CURCUMINOID ANALYSIS OF HAWAII-GROWN TURMERIC (*Curcuma longa*) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND INVESTIGATION OF COLOR RELATIONSHIP WITH CURCUMINOID CONTENT FOR TOTAL CURCUMINOID ESTIMATION

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DEDICATION

I dedicate this thesis to the farmers, scientists, and workers of Hawaii who seek to improve the sustainability and value of Hawaii's agriculture.

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ABSTRACT

An HPLC method was designed to analyze turmeric (*Curcuma longa*) cultivars for curcuminoid contents curcumin, demethoxycurcumin and bis-demethoxycurcumin, and the sum of these were taken as total curcuminoids (**TC**). Of the Hawaii-grown cultivars, ‘Joy’ contained significantly more **TC** than all the others, with exception to ‘BKK’, a low yielding but extremely high **TC** cultivar. ‘Olena’ was the only cultivar to have less **TC** than what is commercially acceptable. Of the Asian cultivars analyzed, Indian cultivars 18-013, 18-014, and 18-010 had the highest **TC** content, comparable to ‘BKK’. Further propagation and in field trials will demonstrate their yield potential. The colors of the Hawaii-grown and Asian cultivars were measures using a standardized color chart to design a color grading chart. The yellow to orange relationship with **TC** content was used to produce approximate colors for different levels of curcuminoid content. These results and future experiments will help guide Hawaii agriculture towards producing high quality and globally competitive turmeric rhizomes.

TABLE OF CONTENTS

Acknowledgements iv
Abstract v
List of Tables viii
List of Figures ix
List of Abbreviations x
Chapter 1 Literature Review 1
1.1 Introduction 1
1.2 History of Turmeric 2
1.3 Modern Global Usage of Turmeric 2
1.4 Turmeric Agronomy 2
1.4.1 General Agronomy 3
1.4.2 Turmeric in Hawaii Agriculture 3
1.5 Curcumin and Curcuminoids 3
1.5.1 Overview 3
1.5.2 Biosynthesis 3
1.5.3 Chemical Properties 4
1.5.4 Medicinal Studies 4
1.5.5 Drug Design Studies 5
1.6 Hypothesis and Objectives 6
1.6.1 Hypothesis 7
1.6.2 Curcuminoid Analysis by HPLC 7
1.6.3 Curcuminoid Estimation by Color 7
References 8
Figures 12
Chapter 2 Quantification of curcuminoids in Hawaii-Grown Turmeric (*Curcuma longa*) by HPLC 15
2.1 Abstract 15
2.2 Introduction 15
2.3 Experimental 16
2.3.1 Reagents and Standards 16
2.3.2 Solvent Preparation 16
2.3.3 Turmeric Samples 16
2.3.4 Curcuminoid Standards 17
2.3.5 HPLC System and Analysis Conditions 17
2.4 Results and Discussion 17
2.5 Conclusion 19
References 20
Tables 22
Figures 24

Chapter 3 Color Chart for Total Curcuminoid Estimation 26
3.1 Abstract 26
3.2 Introduction 26
3.3 Experimental 26
3.3.1 Turmeric samples 27
3.3.2 HPLC Analysis 27
3.3.3 RHS Color Determination 27
3.3.4 Development of Color Grade Chart 27
3.4 Results and Discussion 28
3.4.1 HPLC of Asian turmeric cultivars 28
3.4.2 RHS Color Determination and Color Grade Chart 28
3.5 Conclusion 28
References 30
Tables 31
Figures 32
Chapter 4 Discussion 34
4.1 Quantification of Curcuminoids in Fresh Turmeric Rhizomes by HPLC 34
4.2 HPLC of Asian turmeric cultivars and Development of Color Chart for Curcuminoid Estimation 35
Chapter 5 Future Works 36
Appendix A: SOP for Curcuminoid Analysis of Fresh Turmeric by HPLC 37
Appendix B: Exemplary Chromatograms for Curcuminoid HPLC Analysis 41
Bibliography 43

LIST OF TABLES

Table 2.1 System suitability for curcuminoid quantification 22

Table 2.2 Linear regression parameters for curcuminoid standards 23

Table 2.3 Curcuminoid content in Hawaii grown turmeric cultivars 23

Table 2.4 Relative curcuminoid abundance in *C. longa* cultivars 24

Table 3.1 Total curcuminoid content, RHS Color, and origin of Asian turmeric cultivars 31

Table 3.2 Linear regression parameters for L*, a*, and b* vs total curcuminoids 31

LIST OF FIGURES

Figure 1.1 Structure of curcumin, demethoxycurcumin, and bis-demethoxycurcumin 12

Figure 1.2 Number of publications since 2000, Keywords: “Turmeric” and “Curcumin” 13

Figure 2.1 Rhizome and cross-section of *C. longa* cultivars 24

Figure 2.2 Average curcuminoid content of *C. longa* cultivars grown in three iterations 25

Figure 3.1 Pictorial representation of rhizome color in turmeric cultivars analyzed 33

Figure 3.2 Proposed color grade chart for total curcuminoid estimation 33

LIST OF ABBREVIATIONS

HPLC	High performance liquid chromatography
DMC	Demethoxycurcumin
BDMC	Bis-demethoxycurcumin
TC	Total Curcuminoids
RHS	Royal Horticultural Society

CHAPTER 1 LITERATURE REVIEW

1.1 INTRODUCTION

Turmeric (*Curcuma longa*) is a tropical plant utilized globally in food, textiles, religion, and medicine. It is particularly essential to Indian culture, where it has been used extensively in curries, herbal remedies, cosmetics, and ceremonies. Its origins are unclear, but turmeric has been cultivated in tropical regions of Asia and throughout the Pacific for thousands of years. Today, turmeric plays a vital role in Indian agriculture, where most of the world's turmeric is produced. Turmeric has also become a popular "superfood" in the U.S., an ingredient popularized for its potential health benefits. It has found its way into food products marketed toward more health-conscious consumers, such as snacks, smoothies, and dietary supplements. Furthermore, turmeric is often treated as a panacea amongst many alternative-medicine communities.

Ongoing research into turmeric's medicinal potential point to its diarylheptanoid secondary metabolites referred to as curcuminoids. The main curcuminoids found in turmeric are curcumin, demethoxycurcumin, and bis-demethoxycurcumin. These compounds, and especially curcumin, have demonstrated broad-range therapeutic activity. These benefits include effects against chronic illnesses, such as arthritis, Alzheimer's dementia, and various cancers (see section 1.5.4). However, the design of drug therapies is using turmeric, and curcuminoids are still in its infancy. This deficiency reflects curcuminoids, having demonstrated poor pharmacokinetics, including poor absorption, rapid metabolism, and high excretion. Various drug-design technologies are being explored to help overcome these challenges, such as emulsion and crystallization formulations. Turmeric also contains several terpenoids and oligosaccharides that have demonstrated medicinal potential as well.

The global demand for turmeric has increased significantly in recent years. Hawaii has begun cultivating turmeric at high volume, recently surpassing a previous major crop of Hawaii agriculture, ginger (see section 1.4.2). Shifting from ginger to turmeric cultivation is not difficult for farmers because horticultural practices between turmeric and ginger are conveniently similar, and the value of turmeric has far exceeded ginger. Furthermore, ginger has recently met competition by imports, driving the cost of Hawaii ginger lower. A primary impedance for turmeric cultivation in Hawaii is the standardization of its curcuminoid content. Even the curcuminoid content of Hawaii's industry-standard cultivar 'Hawaiian Red' is mostly unknown. While ginger is cultivated for culinary use and is judged visually and by flavor, turmeric must meet the higher quality standards of pharmaceutical companies. Various analytical techniques can quantify curcuminoids. However, high-performance liquid chromatography (HPLC) is the gold standard of analysis in agriculture and pharmaceuticals (see section 1.6.2). Therefore, an HPLC method has been designed to analyze curcuminoids in turmeric rhizomes to facilitate analysis of Hawaii grown turmeric. In

this study, turmeric cultivated in Waimanalo, Oahu, and Kula, Maui, were analyzed. Additionally, the method was used to analyze cultivars collected from Singapore, India, Myanmar, and Thailand.

HPLC is useful for obtaining accurate results but becomes costly as sample sizes increase. A color grade chart is proposed to help supplement turmeric quality control. Curcuminoids are yellow to orange in solution and red in solid form. Higher curcuminoid cultivars are darker orange, while lighter yellow cultivars contain very little curcuminoids. Using this relationship and a standardized color chart, a color grade chart was designed for estimating curcuminoid content in fresh turmeric. This chart will help rapidly distinguish low and high curcuminoid turmeric and possibly prevent product mislabeling.

1.2 HISTORY OF TURMERIC

Turmeric has been cultivated in tropical Asia for thousands of years. Although turmeric is widely believed to have been domesticated in India, both ethnobotanical and taxonomical studies point to Southeast Asia as the likely region of its progenitor (Veluyadhan et al., 2014; Skornickova et al., 2007). Nonetheless, turmeric finds itself extensively used in Indian culture. A significant system of folk medicine in India known as Ayurveda holds turmeric in high regard, suggesting numerous benefits: aiding digestion, brightening skin, antiseptic, antifungal, and antiparasitic effects (Sasikumar, 2005). Turmeric was also cultivated historically throughout China, Korea, and Japan for herbal teas and remedies. From Southeast Asia, ancient sea voyagers cultivated turmeric and other essential crops throughout the Pacific Islands, all the way to Hawaii. Native Hawaiians used a variety of gingers for ornamental, ceremonial, and medicinal use. They used turmeric ('Olena in Hawaiian) as a yellow textile dye and medicine to treat wounds (Abbott, 1992).

1.3 MODERN GLOBAL USAGE OF TURMERIC

Turmeric (*Curcuma longa*) was first taxonomically classified by the naturalist Linnaeus in 1753 (Linnaeus, 1753). Its genus *Curcuma* contains 100+ species, which has undergone numerous restructures due to its ambiguous nomenclature (Záveská et al., 2016). Turmeric is used globally as a flavoring and coloring agent in various foods and cosmetics. In the U.S., turmeric was granted Generally Recognized as Safe (GRAS) status in powder, oleoresin, and essential oil form (Food and Drug Administration [FDA], 2020a; FDA, 2020b). It is most famously included as a primary component of curry mixes but is also a natural yellow coloring additive in many foods (Ravindran et al., 2007). Turmeric has become increasingly popular in the U.S. as a superfood and is an additive in "health-conscious" marketed foods (Wagoner, 2015). Turmeric contains secondary metabolites that have demonstrated medicinal potential, namely curcuminoids. These compounds have been the focus of therapeutic research and are extracted for use in pharmaceutical supplements. Curcumin-based pharmaceuticals have become a lucrative product, valued at 58.4 million dollars in 2019, and comprising more than half of curcumin's global market share in the overview of Grand View Research's curcumin forecast insights (Grand View Research, 2020).

1.4 TURMERIC AGRONOMY

1.4.1 General Agronomy

Turmeric is a tropical perennial cultivated as an annual crop. It is propagated asexually through the division of its rhizomes and is planted in the summer before or during the summer rainy season, typically in a hill, ridge, or raised bed to help drainage and assist with harvesting. The mother (central bulbous rhizome) is generally reserved as a seed. In contrast, fingers (primary and secondary rhizomes branching from the mother) are used as both seed and sellable material (Ravindran et al., 2007). However, it has been shown that mothers weighing 40-50 g are the most optimal planting material, producing a higher overall yield (Angami et al., 2017). A planting depth of 8-12 cm is recommended for high yield and lower weed competition (Ishimine et al., 2003). Turmeric can also be started in a greenhouse and transplanted out, but the overall benefits to this method are disputed. Turmeric is known as heavy feeding crop, preferentially up-taking potassium, and nitrogen over phosphorus (Nair, P. K., 2013). It was even demonstrated that excess phosphorus could be detrimental to both yield and curcuminoid content (Akamine et al., 2007). A slow-release fertilizer applied multiple times over the growing season is recommended. Its growth season spans 6-9 months long and is harvested in the spring (Mitchell and Green, 2014). Rhizomes harvested earlier are lower yield but suitable for curcuminoid extraction, while those harvested later are for maximum yield due to the late-stage accumulation of starch and fiber (Nair, P. K., 2013; Chavalittumrong and Jurawattanapong, 1992).

