a Clevenger trap (Clevenger, 1928).

Once essential oils are extracted, they are typically evaluated with a Gas Chromatograph (GC). The sample is heated, pressurized and separated into the individual components before passing through a detector. In essential oil analysis, two types of detectors are used. First, a Mass Spectrometer Detector (MS) identifies the compounds qualitatively. The mass spectra of compounds are compiled and organized systematically into libraries such as NIST (National Institute of Standard and Technology). The library is installed onto the data-handling device for the GC-MS and allows for quick identification of compounds during analysis of essential oils by comparing the mass spectrum of the sample compound with the library database (Aromdee, 2012). Once compounds are identified a GC equipped with a Flame-Ionization Detector (FID) is used for quantitative analysis (Rubiolo, Sgorbini, Liberto, Cordero, & Bicchi, 2010), where a hydrogen flame burns compounds as they elute from the column producing ions which are then amplified and recorded by the detector (Nielsen, 2014). Using both methods of analysis is encouraged and even required by such journals as the *Flavor and Fragrance Journal* and

Journal of Agriculture and Food Chemistry and by the International Organization of the Flavor Industry.

For quantitative analysis, it is important that the method for analysis be validated to allow comparisons between research findings and ensure safety and effectiveness between batches. The International Conference on Harmonization (ICH) has published guidelines for method validation, which include testing for accuracy, selectivity, and reproducibility, and meets the requirements for linearity over the specified range of each analyte being studied (2005). Unfortunately, many studies on essential oils only report GC-MS results of percent relative abundance. This provides only the relative amount of an essential oil in an individual sample. It doesn't apply to other samples, thereby, making it difficult to aggregate and compare essential oil composition data from published studies, or to compare a series of samples of the same species (Rubiolo et al., 2010).

Even with properly validated quantitative protocols, it is not feasible to describe all the chemical complexity of an essential oil due to the long analysis time and the fact that many key standards are not available commercially. Another challenge is that many essential oils aren't widely analyzed and don't have published reference ranges for biologically active compounds. Without standard reference ranges, quality control testing on lesser known essential oils is difficult. To add to the confusion, even when there are published reference ranges, plants with the same chemical constituents will vary in composition when grown in different geographical areas or under different environmental conditions. Therefore, the current model is to choose compounds for quantitation based a combination of their abundance in the analyzed oils and their biological relevance. Ultimately, the scope of the analysis and type of quantitation depends on if the results are for someone in the pharmaceutical industry, cosmetics and food industries, for

product characterization, or for establishing a baseline of suspected volatile allergens (Bicchi et al., 2008).

Once qualitative and quantitative information has been obtained, the essential oil can then be described as a chemotype (CT). It typically describes the major compounds of the essential oil. Commonly, there are groups of essential oil constituents that are typical for a given taxon, however the specific CT depends on a number of factors including stage of plant development, geography, environmental factors and plant part (Baser & Buchbauer, 2015). Therefore, any statement about CT needs to be provided in the context of the growing location, time and method of harvest. This information is important because the chemical composition of the essential oil fraction is what gives it economic importance and therapeutic value (Chamorro, et. al., 2012). In the US there is no regulatory framework for quality control of essential oils and it frequently leads to adulteration and fraud in the marketplace. However, there are an increasing number of companies voluntarily providing analytical reports of their essential oils with information about genus, species, CT and country of origin.

Market Trends

With the right marketing, holy basil has a huge potential for horticultural production as a specialty tea crop. It grows well in the southeastern United States, and appears to be relatively tolerant to foliar diseases that often plague summer vegetables. Though holy basil is used for its medicinal properties, growers currently select varieties based on other factors, such as availability of seed, market demand, and harvestable weight (Zhang, et. al., 2012). Unfortunately, few seed suppliers provide varietal information about the holy basil they sell, such as differences in yield or potential difference in known bioactive compounds.

Little information is available on market trends of holy basil sales. However, according to a recent publication by the American Botanical Council, tea sales for the US exceeded 15 billion USD in 2013 and tea drinking in is predicted to steadily increase in future years due to consumer desire for healthy beverages (Keating, et. al., 2014).

Interdisciplinary Research

Growing herbs for medicine impacts a wide variety of people. A successful strategy for producing quality research on medicinal herbs requires involvement and input from many disciplines including agriculture, economics, food science and technology, chemistry, social and environmental sciences. Growers are interested in yield and marketability; food scientists are interested in determining the best methods for analysis of various compounds such as identification and quantification of essential oil constituents; producers/manufacturers are interested in marketability, and due to increased regulations, are looking for quality US growers that are knowledgeable about the plants and follow good agricultural practices.

A critical first step in shaping the body of knowledge around botanicals is developing reference ranges for bioactive compounds, and screening the plants for productivity (Craker, et. al., 2003). Currently, there is a trend for herbal products to be marketed based on a certain percentage of a "marker" compound believed to be the single active constituent of the plant. However, it is more important to start from the ground up to obtain a proper botanical ID of the plant, be aware of the proper time to harvest, and use proper drying and extraction techniques (Winston & Maimes, 2007). There is a need for more research-based information on botanicals, but it needs to arise from herbalists and researchers working together to come up with new standards for quality assurance to benefit a community seeking alternative forms of medicine.

Objectives and Scope

The purpose of this project was to better understand the differences between holy basil varieties to support an increase in domestic production of high quality and marketable holy basil, and contribute to a deeper awareness of the unique chemistry of different holy basil varieties for potential clinical use. This was accomplished by characterizing commercially available varieties and USDA Germplasm accessions of holy basil, by their yield, essential oil content and composition.

There are surprisingly few studies in the US that evaluate these parameters. Studies of holy basil in Mississippi have evaluated yield and essential oils between growing locations and over multiple harvests, however they did not specify which variety of holy basil they evaluated (Zheljazkov, et. al., 2008; Zheljazkov, et al., 2008). The composition of essential oil is influenced heavily by environmental conditions, and no studies have been conducted in Georgia to determine if holy basil is a viable crop with yields and chemical profiles comparable to holy basil grown in India. Additionally, there is a need to identify and apply robust lab analysis techniques for evaluating the phytochemicals in holy basil. A previous study on lavender essential oil reported that the DB-Wax Ultra Inert GC column produced improved peak shape and sensitivity for complex essential oil analysis (Zou, 2016). However, this research has not been done on holy basil essential oil.

There are many ways in which the information provided in this thesis will further public understanding. Primarily, this information will provide valuable information for growers when selecting a holy basil variety that provides the best combination of dry plant yield, and the yield and composition of the essential oil fraction. This will give the farmers a unique opportunity to

grow specific varieties suited to the needs of the industry, allowing them to receive optimal compensation for their products. Secondly, determining the most effective method for extraction and analysis of the essential oil fraction will ensure a robust and accurate investigation, building a foundation for future studies. In a broader context, this information is necessary to inform clinical studies that can be replicated and correlated to CT, to validate the wide-ranging health claims and build a body of knowledge around the scope and effectiveness of this revered herb (Preedy, 2015).

The objectives of this study were to:

1. Characterize the differences in harvestable weight, essential oil yield and essential oil composition between 14 varieties of holy basil.
2. Develop methods for essential oil extraction and analysis using hydrodistillation, and gas chromatography coupled with mass spectrometry and flame-ionization detection.
3. Compare the Agilent J&W DB-Wax Ultra Inert GC and HP-5 capillary columns by GC-FID to evaluate the resolution, sensitivity and peak shape for complex essential oil analysis.

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CHAPTER 2

YIELD AND ESSENTIAL OILS OF HOLY BASIL (*OCIMUM TENUIFLORUM* L., *O. GRATISSIMUM* L.) VARIETIES GROWN IN THE SOUTHEASTERN UNITED STATES

[1]Fuller, N.J., Berle, D. Pegg, R.B., Affolter J.M., To be submitted to HortScience.

Abstract

The use of medicinal plants in North America is on the rise. Holy basil (*Ocimum tenuiflorum* L.*, O. gratissimum* L.*)* is a medicinal herb native to India that has become increasingly popular in the United States. Traditionally, holy basil has been used to promote longevity, reduce stress and restore balance to the body. Because it is easy to grow and adapted to a wide range of growing conditions, there is great potential for holy basil production in the southeastern United States. The purpose of this project was to evaluate holy basil varieties, to facilitate the production of high quality and marketable holy basil. Fourteen varieties of holy basil were grown during the 2015 and 2016 growing seasons and compared for harvestable weight and essential oil content. Eugenol concentration was also compared for each variety since it is the active compound in the holy basil essential oil fraction, and believed to be responsible for many of the health promoting effects. Overall, there were significant differences in harvestable weight and essential oil yield among varieties, but there was no significant difference in the percentage of essential oil among varieties. Additionally, there was a significant interaction between holy basil variety, the number of harvests, and the essential oil percent. Finally, eugenol content was highly variable over the different varieties, with higher eugenol content in 2016 than in 2015. The variety that had the overall highest yield, essential oil, and eugenol content was PI 288779, a USDA accession, suggesting its use in any future breeding work.

Index words:

Ocimum sanctum, *Ocimum tenuiflorum*, holy basil, tulsi, essential oil, hydrodistillation, eugenol

Introduction

The use of herbs in the United States has been overshadowed by dependence on modern medications for the last 100 years. However, due to side-effects of medications, drug-resistance and lack of tools for treating chronic degenerative diseases people are returning to medicinal herbs because they are generally considered safe, economical, and can be obtained easily from many sources, including a home garden (Prakash & Gupta, 2005). This renewed interest in medicinal herbs signals a great opportunity for farmers interested in specialty crops.

Ocimum tenuiflorum L. syn. *O. sanctum,* and *O. gratissimum* L. known as Tulsi or Holy basil, is a medicinal herb native to India with a wide distribution over the subcontinent. It has been used traditionally as an herb that rejuvenates and promotes longevity, and as a remedy for common colds, bronchitis, diarrhea, nausea, headaches, stomach disorders, inflammation, poor memory, ulcers, heart disease and various forms of poisoning and malaria (Kuhn & Winston, 2000; Pattanayak, et. al., 2010; Singh, et. al., 2012). Holy basil can be used in all these capacities, but it is best known for stress-reducing adaptogenic properties, meaning it has the ability to help the body adapt to stress, normalize physiological function, and restore balance regardless of the origin of the stressor. Stress can be a contributing variable to many chronic degenerative diseases such as cardiovascular disease, neurodegenerative diseases and immunological diseases (Esch, et. al., 2002). There is a growing body of evidence indicating that the adaptogenic properties of holy basil have a wide range of therapeutic applications to address these common diseases. For these reasons, it is likely that holy basil will be increasing in popularity and demand in the coming years (Pattanayak et al., 2010).

Holy basil has been broadly researched, and is widely cultivated in India (Aggarwal & Mali, 2015; Raina, et. al., 2013; Sharma, et. al., 2011). However, according to Roy Upton,

Executive Director of the American Herbal Pharmacopeia, with the implementation of Good Manufacturing Practices (GMPs), fewer suppliers can meet the requirements for quality and product verification (Smith, 2011). A potential solution to sourcing quality materials is encouraging domestic cultivation of medicinal herbs where GMP's can be enforced, and materials are more easily traceable.

Growers typically select varieties based on seed availability, market demand and harvestable weight, and not necessarily the presence or concentration of biologically active compounds (Zhang, et. al., 2012). However, with medicinal herbs, an important consideration is the measurable difference in therapeutic constituents, such as essential oils, that are indicators of quality and efficacy. For example, a notable phenolic compound found in holy basil essential oil is eugenol. It has a spicy clove-like scent that has been shown to be effective in reducing blood pressure, and has been used therapeutically for neurological, inflammatory, allergic, and immunological disorders (Sen, 1993).

The cultivars Krishna and Rama are commonly grown in India, and the cultivar Vana is a wild type that grows in the forest in India. However, other commonly sold cultivars that are available domestically, such as Amrita and Kapoor, have little documentation on yield or use. Additionally, there is considerable confusion regarding labeling, where most seed companies only list the Latin name and don't mention information about the cultivar. Thus, it is not well understood how to differentiate the varieties or which ones should be grown to achieve maximum yield and quality.

Unfortunately, there is very little yield or economic data on holy basil grown in the US, and more research needs to be done to determine the market potential. However, with some effective promotion, holy basil has great potential as a high-value crop that can be grown in the

US and doesn't take up much space in the field. According to a recent publication by the American Botanical Council, tea sales for the US exceeded 15 billion USD in 2013 and tea drinking is predicted to increase in future years due to a consumer desire for healthy beverages (Keating, et. al., 2014). To increase production of holy basil in the southeastern US, the first step is to evaluate a selection of holy basil varieties to determine which ones are most suited to the climate, and which have the greatest yield.

The varieties in this study were chosen as a representative sampling of varieties that were commercially available, and germplasm accessions accessible from the USDA. There is some published information about these varieties; however, the differences are still not well understood and this is the first study to examine holy basil production in Georgia.

Materials and Methods

Plant and growth conditions

The experiment was conducted during the 2015 and 2016 cropping seasons at the University of Georgia UGArden farm in Athens, GA, using a randomized complete block design with 3 replications. 9 holy basil (*Ocimum tenuiflorum,* L.*)* accessions (PI 288779, PI 652059, PI 652057, PI, 652056, PI 414201, PI 414202, PI 414203, PI 414204, PI 414205) were obtained from the USDA-ARS National Plant Germplasm System (Ames, IA), and 4 named cultivars (Kapoor, Rama, Krishna and Amrita) were obtained commercially from Strictly Medicinal Seeds (Williams, OR). The cultivar, Vana, was also obtained from Strictly Medicinal Seeds and while it is a different species (*Ocimum gratissimum*), it was included in this study because it is also commonly called holy basil in India and has the same therapeutic applications (Winston & Maimes, 2007). Seeds were planted in June of both years into 50-cell plastic trays filled with

Sunshine Natural and Organic Professional Growing Mix (SunGro, Agawam, MA). Transplants were watered once a day in the greenhouse and thirty-eight day-old seedlings were transplanted into the field in raised beds.

A walk behind tractor (BCS, Abbiategrasso (MI), Italy) was used to shape the raised beds (1 m wide, 0.3 m high, 9 m long, with drip irrigation tubing underneath pine straw mulch) with a total plot size of 9 m^2. Soil is a Cecil sandy clay loam, and soil tests were performed both years to determine pH, Lime Buffer Capacity and available soil nutrients (Table 1). Beds were fertilized with hydrolyzed poultry feathers (13-0-0) (Mason City Byproducts, Inc., Mason City, IA) at the rate of 120 kg N/ha. Additionally, in 2016, pulverized dolomitic lime (Austinville Limestone Co., Austinville, VA) was added, based on a soil test and recommendations from the University of Georgia Soil Test Lab. The recommended amount was of 2,800 kg/ha to raise the pH from 5.69 to 6.0. Varieties were planted in blocks of 6, with 2 rows of plants spaced 30 cm apart, and 60 cm of space between blocks. After planting, seedlings were watered with a fertilizer solution at the rate of 0.18 L of a 4.8 g/L solution of Chilean Nitrate of Soda (16-0-0) (Allganic, Eugene, OR) per plant. Holy basil plants were pruned back to the third node 12 days after planting to encourage branching. Plants were irrigated with approximately 38 mm of water per week using drip irrigation on weeks it did not rain. The University of Georgia Weather Station at the Plant Sciences Research Farm in Watkinsville, Georgia recorded average monthly maximum and minimum temperatures and total monthly rainfall for the area for both years (Table 2).

