THESIS

EFFECTS OF COOKING METHODS ON ANTIOXIDANT PROPERTIES, QUALITY ATTRIBUTES, AND SENSORY CHARACTERISTICS OF SELECTED LEAFY GREENS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2014

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ABSTRACT

EFFECTS OF COOKING METHODS ON ANTIOXIDANT PROPERTIES, QUALITY ATTRIBUTES, AND SENSORY CHARACTERISTICS OF SELECTED LEAFY GREENS

Kale, arugula, spinach, and other types of leafy vegetables are rich sources of vitamins and minerals and well-suited to growing in Colorado's climate. This project focused on the chemical, quality, and sensory assessment of a selection of 6 specialty leafy greens (arugula, cherokee lettuce, mache, pac choi, red kale, and spinach). Total phenolic (TP) content, radical scavenging capacity (1,1-diphenyl-2-picrylhydrazyl, DPPH), color measurement, instrumental texture characteristics, and sensory attributes of raw versus cooked (boiled, microwaved, or steamed) samples of the select taxa were analyzed and evaluated. All 6 varieties were cultivated in a greenhouse using organic planting medium. Samples of the greens were freeze-dried as raw or post-heat treatment, then underwent an extraction procedure, and were analyzed for total phenolics compared to gallic acid standards and radical scavenging using DPPH compared to trolox standards. Fresh and cooked comparisons were tested for color differences using a HunterLab ColorFlex spectrophotometer and changes in texture utilizing a TA-XT2 texture analyzer. Consumer (n=50; n=51) sensory analysis was administered using a 9-point hedonic scale. Fresh, uncooked mache had higher ($p<0.05$) total phenolics and radical scavenging ability than all other cultivars. Cooked samples revealed that spinach values for each test did not differ ($p>0.05$) from fresh samples and microwaved and steamed samples of red kale contained more

total phenolics than fresh, but less ($p<0.05$) was observed in boiled samples. Other significant findings included reductions ($p<0.05$) in lightness of cooked samples and cooked spinach samples after all heating treatments were observed to be more tender than fresh samples ($p<0.05$). This research helps to fill information gaps which exist in leafy greens research. Many studies focus on one cultivar using one testing method, but little research has been conducted on these types of greens using several analytical testing methods to obtain comparable data.

ACKNOWLEDGEMENTS

I would like to thank my two advisors who have been there from the start, Dr. Bunning and Dr. Stone. Without your words of encouragement and motivation this would not have been possible. A big thanks to Dr. Newman for joining my committee and see me through my thesis defense. To my amazing fiancée and bride-to-be, thank you for sharing this journey with me, away from our homes, to better myself. I love you. Lastly, to my entire family, thanks for supporting me through this endeavor and visiting us often.

DEDICATION

To my loved ones, especially my grandparents, who are looking down on me.

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CHAPTER I

INTRODUCTION

The revised USDA Dietary Guidelines advise Americans, who eat less than the daily recommended amounts, to increase their fruit and vegetable consumption by filling at least half their plate with them at every meal (USDA & HHS, 2010). The guidelines recommend increasing dark green and red vegetable intake as fresh or cooked, free of added fats or sugars, to maintain a healthy weight and reduce risks of certain diseases. Along with being a natural source of fiber, vegetables contain vitamins, minerals, and bioactive compounds not associated with diets high in protein. These constituents play parts in various plant functions such as protection against photooxidation and defense against insect predation or pathogens. Radical scavenging and human health benefits have been purported in many scientific studies utilizing green and red leafy vegetables (Bernhardt & Schlich, 2006). The purpose of this study was to investigate whether the nutrients and antioxidants that confer healthfulness upon leafy vegetables remain the same or are altered by various cooking processes used by consumers. This study was part of a larger project with a goal of promoting the production and consumption of underutilized leafy greens in Colorado (Fouladkhah, Bunning et al., 2011).