1.4.2 Turmeric in Hawaii Agriculture

Turmeric has only recently become a significant agricultural crop of Hawaii. It was first included in the USDA NASS 2016 Hawaii Tropical Fruit and Crops Report with the farmgate value of $660,000. In the following 2018 survey, its farmgate value nearly doubled to $1,210,000 (USDA, 2017; USDA 2020). Major commercial turmeric cultivation occurs on the islands of Kauai, Oahu, Maui, and Hawaii. The main cultivars found are 'BKK' – a low yielding oily orange rhizome, 'Hawaiian Red' – a higher-yielding orange rhizome, and 'Olena' – a high yielding yellow rhizome. Of these, 'Hawaiian Red' is considered Hawaii's industry standard for its favorable color, yield, and flavor. Ginger (*Zingiber officinale*) is another major crop of Hawaii that peaked in value at $5,400,000 in 2004. Since then, production and value have declined to $329,000 in 2018 due to increasing competition from China and India (Morita, 2007; USDA, 2020). Horticultural practices between ginger and turmeric are somewhat similar, considering that they are both rhizomatous plants cultivated as annual root crops. The decline of ginger and converse rise of turmeric likely indicates that farmers switched from ginger cultivation to turmeric in favor of higher crop value.

1.5 CURCUMIN AND CURCUMINOIDS

1.5.1 Overview

Curcumin is a diarylheptanoid compound which accumulates as a yellow-orange pigment in the rhizomes of the turmeric plant. It was first identified in the 1800s by Vogel and Pelletier, and later, its chemical structure was elucidated in 1910 as diferuloylmethane (Gupta et al., 2012; Milobedzka et al., 1910). It and

its congeners demethoxycurcumin (**DMC**) and bis-demethoxycurcumin (**BDMC**) are altogether referred to as curcuminoids. Turmeric contains about 1-10% total curcuminoids (**TC**) dry weight. Notably, about 100 other curcuminoid-like compounds have been identified in turmeric and other *Curcuma* species, but their contents are so traced that they are often overlooked in curcuminoid-based studies (Sun et al., 2017). Scientific interest in turmeric and curcuminoids has grown exponentially over the past two decades, as shown in figure 1.2. Most of these studies focus on its medicinal and drug design aspects. Overall, the curcuminoid content produced is measured as a marker for the therapeutic value of the turmeric cultivated.

1.5.2 Biosynthesis

The biosynthetic pathway discussed is shown in figure 1.3 (Ramirez et al., 2006; Katsuyama et al., 2009). Curcuminoids are derived from two phenylpropanoid pathway compounds linked as a diketide with central methylene derived from malonyl CoA. First, intermediate compounds *p*-coumaroyl CoA (**A**) and feruloyl CoA (**B**) are formed from phenylalanine catalyzed by enzymes common to the phenylpropanoid pathway. Then, these two are combined to form curcuminoids in two steps: the first formation of a diketide of intermediates **A** or **B** by reaction with malonyl CoA, then secondly the diketide reacts with another of **A** or **B** to form curcumin [**B**+**B**], **DMC** [**A**+**B**], and **BDMC** [**A**+**A**].

The last two steps are mediated by polyketidesynthase enzymes identified recently as DCS and CURS. Before these enzymes were isolated, it was previously believed that polyketidesynthase enzymes would form **BDMC** as a scaffold for methoxylation to **DMC** and curcumin. However, upon isolation, DCS and CURS demonstrated a preference for **B** over **A**, implying that methoxylation of **BDMC**, who is formed from two **A** molecules, is not a significant pathway for **DMC** and curcumin. Since curcumin is synthesized from two **B** molecules, the preference for **B** explains why curcuminoids are naturally abundant as curcumin>**DMC**>**BDMC**.

1.5.3 Chemical Properties

Curcuminoids, shown in figure 1.1, are diarylheptanoids: two aryl rings connected by a 7-carbon chain. The three main curcuminoids differ only by the absence of methoxyl groups on **DMC** and **BDMC**, hence their name prefixes "demethoxy-"and "bis-demethoxy-." The aryl rings contain a hydroxyl group which is easily deprotonated in alkaline solution or by radical oxidation (Minassi et al., 2013). They also contain a beta-diketone moiety at the center of the 7-carbon chain, which participates in tautomerization using their shared alpha-carbon (Yanagisawa et al., 2010). Enol tautomers in beta-diketone systems are typically favored because of the strong hydrogen bond formed with the carbonyls, and the conjugated 6-membered ring resonance formed (Belova et al., 2010). The beta-diketone moiety allows curcuminoids to chelate with metal ions (Messner et al., 2017). Both tautomers also participate in Michael addition reactions due to their adjacent double bonds. Altogether, these structural features make curcuminoids highly reactive molecules.

Over a hundred other diarylheptanoid compounds have been identified in *Curcuma longa* and other *Curcuma* species as well. These include reduced forms di-, tetra-, and hexa-hydrocurcuminoids, intracyclized forms, so-called cyclocurcuminoids, and other further derived forms (Li et al., 2009; Sun et al., 2017). There are also curcuminoids where the phenolic hydroxyl groups are substituted with a carbohydrate or terpenoid groups. Many of these are trace, and it is unsure whether they are naturally present or degradation products from sample preparation (Typek et al., 2019b).

Curcuminoids are soluble in organic solvents glacial acetic acid, acetone, carbon dioxide, ethyl acetate, dichloromethane, n-butanol, methanol, ethanol, and hexane (National Center for Biotechnology Information, 2020; FAO, 2004). In aqueous solution, curcuminoids are insoluble, only becoming temporarily soluble in an alkaline solution before rapid degradation (Gordon et al., 2015; Kharat et al., 2017). Improving their poor solubility in aqueous systems is a primary focus of drug design research.

1.5.4 Medicinal Studies

Curcuminoids have shown many different potential bioactivities. Over 11,000 medical related articles are currently published on curcumin (WebofSci keyword search: "curcumin"), demonstrating by volume that curcuminoids are a popular topic of medicinal research. Exhaustively reviewing therapeutic research is a challenge. Of these, research on the effects of curcuminoids on Alzheimer's disease and arthritis will be discussed.

Alzheimer's is a neurodegenerative disease whose exact causes are not fully understood. Still, it is widely hypothesized that pathogenesis is either caused by or worsened by the accumulation of misfolded amyloid plaques in the brain (Ballard et al., 2011). Curcuminoids have demonstrated the potential to prevent and treat amyloid plaque by several routes. First, amyloid aggregation can be induced by an imbalance of free metal ions. These ions can be chelated by curcuminoids, thereby ameliorating its effects (Baum and Ng, 2004; Messner et al., 2017). Secondly, amyloid misfolding and plaque formation can be caused by toxigenic amyloid peptides. Curcuminoids have shown binding affinities for amyloid peptides, prevent amyloid accumulation (Yang et al., 2004; Randino et al., 2016). Lastly, curcuminoids increase both amyloid uptake and phagocytotic degradation, possibly leading to the "brain clearing" of amyloid plaques (Zhang et al., 2006). Altogether, these activities show that curcuminoids can potentially be an effective therapeutic in treating Alzheimer's disease.

Arthritis is a joint disorder that causes chronic joint inflammation, as well as degeneration in some cases. The exact cause of arthritis is unknown, so treating arthritis is limited to reducing pain and inflammation with NSAIDs and lifestyle changes to reduce joint stress. However, long-term use of NSAIDs can lead to severe gastrointestinal and cardiovascular issues (Daily et al., 2016). Curcuminoids have shown anti-arthritic potential by inhibiting inflammatory response pathways and mediators such as NF-kB and COX-2 (Ghosh et al., 2015; Kunnumakkara et al., 2017). Excitingly, recent clinical studies have suggested that

curcumin is effective in treating the pain and swelling caused by knee osteoarthritis (Panahi et al., 2015; Daily et al., 2016).

Curcumin and curcuminoids are highly researched in medicinal studies because they have shown "hits" in many bioactivity assays. The FDA has granted turmeric, its extracts, and pure curcumin GRAS-status, further encouraging researchers and pharmaceutical companies to study turmeric and curcumin (FDA, 2020a; FDA 2020b). Research on the medicinal potential of turmeric was initially brought on by anecdotal evidence, citing its prevalence in many different folk medicine systems. However, to date, no double-blind clinical studies have demonstrated reliable therapeutic results in the author's knowledge. Even those employing highly purified curcuminoids have not shown any benefits. Because of this disparity, many researchers have become critical of curcuminoids in medicinal research, grouping it with other false positive "pan-assay interference" molecules (Nelson et al., 2017). The most significant criticism being that curcuminoids do not fit the traditional model for drug-design: it is poorly soluble, highly reactive, shows low-absorption, and degrades rapidly.

To make matters worse, a prominent cancer researcher and pioneer curcuminoids researcher had numerous publications pulled for academic misconduct (Singh Chawla, 2016). Even with these criticisms, many diseases exist that still have not been cured with our current model of "single-drug-single-target" drug discovery. Currently, more research is needed for curcuminoids to elevate to a strong therapeutic potential, but whether it is worth pursuing, it is a matter of perspective.

1.5.5 Drug Design Studies

The simplest and preferred delivery system is as a solid oral tablet consisting of purified crystal drug. However, many drug candidates do not fit this model. A significant difficulty with drug design is solubility. It is estimated that 40% of current drugs are water-insoluble, and 70 to 90% of drug candidates are also insoluble (Thayer, 2010). Curcuminoids have especially challenging physicochemical and reactive properties: not only are they dismally water-insoluble, but they also degrade in light and biological pH, react with alcohol and can bind covalently as a Michael acceptor. Nonetheless, drug formulations to overcome these issues.

In solid delivery systems, curcuminoid solubility is addressed through crystal engineering—modifying either its purified crystal packing structure or doping them with other compounds to form multicomponent systems (Sanphui and Bolla, 2018). The main goal is to produce stable curcuminoid crystals with less intermolecular force to help improve dissolution. Curcumin extracted through classical recrystallization means dissolving in acetone and evaporating. It is the most commonly available and stable polymorph, exhibiting the usual insolubility observed due to its strong intermolecular hydrogen bonding. Two other polymorphs and an amorphous state have been reported. One of the new polymorphs showed three times higher dissolution rates that the other states (Sanphui et al., 2011). Multicomponent systems utilize concomitant compounds to form cocrystals or eutectics with different dissolution and solvation properties.

Successful curcumin cocrystals were formed by recrystallization with phenolic compounds such as resorcinol, pyrogallol, and piperidine (Sanphui and Bolla, 2018). Eutectic mixtures are similar to cocrystals but are much more disordered (Stoler and Warner, 2015). A eutectic mixture of curcumin with nicotinamide at 1:2 exhibited the best dissolution rate in 40% aqueous ethanol of nearly ten times that of curcumin (Gouda et al., 2012).

In liquid delivery systems, curcuminoids can solubilize through emulsions or complexation with macromolecules (Kharat and McClements, 2017; Gupta et al., 2011). Emulsions are stable mixtures of immiscible solvents. In the food and pharmaceutical industry, these are typical of oil and water. Curcuminoids were previously shown capable of forming stable oil in water emulsions with sodium caseinate as an emulsifier (Kharat and McClements, 2017; Kharat et al., 2018) Macromolecules improve solubility by encapsulating curcuminoids and modifying solvent viscosity, preventing curcuminoid recrystallization. Cyclodextrin, modified celluloses, and whey proteins are macromolecules that have shown improved curcumin solubility (Tønnesen et al., 2002; Fan et al., 2018; Mohammadian et al., 2019).

The delivery systems discussed utilize non-covalent interactions. Covalently linking curcuminoids to other compounds, such as nucleotides, peptides, or other structural modifications, is possible and would achieve new physicochemical properties. It would be akin to starting with a new molecule altogether, requiring safety studies and FDA approval.

1.6 HYPOTHESIS AND OBJECTIVES

1.6.1 Hypothesis

Differences in the main quality characteristic of turmeric cultivars, curcuminoid content, can accurately be measured by HPLC analysis. Furthermore, since curcuminoids are orange pigments, dark-orange cultivars should have a predictably higher curcuminoid content, allowing for color-based curcuminoid estimation.

1.6.2 Curcuminoid Analysis by HPLC

This study's first objective is to design an HPLC method for quantifying curcuminoids in fresh turmeric rhizomes so that Hawaii's commercial cultivars can be assessed. This will be achieved by utilizing an isocratic 60:40 2% aqueous acetic acid: acetonitrile with a C18 Kinetex column, a system that has previously shown success for plant metabolites (previous work of Bingham lab; Wichitnithad et al., 2018; Typek et al., 2019a). The Hawaii cultivars analyzed will be grown in 3 iterations to confidently identify the differences in curcuminoids and, thus, quality, between local cultivars.

1.6.3 Curcuminoid Estimation by Color

The second objective of this study is to quantify the correlation between color and curcuminoid content to design a curcuminoid estimating color chart. It was previously demonstrated that there is a robust colorimetric relationship between rhizome color and curcuminoid content (Pal et al., 2020). However, the

colorimeter used did not record colors that were representative of the actual rhizomes. In this study, a color chart will be used to identify realistic colors that can then be correlated to curcuminoid content to design a curcuminoid estimating chart.