Holy basil was harvested by hand approximately 6 weeks after planting, by cutting 15cm above ground. This height allowed for secondary branches to remain intact and enable regrowth in order to take a second harvest (Zheljazkov, et. al., 2008). During the 2015 trial, the second and

final harvest was completed approximately 6 weeks after the first by harvesting the plants 10 cm aboveground, and all the plants were collected at the same time. However, not all varieties were flowering at time of harvest, and harvesting during full flower has been shown to result in the highest quantity of essential oil, as well as the highest concentration of desirable essential oil compounds such as eugenol (Abdel-Hamid, el. al., 2005). Therefore, in the 2016 trial, each variety was harvested based on flowering stage which was determined to be when inflorescence on 80% of the shoots occurred and blooms were opening halfway up the vertical.

For each harvest, plants were weighed and bundled individually and air-dried at ambient temperature in an herb room with a dehumidifier. Plants were considered fully dried when the moisture level in the room maintained a consistent reading of 15% and a test leaf was easily powdered. Dried weights were recorded for each plant. Six plants of each variety were combined and the leaves and flowers stripped from the stems. This was done for three replicates of each variety. The combined mass of the dried leaf and flower of six plants was recorded for each replicate and then packed in food grade aluminum bags and stored at -18 °C.

For each harvest, four samples were collected from different areas of the drying room to perform a moisture analysis. Each moisture analysis was performed in triplicate by preparing a representative mixture from the samples collected in different areas of the drying room. Then the mixture was ground in a coffee grinder. Roughly 2 g of the ground composite was transferred to a tared, aluminum weight pan (VWR International, Radnor, PA) and placed in a preheated Isotemp oven (Fischer Scientific, model 650G, Dubuque, IA) set to 103 °C. Samples were checked intermittently until a constant mass achieved (~4 h). They were removed from the oven and placed in a dessicator. Once the samples had cooled, the masses were recorded, the weight of

the aluminum pans were subtracted from the initial and final values, and the moisture content was calculated using the following equation:

Moisture content = (initial weight – dry weight)/dry weight x 100%.

Additionally, 14 voucher specimens were prepared, one for each variety in the study, and were verified by Dr. James Affolter the director of research at the State Botanical Garden of Athens, Georgia. They are being stored in the University of Georgia Herbarium, Athens, GA.

Hydrodistillation

To extract the essential oils, a Clevenger trap (Wilmad-LabGlass, Kingsport, TN) for oils lighter-than-water was utilized (Clevenger, 1928). A representative sample of dried plant material (leaf and flower, 50 g) was taken from each replicate, for each variety. The plant material was finely ground in a coffee grinder and added to a 2-L round bottom flask with deionized water at a material-to-solvent ratio of 1:13 (w/v) (Charles & Simon, 1990). Distillation was carried out for 180 minutes per sample and was determined by waiting 30 minutes after the last increase in the volume of essential oil in the collecting arm of the Clevenger trap. To facilitate condensation, water passing through the condenser was first chilled to 5 °C and then reprocessed through a water-cooling bath (Fischer Scientific, model 4100 R20, Dubuque, IA). Essential oils were filtered through a glass pipette containing glass wool and 600 mg of anhydrous sodium sulfate (Avantor Performance Materials, Inc., Center Valley, PA) to remove water residue. Mass was recorded, and the collected essential oil fractions stored in ¼ dram (0.92 mL) amber glass vials (Premium Vials, Bristol, PA) at -18 °C.

Recovery rate of essential oils was determined by taking a pre-weighed mass of essential oil and placing it in the collecting arm of the Clevenger trap to simulate the end-point of

distillation. Then it was removed from the Clevenger trap, filtered through sodium sulfate and mass was recorded. This was performed in triplicate and the percent recovery was calculated using the following equation:

% Recovery = recovered mass of essential oil/initial mass x 100%

The percentage of essential oil in each sample was calculated using the following equation:

Essential oil % = mass (g) essential oil/mass (g) of dried sample x 100%

Finally, the essential oil yield (g/plant) was calculated as:

Essential oil yield = concentration of essential oil (g/g) x mass (g) dried leaf and flower of six holy basil plants

Essential oil analysis

Essential oil samples (25 uL) were dissolved in HPLC-grade acetonitrile (Sigma-Aldrich Chemical Company, St. Louis, MO) in a 5 mL volumetric flask giving a final concentration of ~20 mg/mL of the oil. A 1 uL aliquot was injected into a single taper split liner (Agilent Technologies, Santa Clara, CA, USA). All injections were performed in duplicate.

The analysis was carried out with a gas chromatograph (Agilent Technologies model 6890N) equipped with a flame ionization detector and autoinjector (model 7683B). The data was recorded with Chemstation [v. E.02.02]. A non-polar HP-5 fused-silica capillary column (J&W Scientific, Folsom, CA) with 5% phenyl-methylpolysiloxane, 30 m, 0.32 mm i.d., 0.25 μm film thickness was used for the analysis. The oven temperature was held at 50 °C for 5 min, then programmed to increase 3 °C/min to 120 °C, then at 5 °C/min to 250 °C and then 15 °C/min to 300 °C, followed by a 5 min hold for a total run time of 62.67 minutes. The carrier gas was UHP-grade helium at a flow rate of 30 mL/min. Air flow was set to 450 mL/min, UHP-H_2 flow

to 40 mL/min, and makeup gas to 25 mL/min. The inlet pressure was 18.07 psi. The analysis was performed in constant flow mode, and the injector and detector temperatures were set at 250° C, with a split ratio of 80:1. Data was recorded with Chemstation [v. E.02.02], and identification of eugenol in the holy basil essential oils was carried out by comparison of retention time indexes and the injection of a commercial standard.

Quantitative analysis

A solution of 20.29 mg/mL of eugenol (TCI America, Portland, OR) in HPLC-grade acetonitrile was prepared and injected onto the GC at seven different concentrations in a range expected for eugenol in the samples, established by literature values. A calibration curve was created ($y = 132.96x - 4.6827$, $r^2 = 0.99962$) with a concentration range of 1.01 to 20.29 mg/mL. The standard curve allowed for quantification of eugenol in each essential oil sample. The eugenol content was calculated using the following equation:

Eugenol content = mass eugenol/ mass essential oil x 100

Results are expressed as g eugenol/100 g essential oil.

Statistical analysis

The data was analyzed statistically using one-way ANOVA by R 3.2.2 (The R Foundation for Statistical Computing). Significance was determined using Tukey's studentized range test.

Results

Variety evaluation

The fourteen varieties of holy basil had a dry mass range of 77.7 g to 199 g (Figure 1), while essential oil % spanned 0.8 to 1.6%. The essential oil yield range was 0.73 g to 2.52 g (Figure 2). Eugenol content fluctuated from 19.6 to 66.5% among the varieties (Figure 7). The average moisture of the dry leaf and flower was 11.4 ± 0.7% (SD) in 2015 and 11.0 ± 0.1% in 2016, and the recovery rate of essential oil was 90.9% ± 1.02.

In terms of dry mass, there was a significant difference in yield among holy basil varieties (Figure 1). Varieties Rama, Krishna, Amrita, and PI 652057 had lower yields than Vana, PI 414203, Kapoor, PI 414204, PI 652056, PI414205, PI414201, and PI 652059. The yield of the remaining USDA accession, PI 288779, fell between the two groups and was not significantly different than either group.

The average percentage of essential oil per plant was evaluated over both cropping seasons and there was no significant difference of essential oil percent among varieties. For the essential oil yield, the only significant difference was among the lowest and highest: Krishna and PI 288779, respectively (Figure 2). This was due to large variability in essential oil percent and essential oil yield between harvests.

Effect of Growing Year

While the differences among varieties was not as significant as expected, there were significant differences between the 2015 and 2016 growing seasons for yield of dry mass, essential oil percent and essential oil yield. The dry mass yield of holy basil was significantly lower in 2015 than in 2016, 163 g and 143 g respectively ($p < 0.05$). Furthermore, essential oil

percent between growing seasons was significantly different, with mean essential oil percent in 2015 and 2016 0.73% and 1.70%, respectively ($p < 0.001$) (Figure 3). Similarly, there was a significant difference in essential oil yield between years. In 2015 the mean essential oil yield was 1.01 g, and 2.80 g in 2016 ($p < 0.001$) (Figure 4).

Effect of Harvest

Due to the lack of significant differences of the overall essential oil percent among varieties, the essential oil percent between the first and second harvest of both years were evaluated. There was a significant interaction ($p < 0.0001$) among the percentage of essential oil, holy basil variety and time of harvest (Figures 5a and 5b). Even though there was an overall greater essential oil percentage in 2016, the trend was similar over both growing seasons with varieties PI 288779, Amrita, Rama, PI 652057, Vana and Krishna, having lower essential oil percent for the first harvest, and greater for the second harvest. Conversely, in both years essential oil percentage increased for the first harvest and decreased for the second harvest for the remaining 8 varieties- PI 652059, Kapoor, PI 652056, PI 414205, PI 414201, PI 414202, PI 414203, PI 414204. There were two exceptions to this trend. In 2015 USDA accession PI 652057 had the same essential oil percent over both harvests and in 2016 the cultivar Krishna had slightly higher essential oil percent for the first harvest.

Differences in phenotype and physiology

There were several phenotypic and physiological differences observed among the holy basil varieties evaluated. They displayed differences in leaf color, growth habit, and average number of weeks to flowering time (Table 3). The majority displayed green leaves, however two

exceptions where Krishna and Amrita, which developed dark purple leaves as they matured. In terms of growth habit, 6 varieties displayed a more clumping upright growth habit with fewer stems that were more rigid and woody (Figure 6a), and 8 varieties displayed a more sprawling growth habit with greater number of stems that were more herbaceous (Figure 6b). This relates to the same groupings from Figure 5 where the clumping varieties had higher essential oil percentage after the second harvest, and the sprawling varieties had an overall lower essential oil percentage after the second harvest. Finally, the varieties were different in time to reach the flowering stage. Most flowered at approximately 6 weeks, however, notable exceptions were Vana and Krishna which took an average of 9 weeks to flower, and Rama that flowered in 5 weeks.

Differences in Eugenol

Essential oil analysis revealed differences in eugenol content across varieties ($p < 0.0001$) (Figure 7), with Vana and PI 288779 containing the highest eugenol content at 66.5 g eugenol/100 g and 45.23 g/100 g. Conversely, the two lowest yielding varieties were PI 414204 with 19.62 g/100 g, and Amrita that had no detectable amounts of eugenol. Additionally, there was a significant difference in eugenol content between years ($p < 0.0001$), with a mean eugenol content of 21.52 g/100 g in 2015 and 37.04 g/100 g in 2016 (Figure 8). The variety Vana was the only exception to this trend and exhibited similar eugenol content over both years, while all of the other varieties increased in eugenol in 2016 compared to 2015.

Discussion

Surprisingly, there was little difference among the varieties in terms of essential oil yield and essential oil percent. However, there were significant differences for dry mass yield, essential oil percent and essential oil yield between the 2015 and 2016 growing seasons, indicating the environment plays as much, or more, of a role in the yield and quality of holy basil as varietal selection.

Overall, 2016 was perceived as a tough growing season with hot, dry weather. However, these conditions resulted in higher yields, higher percent essential oil and higher eugenol content. While this study was not specifically investigating effects of weather or irrigation, it is clear that temperature, moisture and other environmental factors play an enormous role in the growth, development and physiology of holy basil. Though research about the effect of environmental factors on holy basil is lacking, there have been many studies looking at these effects on culinary basil (*Ocimum basilicum*). To give a few examples, Bekhradi et. al. (2014) reported an increase in antioxidant activity of basil under water stress. Similarly, Ekren et. al. (2012) described an increase in essential oil and improved essential oil composition in purple basil under water stress. However, the study also reported decreased plant height and plant yield under water stress. Another study by Chang et. al. (2005) evaluated the effect of temperature on basil essential oil and found that warmer temperatures increased the ratio of eugenol in essential oils of basil. Additionally, higher temperatures during the two weeks before harvest resulted in increased essential oil content and increased eugenol content. Finally, a study by Chang et. al. (2007) reported on the effect of solar radiation on basil essential oil and found that heavy shading reduced total essential oil content and conversely found that plant height and levels of eugenol were significantly increased by high daily light integrals. It is clear that further research is

needed to evaluate these influences on holy basil to better understand their effect and inform future production practices.

Of the 9 USDA accessions, 7 (PI 652059, PI 652056, PI 414205, PI 414201, PI 414202, PI 414203, PI 414204) were very similar to the commercial variety Kapoor. They had the same sprawling growth habit, higher yields, lower essential oil percent and usually lower eugenol content. This type is also what leads to the most confusion with seed catalogs. It is difficult to botanically confirm this variety, and there is no documentation to suggest that it is regularly cultivated in India. However, due to the presence of eugenol it is considered a suitable alternative to other varieties because it grows well in temperate climates and is very prolific. Two other USDA accessions, PI 288779 and PI 652057, were similar to the Rama/Krishna varieties in growth habit and eugenol content. Finally, the variety Vana had the highest eugenol content and was in the higher yielding group indicating it is a good choice for farmers in the southeastern United States.

Interestingly, the effect of growth habit on the yield and percent of essential oil between harvests was consistent over both years. This could indicate an overall trend that holy basil plants with a more clumping growth habit increase in essential oil percent after the first harvest, and holy basil plants with a more herbaceous growth habit decrease in essential oil percent after the first harvest. This apparent relationship needs further study to understand the best time and number of harvests for this aromatic crop. In a recent study of 3 basil genotypes and the effect of 3 harvests, it was found that the holy basil genotype decreased in yield, essential oils and eugenol after the second harvest but then dramatically increased in all three parameters during the third harvest (Zheljazkov et al., 2008). Further study evaluating more than 2 harvests on these holy basil varieties is needed to determine if the findings of the previous study are consistent.

In addition to the effects of growth habit on essential oil percent, growth habit also affected ease of harvest. Varieties with a more sprawling growth habit (Kapoor-type) had branches that would get heavy and break away from the main stem, reducing total harvest, or getting lower leaves dirty, making it more difficult to harvest. Conversely, varieties with a clumping growth habit had branches that would remain upright and allowed for a more efficient and hygienic harvest.