The focus of this study was to examine the cultivation, nutritional, sensory, and culinary characteristics of six selected leafy vegetables commonly grown in Colorado. The information will be used to guide the development of leafy vegetable preparation materials targeted at parents of pre-schoolers as well as recommended preservation methods aimed at community supported agriculture participants. Future studies will assess possible differences in flavor, appearance,

texture, aroma, and overall acceptability of leafy greens prepared and preserved in different ways.

The six selected leafy greens, arugula, cherokee lettuce, mache, pac choi, red kale, and spinach, were grown in a greenhouse using organic media and harvested. Three of the leafy green samples, spinach, pac choi, and red kale, were subjected to three different heat treatments, microwaving, boiling and steaming. Subsequently, both fresh and cooked greens had various quantitative and qualitative tests performed on samples, including a panelist-based sensory test using a 9-point hedonic scale. The analyses focused on determining differences between quality and functionality of fresh versus cooked leafy greens. This information is important because it will help nutrition advisors determine healthy leafy greens recipes without sacrificing nutrient values.

CHAPTER II

LITERATURE REVIEW

Farmers' markets and community supported agriculture (CSA) shares offer a diverse selection of leafy greens and other vegetables. The greens, in particular, vary in coloration, organoleptic properties, and antioxidant content among types and even within different cultivars of the same species. When desirable, certain cultivars may be cooked by the end user before consuming, which is another factor that may impact the aforementioned characteristics. These quality, sensory, and antioxidant properties of various raw and cooked greens were assessed in this study using instrument-based tests, sensory panels, and chemical assays.

Cooked Greens. Some types of leafy greens, including spinach, kale, and pac choi, are served using a variety of cooking methods in addition to being consumed raw. Methods most commonly used to cook greens are boiling, microwaving, and steaming, though some can be sautéed with oil and used as an ingredient in a variety of dishes. Much research has been carried out to support the claim that these cooking processes bring about a number of changes in physical characteristics and chemical composition such as losing color intensity, nutritive value, and gaining or diminishing antioxidant capacities (Micozzi, Beecher et al., 1990; Turkmen, Sari et al., 2005; Bernhardt & Schlich, 2006; Danesi & Bordoni, 2008; Korus & Lisiewska, 2009; Pellegrini, Chiavaro et al., 2010; Lisiewska, Kmiecik et al., 2011; Mazzeo, N'Dri et al., 2011).

A large variety of vegetables and leafy greens have been analyzed comparing raw, uncooked food with products prepared with thermal processing, resulting in differences depending on cultivar, cooking method, and constituent being analyzed. Bernhardt and Schlich

(2006) examined broccoli and bell peppers to determine whether lipophilic vitamins, β-carotene specifically, increased or decreased depending on cooking method and initial level in the products. The researchers found that when fresh broccoli was cooked using any method (boiled, steamed, stewed, or pressure steamed) the β-carotene amounts increased compared to the raw broccoli. The converse effect was true for the red peppers and no effect was found when cooking frozen peppers. Danesi and Bordoni (2008) also studied cooking effects on various vegetables, but focused on antioxidant activity (AA) for their chemical analyses. The AA of cooked carrots was found to increase or remain the same, tomatoes decreased in AA by boiling, and the green vegetables tested (green beans, zucchini, and peas) all decreased when subjected to cooking.

Of the three cooked leafy green cultivars addressed in this research, spinach has received the most attention, followed by kale and pac choi. Spinach tends to increase in phenolic content (Mazzeo et al., 2011), antioxidant activity (Turkmen et al., 2005), and amino acid profile (Lisiewska et al., 2011) after most cooking methods, though Mazzeo et al. (2011) found that boiling slightly, but significantly, reduced the AA from 4.43 to 4.03 mmol Trolox per 100g dry weight. Kale tended to lose nutritive value as Korus and Lisiewska (2009) demonstrated and reported that cooking kale reduced the Vitamin C content, polyphenols, and AA by 57%, 73%, and 45%, respectively. Of the research studies conducted with pac choi (Franke, Custer et al., 2004; Harbaum, Hubbermann et al., 2007), no one discussed comparisons between cooked and raw material nor gave values for AA or total phenolics.