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FIGURES

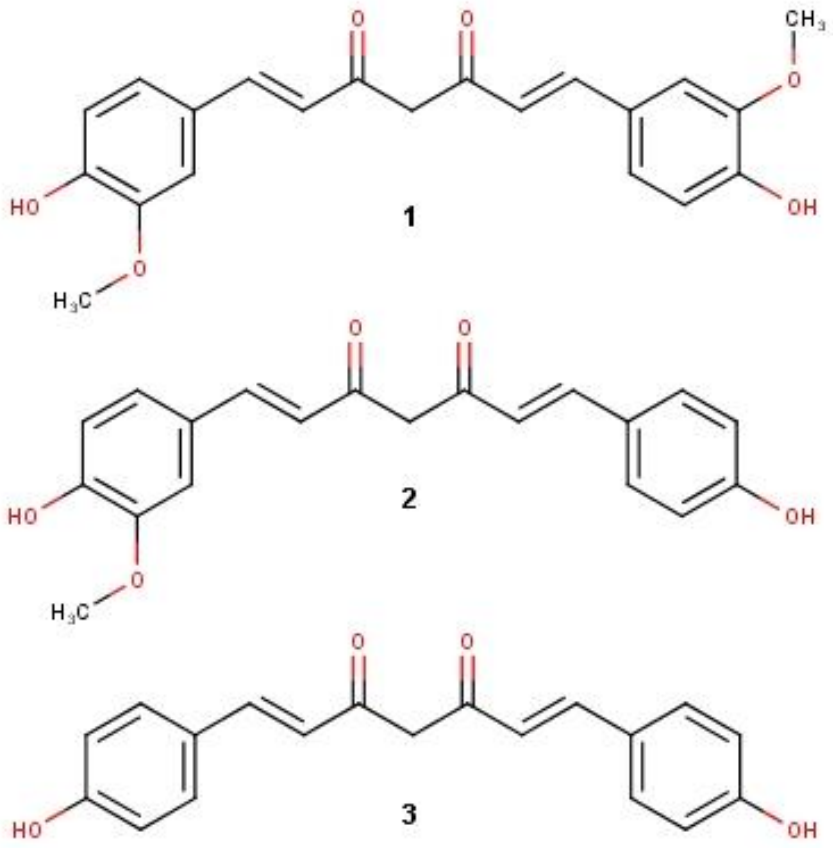

Figure 1.1 Structure of curcumin (**1**), demethoxycurcumin (**2**), and bis-demethoxycurcumin (**3**).

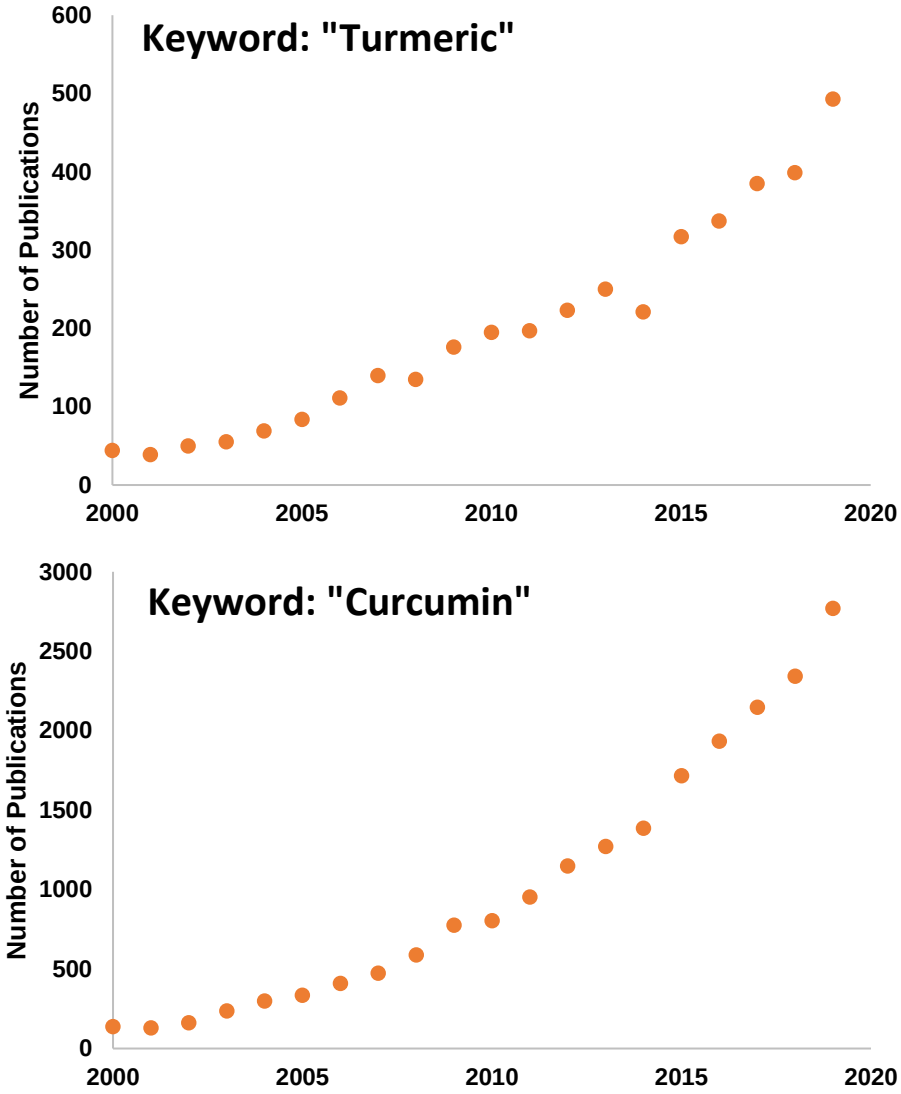

Figure 1.2 Number of publications since 2000 via Web of Science, Keywords: “Turmeric” and “Curcumin”, accessed July 8, 2020. Year 2020 excluded.

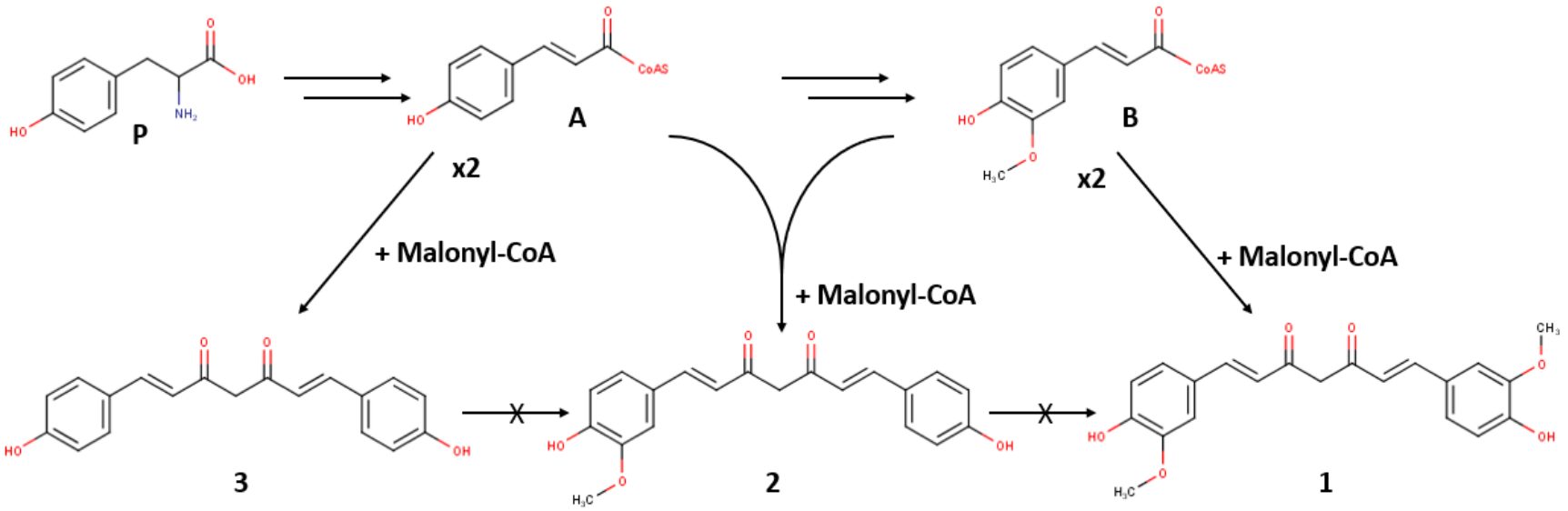

Figure 1.3 Biosynthetic pathway for curcuminoids. Phenylalanine (**P**) is converted to phenylpropanoids *p*-coumaroyl CoA (**A**) and feruloyl CoA (**B**), which are then combined with eachother and a malonyl-CoA to form curcumin [**B**+**B**] (**1**), demethoxycurcumin [**A**+**B**] (**2**), and bis-demethoxycurcumin [**A**+**A**] (**3**). Methoxylation of **3** to form **2** and **1** was demonstrated to not be a major synthetic pathway. Adapted from Ramirez et al., 2006 and Katsuyama et al., 2009.

CHAPTER 2 QUANTIFICATION OF CURCUMINOIDS IN HAWAII-GROWN TURMERIC (*Curcuma longa*) BY HPLC

2.1 ABSTRACT

An HPLC method was designed to analyze Hawaii-grown turmeric (*Curcuma longa*) cultivars for curcuminoid contents curcumin, demethoxycurcumin (**DMC**) and bis-demethoxycurcumin (**BDMC**) and the sum of these were taken as total curcuminoids (**TC**). Five cultivars 'BKK', 'Joy', 'Mystical', 'Hawaiian Red', and 'Olena' were grown in 3 iterations on Oahu (Waimanalo Research Station, 2018 and 2019) and Maui (Maui Agricultural Research Center, 2019). The results showed a wide range of **TC** between cultivars (1.084 – 10.962% w/w dw). Across all 3 iterations, 'BKK' had the highest **TC** (9.531±0.125%) while 'Olena' had the lowest (1.271±0.052%). 'BKK', although high in **TC**, suffers low yields and is therefore not commercially viable. Conversely, 'Olena' is high yielding but suffers low **TC**. 'Joy' was significantly higher in **TC** that 'Hawaiian Red', Hawaii's standard industry cultivar (6.504±0.079 and 5.896±0.178, respectively). 'Caribbean' was a cultivar present in the Waimanalo 2018 and 2019 iterations and demonstrated high variability in **TC** content (27.9% increase from 2018 to 2019). 'Roma', a cultivar that was present only in the Maui 2019 iteration, showed a high **TC** content as well, being statistically similar to 'Joy'. 'Joy' and potentially 'Roma' are good candidates for production as a high-curcuminoid cultivars.

2.2 INTRODUCTION

Turmeric (*Curcuma longa*) is a globally consumed spice and medicinal herb that has grown in demand in recent years. India, the major exporter of turmeric, exported 133600 tonnes valued at 204 million USD in the financial year 2018-2019, up by 55.3% in mass and 67.6% in value from 2014-2015 (Spices Board of India, 2019). The demand for turmeric follows numerous scientific publications on the potential medicinal effects of turmeric and its constituents, leading to the development of various turmeric-based supplements and nutraceuticals (Priyadarsini, 2014). Due to the increased global interest in this spice, Hawaii has also become a recent turmeric producer, producing 220,000 lbs and allotting 40 acres of land to its cultivation in 2018 (USDA, 2020). Much of this land was previously allocated to ginger (*Zingiber officinale*), a major crop of Hawaii agriculture (Morita, 2007). Compared to ginger, turmeric has demonstrated higher resistances to tropical diseases and higher profitability with the recent boost in demand (K P Prabhakaran Nair, 2013). However, a major hurdle remains for turmeric in Hawaii agriculture, which is to determine the content of the medicinal pigments present in turmeric rhizomes called curcuminoids.

Curcumin, demethoxycurcumin (**DMC**), and bis-demethoxycurcumin (**BDMC**) are phenolic secondary metabolites unique to *Curcuma* species (*fig. 1.1*). Although other curcumin-like compounds naturally

exist, they are trace and the sum of the main three compounds are in literature taken as total curcuminoids (**TC**) (Sun et al., 2017; Jayaprakasha et al., 2002; Rafi et al., 2015; Cao et al., 2014). Curcuminoids have demonstrated a broad range of potential medicinal benefits including preventative effects against the development of chronic illnesses such as Alzheimer's, arthritis, and various cancers (Priyadarsini, 2014; Anand et al., 2008; Yang et al., 2005; Ghosh et al., 2015; Bashang and Tamma, 2019). Most studies focus either on individual curcumin, the highest produced of the curcuminoids, or on **TC**. Confusingly, "curcumin" is a term also taken to describe to total pigment extracted from turmeric, or essentially **TC** (BIS, 2010; FAO, 2004). Therefore, the differences in bioactivity between individual curcuminoid is poorly understood (Minassi et al., 2013; Gordon et al., 2015; Nelson et al., 2017).