Another factor affecting harvest is the differences in flowering time among varieties. This is another important consideration for farmers. Varieties that flower quickly constrain the farmer to a tight window where a harvest needs to be taken before the plant goes to seed. Conversely, an extended time to flower also reduces the number of harvests a farmer can take before a killing frost. These are all important points that should be considered when making decisions about which variety to grow.

Conclusion

Of the commercially available varieties, Amrita was difficult to grow, was the least productive, and had no detectable amounts of eugenol, making it an undesirable choice for farmers. In India, varieties Rama and Krishna are cultivated for their high eugenol content, however Rama had the lowest harvestable weight and Krishna had the lowest essential oil yield. Vana had the highest eugenol content and high yield, but the essential oil yield was lower than many other varieties. Finally, Kapoor had high yields, but essential oil content and eugenol content was highly variable. Due to these challenges, it would be advisable for a farmer to grow a combination of these to sell to different markets. A grower able to specify and verify they are growing a certain variety could possibly receive higher compensation, especially for consumers

that are knowledgeable about the differences and are looking for specific qualities. Conversely, a grower could grow several varieties and mix them together to achieve a high quality holy basil product with advantages from each commercial variety. Additionally, holy basil produced superior harvestable weight and essential oils with increased eugenol content after exposure to lack of rain and extended heat conditions in 2016. This suggests that growing holy basil would be a great option for growers in the southeastern US with increasingly variable weather conditions.

In terms of USDA accessions, there was a clear leader; accession PI 288779. It is a landrace collected from India with an upright growth habit, making it easy to harvest. PI 288779 had a reasonable harvestable weight, gave the greatest essential oil yield, and was the second highest in eugenol content. Additionally, PI 288779 increased in essential oil percent after the first harvest, making it an ideal choice for a crop where more than one harvest is usually taken. Being a USDA accession, a logical next step would be to work with this variety over several years to prepare it for commercial production as a high quality holy basil variety that grows exceptionally well in Georgia.

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Table 2.1: The pH, LBC, and plant available nutrients in the 2015 and 2016 growing seasons.

Year	pH	LBC[1]	Ca	K	Mg	Mn	P	Zn
		ppm CaCO3/pH			ppm[2]			
2015	5.61	447	1243	446.4	268.9	21.4	103.2	17.68
2016	5.69	540	1057	189	193	20.29	44	14.52

[1]LBC=Lime Buffer Capacity
[2]1 ppm = 1 mg kg-1, Ca = calcium, K = potassium, Mg = magnesium, Mn = manganese, P = phosphorus, Zn = Zin

Table 2.2: Average daytime and night time air temperatures, and total monthly rainfall for the 2015 and 2016 growing seasons.

	Avg. Max Temp (°C)		Avg. Minimum Temp (°C)		Rainfall (mm)	
	2015	2016	2015	2016	2015	2016
Month						
July	33.3	33.4	21.8	21.6	106	41.7
August	31.8	32.3	20.7	21.9	188	99.8
September	27.8	31.4	17.7	18.8	133	31.5
October	23.3	26.6	11.4	12.3	183	0
November	19.5	20.5	9.1	6.6	250	69.9

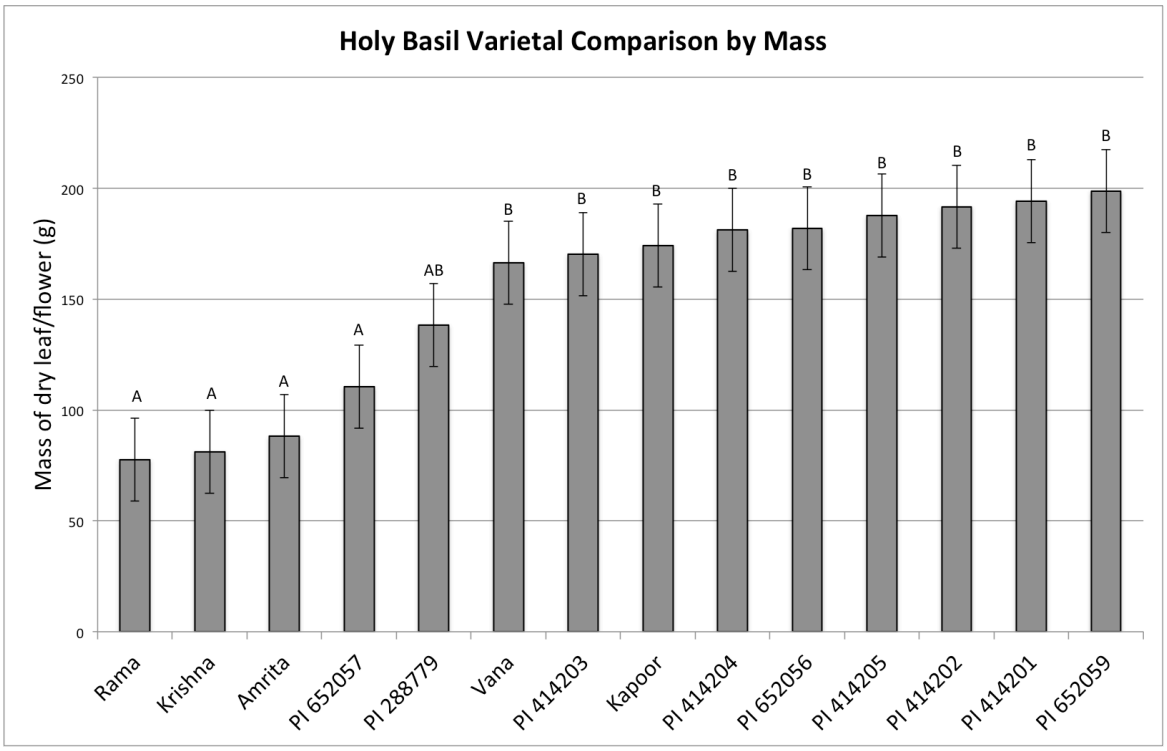


Fig. 2.1: Mean cumulative yield of combined dry mass of 6 holy basil plants across both harvests of both growing seasons. Significance is indicated by a p-value ≤ 0.05. Similar letters above bars indicate no significant difference between varieties.

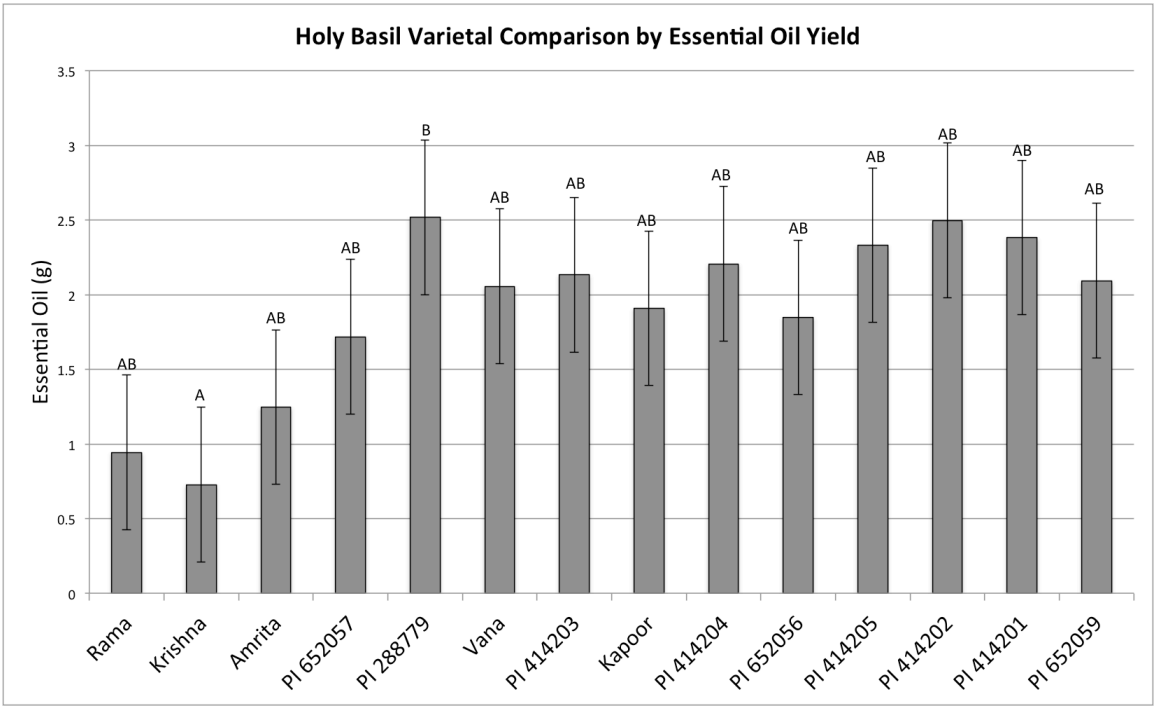


Fig. 2.2: Mean cumulative yield of essential oil per holy basil variety. Significance is indicated by a p-value ≤ 0.05. Similar letters above bars indicate no significant difference between varieties.

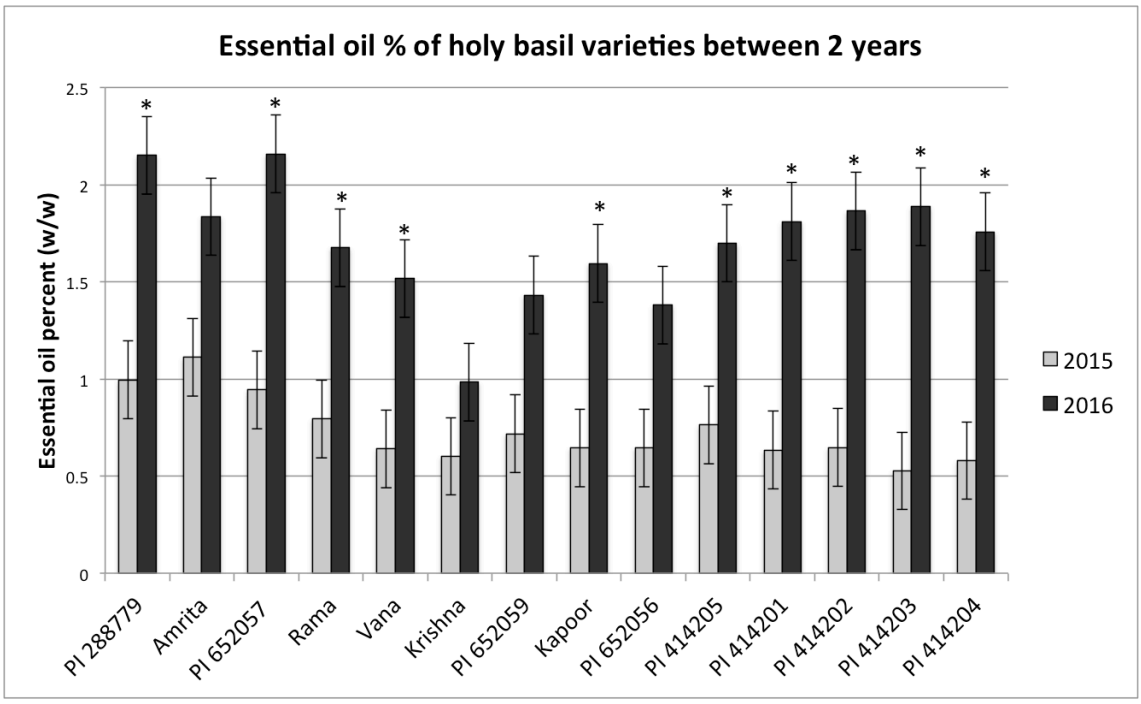


Fig. 2.3: Mean percent of essential oil between the 2015 and 2016 growing season. An asterisk (*) denotes the varieties with a significantly higher essential oil percentage (p-value ≤ 0.05) in 2016, as compared to 2015.

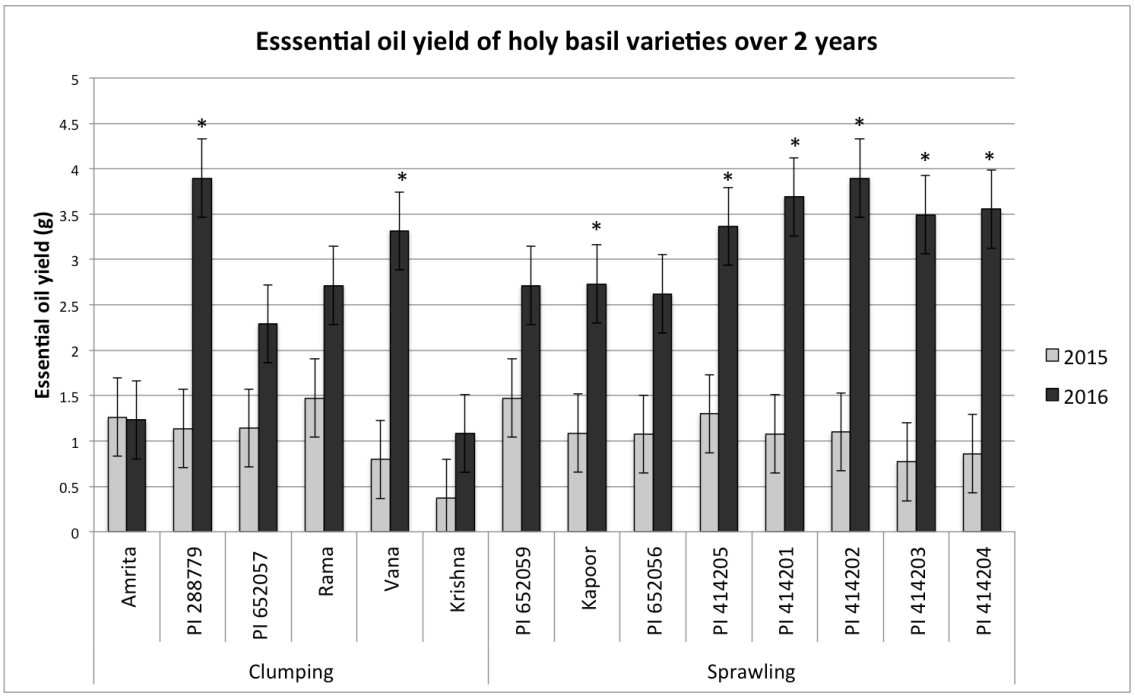
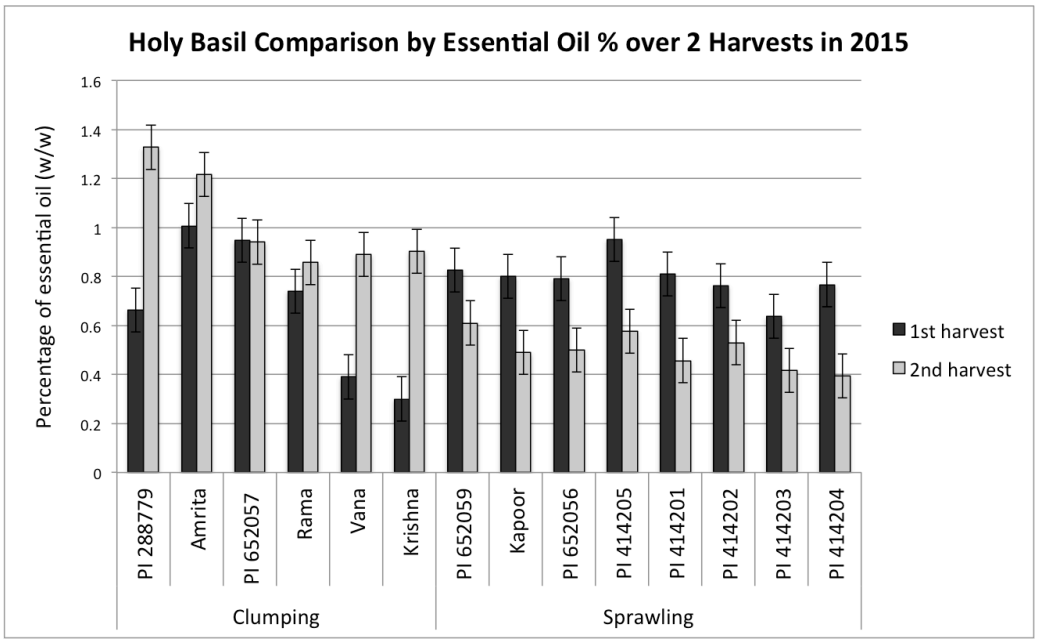


Fig. 2.4: Mean yield of essential oil between the 2015 and 2016 growing season, calculated as concentration of essential oil (g/g), multiplied by the combined mass of dried leaf and flower of six holy basil plants (g). An asterisk (*) denotes the varieties with a significantly higher essential oil yield (p-value ≤ 0.05) in 2016, as compared to 2015.

a.



b.