Wide variations in cooking times were implemented in research examining spinach and kale. Spinach ranged from 5 to 10 minutes for boiling, 7.5 to 20 minutes for steaming, and 1

minute for microwaving and kale ranged from 6 to 10 minutes for boiling times (Micozzi et al., 1990; Turkmen et al., 2005; Korus & Lisiewska, 2009; Mazzeo et al., 2011).

Assessment of Color Intensity. Color quality and appearance are key factors for almost all foods, especially produce, as it conveys freshness and overall quality to the consumer (Lawless, 2010). For instance, paleness or yellowing may indicate nutrient deficiencies in spinach and browning in any green usually means it is past its prime. In a study by Dubose et al. (1980), color was even linked to sensory perceptions such as flavor, aroma, and taste. This research study examined orange and cherry flavored beverages where colorants and flavorants could be changed independently. The perceived intensities were observed to increase as the colorants increased, without a change in flavorant levels (DuBose et al., 1980).

Color is the outward appearance of energy being absorbed, reflected, refracted, and transmitted from an object that is being struck by light. Objects that we can visualize in the physical world are in three-dimensions and likewise, the color of an object is three-dimensional as well. These dimensions are defined as hue (perceived color, i.e. blue), lightness or brightness, and saturation, which is defined as the 'purity' of the perceived color (pure green vs. grayish green) (Lawless, 2010).

Accordingly, the instrument used to assess color, the Hunter Colorimeter, uses these three dimensions to describe the color of an object in an L*, a*, b* system. This system is modified from A.H. Munsell's color solid developed around 1900 that displayed hue, value (brightness), and chroma (color) as a 3-d diagram. The advantage to the L*, a*, b* designations compared to other systems is the linear characterization of the results plotted with the rectangular Cartesian coordinates (a, b) instead of a horseshoe shape that is difficult for linear calculations. In the L*, a*, b* system, +a represents red color (min=0, max=100), -a represents green color (min=0,

max= -100), +b represents yellow color (min=0, max=100), and –b represents blue (min=0, max= -100). L* signifies the degree of whiteness or blackness, where a value of 100 is pure white and 0 is black (Lawless, 2010). Further calculations of the L*, a*, and b* values can determine the chroma (C) and hue angle (H) by combining the one-dimensional values into 3-d color space standards. These calculated values better define or assess real-world descriptions of perceived color by the human eye using the equations $C = (a^{*2}+b^{*2})^{1/2}$ and $H = \tan^{-1}(b^*/a^*)$ (Setser, 1984; Thai & Shewfelt, 1991; Han, Gomes-Feitosa et al., 2004).

Another aspect of instrumental analysis is the natural inconsistency of samples used for color determination. Most instruments are used to estimate some factor(s) based upon measurements mathematically calibrated to known standards. Leafy greens and most foods are heterogeneous products of nature, where color and pigmentation are varied and irregularly textured surfaces scatter light in random directions. These characteristics create a test surface that includes none of the characteristics composing an ideal sample, namely homogeneous pigmentation, flat, evenly light scattering, and opaque (Lawless, 2010).

Texture Analysis. The importance of texture for consumer acceptability is highly valuable as it has been included in the four principal quality factors in foods (Schiffman, 1977). The three other factors are appearance, flavor, and nutrition, all of which are addressed in other sections of this study. Texture is described as the tactile response between some body part, usually the mouth, teeth, and tongue, and the food being consumed. Other important components of texture are derived from the kinesthetics (the ability to feel how something moves or positions), sight (i.e. used to discern flow), and sounds (important characteristics for such descriptors as crunchy, crackly, and crisp) (Bourne, 2002). Classic examples of the bearing of texture on food recognition are studies performed by Schiffman (1977) and Schiffman and others (1978) using

29 different foods that were pureed and strained, and subjects who were blindfolded. The subjects were asked to identify the food based solely on flavor. The food items identified correctly most often by subjects were apples at approximately 80% and the least often was cabbage, which attained only 4% correct identifications. On average, only 40% correct identifications were provided from normal weight, young adults (Schiffman, 1977; Schiffman et al., 1978).