TC content can vary between 1-10% w/w dry weight depending on genetics, horticultural practices, and environment, but industry needs are mostly limited to medium and high curcumin cultivars (3.5 – 5.0% curcumin) (Ravindran et al., 2007). Thus, cultivars producing in and above this range should be considered for commercial farming. The curcuminoid content of common commercial turmeric cultivars used in Hawaii remain to be determined. An HPLC method has been developed to quantify the content of each curcuminoid present in fresh turmeric rhizomes. This method has been tested on different cultivars of turmeric grown in three iterations across Hawaii and will be useful in the future for the quality control of Hawaii's turmeric crops.

2.3 EXPERIMENTAL

2.3.1 Reagents and Standards

Glacial acetic acid (99.7% w/w) and HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). P-grade standards of curcumin, demethoxycurcumin, and bis-demethoxycurcumin were purchased from Chromadex (Los Angeles, CA, USA). Ultrapure water was prepared inhouse using a Barnstead Nanopure Infinity ultrapure water system.

2.3.2 Solvent Preparation

A 2 % v/v aqueous solution of acetic acid was prepared from glacial acetic acid and purified water. The 2% v/v aqueous acetic acid and acetonitrile were degassed separately by filtering through a 0.45-µm nylon membrane attached to the vacuum freeze dryer (Alpha 2-4 LDplus, Christ, Germany). The solvents were combined at 60:40 2% v/v aqueous acetic acid: acetonitrile (**60:40**).

2.3.3 Turmeric Samples

Turmeric (*Curcuma longa*) varieties were cultivated over a single growth season (June/July – March/April) and harvested in 3 iterations over 2 locations: Waimanalo Research Station, Oahu County, HI in 2018 and 2019, and Maui Agricultural Research Center, Maui County, HI in 2019. In the Waimanalo 2018 and Maui 2019 iterations, turmeric was planted in single row plantings with 1 foot spacing within rows and 5 feet between rows. In the Waimanalo 2019 iteration, turmeric was planted in raised beds which contained

3 rows spaced 30 cm by 30 cm with 4 feet between their edges to favor mechanical harvesting. Although seven cultivars were analyzed in total, only five were retained across all three iterations, shown in fig. 2.2.

Fresh rhizomes were processed for extraction promptly after harvesting: 4 g of 1 mm thick cross-sectional discs were cut from each sample and freeze dried until completely dry (24-36 hours). The dried rhizomes were then crushed to a fine powder by mortar and pestle. The powder was extracted with **60:40** at 5 mg powder/mL solvent in a 2-mL microfuge tube. The mixture was sonicated (Branson 1210 Ultrasonic Cleaner) for 5 min and centrifuged (Sorvall MC 12V) at 13 krpm for 3 min. The supernatant was transferred to a clean 2-mL microfuge tube and sonication and centrifugation was repeated. A 100-µL aliquot of the resulting supernatant was transferred a 1-mL brown vial fitted with a glass HPLC insert.

2.3.4 Curcuminoid Standards

Standards were prepared by the mass difference method as suggested by the manufacturer. In summary, each standard was emptied into a separate container, followed by multiple rinses with **60:40** to ensure complete transfer. The loss between the filled and emptied vial was then used as the mass of standard. This mass divided by the total solvent required to solvate the standard gave the concentration of the standard stock (25-35 mL **60:40** per 5 mg standard). Standards were then serial diluted to approximately 0.05 mg/mL and analyzed in triplicate at 10, 20, 30, 40, and 50 µL.

2.3.5 HPLC System and Analysis Conditions

HPLC analysis was performed using a Water 2695 Separations Module fitted with a Phenomenex Kinetex C_{18} column (150 x 4.6 mm i.d.; 5 µm; Phenomenex Inc, Torrance, CA, USA). A Waters 996 Photodiode Array Detector was used for detection at 450 nm.

Samples were analyzed in 5-µL injections. An isocratic flow of 60:40 2% v/v aqueous acetic acid: acetonitrile at 1.8 mL/min was used for separation. Curcuminoids were separated and detected between 5 and 7 minutes: bis-demethoxycurcumin at 5.819 ± 0.040 min, demethoxycurcumin at 6.354 ± 0.040 min, and curcumin at 6.928 ± 0.039 min. Total runtime is 10 min, including a 2 min delay between injections. Suitability and regression parameters are shown in Tables 2.1 and 2.2.

2.4 RESULTS AND DISCUSSION

Turmeric cultivars were analyzed in 3 iterations over 2 locations: Waimanalo Research Station in 2018 and 2019 and Maui Agricultural Research Station in 2019. The content of the individual curcuminoids curcumin, demethoxycurcumin (**DMC**), and bis-demethoxycurcumin (**BDMC**) as well as their sum presented as total curcuminoids (**TC**) were determined for each cultivar by HPLC and the results are shown in Table 2.3. **TC** is the main quality characteristic measured commercially and is discussed primarily.

In the Waimanalo 2018 iteration, six *Curcuma longa* cultivars were analyzed in this iteration: 'BKK', 'Hawaiian Red', 'Caribbean', 'Joy', 'Mystical', and 'Olena'. 'BKK' had the highest **TC** content of 10.023±0.023% w/w dw, while 'Olena' had the lowest at 1.584±0.099% w/w dw. Four homogenous groups were identified among the mean **TC** content of each cultivar through an LSD All-Pairwise Comparisons Test: A ('BKK'), B ('Joy', 'Mystical'), C ('Hawaiian Red'), D ('Caribbean'), and E ('Olena'). 'Hawaiian Red' is considered Hawaii's standard commercial cultivar. Groups A and B were higher than C, suggesting that 'BKK', 'Joy', and 'Mystical' are commercially competitive with respect to **TC**. Although 'BKK' has the highest **TC** content, it suffers from low yield. Conversely, 'Olena' has the lowest **TC** content but is high yielding (data not shown). Thus, they both are not considered viable commercially. They are included in further iterations to function as extreme high and low **TC** controls. The individual curcuminoid contents of each cultivar also followed the same trend with 'BKK' at the highest and 'Olena' at the lowest. Other *Curcuma* species were also grown and analyzed: *Curcuma aeruginosa, C. zanthorrhiza, C. caesia, C. mangga, C. zedoaria* and *C. aromatica.* Their **TC** contents were not-detectable, except in the case of *C. zanthorrhiza* and *C. mangga* with total curcuminoid contents <1% (data not shown). The low curcuminoid content results of these non-*C. longa* cultivars are consistent with literature (Rafi et al., 2015; Bos et al., 2007; Variappan et al., 2013). Due to these extremely low results, they were excluded in the remaining iterations.

In the Waimanalo 2019 iteration, a new cultivar 'Roma' was also cultivated and analyzed in addition to the previous six. Overall, the same trend was observed with 'BKK' at the highest **TC** with 8.211±0.180% w/w dw and 'Olena' at the lowest with 1.084±0.082% w/w dw. Four homogenous groups A'–D' were identified: A' ('BKK'), B' ('Joy' and 'Roma'), B'C' ('Caribbean' and 'Mystical'), C' ('Hawaiian Red'), and D' ('Olena'). The **TC** content of 'Joy' and 'Roma' were significantly higher than 'Hawaiian Red', while 'Mystical' was not significantly different from 'Hawaiian Red'. 'Caribbean' was not significantly different from 'Hawaiian Red', while in the Waimanalo 2018 it was significantly lower. The **TC** of 'Caribbean' was higher than in the 2018 iteration (28.9% relative increase in **TC** from 2018 to 2019). The 'Caribbean' rhizomes received in 2019 were larger in diameter than those in 2018, which may imply better overall health of the plant in this iteration. 'BKK', 'Joy', 'Hawaiian Red', and 'Joy' all showed lower mean **TC** contents compared to the 2018 iteration. With these cultivars, it was also observed that while the curcumin and **DMC** content decreased, the **BDMC** content increased between the 2018 and 2019 iterations. Time of harvest may have played a role in this discrepancy, with 2018 iteration harvested in January and 2019 iteration harvested in April because the production of curcuminoids is has been demonstrated to peak early on in development, while starch and fiber accumulation continues (Chavalittumrong and Jurawattanapong, 1992; Li, 2011; Mitchell and Green, 2015).

The Maui 2019 iteration included 5 *C. longa* cultivars: 'BKK', 'Hawaiian Red', 'Mystical', 'Joy', and 'Olena'. The cultivar 'Caribbean' was excluded due limited planting space and its low **TC** in the Waimanalo 2018 iteration. The same trend was observed in this iteration as well with 'BKK' at the highest **TC**

(10.962±0.287% w/w dw) and ‘Olena’ at the lowest (1.208±0.091% w/w dw). Three homogenous groups A’”—C’” were identified: A’” (‘BKK’), B’” (‘Hawaiian Red’, ‘Mystical’, and ‘Joy’), and C’” (‘Olena’). The **TC** content of ‘Joy’ and ‘Mystical’ were not significantly different from ‘Hawaiian Red’ as they were in the Waimanalo 2018 iteration. ‘BKK’ had the highest **TC** content in this iteration compared to the previous iterations (12.6% difference from average of all 3 iterations), while ‘Hawaiian Red’, ‘Mystical’, and ‘Joy’ were the lowest (2 to 8% difference from average). This may suggest that Maui as a location positively affects the **TC** of ‘BKK’ while negatively impacting the others, but more Maui location trials are needed.

The average **TC** content of the five common cultivars across all three iterations, ‘BKK’, ‘Joy’, ‘Mystical’, ‘Hawaiian Red’, and ‘Olena’ are shown in fig. 2.3. Analyzing these cultivars across all three iterations, the interactions between curcumin, **DMC**, **BDMC**, and **TC** content and cultivar, iteration, and cultivar*iteration were highly significant. Four homogenous groups **A** – **D** were identified: **A** (‘BKK’, 9.531±0.125), **B** (‘Joy’, 6.504±0.079), **BC** (‘Mystical’, 6.100±0.148), **C** (‘Hawaiian Red’, 5.896±0.178), and **D** (‘Olena’, 1.271±0.052). ‘Joy’ was significantly higher in **TC** content than ‘Hawaiian Red’, while ‘Mystical’ was not, differing from ’Hawaiian Red’ by less than 0.2% w/w dw **TC**. ‘BKK’ and ‘Olena’ were in all iterations the highest and lowest in **TC** content, respectively.

The relative abundances of curcuminoids in each cultivar for the Waimanalo 2019 iteration are shown in table 2.4. This iteration was selected to best compare each cultivar because all *C. longa* cultivars analyzed were present, and harvest was performed at a typical time. On average, the relative abundance of curcuminoids is 68.8±2.3: 17.2±1.3: 13.9±0.7 curcumin: **DMC**: **BDMC**. ‘BKK’ had the highest absolute and relative **BDMC** content of 1.638±0.078 and 19.9±1.0, respectively. This cultivar was also the only one to demonstrate **BDMC** content higher than **DMC**, (13.5±0.4: 19.9±1.0 **DMC**: **BDMC**). This relationship was present for ‘BKK’ in the Maui 2019 iteration as well, but not in the Waimanalo 2018 iteration. As previously discussed, the decrease in **DMC** and increase in **BDMC** may be due to differences in harvest times. ‘Olena’ had the highest relative curcumin content (82.8±6.0) and lowest relative **BDMC** content (2.3±0.4). Although the absolute **TC** is the lowest, ‘Olena’ could still be useful for producing curcuminoid extracts with higher relative curcumin and **DMC** content. The differences in relative abundance of individual curcuminoids between cultivars could be used as an inter-cultivar fingerprint for quality control, such as verifying the source of curcuminoid extracts. Similar fingerprinting was proposed for inter-species authentication between *C. longa* and *C. zanthorrhiza* (Rafi et al., 2015).

2.5 CONCLUSION

Turmeric cultivars ‘BKK’, ‘Joy’, ‘Mystical’, ‘Hawaiian Red’, and ‘Olena’ were grown in Hawaii in 3 iterations across 2 locations have been analyzed by HPLC for total curcuminoid (**TC**) content, taken as the sum of curcumin, demethoxycurcumin, and bis-demethoxycurcumin content. The results have shown a wide range of **TC** content (1.084 – 10.962% w/w dw), with cultivar ‘BKK’ producing the highest (8.211 – 10.962%), intermediate producing cultivars: , ‘Joy’ (5.686 – 7.170%) ‘Mystical’ (5.571 – 7.102%),

'Hawaiian Red' (4.946 – 6.288%) and 'Olena' at the lowest with (1.084 – 1.584%). 'Hawaiian Red' is considered the Hawaii's industry standard cultivar. 'BKK' and 'Joy' were significantly higher in **TC** than 'Hawaiian Red' overall, while 'Mystical' was statistically similar. 'Joy' was also high yielding, demonstrating high commercial viability as a high-curcuminoid cultivar. 'Roma', a cultivar that was present only in the Waimanalo 2019 analysis showed promising results as it was statistically the same as 'Joy' and higher than 'Hawaiian Red' in that iteration. These results and future studies will help guide Hawaii agriculture towards producing high quality turmeric for the global market.