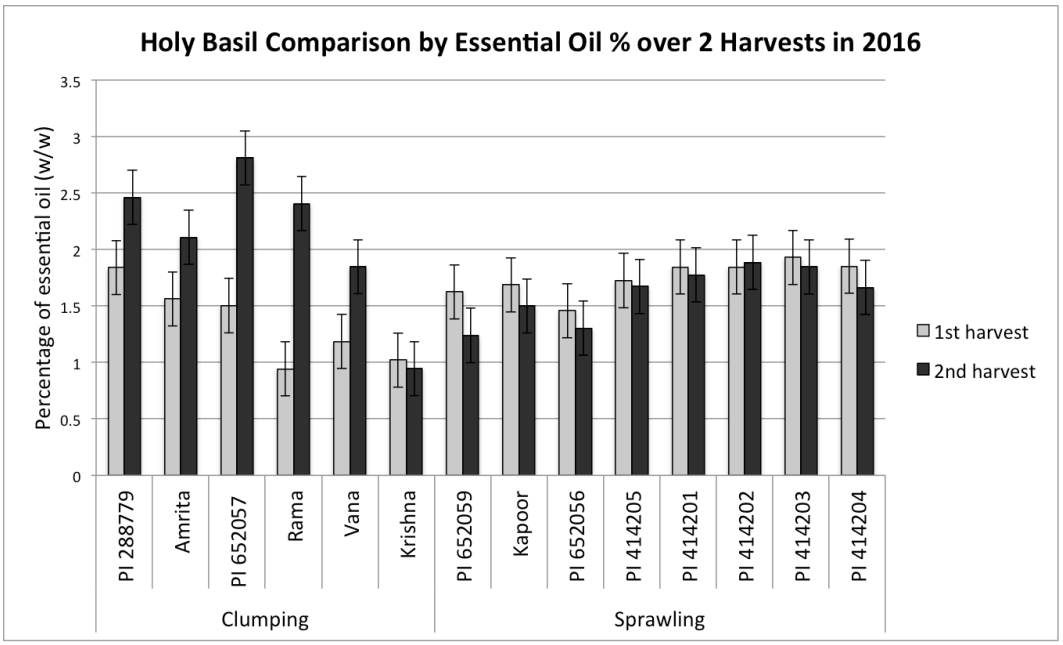


Fig. 2.5: a = mean essential oil percent of holy basil varieties between the first and second harvest during the 2015 growing season; b = mean essential oil percent of holy basil varieties between the first and second harvest during the 2016 growing season.

a.

b.

Fig. 2.6: a = example of clumping growth (PI 652057); b = example of sprawling growth (PI 414205)

Table 2.3: Varietal comparison by leaf color, growth habit and the average number of weeks it took for the holy basil plants to reach full bloom stage for both harvests.

Phenotypic differences between varieties			
Variety	Leaf color	Growth habit[1]	# wks to flower
Vana	Green	Clumping	9
Krishna	Purple	Clumping	9
Rama	Green	Clumping	5
Amrita	Purple	Clumping	7
PI 288779	Green	Clumping	7
PI 652057	Green	Clumping	6
Kapoor	Green	Sprawling	6
PI 652059	Green	Sprawling	6
PI 652056	Green	Sprawling	6
PI 414205	Green	Sprawling	6
PI 414201	Green	Sprawling	6
PI 414202	Green	Sprawling	6
PI 414203	Green	Sprawling	6
PI 414204	Green	Sprawling	6

[1]Clumping = upright growth, fewer branches, stems were more tough and woody; sprawling = stems would bend down to the ground and then grow vertically, branches were more numerous, stems were tender and more herbaceous.

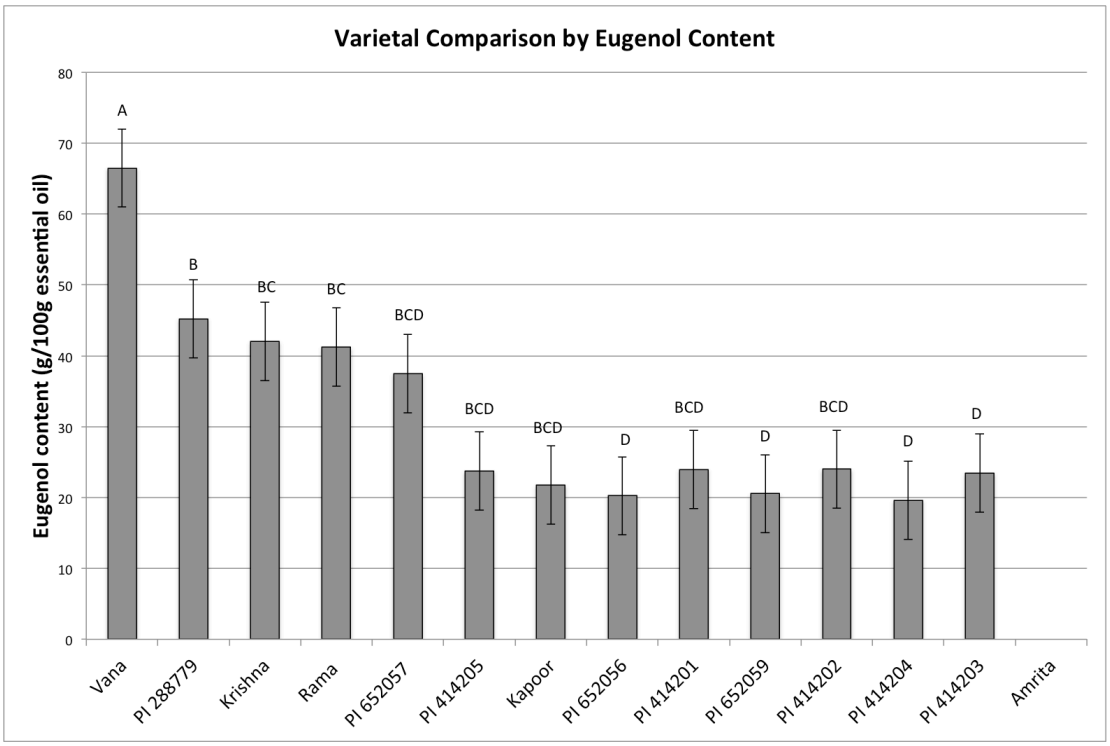


Fig. 2.7: Mean eugenol content of each holy basil variety. Significance is indicated by a p-value ≤ 0.05. Similar letters above bars indicate no significant difference between varieties.

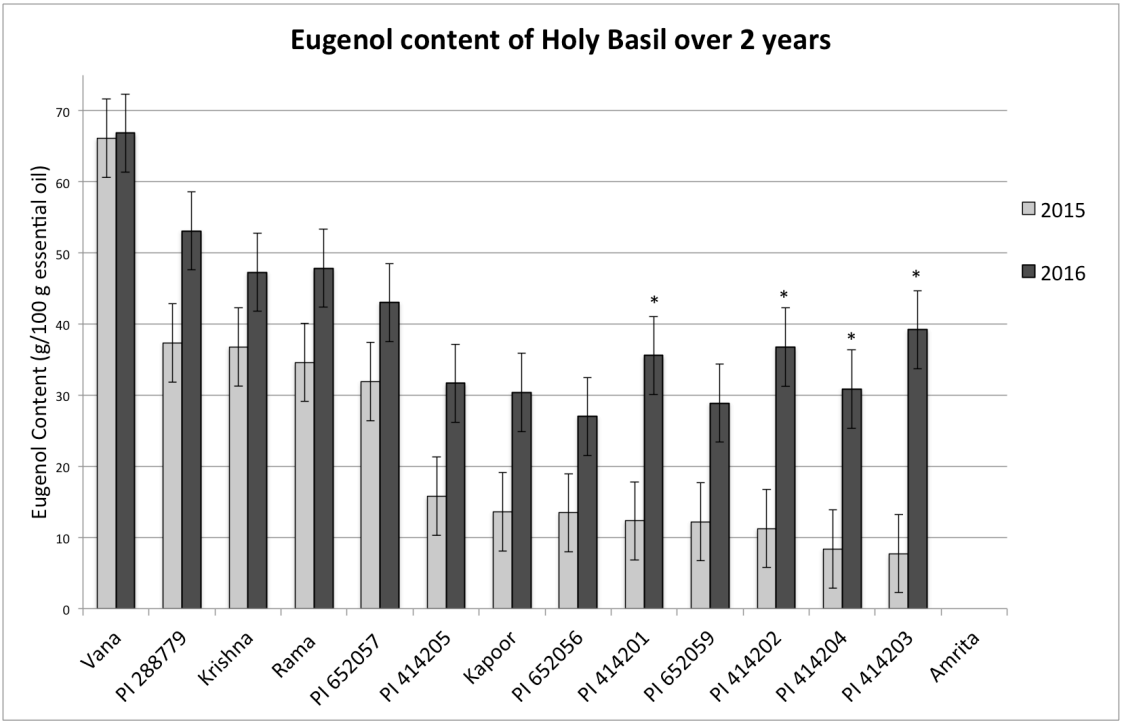


Fig. 2.8: Mean eugenol content of essential oil between the 2015 and 2016 growing seasons. An asterisk (*) denotes the varieties with a significantly higher eugenol content (p-value ≤ 0.05) in 2016 compared to 2015.

CHAPTER 3

QUANTITATIVE ANALYSIS OF VARIOUS HOLY BASIL ESSENTIAL OILS THROUGH A VALIDATED GAS CHROMATOGRAPHIC METHOD AND PREDICTED RELATIVE RESPONSE FACTORS[1]

[1]Fuller, N.J., Pegg, R.B., Berle, D., Affolter, J.M. To be submitted to the Flavour and Fragrance Journal.

Abstract

Holy basil (*Ocimum tenuiflorum* L.*, O. gratissimum* L.) is a medicinal herb traditionally used in the Indian healing tradition Ayurveda. It has been shown to reduce stress, promote physiological balance, stimulate healthy metabolism, support the immune system, and function as an anti-inflammatory. It is believed that much of the therapeutic potential of this herb is attributed to its essential oils, but information is lacking concerning the holy basil varieties available and their essential oil profiles. This study reports on the essential oil profiles of 14 varieties of holy basil, and a reliable method for quantification of the essential oil constituents extracted via a combination of external and internal standardization approaches using predicted relative response factors (RRFs). In terms of essential oil profiles, there was one variety composed primarily of methyl eugenol (~65 %), and one of eugenol (67%). Some varieties were moderately high in eugenol (~37-45%) and β -caryophyllene (~23-37%), while others possessed equally moderate quantities of eugenol, eucalyptol, estragole, and β-bisabolene. The developed method showed good precision and repeatability with a relative standard deviation (RSD) less than 4% for all tests. The reliability for using predicted RRFs was evaluated with a difference of 0.96 ± 0.25 to 11.21 ± 1.34% between the two quantification methods. Compounds detected with moderate to low concentrations showed a smaller difference, while high concentration compounds showed greater differences between calculated and predicted values. A combination of these strategies is likely the best way to achieve an efficient, reliable and accurate quantification of the chemical constituents in the essential oils of the 14 holy basil varieties.