Human-based texture analysis tends to be very subjective due to the variety of descriptors for food texture characteristics. One study, out of many reported in the literature, conducted by Szczesniak (1971) administered a word association test to 150 subjects. The principal findings indicated the use of 78 different words to describe different facets of texture in food.

Due to the variety of descriptive words and lack of methods to quantify levels of crispness, crunchiness, or smoothness, instrument-based analyses are used by researchers to define texture characteristics. An instrumental texture analyzer, designed to be affixed with different attachments, such as a 13-pin probe or knife-edge probe, measures the force required to puncture an amount of product or shear through a single leaf, respectively. The procedure for testing varies widely throughout the literature primarily due to the vast variety of substrates being tested. Number of sample replicates is one aspect of testing that varies among studies. For example, Toole et al. (2000) tested up to 360 sample replicates to determine the mechanical properties of lettuce due to vein orientation in the instrument whereas Baur et al. (2005) tested iceberg lettuce after alternative washing methods in sextuplicate. Martin-Diana et al. (2005a; 2005b) have suggested that a minimum of 20-25 samples are necessary to obtain a lognormal curve. Other parameters that have been found to vary between studies focused on testing leafy greens for texture are sample preparation, test speed of the instrument, and the type of probe used

(Toole et al., 2000; Baur et al., 2005; Martin-Diana et al., 2005a; Martín-Diana et al., 2005b; Martin-Diana, Rico et al., 2006; Rico, Martín-Diana et al., 2006; Akbas & Ölmez, 2007; Wei, Zhou et al., 2007).

Analyses of textural properties have used sample preparations of rectangular, 1 cm^2, shredded sections of various gram weights, and whole leaves using a 3, 5, 8, and 10-blade Kramer shear cell (Tay & Perera, 2004; Baur et al., 2005; Martin-Diana et al., 2006; Rico et al., 2006; Akbas & Ölmez, 2007; Wei et al., 2007). Interestingly, research concerning texture analysis of cooked greens was not found in any literature reviewed. Test speeds in various studies ranged from 0.5 mm/s (Toole et al., 2000) to 17 mm/s (Martin-Diana et al., 2005a; Martín-Diana et al., 2005b) with an average test speed of 1.7 mm/s (Tay & Perera, 2004; Baur et al., 2005; Martin-Diana et al., 2006; Rico et al., 2006; Akbas & Ölmez, 2007).

Antioxidant Assessment

Total Phenolic Content. The term 'phenolic' refers to the immense amount of naturally found plant metabolites and by-products including natural phenols and polyphenols. Polyphenols are recognized to be plant defense mechanisms and their production may correlate to UV light exposure, insect herbivory, and bacterial or fungal infections. These compounds all have in common one to many benzene ring structures composed of only carbon, hydrogen, and oxygen (Belitz, 2009). An increased interest concerning these chemicals can be attributed to the recognition of antioxidant properties and possible health benefits derived from consuming polyphenol-rich foods (Asami, Hong et al., 2003; Manach, Scalbert et al., 2004).

Singleton and Rossi (1965) developed the Folin-Ciocalteu assay, which is commonly used to assess total phenolic content in foods. The assay utilizes a Folin-Ciocalteu reagent which reduces phenolic compounds from an extracted plant solution. A recent modification to the

method was proposed by Ronald et al. (2005) to correct for ascorbic acid interference during the reaction.