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TABLES

Table 2.1 System suitability for curcumin, demethoxycurcumin, and bis-demethoxycurcumin quantification

Compound	Retention Time (min)	Tailing Factor	Theoretical Plates
Curcumin	6.928 ± 0.039	1.028 ± 0.012	10432 ± 771
Demethoxycurcumin	6.354 ± 0.040	1.025 ± 0.056	10045 ± 912
Bis-demethoxycurcumin	5.819 ± 0.040	1.031 ± 0.030	10622 ± 1533

Table 2.2 Linear regression parameters for curcuminoid standards

Standard	Slope	Y-intercept	r^2	Range, µg	LOQ, µg	LOD, µg
Curcumin	2276146095	101488	0.9997	0.154 - 2.500	0.079	0.026
Demethoxycurcumin	3796132900	21253	0.9996	0.161 - 0.806	0.027	0.009
Bis-demethoxycurcumin	3124717333	16262	0.9992	0.100 - 0.500	0.026	0.009

Table 2.3 Curcuminoid content in turmeric cultivars grown in Waimanalo (2018 and 2019) and Maui (2019).

Iteration	Variety	Curcumin	DMC	BDMC	Total
		----------% w/w dw----------			
Waimanalo 2018	'BKK'	6.680 ± 0.027	2.265 ± 0.026	1.078 ± 0.010	10.023 ± 0.023
	'Caribbean'	3.338 ± 0.238	1.171 ± 0.118	0.436 ± 0.017	4.946 ± 0.371
	'Hawaiian Red'	4.409 ± 0.228	1.429 ± 0.108	0.450 ± 0.024	6.288 ± 0.359
	'Mystical'	4.872 ± 0.123	1.634 ± 0.076	0.597 ± 0.025	7.102 ± 0.147
	'Joy'	4.723 ± 0.009	1.841 ± 0.059	0.605 ± 0.008	7.170 ± 0.060
	'Olena'	1.288 ± 0.072	0.263 ± 0.028	0.033 ± 0.002	1.584 ± 0.099
Waimanalo 2019	'BKK'	5.467 ± 0.073	1.107 ± 0.034	1.638 ± 0.078	8.211 ± 0.180
	'Roma'	4.082 ± 0.066	1.345 ± 0.076	0.950 ± 0.059	6.377 ± 0.196
	'Caribbean'	4.045 ± 0.038	1.276 ± 0.139	1.007 ± 0.042	6.328 ± 0.199
	'Hawaiian Red'	4.023 ± 0.189	0.945 ± 0.098	0.792 ± 0.048	5.760 ± 0.322
	'Mystical'	4.062 ± 0.179	1.022 ± 0.095	0.913 ± 0.037	5.996 ± 0.309
	'Joy'	4.339 ± 0.074	1.141 ± 0.038	1.012 ± 0.026	6.491 ± 0.116
	'Olena'	0.898 ± 0.065	0.161 ± 0.016	0.025 ± 0.004	1.084 ± 0.082
Maui 2019	'BKK'	7.173 ± 0.160	1.456 ± 0.047	2.332 ± 0.081	10.962 ± 0.287
	'Hawaiian Red'	4.215 ± 0.163	0.786 ± 0.029	0.685 ± 0.024	5.686 ± 0.201
	'Mystical'	3.900 ± 0.020	0.907 ± 0.032	0.764 ± 0.019	5.571 ± 0.065
	'Joy'	4.104 ± 0.108	0.907 ± 0.058	0.852 ± 0.040	5.864 ± 0.206
	'Olena'	1.025 ± 0.084	0.149 ± 0.005	0.033 ± 0.006	1.208 ± 0.091
Cultivar (*C*)		P<0.001	P<0.001	P<0.001	P<0.001
Iteration (*I*)		P<0.001	P<0.001	P<0.001	P<0.001
*C*I*		P<0.001	P<0.001	P<0.001	P<0.001

Table 2.4 Relative curcuminoid abundance in Waimanalo 2019 C. longa cultivars, as percent of total.

Cultivar	Curcumin	DMC	BDMC
'BKK'	66.6 ± 0.9	13.5 ± 0.4	19.9 ± 1.0
'Caribbean'	63.9 ± 0.6	20.2 ± 2.2	15.9 ± 0.7
'Hawaiian Red'	69.8 ± 3.3	16.4 ± 1.7	13.8 ± 0.8
'Joy'	66.8 ± 1.1	17.6 ± 0.6	15.6 ± 0.4
'Mystical'	67.7 ± 3.0	17.0 ± 1.6	15.2 ± 0.6
'Olena'	82.8 ± 6.0	14.9 ± 1.5	2.3 ± 0.4
'Roma'	64.0 ± 1.0	21.1 ± 1.2	14.9 ± 0.9
Average	68.8 ± 2.3	17.2 ± 1.3	13.9 ± 0.7

FIGURES

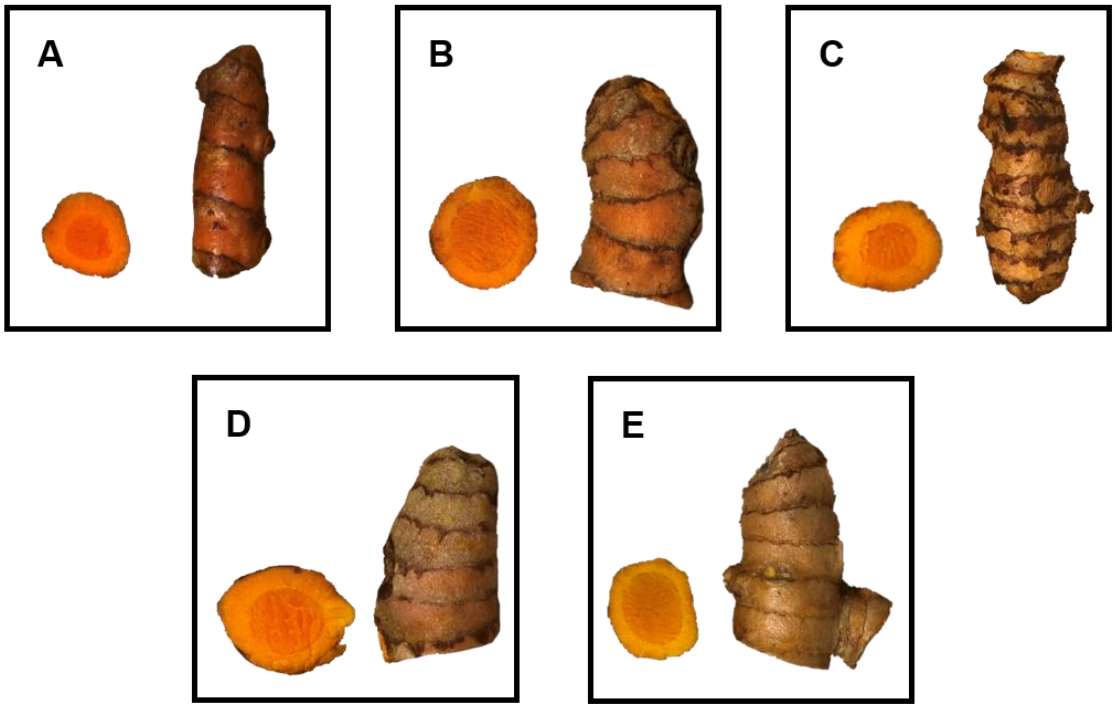

Figure 2.1 Rhizome and cross-section of *C. longa* cultivars: 'BKK' (A), 'Hawaiian Red' (B), ‘mystical’ (C), ‘Joy’ (D), and ‘Olena’ (E).

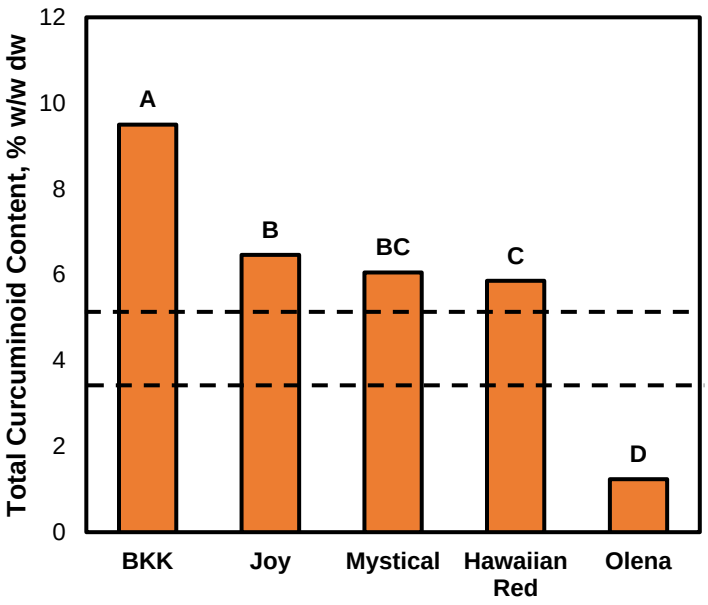

Figure 2.2 Average curcuminoid content of 5 C. longa cultivars grown in three iterations. Contents of commercial Indian cultivars shown for comparison: Upper dotted line: 'Alleppey' at 5%; lower dotted line: 'Madras' at 3.5% (adapted from Ravindran, et al., 2007).

CHAPTER 3 HPLC ANALYSIS OF ASIAN TURMERIC CULTIVARS AND COLOR CHART FOR TOTAL CURCUMINOID ESTIMATION

3.1 ABSTRACT

Turmeric (*Curcuma longa*) is a spice valued as a culinary pigment and medicinal herb due to its total curcuminoid (**TC**) content. Asian turmeric cultivars obtained from Singapore, India, Myanmar, and Thailand, as well as Hawaii-grown cultivars were analyzed for **TC** content by HPLC as the sum of the major curcuminoids: curcumin, demethoxycurcumin, and bis-demethoxycurcumin. Their colors were determined using a Royal Horticultural Society Color Chart and were plotted separately as L*, a*, and b* vs. **TC** to produce a range of colors for 1 to 10% **TC**. Of these, 1, 3, 5, and 10% were selected for use in the color grade chart for **TC** estimation. The chart would benefit from more cultivars and more observers. Overall, the chart as is could be useful for **TC** estimation but would benefit from more cultivars and more observers.

3.2 INTRODUCTION

Turmeric is a tropical plant valued globally as a culinary spice and for the medicinal uses of the pigment compounds stored in its rhizomes called curcuminoids (*fig.* 1.1). The three significant curcuminoids present in *C. longa* are curcumin, demethoxycurcumin (**DMC**), and bis-demethoxycurcumin (**BDMC**), in total comprising 1-10% w/w dry weight of the rhizome (Ravindran et al., 2007). These compounds are yellow to red and reported to have potential medicinal effects against the development of chronic inflammation, Alzheimer's, and various cancers (Ghosh et al., 2015; Randino et al., 2016; Chun et al., 1999). The sum of these curcuminoids comprises most of the total pigment present in turmeric and is referred to as the total curcuminoid (**TC**). Different government entities set minimum **TC** contents as a quality standard for turmeric products. For example, the Board of Indian Standards requires a minimum **TC** 2.0% w/w for exported ground powders (BIS, 2010). Additionally, pharmaceutical customers may have further specifications on the relative abundance of individual curcuminoids. It was previously demonstrated that there is a variation in relative abundance between cultivars (see Table 2.4).

The global demand for turmeric has increased dramatically over the past couple of years. India, the major global producer of turmeric, increased bulk exports of turmeric by 55.3% from 2014 to 2019, valued at 204 million USD in the financial year of 2018-2019 (Spices Board of India, 2019). Hawaii has recently developed its agricultural market for turmeric, producing 520,000 lbs. of fresh rhizomes in 2018 (USDA, 2020). However, a significant hurdle for Hawaii's growing market remains to standardize the curcuminoid content of commercial cultivars produced. Previous work has demonstrated a wide range of **TC** contents between commercially cultivated turmeric cultivars in Hawaii through high-performance liquid chromatography (HPLC) (see Table 2.3). HPLC was useful for determining exact quantities of individual curcuminoids, but the process is costly and difficult. A color grade chart is proposed for estimating

curcuminoid content in turmeric rhizomes that are fast and accessible to supplement commercial turmeric quality control.