Keywords: holy basil, tulsi, *Ocimum tenuiflorum*, *Ocimum sanctum*, *Ocimum gratissimum*, essential oils, gas chromatography, relative response factors

3.1 Introduction

Essential oils are volatile liquids of a plant, containing complex mixtures of chemical constituents, usually of low-molecular-weight and similar physicochemical characteristics.[1] Between 40 to 60,000 tons of essential oils are produced annually with a market value of $700 million USD. They are used commonly as flavors and additives in the food industry, as well as in the cosmetic, health, and various agricultural industries.[2] The International Organization for Standardization (ISO) defines an essential oil to be a product recovered by distillation with either water or steam, or by mechanical processing of citrus rinds, or by dry distillation of natural materials.[3]

Essential oils are typically diluted and separated into their respective constituents by gas chromatography. Then, the compounds can be identified and quantified by mass spectrometry and a flame ionization detector, respectively.[1] Moreover, the International Conference on Harmonisation (ICH) has published guidelines for method validation, which include testing for accuracy, reproducibility and meet the requirements for linearity over the specified range of each analyte being examined.[4]

Unfortunately, many studies on essential oils only report GC-MS results of percent relative abundance. This provides only the relative amount of an essential oil in an individual sample. It doesn't apply to other samples, thereby, making it difficult to aggregate and compare essential oil composition data from different published studies, or to compare a series of samples of the same species.[1] Even with properly validated quantitative protocols, it is not feasible to describe all of the chemical complexity of an essential oil due to the long analysis time required and the fact that many key standards are not available commercially. Therefore, a new method is

reported that allows for the quantification of volatile compounds with GC-FID by predicting the RRFs from the molecular formula of the compound in question.[5]

Ocimum tenuiflorum syn. *O. sanctum,* known as "tulsi" or "holy basil" is a medicinal herb commonly used in Ayurveda, the traditional healing system of India for over 5,000 years. It is an aromatic plant in the Lamiaceae family, and grows as a small erect shrub with hairy stems. It is a sacred plant to the Hindu people and has been used customarily as an herb that rejuvenates and promotes longevity. Due to its large popularity in India, there have been many research studies on the medicinal action of holy basil. These studies have demonstrated that it can be used to reduce stress, promote physiological balance, promote healthy metabolism, support the immune system, function as an antioxidant and anti-inflammatory, act as a neuroprotective, as well as protect against the negative effects of ionizing radiation.[6-9]

The leaves and flowers of holy basil contain a variety of phytochemicals believed to have therapeutic activity. These include saponins, flavonoids, alkaloids, glycosides, tannins, and ascorbic acid (vitamin C). Yet, the bulk of the therapeutic potential of holy basil is commonly attributed to its essential oil fraction.[10] Studies on the essential oils of holy basil have shown they can be effective in reducing blood sugar levels and improving blood lipid profiles, as well as possessing antidiabetic, cardioprotective, hepatoprotective, antimicrobial, antifungal, and antiviral properties.[10] For instance, eugenol (2-methoxy-4-(prop-2-en-1-yl)phenol; CAS No. 97-53-0) is a notable phenolic compound in holy basil's essential oil fraction, and has been shown to be an efficient vasodilator, and therapeutic for nuerological, inflammatory, allergic, and immunological disorders.[11] Other major compounds reported in the literature include estragole (methyl chavicol), methyl eugenol, β-elemene, β-caryophyllene, β-bisabolene, and eucalyptol (1,8-cineole).[12]

There are many challenges associated with the study of holy basil. Currently, there are no published standard reference ranges for known biologically-active compounds, and the mechanism of action of the holy basil plant is not at all well understood.[8] Additionally, there are many different cultivars of holy basil, and the differences among these are unclear. This information is important because the chemical composition of the essential oil fraction is what gives it economic importance and therapeutic value.[13] This is the first study to our knowledge to investigate a representative selection of the available holy basil varieties grown in the southeastern United States.

Thus, the purpose of this study is to characterize the essential oils of 14 holy basil varieties of potential commercial importance. The research entailed developing a validated analytical method for essential oil profiling, comparing the performance of different analytical columns and quantification strategies, and reporting the ranges of dominant phytochemicals present in the extracted essential oils. This work is important for improving the quality of essential oil reporting and understanding the essential oil composition of holy basil grown in the southeastern United States. This will assist horticulturalists in determining if holy basil can be grown commercially in the southeastern U.S. with similar quality characteristics to its counterpart grown in India.

3.2 Experimental

3.2.1 Plant Material

Fourteen varieties of holy basil (*Ocimum tenuiflorum* L.*, O. gratissimum* L.) were grown during the 2015 and 2016 growing seasons at the University of Georgia UGArden farm in Athens, GA. The study design consisted of a randomized complete block design with three

replications, and a plot size of 9 m^2. Nine holy basil accessions (PI 288779, PI 652059, PI 652057, PI, 652056, PI 414201, PI 414202, PI 414203, PI 414204, PI 414205) were obtained from the USDA-ARS National Plant Germplasm System (Ames, IA, USA), and five named cultivars (Kapoor, Rama, Krishna, Amrita, and Vana) were obtained from Strictly Medicinal Seeds (Williams, OR, USA). Vana is the only (*Ocimum gratissimum* L.) cultivar, but it is also commonly referred to as holy basil and is believed to have the same therapeutic uses.[6,9] Therefore, it was included in this study to determine the essential oil composition compared to *O. tenuiflorum*.

Seeds were sown in 50 count plastic trays using Sunshine Natural and Organic Professional Growing Mix (SunGro, Agawam, MA, USA) in June of both years in a temperature-controlled greenhouse. Thirty-eight day-old seedlings were transplanted into raised beds (1 m wide, 0.3 m high, 9 m long, with drip irrigation tubing underneath pine straw mulch), with six plants of each variety per replicate. Seedlings were planted in two rows, with 30 cm spacing between rows and plants, and a 60 cm divide between varieties. On weeks it did not rain, the irrigation system supplied roughly 38 mm of water per week during the growing season.

Before planting, the raised beds were fertilized with hydrolyzed poultry feathers with a nitrogen-phosphorus-potassium ratio of (13-0-0) (Mason City Byproducts, Inc., Mason City, IA, USA) at the rate of 120 kg N/hectare. Additionally, in 2016, pulverized dolomitic lime (Austinville Limestone Co., Austinville, VA, USA) was added at a recommendation level of 2,800 kg/ha to raise the soil pH from 5.69 to 6.0. After planting, individual seedlings were watered with a fertilizer solution at the rate of 0.18 L of a 4.8 g/L solution of Chilean nitrate of soda (16-0-0) (Allganic, Eugene, OR, USA). Holy basil plants were pruned back to the third node 12 days after planting to encourage branching.

The holy basil was harvested twice during each growing season, when the plants were in full flower: with an inflorescence on 80% of the shoots and blooms opening halfway up the vertical.[14] The weeks to maturity varied slightly for certain varieties of holy basil, but they were all ready to harvest within six to nine weeks of planting. The first harvest was taken at a height of 15 cm to allow the branches to regrow,[15] and six to nine weeks after the first harvest (depending on the variety), the second cuttings were taken at a height of 10 cm.

Plants were dried at ambient temperature in an herb room with a dehumidifier. The plants were ready for processing once the moisture in the room was maintained at 15% and a test leaf was easily powdered by hand. For processing, the leaves and flowers were stripped from the stems. There were six plants per rep for each variety, and the dry leaves and flowers of these six plants were combined to yield a representative mixture for each rep. Then, 100 g of this representative composite was weighed out and stored in food-grade aluminum bags in a -18°C freezer until further analysis. There were three replicates for each variety for each harvest.

Additionally, after each harvest, four samples were collected from different zones of the drying room to determine the moisture content of dried holy basil. There were four total harvests; so, four moisture analyses were conducted. Each moisture analysis was performed in triplicate by mixing 10 g of dried leaves from each of the four collection zones, and grinding them in a coffee grinder in 2 s bursts. Roughly 2 g of the ground mixture were transferred to a tared, aluminum weigh pan (VWR International, Cat No: 25433-008, Radnor, PA, USA) and placed in a preheated Isotemp oven model 650G (Fischer Scientific, Dubuque, IA, USA) set to 103 °C. Samples were checked periodically until a constant mass was achieved (~4 h). The test samples were removed from the oven and placed in a dessicator. Once the samples had cooled, the

masses were recorded, the weights of the aluminum pans were subtracted from the initial and final values, and the moisture contents were calculated using the following equation:

Moisture content = (initial weight – dry weight)/dry weight x 100%

Additionally, 14 voucher specimens, one for each of the 14 varieties evaluated, were prepared and then verified by Dr. James Affolter, the Director of Research at the State Botanical Garden of Athens, GA, USA. They are stored in the University of Georgia Herbarium Athens, GA.

3.2.2 Reagents and Standards

The external standard eugenol (99%) was purchased from TCI America (Portland, OR, USA), β-caryophyllene (99%) was obtained from Fluka Analytical Co. (St. Louis, MO, USA), while estragole (98.5%), eucalyptol (99%), methyl eugenol (99%) and HPLC-grade acetonitrile (99%) were acquired from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). The internal standard methyl octanoate was purchased from Acros Organics (Morris Plains, NJ, USA). Methyl octanoate was chosen as the internal standard based on the protocol advised by Cachet et. al. for utilizing predicted RRFs for rapid quantification of essential oil constituents by GC-FID.[5]

3.2.3 Hydrodistillation

To extract the essential oils, a Clevenger trap (Wilmad-LabGlass, Kingsport, TN, USA) for oils lighter-than-water was employed.[16] Dried plant material (leaf and flower, 50 g) was finely ground in a coffee grinder and transferred to a 2-L round bottom flask with deionized water at a material-to-solvent ratio of 1:13 (w/v),[17] and a distillation time of 180 minutes per

sample. To facilitate condensation, water passing through the condenser was first chilled to 5 °C and then recycled through an Isotemp™ Refrigerated/Heated Bath Circulator model 4100 R20 (Fischer Scientific, Dubuque, IA, USA). The distillation time was determined by waiting 30 min after the last increase in volume of essential oil in the collecting arm of the Clevenger trap was observed.

The essential oil was then passed through 600 mg of anhydrous sodium sulfate (Avantor Performance Materials, Inc., Center Valley, PA, USA) to remove any water residue. The collected oil fractions were stored in ¼ dram (0.92 mL) amber glass vials (Cat No: 1-4DAOR00144, Premium Vials, Bristol, PA, USA) at -18 °C.

3.2.4 Gas Chromatographic conditions

Essential oil samples were dissolved in HPLC-grade acetonitrile in a 5 mL volumetric flask giving a final concentration of ~20 mg/mL. Sample aliquots were transferred to 2 mL GC vials and then capped with a manual crimper (Agilent Technologies, Cat No: 8710-0979, Santa Clara, CA, USA). A 1 µL aliquot was injected into a single taper split liner (Agilent Technologies, Cat No: 5183-4647, Santa Clara, CA, USA). All injections were performed in duplicate.

The separations were carried out with an Agilent Technologies Gas Chromatograph model 6890N equipped with a flame ionization detector and autoinjector model 7683B. The data was recorded with Chemstation [v. E.02.02]. A non-polar HP-5 fused-silica capillary column (J&W Scientific, Folsom, CA, USA; 5% phenyl-methylpolysiloxane, 30 m, 0.32mm i.d., 0.25 µm film thickness) was used for the analysis. The oven temperature was held at 50° C for 5 min, then programmed to increase 3 °C/min to 120 °C, then at 5 °C/min to 250 °C and then 15 °C/min

to 300° C, followed by a 5 min hold for a total run time of 62.67 minutes. The carrier gas was UHP-grade helium at a flow rate of 30 mL/min. Air flow was set to 450 mL/min, UHP-H_2 flow to 40 mL/min, and makeup gas to 25 mL/min. The inlet pressure was 18.07 psi. The analysis was performed in constant flow mode, and the injector and detector temperatures were set at 250 °C, with a split ratio of 80:1.

Additionally, a polar DB-WAX UI column (Agilent J&W, Agilent Technologies, Folsom, CA, USA; bonded polyethylene glycol (PEG), 30 m length, 0.25 mm i.d., 0.25 μm film thickness) was employed to compare separations. The oven temperature was held at 50 °C for 5 min, then programmed to increase 3 °C/min to 120 °C, then at 5 °C/min to 250 °C, proceeded by a 0.67 min hold for a total run time of 55 minutes. The carrier gas was UHP-grade helium at a flow rate of 30 mL/min. Air flow was set to 300 mL/min, UHP-H_2 flow to 25 mL/min, and makeup gas to 25 mL/min. The inlet pressure was 11.8 psi. The analysis was performed in constant flow mode. The sample preparation, injector and detector temperatures, and split ratio were the same as those described in the GC-FID analysis with the HP-5 column.

An Agilent Technologies Gas Chromatograph, model 6890N equipped with a quadropole Mass Spectrometer Detector model 5973, was used to identify the compounds present in holy basil essential oils. The mass spectral data was recorded with Chemstation [v. E.02.02]. A non-polar HP-5MS fused-silica capillary column (J&W Scientific, Folsom, CA, USA; 5% phenyl methylsiloxane, 30 m, 0.25mm i.d., 0.25 μm film thickness) was employed for the analysis. Samples were prepared by diluting ~100 mg of essential oil in 5 mL of acetonitrile. Samples were transferred to a 2 mL GC vial following the procedure described for the GC-FID analysis. A 1 μL aliquot of the solution was manually injected into a single taper split liner (Agilent Technologies, Cat No: 5183-4647, Santa Clara, CA, USA). The oven temperature, inlet

temperature, split ratio and carrier gases were the same as those described for the GC-FID analysis using the HP-5 column. The analysis was performed in constant flow mode and the inlet pressure was 10.99 psi. The electron multiplier voltage was set at 70 eV under scan acquisition mode. MS source and MS Quad temperatures were set to 230 and 150 °C, respectively. The analysis was carried out in the positive ion mode, with a velocity of 42 cm/s, a mass range of 40-650 amu, and a scan rate of 2.42 scans/s.

The compounds present in holy basil essential oils were identified through a combination of mass spectral data, retention time indices, injection of commercial standards, and employment of the NIST mass spectral library (NIST Database, version 2.2).

3.2.5 Standard Solution

A standard solution containing 20.29 mg/mL eugenol, 15.27 mg/mL β-caryophyllene, 6.45 mg/mL eucalyptol, 9.33 mg/mL estragole, and 20.87 mg/mL methyl octanoate (IS) in acetonitrile was prepared in a 10 mL volumetric flask. Due to co-elution of methyl eugenol with β-caryophyllene (see results section), a separate standard of methyl eugenol was prepared with 20.46 mg/mL methyl eugenol and 19.76 mg/mL of the IS. Additionally, a standard solution containing 28.72 mg/mL of methyl eugenol, 8.87 mg/mL of β-caryophyllene, and 21.79 mg/mL of the IS was prepared for the DB-WAX UI column.

3.2.6 Validation

Analytical calibration curves were assessed for linearity and generated by the injection of the standard solutions at seven different concentrations in the range expected for each compound in the essential oil. Precision was evaluated for repeatability (intra-day precision) by injecting

five replicate analyses of a single sample on the same day, and inter-day precision by preparing and analyzing five replicates of a given sample over multiple days. This evaluation was performed on two different essential oil samples at different times during the analysis process on the HP-5 column, and once on the DB-WAX UI column. Limit of detection (LOD) and limit of quantification (LOQ) values were measured by using the following equations:

$$LOD=3*\sigma(blank);\ LOQ=10*\sigma(blank)$$

Additionally, resolution was calculated between peaks on the GC-FID chromatograms using the equation:

$$R_s= 2\Delta t/w_2 + w_1$$

Where Δt is the difference between the retention times of peaks 1 and 2, w_2 is the width of peak 2 at the base line, and w_1 is the width of peak 1 at the baseline in time units. R_s should be greater than 1.5.

3.2.7 Quantification by Gas Chromotography-Flame Ionization Detection

A study by Cachet et al. found that using predictive RRFs can reliably quantify a compound down to a concentration of ~1% in the essential oil fraction with a mean accuracy of 6%.[5] Therefore, when a compound was found to be greater than 1% in an essential oil and external standards were not commercially available, it was quantified by internal standardization using predicted RRFs. The predicted RRF of an analyte is calculated by its molecular formula using a simplified equation for the atoms occurring in holy basil essential oil constituents:

$$RRF_i = 10^3(MW_i/MW_{ISTD})(-61.5 + 88.8n_C + 18.7n_H, - 41.3n_O + 3.8n_N)^{-1}$$

where n_C, n_H, n_O and n_N are the number of carbon, hydrogen, oxygen and nitrogen atoms in the compound and MW_i and MW_{ISTD} are the molecular masses of the analyte and the internal standard (methyl octanoate), respectively.