DPPH. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay is commonly used due to its reproducibility, stability, and simplicity (Katsube, Tabata et al., 2004). DPPH is characterized as a stable free radical because of the delocalization of the spare electron over the molecule, so that the molecules do not bond with each other, which happens with most other free radicals (Molyneux, 2004). This delocalization results in the deep violet color, creating an absorption band at 520 nm using ethanol solutions (Molyneux, 2004). When samples containing hydrogen donating substances extracted in solvent are mixed with DPPH solution, the DPPH is reduced resulting in the loss of the deep violet color (although a residual pale yellow color from the picryl group is normally still present) (Molyneux, 2004). The reduction of DPPH in the assay is therefore a measurement of the oxidizing potential in the samples tested.

Some variations exist in the literature for absorption values used, type of solvent, and the reaction time allowed before reading the microplate. Absorption values presented in research methods range from 515 nm (Bondet, Brand-Williams et al., 1997; Salandanan, Bunning et al., 2009; Aldrich, Salandanan et al., 2010) and 517 nm (Yu, 2001; Zhou, Laux et al., 2004) to 550 nm (Katsube et al., 2004). Solvents used for extraction and dilution purposes were either 100% methanol or ethanol. The reaction time is an important parameter to consider for this test as a longer time will let the reaction go to completion whereas shorter times will give you a snapshot. The range for times found in the literature varied from 3 minutes (Aldrich et al., 2010) to 40 minutes (Zhou et al., 2004).

Sensory Analysis

Among the various tests utilized by researchers and the food production industry is a collection of test methods known as sensory analysis, which uses human subjects in order to gain data to determine acceptance by consumers. As defined by Stone and Sidel (2004), and cited by Lawless (2010), sensory analysis is the "scientific method used to evoke, measure, analyze, and interpret those responses to products as perceived through the senses of sight, smell, touch, taste, and hearing." Each of the actions in the definition are key components to any sensory analysis study. By using randomly numbered labels, controlling the preparation and serving methods, and other variables, the subjects of the evaluation are generating their own judgments about the food without researcher or environmental bias. Test methods based on hedonic scales are used to quantify the measured judgments of the subjects for later analysis of data. Lastly, numbers are only useful when interpretation of the results is made in the context of a hypothesis (Lawless, 2010).

Certain characteristics such as perishable nature, non-homogeneity, color, and texture variability of leafy greens must be carefully considered. The sensory analysis test design and analysis of results in addition to the precautions by the researcher to maximize the accuracy of results obtained include keeping the greens refrigerated until the test, using fresh-harvested samples, and ensuring consistent size and shapes of samples administered. Four common attributes tested using a hedonic scale for leafy greens are appearance, texture, flavor, and overall acceptability (Bunning, Kendall et al., 2010). Bitterness is a fifth attribute (Aldrich et al., 2010); however, in this study bitterness was considered to be a component of flavor and was not tested separately.

Appearance. Appearance is the first sensory characteristic encountered by the consumer when choosing leafy greens and is considered one of the most important attributes for quality

discrimination (Shewfelt, 1990; Lawless, 2010). Any visual imperfections such as discoloration, wilting, or physical contamination can impair perceptions of leafy greens by the consumer (Bunning et al., 2010).

Texture. Fresh vegetable texture is primarily derived from the cell wall structure and turgor pressure of the plant (Waldron, Parker et al., 2003). Sensory adjectives for texture of leafy greens include crispiness and crunchiness, which are associated with the freshness and overall quality of the food (Bourne, 2002). Though most greens are 95% water, they are expected to have a crisp texture when tasted (Bunning, 2007); a lack of crispiness usually signals a loss in the quality or freshness of leafy greens.

Flavor. The flavor of leafy greens is derived from a combination of naturally present substances including, but not limited to, phenolic acids and sugars (Delaquis, Stewart et al., 2000; Manach et al., 2004). These constituents provide leafy greens with their two major flavor profiles: sweetness and bitterness (Delaquis et al., 2000).