Curcuminoids are orange-colored pigments that are responsible for the yellow to the orange color of turmeric rhizomes. A recent study has demonstrated that orange rhizomes, rather than lighter yellow, have higher curcuminoid, iron, and total phenolic contents (Pal et al., 2020). The authors of that study used L*a*b* color space values to quantify color in turmeric cultivars and, as expected, found a strong correlation between curcuminoid content and redness (a*). In this study, the color of turmeric cultivars will be analyzed semi-quantitatively by matching rhizome colors to a standardized color chart. The Royal Horticultural Society (RHS) Color Chart is a reputable collection of colors used, especially for describing plant varieties in floriculture. The RHS Color Chart contains a Yellow to Red series of colors, appropriate for differentiating yellow to dark orange-colored rhizomes. The correlation between RHS color and **TC**, as determined by HPLC, will then be used to design a color chart for estimating curcuminoid content.

3.3 EXPERIMENTAL

3.3.1 Turmeric samples

Asian turmeric cultivars were collected from Singapore, India, Myanmar, and Thailand and grown in aquaponics in Waimanalo, HI. Hawaii-grown cultivars were also included in this study: the Maui 2019 cultivars from chapter 2 and a cultivar obtained from Molokai, HI. Samples were processed in the same manner as in section 2.3.3.

3.3.2 HPLC Analysis

HPLC was performed as in section 2.3.5.

3.3.3 RHS Color Determination

A First Edition RHS Color Chart was used for color determination. Fresh rhizome cross-sections of each cultivar were compared against the color chart and the colors were recorded, shown in Table 3.1. Each cross-section showed a darker inner cortex and lighter outer cortex, so each cultivar was assigned two colors (see figure 3.1).

3.3.4 Development of Color Grade Chart

The colors identified for each cultivar were analyzed as L*a*b* CIELAB color space values using the values provided with the RHS Color Chart. The values for the inner and outer cortex were then averaged, and a linear regression was obtained for the individual L*, a*, and b* vs **TC**. These regressions were then used to produce color grade chart for theoretical colors corresponding to 1-10% w/w dw **TC**, shown in fig. 3.2.

3.4 RESULTS AND DISCUSSION

3.4.1 HPLC of Asian turmeric cultivars

The Asian turmeric cultivars analyzed showed a wide range of **TC** content (3.362 – 10.922% w/w dw). The three highest **TC** cultivars overall were Indian cultivars 18-013 (10.922 ± 0.108), 18-014 (9.884 ± 0.123), and 18-010 (9.871 ± 0.161). 18-013 had nearly the same **TC** as Maui 2019's 'BKK' (10.962 ± 0.287). Nearly all cultivars, except for 18-023 and 18-032, had **TC** above that of the high curcuminoid commercial Indian cultivar 'Alleppey' (5% w/w dw). These cultivars, and especially the top three 18-013, 18-014, and 18-010, are recommended for field trials to determine overall commercial viability.

3.4.2 RHS Color Determination and Color Grade Chart

Colors were determined for turmeric cultivars using an RHS Color Chart. Color analysis of turmeric was done recently quantitatively using a colorimeter (Pal et al., 2020). The authors of that study found a strong correlation between curcuminoid content and redness, denoted as +a* in the L*a*b* color space. Colorimeters have been used on other crops to estimate chemical composition (Pathare et al., 2013). Colorimeters assign an objective and quantitative value but are prone to interferences from the sample's shape and glossiness.

Additionally, colorimeters are better at identifying changes in color rather than quantifying the exact color. The RHS Color Chart, at the expense of more robust quantitative data, allowed for better visual, albeit subjective, color identification. However, the RHS Color Chart identification is also subject to the observer's color vision and visual fatigue. Subjectivity with the RHS Color Chart is outweighed by the realistic colors identified for this study because the data will be used in a color grade chart.

Each rhizome cross-section showed a distinct color for the inner and outer cortex, and their areas were about 1:1. To approximate their overall color, the L*a*b* values to the RHS colors identified were averaged. These values were then plotted against **TC** to obtain individual linear regressions, detailed in table 3.2. The linear regressions obtained gave a coefficient of determination values (R2) of 0.677, 0.690, and 0.464 for L*, a*, and b* vs. **TC**, respectively. The strongest correlation was between a* and **TC**, just like the findings of Pal et al., 2020. The weak relationship between b* and **TC** and the negative slope is possibly due to how curcuminoids interact with light at low concentrations. Since curcuminoids are poorly soluble in aqueous solutions, it is possible that condensed crystalline structures are accounting for the darker and redder color of high **TC** rhizomes, while solubilized and smaller crystal curcuminoids account for the lighter color of low **TC** rhizomes. The literature also noted that redness also correlated with the presence of iron and other phenolic compounds (Pal et al., 2020). Light scattering caused by larger curcuminoid crystals may also be responsible for the negative L* slope (-0.471).
Using each regression, L*a*b* values were approximated for **TC** 1 through 10%. Differences in L*a*b* colors are calculated as delta E (square root of the sum of square differences between L*, a*, and b*). The delta E between 1% **TC** was 1.48, while the minimum delta E for differentiating colors is about 2.3

(Sharma, 2003). This means that the theoretical precision of the **TC** estimated is about 1.55%. **TC** 1%, 3%, 5%, and 10% were selected for the color chart. To use the chart, the **TC** would be the average of the two colors identified for the inner and outer cortex. For example, a rhizome with a 5% colored inner cortex and 3% colored outer cortex would have an estimated **TC** of 4%.

3.5 CONCLUSION

HPLC has analyzed Asian turmeric cultivars, and a wide range of total curcuminoid (**TC**) content has been shown for these cultivars, with the highest being 18-013 (10.922 ± 0.108), 18-014 (9.884 ± 0.123), and 18-010 (9.871 ± 0.161). Most cultivars exceeded commercial expectations for TC, and they are recommended for testing for commercial viability. The colors of these and Hawaii-grown cultivars were measured using an RHS Color Chart, and the corresponding L*a*b* color space values were used to design a TC estimating color grade chart for 1, 3, 5, and 10%, shown in fig. 3.2. Further testing with more observers and varied TC cultivars will help improve the accuracy of the chart. An alternative use for color grading could be to confirm the identity of turmeric cultivars. For example, 'Olena' is a high yielding but very low curcuminoid content cultivar. It could be mislabeled as a more valuable cultivar such as 'Hawaiian Red' and be sold incorrectly. Overall, color grade charts for turmeric cultivars could help reduce reliance on HPLC for curcuminoid content determination.

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TABLES

Table 3.1 Total curcuminoid content (TC), RHS Color, and origin of turmeric cultivars analyzed.

Code	Country	Region	TC			RHS Color	
						Inner	Outer
18-002	Singapore	Geylan	7.752	±	0.129	25A	25B
18-007	Singapore	Geylan	6.569	±	0.151	25A	25B
18-008	Singapore	Geylan	9.135	±	0.115	28A	28B
18-010	India	Wayanad	9.871	±	0.161	28A	28B
18-012	India	Wayanad	8.355	±	0.140	28B	25A
18-013	India	Sittilingi	10.922	±	0.108	28A	28B
18-014	India	Mysore	9.884	±	0.123	28A	28B
18-015	India	Mysore	7.889	±	0.164	25A	25B
18-016	India	Mysore	7.427	±	0.062	28B	25B
18-020	Myanmar	Taungbohla	6.308	±	0.151	25A	24A
18-023	Myanmar	-	4.034	±	0.154	25A	25B
18-024	Myanmar	-	5.528	±	0.143	25A	24A
18-027	Thailand	Chiang Mai	7.733	±	0.158	28A	25A
18-032	Thailand	Chiang Mai	3.362	±	0.175	28B	25A
18-034	USA	Molokai, HI	7.851	±	0.121	28A	28B
BKK	USA	Maui, HI	10.962	±	0.287	28A	25A
Hawaiian Red	USA	Maui, HI	5.686	±	0.201	25A	25B
Joy	USA	Maui, HI	5.571	±	0.065	25A	24A
Mystical	USA	Maui, HI	5.864	±	0.206	25A	24A
Olena	USA	Maui, HI	1.208	±	0.091	24A	23A
E1	USA	Waimanalo, HI	6.788	±	0.168	28A	28B
E2	USA	Waimanalo, HI	1.992	±	0.177	25A	23A

Table 3.2 Linear regression parameters for L*, a*, and b* vs Total Curcuminoids

	Slope	Intercept	R2
L*	-0.471	75.469	0.677
a*	1.158	23.330	0.690
b*	-0.794	73.281	0.464

FIGURES

Figure 3.1 Turmeric cultivar ‘Hawaiian Red’ cross-section showing a typical darker pigmentation of the inner cortex compared to the lighter outer cortex.

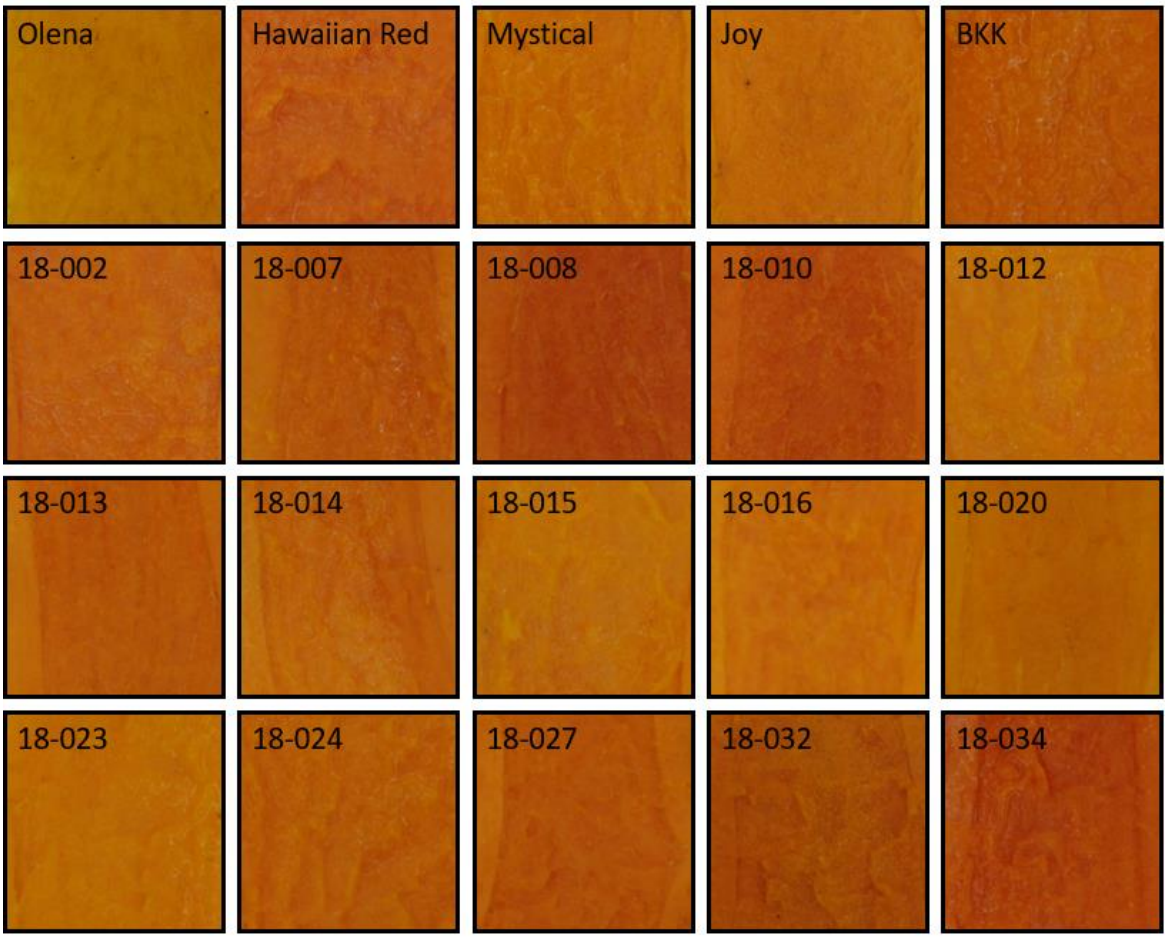


Figure 3.2 Pictorial representation of rhizome color in turmeric cultivars analyzed.

	1%	3%	5%	10%
L*:	75.0	74.1	73.1	70.8
a*:	24.5	26.8	29.1	34.9
b*:	72.5	70.9	69.3	65.3

Figure 3.3 Proposed color grade chart for total curcuminoid estimation.