3.3 Results and Discussion

3.3.1 Mass Spectrometry Analysis

Positive identification of the constituents contained in the essential oil fraction of holy basil was carried out by comparing mass spectral data, matching of t_R values and confirmation with authentic commercial standards. The constituents present in holy basil essential oils could be divided into distinct chemical classes: terpenes (mono- and sesquiterpene hydrocarbons), alcohols, oxides, esters and phenylpropanoids. The individual chemical constituents within these chemical classes showed similarities in their patterns of fragmentation. Table 3.1 presents the results, and lists the parent molecular ion (M^+) and major fragmentation ions present in the mass spectra for each constituent in order of elution on the HP-5 capillary column.

The compounds β-pinene, (-)-β-ocimene, and β-ocimene yielded a molecular ion at *m/z* 136, and exhibited a diagnostic pattern of neutral losses at [M-15, M-29, M-43, M-57, M-71, M-85]. According to McLafferty, this indicates a stepwise loss of alkyl groups (C_nH_{n-1}) supporting their classification as monoterpene hydrocarbons. [18]

Similarly, the compounds α-bisabolene, β-bisabolene, β-caryophyllene, α-humulene, β-elemene, germacrene D, α-copaene, (-)-α-bergamoetene , β-farnesene , and 8-isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene produced the same pattern of neutral losses as the monoterpenes but had a molecular ion at a *m/z* of 204, and these were confirmed by the GC-MS library to be

sesquiterpene hydrocarbons. The identity of β-caryophyllene was also confirmed by an authentic commercial standard.

The compounds methyl eugenol, eugenol and estragole were tentatively identified, belonging to the group of phenylpropanoids. They had molecular ions at a *m/z* ratio of 178, 164, and 148, respectively. They all exhibited key fragmentation ions at *m/z* of [M-15; $\{CH_3\}]^+$, and a *m/z* peak of 91 indicating the presence of a benzyl group. This supports the classification as phenylpropanoids. Furthermore, identities of these three were confirmed by commercial standards.

A molecular ion at *m/z* 130 was provided by ethyl 3-methylbutanoate. It is likely an ester. The most abundant fragmentation peaks occurred at *m/z* 88 [M-15; $\{C_4H_8O_2\}]^+$, and 57 [M-73; $\{C_4H_9\}]^+$, supporting the identification. Another tentative identification was made for caryophyllene oxide with a molecular ion at *m/z* 220. The most abundant fragment ion occurred at *m/z* 79 [M-141; $\{C_6H_7\}]^+$, and the fragmentation data matched those reported by El-Gohary.[19]

Finally, eucalyptol, endo-borneal and linalool were tentatively identified as alcohols. They all produced a molecular ion at a *m/z* of 154 and all displayed characteristic fragmentation of [M-15; $\{CH_3\}]^+$. Endo-borneal had an abundant fragment ion at *m/z* 95 [M-59; $\{C_6H_7O\}]^+$. Linalool exhibited diagnostic fragment ions at m/z of 93 [M-61; $\{C_7H_9\}]^+$ and 71 [M-83; $\{C_4H_7O\}]^+$. Finally, eucalyptol had abundant fragmentation peaks at *m/z* 43 [M-111; $\{C_3H_7\}]^+$, 81 [M-73; $\{C_6H_9\}]^+$ and 108 [M-46; $\{C_7H_8O\}]^+$, respectively. Additionally, the identity of eucalyptol was confirmed by an authentic commercial standard.

3.3.2 Linearity, precision, and accuracy

Table 2 gives the results for the linearity of the chemicals employed as standards with the HP-5 and DB-WAX UI columns. The linear dynamic range was suitable for all of the compounds analyzed on both columns, and the table indicates that the r^2 coefficients of variation for all compounds were >0.999 on the HP-5 column, and >0.998 on the DB-WAX UI column. Table 1 also reports that the linear dynamic range was suitable for all the compounds quantified by external standardization throughout this study.

Tests for precision include repeatability, which is the measure of variation between a series of measurements taken on the same sample, and intermediate precision (inter-day), which is the measure of variation between multiple preparations of a common sample over several days. Tables 3a, 3b and 3c give the results for precision tests on two samples of holy basil essential oil (Kapoor and PI 652057) evaluated at two different instances during the 5-week analysis period. Additionally, repeatability and intermediate precision were evaluated for the standards β-caryophyllene, methyl eugenol, and the IS on the DB-WAX UI column. The variations are expressed as relative standard deviations (RSD%).

For the Kapoor variety, the variation in repeatability for retention time, peak area and concentration were 0.00 to 0.01, 0.7 to 1.04, and 0.71 to 0.92%, respectively; and variation in inter-day precision was 0.00 to 0.01, 0.5 to 1.07, and 0.13 to 0.99%, respectively. For accession PI 652057, the variation in repeatability for retention time, peak area, and concentration were 0.00 to 0.01, 0.98 to 1.33, and 0.49 to 1.08%, respectively; while, the variation in inter-day precision was 0.00 to 0.04, 1.09 to 3.18, and 0.12 to 1.13%, respectively. Finally for the DB-WAX UI column, the results for variation in repeatability for retention time, peak area, and concentration, respectively, were 0.01, 0.58 to 1.58, and 0.49 to 0.59%, with 0.00 to 0.01, 1.1 to

1.18, and 1.08 to 1.14% for the variation in inter-day precision. All RSD values were below 4%, with most below 1.5%. This demonstrates that the method employed for analysis on both the HP-5 and DB-WAX UI capillary columns has satisfactory precision. The average moisture of the dry leaf and flower was 11.4 ± 0.7% and 11.0 ± 0.1% (SD) in 2015 and 2016, respectively. The LOD and LOQ values were 0.23 and 0.78, respectively.

3.3.3 Predictive RRFs

Table 4 lists the chemical class and predictive RRFs for the compounds identified in the essential oil samples. The chemical classes include mono and sesquiterpene hydrocarbons, alcohols, oxides, esters, phenylpropanoids and ethers with a range of RRFs from 0.75 to 1.49. The largest group, with eleven compounds, was the sesquiterpenes. These compounds consist of three isoprene units (C15), which lead to increased cyclization and a greater a diversity of structures.[20] Unfortunately, many sesquiterpenes, as well as other classes of essential oil compounds, are difficult to find commercially, or are unaffordable and difficult to isolate or synthesize. Therefore, the development of a new technique to quantify these difficult-to-find standards allowed for a more thorough, precise, and efficient analysis of the essential oils.

To corroborate the findings of Cachet et al., predictive RRFs were calculated for the compounds with available standards and both quantification methods were compared.[5] Figure 1 depicts the results, expressed as the average difference and standard deviation between the concentrations calculated through calibration curves and RRFs with an internal standard. For all five compounds, the quantification results from the predicted RRFs were slightly higher. The compound eucalyptol showed the least variance, with an average difference of 0.96 ± 0.25 (SD) between the two quantification methods. Interestingly, the compound methyl eugenol showed the

greatest difference between methods at 11.21 ± 1.34. It seemed that quantification by predicted RRFs was less accurate for compounds that make up a larger percentage of the essential oil. Cachet et al. cites a relative accuracy of ~6% using this method; however, it might not be the best approach for quantifying major compounds in the essential oil, where their high concentration can lead to greater inaccuracies. A reasonable approach to essential oil quantification would be to find external standards for compounds that comprise >30% of the essential oil fraction and use predictive RRFs for the remaining compounds that are above the LOQ. A combination of these strategies is most likely the best way to get a reliable and accurate quantification of these complex mixtures of phytochemicals.

3.3.4 Gas Chromatography

Methyl eugenol and β-caryophyllene were two dominant constituents of the holy basil culivar Amrita. When the pure compounds were mixed together in the standard solution, they, unfortunately, co-eluted on the HP-5 column. Therefore, standard curves of methyl eugenol and β-caryophyllene were generated separately. Due to the co-elution issue, it led to challenges in terms of quantification of these two compounds in the Amrita essential oil. A second, more polar GC column was employed to test each of the essential oils and determine if there were additional compounds that had not eluted, been detected, or had improved resolutions. The chromatography revealed that the essential oil of Amrita exhibited no co-eluting peaks; all were fully resolved. Figure 2 depicts the chromatograms of the 'Amrita' essential oil on the HP-5 and DB-WAX UI columns and confirms that the DB-WAX UI column results in excellent resolution (R_s >1.5) between the methyl eugenol and β-caryophyllene peaks as compared to their co-elution (R_s <1.0) on the HP-5 column. A standard curve was then prepared with methyl eugenol and β-

caryophyllene to be able to accurately quantify and compare the results between the HP-5 and DB-WAX UI columns. Table 5 reports these findings.

The greatest difference in performance between these two columns was discovered in the analysis of the essential oils from the second harvest in 2015. β-Caryophyllene was completely hidden by the methyl eugenol peak and was not detected on the HP-5 column, suggesting that it was devoid in the sample. However, separation and quantification with the DB-WAX UI column revealed a mean β-caryophyllene content of 12.54 ± 0.18%. Additionally, data from the first harvest of 2015 and both harvests of 2016 indicate that co-elution on the HP-5 column led to perceived lower quantities of β-caryophyllene and perceived higher quantities of methyl eugenol than were actually present in the samples. It is clear that using a polar column such as a DB-WAX UI is a better choice for analyzing samples that contain both methyl eugenol and β-caryophyllene. All of the other compounds showed excellent resolution (R_s >1.5) between peaks on the HP-5 column.

3.3.5 Quantification of holy basil essential oils

Table 6 gives the mean essential oil percentages of compounds over both growing seasons for all 14 varieties of holy basil. Previous findings have reported variations in the dominant marker compounds between varieties, which was further supported by this study.[12] The variety Amrita was composed primarily of methyl eugenol, 64.71 ± 4.04%, and was the only cultivar containing the methyl ether of eugenol. Other constituents include β-elemene, β-caryophyllene, α-humulene and Germacrene D which is supported by the findings of Bakkali et. al. of methyl eugenol rich varieties grown in India.[20] All other varieties exhibited varying quantities of eugenol, with Vana being the highest in this phytochemical at 66.50 ± 7.29%. This

cultivar was also the only *Ocimum gratissimum* variety. Its high eugenol content offers a possible explanation for why this variety of basil is used interchangeably with the other *O. tenuiflorum* varieties. The varieties Krishna, Rama, PI 288779 and PI 652057 were moderately high in eugenol (~37 to 45%) and β-caryophyllene (~23 to 37%). Reina et. al. evaluated 32 accessions of holy basil grown in India and reported that 28 contained a profile similar to these four previously mentioned varieties.[22] The only differences were between minor compounds where Reina et. al. reported minor amounts of β-selenene and α-selenene, that were not identified in our study and we identified 8-isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene in quantities ranging from 6.43 ± 1.33% to 17.28 ± 3.41% between the four varieties that were not previously reported. Finally, the varieties Kapoor, PI 652059, PI 414203, PI 652056, PI 414205, PI 414201, PI 414202, and PI 414204, were similar in composition with an essential oil profile comprising moderate amounts of eugenol, eucalyptol, estragole, and β-bisabolene. Zheljazkov et. al. reported on this holy basil essential oil type but our study reported additional compounds including β-bisabolene, α-bisabolene, β-pinene, β-ocimene (trans)-α-bergamotene, and germacrene D that were not formerly described.[23] Therefore, to our knowledge, this is the most comprehensive reporting of this holy basil essential oil type.

There are many ways that this work was novel, and signifies a valuable contribution to the literature regarding holy basil essential oils. To begin, this is the first study, to our knowledge, to report on a validated lab method for analyzing holy basil essential oils. To demonstrate the challenges, typically the literature on holy basil essential oil primarily reports on the percent relative abundance of constituents in the essential oil from GC-MS analysis; which eliminates the ability to reliably compare between studies.[14] If quantification was performed through an internal standard, it was typically done without the use of response correction factors,

decreasing the accuracy of the analysis.[20] Or, if external standards were used, it was not a comprehensive analysis. Furthermore, there were very few studies done on holy basil in the United States. There have been previous studies on holy basil in Mississippi, however, they only evaluated one variety of holy basil and achieved only ~40-70% quantification using commercial standards.[15, 22] Therefore, this study was innovative in that it evaluated a selection of holy basil varieties available domestically, a validated lab method was developed, and a more comprehensive quantification of the essential oils, including reporting on many of the minor compounds not found in previous studies, was performed.

3.4 Conclusions

The developed analytical method is efficient, straightforward, and reliable for quantifying complex holy basil essential oils. The use of predicted RRFs is an excellent way to facilitate rapid quantification of essential oil phytochemicals, especially when pure compounds are not available commercially. However, it is still best to source external standards for quantification of the phytochemicals that comprise the bulk of the essential oil fractions, as higher concentrations can lead to greater inaccuracies. An HP-5 column provides excellent resolution for many compounds in holy basil essential oil, except for those containing methyl eugenol and β-caryophyllene. In which case, a polar column is much better suited for the complete separation of these two constituents. Ultimately, this study showed that holy basil could be grown in the southeastern US with similar quality characteristics to holy basil grown in India. This opens up greater opportunities for domestic production. Ideally, this will increase awareness of holy basil as a medicinal herb with useful applications in the US.