Overall Acceptability. Considered to be the sum total of quality attributes in a food, overall acceptability helps in determining the willingness to purchase a product that meets the consumer's expectations (Bunning, 2007). Important factors of consumers' decisions to buy products include food quality, value, experience, product characteristics, and purchase motives (Waldron et al., 2003). Aspects of food quality, experience, and product characteristics can all be affected by the previous sensory attributes of appearance, texture, and flavor, so meeting the expectations of a consumer is important for product sales.

This study was designed to take the research of Fouladkhah et al. (2011) further by examining leafy greens in a cooked state compared to fresh greens. Fouladkhah and others (2011) conducted much research on lesser known leafy vegetables; whereas this study identified

six species that are easily available to the average consumer. Examination of the greens was performed using procedures available that allowed for comparison with prior studies completed at CSU and elsewhere.

CHAPTER III

MATERIALS AND METHODS

This project focused on the chemical, quality, and sensory assessment of a selection of 6 specialty leafy greens (arugula, cherokee lettuce, mache, pac choi, red kale and spinach). Total phenolic content, radical scavenging capacity, color measurement, instrumental texture characteristics, and sensory attributes analysis of raw versus cooked samples of the select taxa were analyzed and evaluated. Greens were chosen based upon relevance in regional community supported agriculture shares or because they are commonly served cooked as well as raw. The six taxa, arugula (*Eruca sativa),* cherokee lettuce (*Lactuca sativa),* mache (*Valerianella locusta),* pac choi (*Brassica rapa* (-Chinensis group), red kale (*Brassica napus* var *pabularia)* and spinach (*Spinacia oleracea),* were cultivated in a greenhouse using organic growing media. Upon harvest, greens were divided; a portion was freeze-dried for chemical evaluation and the remaining plants were kept fresh in a 4°C refrigerator in gallon-size zip-top bags for sensory and instrumental evaluation.

Cultivation of the Leafy Greens. Three planting trials of the six selected taxa were performed on campus in Colorado State University's Plant Growth Facility (PGF) greenhouse in 2011. Plastic planting trays approximately 51x35.5x10 cm were initially washed, sanitized with a 0.5% chlorine bleach solution, and treated with an algicide (Green-Shield® CA, Whitmire Micro-Gen Research Laboratories, Inc., St. Louis, MO) to remove fungal spores. An organic planting mix was used in an 8:2:1 ratio of organic medium (Sunshine® Professional Growing Mix, Sun Gro Horticulture Ltd., Bellevue, WA), worm compost (local source, Fort Collins, CO), and coconut

fiber (Cocotek, General Hydroponics-USA, Sebastopol, CA). The six different taxa were planted in duplicate (2 trays per taxa) in the first trial and triplicate (3 trays per taxa) in the second and third trials, for a total of 8 trays per taxon. Seeds (Johnny's Selected Seeds, Winslow, ME) were sowed according to standard growing recommendations and watered as needed. After germination, the seedlings were thinned to allow space for plant growth, from 5-10 cm apart. During each planting trial period, the PGF staff refrained from using pesticides in order to conform to our organic growing conditions. The trials lasted an average of 35 days from seed to harvest. Indoor climate information, tracked in each greenhouse section, was acquired from the PGF manager. Temperature and humidity data corresponding to each planting trial within the greenhouse are presented in Table 3.1.

Harvesting. Each harvest took place in the morning hours between 7:00 and 10:00 am. Random sampling of 3 to 6 plants from each tray resulted in composite samples representative of all trays. The plants were collected by using scissors to cut plants above soil level in order to reduce damage to the plants. Samples were transferred to labeled, gallon-size, zip-top plastic bags and immediately stored in coolers with ice packs. The coolers were transported to a lab to prepare samples for freeze drying.