CHAPTER 4 DISCUSSION

4.1 QUANTIFICATION OF CURCUMINOIDS IN FRESH TURMERIC RHIZOMES BY HPLC

High curcuminoid content is a significant quality factor for commercial turmeric production, but the cultivars produced in Hawaii have yet to be analyzed. Therefore, a novel HPLC method was developed to analyzed curcuminoids curcumin, demethoxycurcumin (**DMC**), and bis-demethoxycurcumin (**BDMC**) content in fresh turmeric rhizomes. Turmeric cultivars were grown in three iterations at two locations (Waimanalo Research Station, Oahu, HI, and Maui Agricultural Research Center, Maui, HI) across Hawaii. The cultivars present in all iterations and their total curcuminoid (**TC**) content, taken as the sum of curcumin, **DMC**, and **BDMC**, are as follows: 'BKK' (8.211 – 10.962%), 'Joy' (5.686 – 7.170%) 'Mystical' (5.571 – 7.102%), 'Hawaiian Red' (4.946 – 6.288%) and 'Olena' (1.084 – 1.584%), shown graphically in figure 2.2. Additional cultivars 'Caribbean' (4.946—6.328%, two iterations) and 'Roma' (6.377±0.196%, one iteration) were also analyzed (see table 2.3 for all HPLC results). Homogenous groups as assigned by an LSD All-Pairwise Comparisons Test showed that 'BKK' (**A**) was significantly higher than the rest, followed by 'Joy' (**B**), 'Mystical' (**BC**), 'Hawaiian Red' (**C**), and lastly Olena (**D**). The relative abundance of curcuminoids produced was 68.8±2.3: 17.2±1.3: 13.9±0.7 % w/w dw curcumin:**DMC**:**BDMC**. No directional cultivar, iteration, or cultivar*iteration interactions were observed.

'BKK' and 'Olena' are both cultivars with low commercial viability: 'BKK' because, although it has the highest **TC**, it has low yield and 'Olena' because of its low **TC** content. 'Hawaiian Red' is a popular commercial cultivar because of its nice color and yield, and it is currently considered the commercial standard of Hawaii. Its **TC** content was shown to be close in range to commercial Indian cultivar 'Alleppey' (5-6%). However, analyses showed that 'Joy' was significantly higher in **TC** content than 'Hawaiian Red,' which is almost 1% higher. 'Roma' and 'Caribbean' were also promising because they were similar to 'Joy' in the Waimanalo 2019 iteration, although 'Caribbean' was low in the Waimanalo 2018 iteration. Overall, 'Joy' can most confidently be recommended for commercial cultivation in Hawaii, while 'Mystical' and 'Hawaiian Red' can remain as acceptable cultivars. 'Roma' and 'Caribbean' are also cultivars that show potential, but more iterations will help understand their commercial value more confidently.

4.2 HPLC OF ASIAN TURMERIC CULTIVARS AND DEVELOPMENT OF COLOR CHART FOR CURCUMINOID ESTIMATION

Fourteen reportedly high curcuminoid Asian turmeric cultivars were obtained from Myanmar, India, Thailand, and Myanmar to help expand Hawaii's high curcuminoid turmeric germplasm (table 3.1). To prevent losses, they were cultivated in an aquaponic system. The HPLC method designed for analyzing curcuminoids in fresh turmeric was used to analyze these cultivars. The highest **TC** content cultivars were 18-013 (10.922 ± 0.108), 18-014 (9.884 ± 0.123), and 18-010 (9.871 ± 0.161) and they are recommended for field trials to observe their viability in Hawaii's environment. The colors of the Asian cultivars, as well as those from Hawaii, were also identified using an RHS Color Chart (**TC** and RHS Colors shown in Table 3.1). The L*a*b* value associated with the identified colors were then plotted against **TC** to obtain three linear regressions (R^2 = 0.677, 0.690, and 0.464 for L*, a*, and b* vs **TC,** respectively). These regressions were then used to generate colors for **TC** 1, 3, 5, and 10%, as shown in figure 3.3.

The chart designed can be used for estimating turmeric curcuminoid content in the field. The benefits of such a tool are its accessibility and ease of use. It can be used either to quickly grade turmeric, monitor the health of turmeric growing in the field, or even confirm cultivar labeling. However, there are at least two challenges with this chart as it is. First, the linearity of the regressions, especially b* vs. **TC**, is low. This approach can be improved by increasing observers and observations, using a different standardized color chart with more color choices in the orange region (such as Pantone), or using a higher-order regression rather than a linear one. Secondly, color charts are subjective, and some viewers may lack the necessary color discrimination ability. At the very least, it can confidently be said that yellow to yellow-orange turmeric does not have commercially viable **TC** content and can be sorted out. In contrast, orange turmeric can have anywhere from 3 to 10% **TC** content.

CHAPTER 5 FUTURE WORKS

The HPLC work and color chart design have helped set the stage for future turmeric studies in Hawaii. We still need to standardize further horticultural methods such as soil type, timing, planting strategies, harvesting, and post-harvest processing. Other future work might also include large scale dormancy breaking of rhizomes for commercial seed, experimenting with treatments or soil additives to improve curcuminoid production, improving shelf life of dormant rhizomes, and characterizing other novel bioactive compounds. An alternative spectrophotometric method to compliment HPLC should also be considered for regular quality control services to be available through CTAHR Agricultural Diagnostics Services. If largescale turmeric extraction processing occurs in Hawaii, then byproducts such as starches, fibers, juices, and leaves could also be useful resources in food and agriculture.
Food product development with Hawaii grown produce should also be a significant focus of research. Turmeric can be processed into dry powders, liquid extracts, or purees. These can be incorporated into other existing recipes to add bitter and earthy notes and bright yellow color, such as alcoholic beverages. A local curry mix might be an interesting venture, utilizing turmeric, Maui onions, and sweet potatoes. Or a turmeric chocolate product, using cacao butter to extract and suspend turmeric solids. Public service efforts to educate the public on utilizing turmeric in household cooking should be implemented too.

Turmeric is a major global agricultural commodity that is growing in demand due to its potential medicinal benefits. As clinical studies come to fruition and effective therapeutics are designed, its demand should increase even more. In my opinion, Hawaii agriculture can benefit from partaking in this global phenomenon. India will always be the dominant global producer of turmeric, but Hawaii is still positioned for success. First, turmeric is a tropical plant and grows well in our climate. Second, the locally grown aspect allows us to sell turmeric with fewer restrictions and higher value. We are also capable of supplying fresh “green” turmeric to local and mainland food companies to utilize in foods and beverages, rather than the dry powders and oleoresins imported from India and China. And last, turmeric would not be the first root crop grown in Hawaii; taro, sweet potato, and ginger are also significant crops of Hawaii. This means that the infrastructure for typical root crop harvest and post-harvest processing such as excavators and curing racks already exist. Overall, I believe that turmeric is beyond a flavor-of-the-month type of crop for Hawaii agriculture and will continue to grow in value and importance globally.

APPENDIX A: SOP FOR CURCUMINOID ANALYSIS OF FRESH TURMERIC BY HPLC

Overview

This appendix details the entire procedure for fresh turmeric HPLC analysis. This includes freeze drying, solvent preparation, extraction, HPLC analysis, curcuminoid standard preparation, and curcuminoid content calculation. Detailed operation and maintenance of the HPLC machine are not included.

Equipment

- Knife and cutting board
- Vacuum Freeze Dryer (Alpha 2-4 LDplus Christ)
- Mortar and pestle
- Analytical balance with 0.0001 g precision
- Ultrasonic Bath with floating rack (Branson 1210 Ultrasonic Cleaner)
- Microcentrifuge (Sorvall MC 12V)
- 1000-µL and 200-µL pipettes
- Waters 2695 Separations Module equipped with a Waters 996 Photodiode Array
- Phenomenex Kinetex C_{18} Column (150 x 4.6 mm i.d.; 5 µm)

Glass and disposable wares

- 1000-mL graduated cylinder
- HPLC solvent filter with side-armed flask
- 2 1-L screwcap glass bottles
- 500-mL beaker
- 250-mL screwcap bottle
- Aluminum foil
- 20-mL scintillation vials
- 1000-µL and 200-µL pipette tips
- 2.5-mL microfuge tubes
- 2-mL brown HPLC vials with glass inserts
- Glass container for HPLC waste (typically an empty reagent bottle)
- 50 mL falcon tubes

Reagents

- Ultrapure water (prepared inhouse with Barnstead Nanopure Infinity ultrapure water system)
- Glacial acetic acid (99.7% w/w)
- HPLC-grade acetonitrile

1. Freeze Drying

Preparation of samples for freeze drying: Obtain 3-4 representative rhizomes for each sample. Cut 1-mm cross-sectional discs from the rhizomes adding up to approximately 4 grams using a knife and cutting board. Place the slices in the center of a weighed labeled 6x6 inch piece of aluminum foil (recorded as "FE") and fold each side over to form a lightly packed 2 by 2 inch square. Poke the surface of the aluminum packet with a yellow disposable pipette tip to create airholes and record the weight (recorded as "FF"). Repeat for all samples, cleaning cutting board and knife with a methanol rinse and paper towel. Store packets in the freezer until completely frozen (4 hours – overnight). Alternatively, they can be frozen quickly using liquid nitrogen. Samples are now ready to be freeze dried.

Handling of Christ freeze dryer: Transfer frozen samples to an appropriately sized freeze drying flask (either 500-mL or 1-L depending on sample size). Ideally, the flask and samples should be chilled in a liquid nitrogen bath for 10-15 min to reduce stress on freeze dryer. Attach rubber lid to the flask and connect to the freeze dryer. Seal the connection from the lid to the metal inlet wih parafilm tape and slowly open the valve to the freeze dryer until completely open (direction doesn't matter). Stop turning the valve if the freeze dryer internal pressure rises to quickly (greater than 20 mbar increase on the gauge and loud motor hum), and proceed when the pressure normalizes. The valve is completely open at 180 degrees from closed position. Allow samples to freeze dry until brittle, 24-48 hours depending on sample size. Sufficient drying is indicated by calculated moisture content of around 80% (see eq. 1).

Powderization: Weigh dried samples (FD) and transfer contents to a clean dry mortar and pestle. Grind down to a fine powder and collect into a 50-mL scintillation vial.

Equation 1: $$Moisture\ content\ \% = \left(1 - \frac{FD-FE}{FF-FE}\right) \times 100\%$$

2. Solvent Preparation

2% Aqueous Acetic Acid: Measure 1000-mL of ultrapure water in a 1000-mL graduated cylinder. Water was prepared inhouse using the Barnstead Nanopure Ultrapure Water System. Remove 20 mL of water using a 1000-µL pipette (20 x 1000 µL aliquots), then add 20 mL of glacial acetic acid (20 x 1000 µL aliquots). Prevent acid from splashing by expelling the acid below the surface of the water. Transfer entire contents to a clean 1-L screwcap flask and combine well, unscrewing slightly intermitently to prevent pressure build-up.

Acetonitrile: HPLC grade acetonitrile can be directly filtered without further preparations.

Solvent Degassing with Christ freeze dryer: The set-up can be top heavy and should be watched carefully the entire time. Clamp the HPLC filter funnel to the side-armed flask and connect flask to the freeze drying system. Wash the set-up before use: pour 75 mL of solvent (preferably acetonitrile because its

easier to prepare) into the filter and open the freeze dryer valve slowly. If solvent leaks out the the set-up, either the clamp is loose, the pieces are not aligned, or the filter paper needs to be replaced. After the solvent has been pulled through, close the valve and disconnect set-up from the freeze dryer. Swirl solvent around and pour into a discard beaker. Repeat for a total of 3 rinses. Now that the set-up has be washed, solvent can be degassed. Fill filter funnel with solvent and open the valve, pulling solvent down into the side-armed flask. Top off the solvent before it reaches the bottom the maintain a steady pressure and flow. After all of the solvent has been filtered, disconnect set-up from the freeze dryer. Rinse a clean 1-L screwcap bottle with three 75 mL aliquots of degassed solvent, careful to empty solvent into the discard beaker in-between rinses. Transfer remaining degassed solvent to the bottle. If filtering both solvents, the next solvent can be filtered immediately after the first.

Preparation of **60:40** extraction solvent with the HPLC: Disconnect column inlet and cap the column inlet. Do not keep the system in this set-up for more than 2 hours to prevent column damage. Run HPLC system with an isocratic flow of 60:40 2% acetic acid: acetonitrile at 3 mL/min. Flush line for a minute by allowing effluent from the column inlet to flow into a discard beaker, then collect effluent into a 250-mL screwcap bottle. Around 100 mL of **60:40** can extract 80 samples. After collection, drop flow rate to 1.8 mL/min and reconnect column to the inlet. Run for a minute then turn off flow.

3. Extraction

Multiple samples can be processed at a time. Weigh around 5-8 mg of dry powdered turmeric in a 2.5-mL microfuge tube and record weight added (M). Add 1000 µL of **60:40** to the tube and sonicate for 5 minutes. Transfer tubes to microcentrifuge and spin down at 13 krpm for 3 min. Transfer 500 µL of supernatant to a new 2.5-mL microfuge tube. Repeat sonication and centrifugation. Transfer 120 µL to a 2-mL brown HPLC vial with glass insert. Tap glass insert gently to remove air bubbles and replace the vial cover. Samples should be analyzed on the same day as extraction.