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Table 3.1 Molecular ion (M^+) and fragmentation ions from mass spectra of gas chromatography mass spectrometry analysis

Tentative identification	t_R[a]	M^{+}[b]	Fragmentation ions, *m/z* (RI)[c] Largest fragmentation ions
ethyl 3-methylbutanoate	3	130(2)	**88**[d], 87(17), 85(74), 70(21), 61(20), 60(36), 57(52), 43(23), 41(31)
β-pinene	6.5	136(11)	121(14), 94(12), **93**, 91(26), 79(21), 77(21), 69(27), 53(15), 41(22)
eucalyptol	8.9	154(72)	139(62), 111(70), 108(81), 93(54), 84(60), 81(87), 72(64), 69(51), **43**
trans-β-ocimene	9.5	136(2)	121(12), 105(17), **93**, 92(37), 91(45), 80(15), 79(34), 77(30), 41(16)
β-ocimene	9.9	136(7)	121(19), 105(20), **93**, 92(23), 91(48), 80(35), 79(42), 77(33), 41(17)
linalool	12.4	154(.3)	121(28), 93(93), 80(33), **71**, 69(45), 67(21), 55(48), 43(48), 41(49)
endo-borneal	15.3	154(0.5)	139(8), 121(7), 110(20), 96(8), **95**, 93(8), 67(8), 55(7), 43(6), 41(10)
estragole	17.1	**148**	147(56), 133(21), 121(36), 117(33), 115(21), 105(19), 91(19), 77(22)
eugenol	24.5	**164**	149(33), 137(18), 133(17), 131(26), 104(17), 103(26), 91(21), 77(24)
α-~~e~~opaene	25.1	204(21)	**161**, 120(23), 119(94), 105(83), 93(40), 92(23), 91(35), 81(18), 77(16)
β-elemene	25.8	204(2)	147(56), 121(50), 107(68), 105(43), **93**, 81(89), 79(58), 68(61), 67(64)
methyl eugenol	26.8	**178**	179(13), 163(29), 147(29), 107(22), 103(25), 91(28), 79(11), 77(12)
β-caryophyllene	26.9	204(10)	**133**, 120(46), 107(46), 105(63), 93(99), 91(86), 79(72), 69(69), 41(61)
(*E*)-α-bergamotene	27.8	204(3)	**119**, 107(34), 105(25), 93(98), 91(38), 79(25), 77(24), 69(33), 41(31)
α-humulene	28.3	204(7)	147(20), 121(31), 107(17), 94(13), **93**, 92(17), 91(20), 80(31), 79(17)
(*E*)-β-farnesene	28.8	204(5)	133(34), 120(23), 93(68), 91(23), 81(22), 79(26), **69**, 67(23), 41(52)
germacrene D	29.5	204(17)	**161**, 120(20), 119(30), 105(45), 93(19), 91(39), 81(24), 79(23), 77(19)
8-isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene	30.4	204(14)	189(52), 147(54), 107(80), 105(60), **93**, 91(52), 81(66), 68(76), 67(80)
β-bisabolene	30.8	204(35)	161(32), 109(34), 94(38), **93**, 91(37), 79(41), 69(95), 67(37), 41(59)
(*E*)-α-bisabolene	31.9	204(21)	121(30), 119(35), 109(31), 109(23), 107(20), **93**, 91(24), 80(24), 79(23)
caryophyllene oxide	32.9	220(3)	109(54), 107(60), 95(62), 93(89), 91(82), **79**, 69(64), 43(59), 41(77)

[a] t_R = retention time (min) of compounds eluting on the HP-5 capillary column
[b] M^+ = molecular ion
[c] RI = relative intensity to base peak (%)
[d] Base peak (relative intensity = 100%)

Table 3.2: Results for linearity, with data on linear dynamic range, regression equations, r^2 values and number of replicates

		HP-5 column		
	LDR[a]	LRE[b]	$r^{2\,c}$	n[d]
Eucalyptol	0.32-6.45	y = 157.89x + 6.1638	0.99972	7
Estragole	0.47-9.33	y = 159.88x + 3.8305	0.99961	7
Eugenol	1.01-20.29	y = 132.96x - 4.6827	0.99962	7
Methyl Eugenol	0.76-15.27	y = 144.62x - 7.8895	0.99981	7
β-caryophyllene	1.02-20.46	y = 191.21x + 1.3022	0.99965	7
Methyl octanote (IS)	1.04-20.87	y = 128.17x + 8.0802	0.99963	7
		DB-WAX UI column		
Methyl octanoate (IS)	1.09-21.79	y = 115.82x - 36.606	0.99859	7
β-caryophyllene	0.44-8.87	y = 177.49x - 25.069	0.99862	7
Methyl eugenol	1.44-28.72	y = 128.15x - 63.533	0.9984	7

[a]LDR = linear dynamic range
[b]LRE = linear regression equation, regression equation as y=mx+b, where x is the concentration in mg/mL and y is the peak area
[c]r^2 = is a measure of how well the regression line approximates the real data points
[d]n = the number of points in each calibration curve.

Table 3.3a: Results for precision and accuracy of the holy basil variety, Kapoor.						
Precision	t_R [a]	RSD (%)[b]	Peak Area	RSD (%)	Conc.[c]	RSD (%)
Repeatability (n=5)						
β-pinene	6.5±0.0	0.00	43.2±0.30	0.7	1.16±0.01	0.83
Eucalyptol	8.9±0.0004	0.01	348.5±2.92	0.84	9.90±0.07	0.68
β-ocimene	9.9±0.0004	0.01	118.5±0.96	0.81	3.17±0.02	0.78
Methyl octanoate (IS)	13.8±0.001	0.01	2672.4±18.9	0.71	101.0±0.71	0.71
Estragole	17.0±0.0004	0.00	497.1±4.71	0.95	14.09±0.12	0.82
Eugenol	24.5±0.0015	0.01	1196.2±12.46	1.04	40.93±0.38	0.92
β-caryophyllene	26.9±0.0008	0.00	70.6±0.63	0.89	1.66±0.01	0.74
(-)-α-bergamotene	27.8±0.0011	0.00	64.7±0.59	0.92	1.71±0.01	0.75
α-humulene	28.3±0.0005	0.00	84.7±0.81	0.95	2.24±0.02	0.72
Germacrene D	29.5±0.0009	0.00	78.3±0.69	0.88	2.07±0.02	0.76
β-bisabolene	30.7±0.0013	0.00	476.6±4.36	0.91	12.60±0.10	0.78
α-bisabolene	31.9±0.0005	0.00	414.7±4.07	0.98	10.96±0.80	0.73
Inter-day						
b-pinene	6.5±0.0	0.00	43.5±0.22	0.5	1.16±0.01	0.39
Eucalyptol	8.9±0.0004	0.01	351.4±2.12	0.6	9.98±0.06	0.6
β-ocimene	9.9±0.0004	0.01	119.3±0.70	0.58	3.19±0.01	0.14
Methyl octanoate (IS)	13.8±0.001	0.01	2636.4±28.3	1.07	100.8±0.99	0.99
Estragole	17.0±0.0005	0.00	502.0±3.86	0.77	14.22±0.11	0.78
Eugenol	24.5±0.0008	0.00	1210.2±10.25	0.85	41.37±0.35	0.85
β-caryophyllene	26.9±0.0007	0.00	71.4±0.55	0.78	1.68±0.01	0.8
(-)-α-bergamotene	27.8±0.0008	0.00	65.3±0.50	0.76	1.72±0.002	0.13
α-humulene	28.3±0.0008	0.00	85.6±0.43	0.71	2.26±0.004	0.18
Germacrene D	29.5±0.0005	0.00	79.1±0.63	0.79	2.08±0.003	0.17
β-bisabolene	30.7±0.0005	0.00	482.3±4.23	0.88	12.71±0.04	0.3
α-bisabolene	31.9±0.0009	0.00	419.2±3.36	0.8	11.05±0.01	0.13

[a] t_R = retention time (min)
[b] Conc.= concentration (mean percentage in the oil)
[c] RSD% = relative standard deviation

Table 3.3b: Results for precision and accuracy of the holy basil accession, PI 652057.

Precision	t_R [a]	RSD (%)[b]	Peak Area	RSD (%)	Conc.[c]	RSD (%)
Repeatability (n=6)						
Methyl octanoate (IS)	13.8±0.001	0.01	2412.4±23.6	0.98	100.0±0.98	0.98
Eugenol	24.5±0.002	0.01	1272.4±16.59	1.3	41.81±0.45	1.08
β-elemene	25.9±0.002	0.01	236.7±3.024	1.28	6.11±0.03	0.5
β-caryophyllene	26.9±0.001	0.01	1440.4±18.21	1.26	33.00±0.33	1.01
8-isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene	30.4±0.001	0.00	550.5±7.34	1.33	14.23±0.07	0.5
α-humulene	28.3±0.001	0.00	85.4±1.06	1.25	2.21±0.01	0.49
Inter-day						
Methyl octanoate (IS)	13.8±0.002	0.01	2434.3±77.52	3.18	98.93±1.09	1.1
Eugenol	24.5±0.002	0.01	1292±14.51	1.23	42.09±0.47	1.13
β-elemene	25.9±0.001	0.00	240.4±2.96	1.23	6.09±0.02	0.28
β-caryophyllene	26.9±0.002	0.01	1462.8±15.95	1.09	33.23±0.36	1.09
8-isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene	30.4±0.0005	0.00	558.7±6.14	1.1	14.15±0.02	0.14
α-humulene	28.3±0.001	0.004	86.7±0.95	1.09	2.19±0.01	0.12

[a] t_R = retention time (min)
[b] Conc.= concentration (mean percentage in the oil)
[c] RSD% = relative standard deviation

Table 3.3c: Results for precision and accuracy of the holy basil variety Amrita, on the DB-WAX UI column

Precision	t_R [a]	RSD (%)[b]	Peak Area	RSD (%)	Conc.[c]	RSD (%)
Repeatability (n=5)						
methyl octanoate (IS)	21.6±0.002	0.01	2113.1±33.3	1.58	0.99±0.01	0.49
β-caryophyllene	29.7±0.002	0.01	724.2±4.3	0.59	18.58±0.10	0.54
methyl eugenol	40.6±0.002	0.01	1852.4±10.7	0.58	65.82±0.39	0.59
Inter-day						
methyl octanoate (IS)	21.6±0.002	0.01	2055.6±22.6	1.1	0.99±0.01	1.08
β-caryophyllene	29.7±0.002	0.01	740.2±8.4	1.13	18.46±0.20	1.09
methyl eugenol	40.6±0.001	0.00	1895.4±22.3	1.18	65.44±0.74	1.14

[a] t_R = retention time (min)
[b] Conc.= concentration (mean percentage in the oil)
[c] RSD% = relative standard deviation

Table 3.4: Predicted RRFs for chemical groups found in holy basil essential oils	
Compounds	Predicted RRF[a]
Monoterpene hydrocarbons	0.76
β-pinene	
(-)-β-ocimene	
β-ocimene	
Sesquiterpene hydrocarbons	0.75
α-bisabolene	
β-bisabolene	
β-caryophyllene	
α-humulene	
β-elemene	
Germacrene D	
α-copaene	
(-)-α-bergamoetene	
β-farnesene	
8-isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene	
Alcohols	0.87
Linalool	
Endo-borneal	
Eucalyptol	
Oxides	0.83
Caryophyllene oxide	
Esters	1.49
ethyl 3-methylbutanoate	
Phenylpropanoids	
Eugenol	1.07
Methyl eugenol	1.03
Estragole	0.93
[a]RRF= relative response factor	

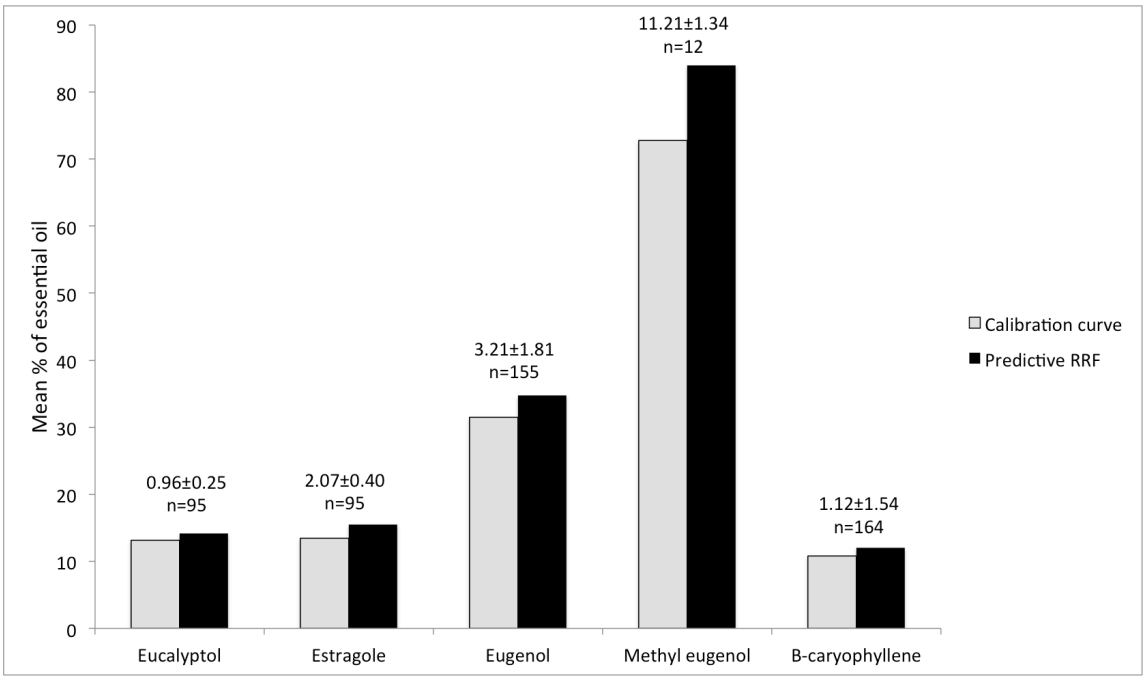

Figure 3.1: Mean percent quantification of essential oil compounds by calibration curves and predictive relatives response factors. Expressed as the average difference and standard deviation between the concentrations.