Sample Preparation for Total Phenolics and DPPH Analyses. Samples of each taxon were washed with distilled water, if necessary, and blotted dry with paper towels. A target weight of 36 grams of fresh greens was used, however, due to low yield in some taxa and trials the average weight of fresh samples was 25.9 g with a standard deviation of 9.3. Weigh 'boats' in the form of modified paper bags (Target Corporation, Minneapolis, MN) with dimensions 13 x 8 x 8 cm were labeled and used for containing the greens in the freeze dryer. For consistency, vascular tissues were removed from certain taxa to represent the typical edible portion of the green used

for freeze-dried samples. Weight values of empty, labeled, paper bags, and bags with fresh sample greens were recorded and used for calculations of percent dry weight, necessary for total phenolics and DPPH determinations.

Cooking Procedures for Cooked Greens Comparison. Three different cooking methods were utilized: steaming, boiling, and microwaving. A target weight of 36 g fresh weight for each replicate was set; however, due to low yields during certain plant trials of different taxa the average sample weight used was 20.7 ± 11.1 g fresh greens. Inedible vascular tissue was removed and discarded from red kale only; spinach and pac choi are usually cooked and consumed with the entire leaf intact. Steaming equipment consisted of a stainless steel, 7.57 L pot (Chefmate® Multi-Cooker, Target Corporation, Minneapolis, MN) with a steamer insert and glass lid. One liter of distilled water was used as the steam source and replaced between each replicate. Pre-experimental trials determined that pac choi, red kale, and spinach took 6, 2.5, and 2 minutes of steam cooking until tender, respectively. Boiling equipment consisted of a 2.36 liter pot (Chefmate®, Target Corporation, Minneapolis, MN) with a glass lid. One liter of distilled water was brought to a rolling boil, at which time greens were added. Pac choi, red kale, and spinach were cooked for a total of 4, 4, and 2 minutes until tender, respectively. Microwaving methods utilized a microwave oven (Sharp® Carousel, Sharp Electronics Corp., Osaka, Japan) a large, microwave-safe, glass bowl, and microwave-safe plastic wrap. A total of 200 mL distilled water was added to the bowl, raw greens were added, then covered and sealed with plastic wrap. All taxa were cooked for 60 seconds, removed from the microwave oven and stirred, re-covered, and cooked for an additional 60 seconds. All replicates from each cook method were transferred to plastic food strainers immediately after the appropriate length of cooking and set out to drain for 30 minutes. Extra water on the samples was blotted dry with

paper towels, the samples re-weighed, wrapped in plastic wrap, bagged in plastic zip-top bags and frozen in a -20°C freezer.

Freeze Drying Operation. After preparation and weighing, samples were immediately transferred to a Genesis Freeze Dryer (Virtis Inc., Gardner, NY) pre-cooled to -25°C. Samples were left in the freeze dryer for 2 hours to ensure a complete freeze before the vacuum was applied. After 48 hours, refrigeration was discontinued and within 72 hours the samples reached +25°C, signifying complete dryness. The freeze-dried samples were removed, immediately weighed, packaged in separate zip-top bags, and stored in a -20°C freezer.

Grinding of Freeze-Dried Samples. Each freeze-dried replicate was ground using a coffee grinder (Fresh Grind™ Coffee Grinder, Hamilton Beach/Proctor-Silex, Inc., Washington, NC), and mortar and pestle, then sieved with a number 40 sieve (American US STD/ASTM E 11 standard of 1995 equal to 425 μm based on ISO 565 standard of 1987). The resulting uniform samples were transferred to 15 mL conical centrifuge tubes and stored in a -20°C freezer.

Extraction Procedure. Lyophilized ground samples were weighed to 200 ± 1 mg and transferred to labeled 15 mL conical centrifuge tubes. Ten milliliters of 80% acetone (EMD Millipore, Billerica, MA) were added to each tube. Samples were vortexed for 30 seconds and placed in a dark refrigerated rotator for 15 minutes. Samples were vortexed again and centrifuged at 4°C and 3800 revolutions per minute for 15 minutes. After centrifugation, three 1 mL aliquots of supernatant were transferred to separate labeled Eppendorf tubes for total phenolics, DPPH analysis, and one tube was reserved for additional tests if needed. Total phenolics analysis was conducted on the same day of analysis; therefore, one Eppendorf tube was placed in a refrigerator and two of the three tubes were placed into a vacufuge concentrator

with caps open under vacuum for approximately 3 hours. The concentrated samples were stored in a -20°C freezer.