Equation 2: $Sample\ concentration\ (C), mg/\mu L = \frac{M}{1000}$

4. HPLC Analysis

HPLC conditions: Isocratic flow 60:40 2% acetic acid: acetonitrile, flow rate 1.8 mL/min, PDA detector scanning 210-600 nm, Phenomenex Kinetex C_{18} Column (150 x 4.6 mm i.d.; 5 µm). 5-µL of sample injected per run. Runtime 8 minutes with 2 minute delay. Samples should be run with a known sample or curcuminoid standard.

Troubleshooting:

- Peaks splitting: column is damaged and should be replaced
- Large peak overlap: solvents are possibly contaminated and should be remade
- Delayed peaks: lengthened tubing will delay peaks

5. Standard Preparation and Analysis

Analytical grade 5 mg standards for curcumin, demethoxycurcumin, and bis-demethoxycurcumin can be obtained through Chromadex. Standards should be prepared through mass-transfer: obtain the initial weight of the bottle (I), then repeatedly rinse the the contents with 1000 µL aliquots of **60:40** into a 50-mL falcon tube until a final volume of 30 mL is reached. Air dry the emptied bottle and weigh (E). Transfer 1000 µL to a brown HPLC vial and analyze same day. Analyze same day using the same HPLC conditions as with the sample analysis but injecting over a range of 1 µL, 10 µL, 20 µL, …, 50 µL. Dried 1 mL aliquots of standard can be stored in a freezer and reconstituted for recalibration. New standards and column should be obtained yearly.

Equation 3: $Standard\ concentration\ (SC), mg/uL = \frac{I-E}{30{,}000\ (or\ total\ volume\ used\ for\ transfer)}$

6. Calculation of curcuminoid content

Standard curve: Convert injected volumes to mass (mg) using eq. 3. Take the linear regression of absorption vs mass, obtaining separate regression parameters slope (m) and y-intercept (b) for each curcuminoid standard. The coefficient of determination (r^2) for each curve should be 0.999.

Sample calculation of curcumin content for unknown sample:

Given:

- Sample concentration (C) = 5.0 mg/1000 µL
- Injection volume (V) = 5 µL
- Absorbance of curcumin peak = 2800000 AU
- Curcumin regression parameters: m = $2.28*10^9$, b = $1.01*10^5$

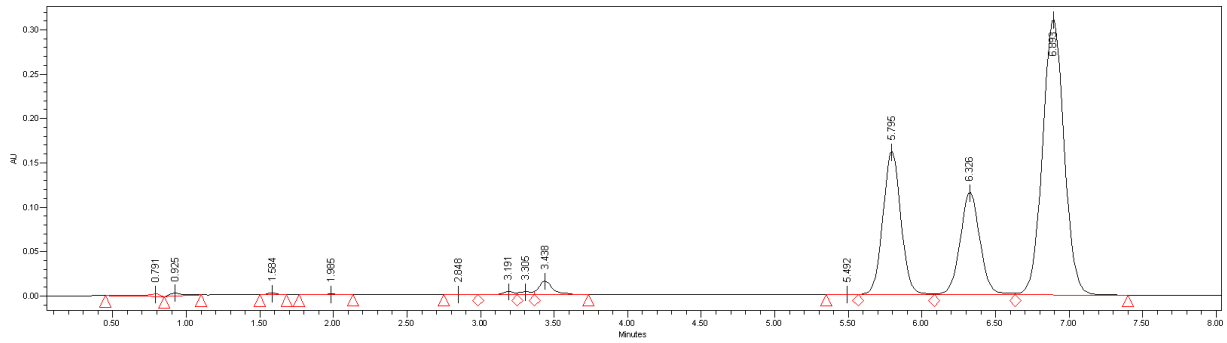
Calculations:

$$Curcumin\ injected = \frac{2800000 - 1.01*10^5}{2.28*10^9} = 0.00118\ mg$$

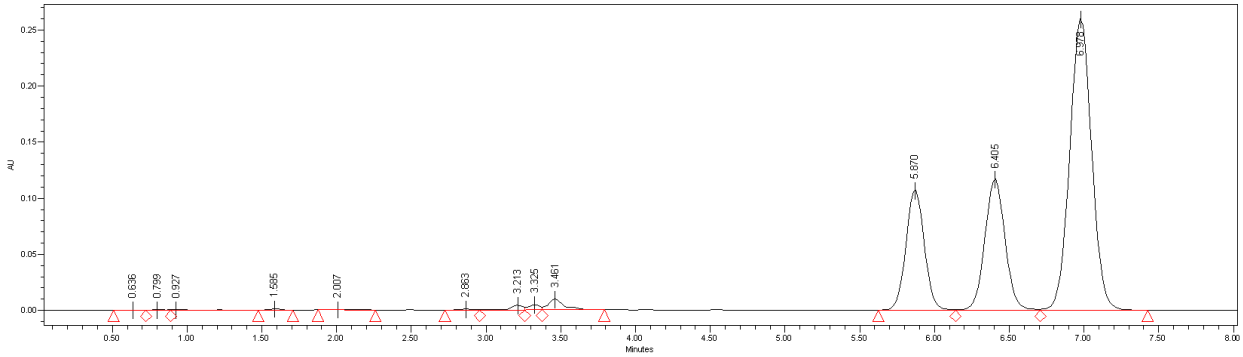
$$Curcumin\ content = \frac{0.00118\ mg}{5\ \mu L \times {}^{5\ mg}/_{1000\ \mu L}} = 0.0474\ w/w\ dry\ weight$$

APPENDIX B: EXEMPLARY CHROMATOGRAMS FOR CURCUMINOID HPLC ANALYSIS

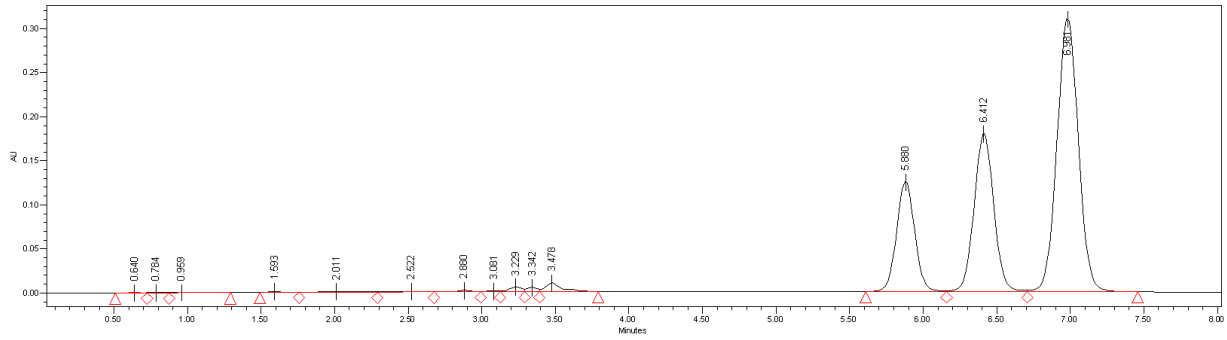
Curcuma longa 'BKK':



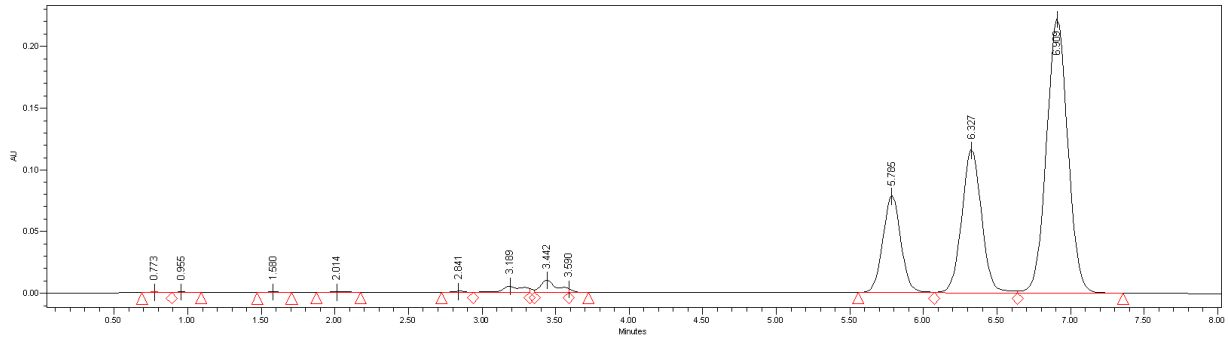
C. longa 'Joy':



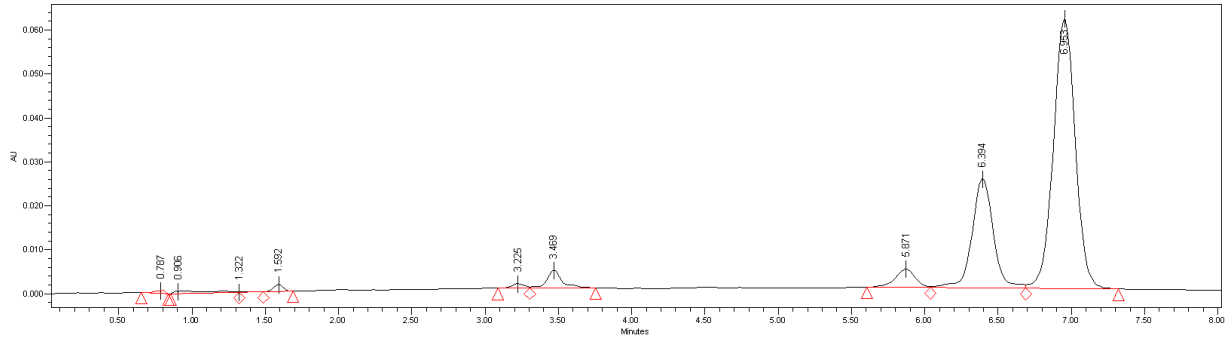
C. longa 'Mystical':



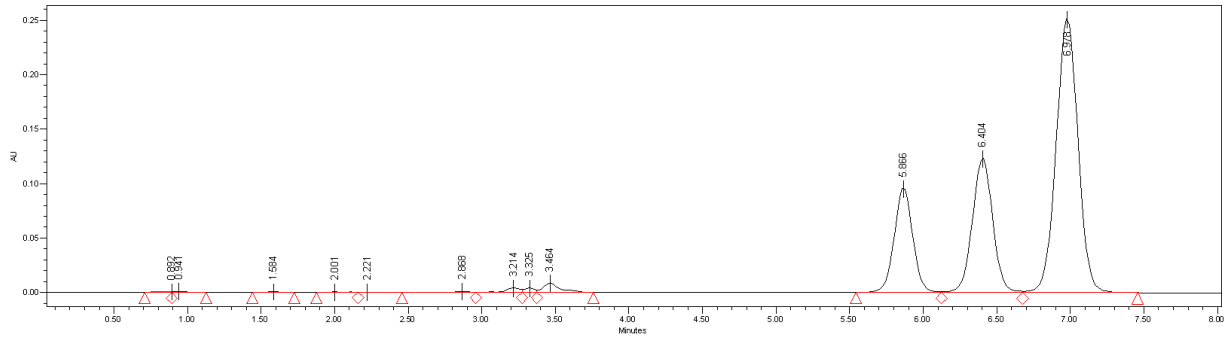
C. longa ‘Hawaiian Red’:



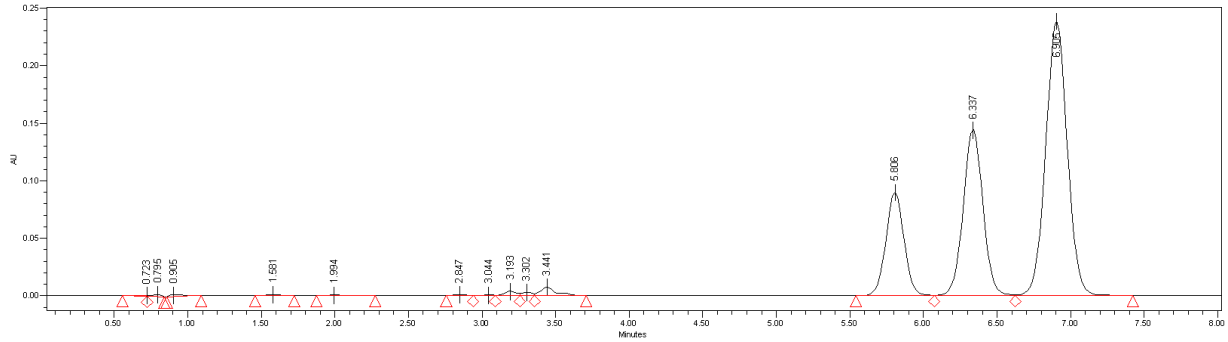
C. longa ‘Olena’:



C. longa ‘Caribbean’:



C. longa ‘Roma’:



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