Table 3.5: Comparison of methyl eugenol and β-caryophyllene quantification between HP-5 and DB-WAX UI capillary columns

		Methyl Eugenol		β-caryophyllene	
		DB-WAX UI	HP-5	DB-WAX UI	HP-5
2015	1[a]	59.94±1.83[b]	63.60±2.79	18.93±1.95	14.75±2.20
	2	70.28±1.22	85.71±3.14	12.54±0.18	0.00
2016	1	63.62±1.28	69.64±2.24	19.39±0.48	14.08±0.96
	2	65.01±0.9	72.22±0.50	18.80±0.39	12.84±0.47

[a]two harvests of holy basil were taken each growing season
[b]expressed as mean percentage in the essential oil with standard deviation

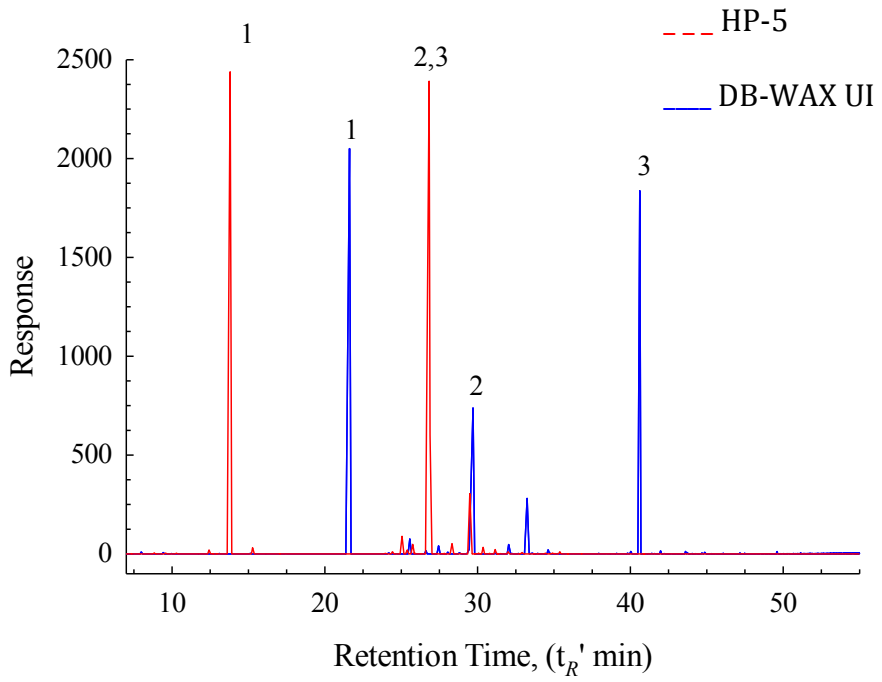

Figure 3.2: HP-5 and DB-WAX UI chromatograms of 'Amrita' essential oil. 1, methyl octanoate (IS); 2, β-carophyllene; 3, methyl eugenol

Table 3.6: Quantitative determination of holy basil essential oil expressed as mean percentage in the oil with standard deviation (n=12)

	t_R[a]	t_R[b]	LRI[c]	LR[d]	Amrita	Amrita[e]	Vana	Krishna	Rama	PI 288779	PI 652057
ethyl 3-methylbutanoate	3.0		854		–	–	–	–	–	–	–
β-pinene	6.5		979		–	–	–	–	–	–	–
eucalyptol	8.9		1032		–	–	–	–	–	–	–
(E)-β-ocimene	9.5		1049		–	–	6.72±3.36	–	–	–	–
β-ocimene	9.9		1037		–	–	–	–	–	–	–
linalool	12.4		1099		–	–	–	2.21±0.85	–	–	–
endo-borneal	15.3		1167		–	–	–	1.54±0.49	–	–	–
estragole	17.1		1196		–	–	–	–	–	–	–
eugenol	24.5		1357		–	–	66.50±7.29	42.03±16.33	41.24±13.31	45.23±19.98	37.48±13.52
α-copaene	25.1		1221		2.88±0.56	–	2.10±0.49	–	–	0.75±0.82	–
β-elemene	25.8		1391		1.52±0.24	–	–	2.48±0.51	6.93±1.50	6.09±1.69	6.84±1.31
methyl eugenol	26.8	29.7	1402	2013	72.79±8.69	64.71±4.04	–	–	–	–	–
β-caryophyllene	26.9	40.6	1419	1595	10.42±6.41	17.41±3.07	5.04±1.70	37.25±10.04	27.14±5.62	23.20±6.72	32.78±6.31
(E)-α-bergamotene	27.8		1435		–	–	–	–	–	–	–
α-humulene	28.3		1454		0.91±0.57	–	–	2.43±0.70	1.78±0.39	1.53±0.48	2.12±0.43
(E)-β-farnesene	28.8		1457		–	–	–	–	–	–	–
germacrene D	29.5		1481		7.41±0.92	–	9.03±4.16	–	1.61±1.42	2.86±0.70	–
8-isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene	30.4		1570		–	–	–	6.43±1.33	16.66±3.35	13.97±4.05	17.28±3.41
β-bisabolene	30.8		1509		–	–	–	–	–	–	–
(E)-α-bisabolene	31.9		1512		–	–	–	–	–	–	–
caryophyllene oxide	32.9		1581		–	–	2.21±3.12	3.32±3.26	1.39±1.59	3.06±2.89	1.17±1.3

Table 3.6: (Continued)

	t_R[a]	LRI[c]	Kapoor	PI 652059	PI 414203	PI 652056	PI 414205	PI 414201	PI 414202	PI 414204	Identification[f]
ethyl 3-methylbutanoate	3.0	854	2.67±1.36	3.46±1.30	2.16±0.55	2.88±1.12	2.56±0.81	2.37±0.92	2.61±1.20	2.77±1.05	LRI, GC-MS
β-pinene	6.5	979	1.46±0.41	1.55±0.40	1.39±0.38	1.19±0.78	1.38±0.38	1.03±0.71	1.19±0.44	1.24±0.65	LRI, GC-MS
eucalyptol	8.9	1032	12.63±2.69	13.93±3.24	13.65±4.26	13.33±2.97	12.79±2.95	12.54±4.05	12.40±3.32	14.08±3.33	LRI, GC-MS
(E)-β-ocimene	9.5	1049	–	–	–	–	–	–	–	–	CAS, LRI, GC-MS
β-ocimene	9.9	1037	1.97±1.45	1.62±1.11	2.34±1.87	1.90±1.31	2.31±1.67	2.16±1.81	2.15±1.76	1.48±1.53	LRI, GC-MS
linalool	12.4	1099	–	–	–	–	–	–	–	–	LRI, GC-MS
endo-borneal	15.3	1167	–	–	–	–	–	–	–	–	LRI, GC-MS
estragole	17.1	1196	13.97±2.29	13.42±1.64	13.50±3.56	13.52±2.00	13.47±2.86	13.18±3.84	13.17±3.94	13.57±3.45	LRI, GC-MS
eugenol	24.5	1357	21.77±11.1	20.55±11.34	23.46±16.85	20.25±9.32	23.75±10.05	23.97±13.58	14.8±9.67	19.62±12.98	CAS, LRI, GC-MS
α-copaene	25.1	1221	–	–	–	–	–	–	–	–	CAS, LRI, GC-MS
β-elemene	25.8	1391	–	–	–	–	–	–	–	–	LRI, GC-MS
methyl eugenol	26.8	1402	–	–	–	–	–	–	–	–	LRI, GC-MS
β-caryophyllene	26.9	1419	1.62±0.19	1.59±0.13	1.58±0.16	1.65±0.17	1.62±0.12	1.63±0.10	1.56±0.14	1.59±0.11	CAS, LRI, GC-MS
(E)-α-bergamotene	27.8	1435	2.58±0.57	2.63±0.47	2.42±0.60	2.62±0.44	2.52±0.43	2.55±0.51	2.49±0.54	2.61±0.49	CAS, LRI, GC-MS
α-humulene	28.3	1454	2.10±0.40	1.86±0.27	1.78±0.31	1.90±0.21	1.81±0.25	1.84±0.27	1.84±0.31	1.87±0.29	LRI, GC-MS
(E)-β-farnesene	28.8	1457	0.63±0.68	1.17±0.15	0.55±0.58	1.17±0.14	1.13±0.15	1.20±0.20	1.11±0.19	1.10±0.16	LRI, GC-MS
germacrene D	29.5	1481	1.22±0.38	1.09±0.87	1.18±0.89	1.40±0.91	1.35±0.88	1.43±0.92	1.30±0.86	1.16±0.80	LRI, GC-MS
8-isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene	30.4	1570	–	–	–	–	–	–	–	–	LRI, GC-MS
β-bisabolene	30.8	1509	19.58±4.81	20.08±4.53	18.63±5.82	20.67±4.06	18.27±3.82	19.27±4.52	19.35±4.74	20.18±5.04	LRI, GC-MS
(E)-α-bisabolene	31.9	1512	8.91±2.39	8.77±2.07	8.97±2.15	9.32±2.67	9.79±2.26	9.81±2.42	9.32±2.17	8.74±2.04	LRI, GC-MS
caryophyllene oxide	32.9	1581	0.81±0.89	1.03±0.69	0.97±1.05	0.78±0.89	0.75±0.88	0.82±0.88	0.88±0.96	0.99±1.05	LRI, GC-MS

[a]$t_{R,}$= retention time on the HP-5 column; [b]t_R = retention time on the DB-WAX UI column
[c]LRI=linear retention index, calculated relative to the C9-C23 n-alkanes on a non-polar column; [d]LRI = linear retention index calculated on a polar column
[e]Amrita, quantification of methyl eugenol and β-caryophyllene based on a DB-WAX UI column
[f]identification of essential oil compounds; LRI=linear retention index, GC-MS=gas chromatography-mass spectroscopy, CAS=commercially available standard

CHAPTER 4

CONCLUSIONS

There is a pressing need in the current healthcare model in the United States for alternative therapies that address issues of health promotion, disease prevention and support for chronic conditions. As an herb that reduces the negative effects of stress, supports healthy immune function, and normalizes blood sugar, holy basil is well suited to the needs of this country. Developing a better understanding of this plant is crucial for increasing awareness and acceptance of this herb in the US. A critical first step in growing the body of knowledge around botanicals like holy basil, is developing reference ranges for bioactive compounds, and screening the plants for productivity (Craker, Gardner, & Etter, 2003). Therefore, the purpose of this research is to understand the differences in holy basil varieties by their yield and the composition and yield of the essential oil fraction, and determine the best methods for extraction and analysis of the essential oil fraction of holy basil.

Field trials

In the midst of collective confusion, it is not well understood how to differentiate the varieties of holy basil and which ones should be grown to achieve maximum yield and quality. Based on the results of this study, there are indeed measurable differences among holy basil varieties based on yield of dry mass and essential oils. Though, the majority of the differences

resulted from the influence of growing season and harvest time, indicating that the environment can play as important a role as genetics.

Most of holy basil is grown in India, but as demand for holy basil in the US increases, and more strict regulations are passed down from the USDA and FDA regarding Good Manufacturing Practices (GMPs), there will be a need for domestically produced herbs that can be easily sourced and tracked to ensure safety (Smith, 2011). Unfortunately, there is very little yield or economic data on holy basil grown in the US, and more research is needed to determine market potential. However, with the right marketing, holy basil has great potential as a high-value crop that can be grown in the US and doesn't take up much space in the field.

This research was unique in the number of varieties of holy basil that were studied and how they were grouped by yield as well as their essential oil fractions. A study by Zheljazkov et. al. looked at holy basil and yield differences over various growing locations, however the variety is not specified so it isn't clear which holy basil they used for their research (Zheljazkov, Cantrell, Evans, Ebelhar, & Coker, 2008). Another study by Zheljazkov et. al. showed differences in harvests of holy basil, but again, they didn't specify the variety, making it difficult to compare results between the reports (Zheljazkov et al., 2008). Additionally, many studies have been done on holy basil in India, but they usually only include the two commonly cultivated varieties Rama and Krishna and it is difficult to make comparisons. Therefore, this research contributed to the body of knowledge around holy basil by showing a representative sample of holy basil varieties that are available domestically and their performance in terms of growth and essential oils in the southeastern US.

There are several things I would have done differently for my field trials. First, I would have taken data on germination rates. Germination rates are an important factor for farmers when

choosing a variety, and determining the amount of seed to purchase. Second, I would increase my plot size. Some papers require a minimum of 1mL of essential oil per sample when comparing essential oil content between varieties. Because of my small plot size, I didn't have enough plant material to extract that quantity of essential oil, and therefore became limited in where I was able to report this information. A larger plot size would have also facilitated a better yield estimate per acre.

There are numerous ways that this research could be continued to expand upon the results of this study. There was a clear effect of weather on the holy basil varieties. It would be beneficial to design an experiment investigating the effects of drought and sun exposure on growth and quality of holy basil. Another possibility is looking at the genetic differences in these holy basil varieties, and perform a breeding study to identify genetic strategies for increasing yields while maintaining or increasing the essential oils or other biologically active compounds in the plant.

Lab analysis

In terms of essential oil composition, we observed differences among the varieties. There have been studies produced in India that report on variability in the essential oil fraction of holy basil, but this is the first study, to our knowledge, to report on the essential oil composition of the varieties that are available and growth domestically. This study showed four different essential oil profiles among the 14 varieties. There was a variety composed primarily of methyl eugenol, and one composed primarily of eugenol. A group of varieties were composed mainly of eugenol and β -caryophyllene, and another group contained equally moderate amounts of eugenol, eucalyptol, estragole, and β-bisabolene.

This work contributed to the body of research on quantitative analysis of essential oils, building upon the findings of Cachet et. al. and using a combination of external standards and an internal standard with predicted RRFs for quantification of complex essential oil mixtures (Cachet et al., 2016). From the results of this study, dominant phytochemicals in the essential oil fraction exhibited a larger error than phytochemicals in small quantities. This is something that could be explored further in a future study. Also, this study demonstrated that methyl eugenol and β-caryopyllene co-elute on more non-polar capillary GC columns. Therefore, a more polar GC column is better for achieving full separation when an essential oil contains both of these phytochemicals.

For the scope of this study, only the essential oil fraction could be evaluated. However, as for future research, it would be helpful to evaluate other compounds in the holy basil varieties such as antioxidants or vitamin and mineral content. Since the biological action of holy basil is not well understood, it is likely that a combination of these substances contribute to the therapeutic potential of holy basil. Another study could evaluate the differences in water-soluble compounds between varieties and explore how those differences could impact biological activity. There are countless possibilities for research on holy basil that could serve to improve our understanding and facilitate increased quality and awareness of this important medicinal herb.

Putting all of this information together it was determined that the variety Kapoor is the most commonly available variety in the US and is the easiest to grow. Additionally, the majority of the USDA accessions were similar to this variety. The essential oil fractions contained ~20% each of eugenol, eucalyptol, estragole, and β-bisabolene. Even though it was not as high in essential oils as the other varieties and does not have high levels of eugenol; it's ease of growing, high yields and pleasant taste make it a good choice for farmers. The variety Amrita was high in

methyl eugenol with no detectable eugenol and was difficult to grow. The variety Vana was easy to grow and had the highest content of eugenol and grows wild in India. The varieties 'Rama' and 'Krishna' were high in eugenol and β-caryophyllene, two phytochemicals with high biological activity, and are the varieties traditionally cultivated in India and highly prized by herbalists. The only disadvantage to these varieties is that they are difficult to grow and had low yields in this study compared to others. Finally, the USDA accession PI 288779 was high in eugenol and β-caryophyllene but had superior yield of dry mass and essential oils making it a highly prized variety that grows well in the southeastern US. A future study could focus preparing this USDA accession to be released commercially.

Overall, growers should plant a collection of varieties so the strengths and weaknesses between them could be offset. To support this, it is becoming increasingly popular for herbalists and tea companies to serve a mixture of holy basil in their teas. Otherwise, the variety they grow is highly dependent on consumer taste, the needs of the buyer, and personal preference, and should be chosen accordingly.

Final thoughts

Personally, the knowledge I gained from working on an interdisciplinary project has been overwhelmingly valuable. I learned from being able to work with these plants on a physical level; experiencing how they grow in the field, learning about the best time to harvest, post harvest practices and what to consider when preparing samples for lab analysis. On the flip side, from the analytical perspective I learned about the importance of different quantification strategies, and testing for precision and accuracy. While at times overwhelming, the opportunity to work in two fields gave me perspective, and ultimately led to a more balanced and

comprehensive project. I also learned about the limitations of research. I chose the essential oil fraction of holy basil as a marker for medicinal quality, but the reality is that the action of holy basil comes from a synergistic interaction of many different phytochemicals that would take a lifetime to understand. I learned that trying to evaluate or measure a complex system is difficult and there are limitations to the conclusions I draw from my research. Finally, I learned that a good scientist is humble and curious. Always questioning how the world works and holding space for new ideas.

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