Total Phenolics Assay. The total phenolics assay protocol used was first developed by Singleton and Rossi (1965) and later adapted by Spanos and Wrolstad (1990). Triplicate aliquots of 35 μL of freshly extracted samples were pipetted into a 96-well microplate, which is a modification suggested by Salandanan et al. (2009), and 150 μL of 0.2 Folin-Ciocalteu reagent (VWR, Radnor, PA) was then added. The microplate was vortexed for 30 seconds at 400 revolutions per minute and rested at room temperature for 5 minutes. To each well 150 μL of 75ppm sodium carbonate (EMD Millipore, Billerica, MA) were added, vortexed again for 30 seconds at 400 revolutions per minute then transferred to a 45°C incubator for 30 minutes. After removing the plate from the incubator, it was covered and allowed a 60 minute rest at room temperature. Absorbance values were measured at 765 nm at 25°C using a spectrophotometer (Spectra Max Plus 384, Sunnyvale, CA). The calculation of total phenolics from absorbance values required that 7 gallic acid (Sigma-Aldrich Corporation, St. Louis, MO) standard solutions from 0 to 100 μg/mL (ppm) be loaded with each microplate. Reliability and reproducibility was evaluated using coefficient of variation (C.V.); analyses above a 5% criterion for the replicate were repeated. Total phenolic calculations were based on fresh, dry, and extraction weights utilizing a standard curve correction factor generated using Microsoft Excel 2010.

DPPH$^{\bullet}$ Radical Scavenging Activity. This assay, utilizing the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, was first reported by Yu (2001) and Zhou et al. (2004), then later used by Liu et al. (2007), Salandanan et al. (2009), and Aldrich et al. (2010). Previously vacufuged, extracted samples were reconstituted with 1.0 mL of 5.0 mmol phosphate buffer solution (PBS) (Thermo Fischer Scientific Inc., Waltham, MA) and sonicated for 10 minutes to

make a complete solution. A stock solution of 7.89 mg DPPH (Calbiochem, EMD Millipore, Billerica, MA) and 20 mL 100% methanol was prepared and sonicated for 30 minutes. An additional 80 mL methanol was added to the solution then placed on the plate mixer for 3 hours at 400 rpm and stored in a 4°C refrigerator until used. A trolox solution was used for standard curve analysis and was made by mixing 50 mL of 5 mmol PBS with 12.52 mg trolox (Calbiochem, EMD Millipore, Billerica, MA). The standards used on the microplates were made by mixing volumes of stock trolox solution with PBS solution, creating standards with 0, 20, 30, 40, 50, 60, 70, 80, and 90 μmol trolox. Absorbance was read in the spectrophotometer using Softmax Pro software at 515 nm and adjusted to 0.95 AU by diluting the DPPH stock solution with additional methanol. To the microplates, 15 μL of reconstituted samples were mixed with 285 μL of the DPPH solution in the microplate, held for exactly 3 min at 25°C, and read by the spectrophotometer. The results were determined by regression from the trolox standard curve utilizing percent dry matter of corresponding extracted samples and absorption values, and expressed as μmol TEAC kg^{-1} DW.

Color Measurement. To measure the color attributes of leafy green samples, a spectrocolorimeter (Hunterlab Colorflex, Firmware versions 1.1, Reston, Virginia) was used with a measuring aperture of 36 mm. Calibration was accomplished prior to each trial with manufacturer supplied white, green, and black tiles. A circular glass cuvette was used to contain the sample leaves for measurements. A random sampling of at least 6 leaves per test replicate were arranged in the bottom of the cuvette, placed on the reading lens, and tested. A single reading of each replicate (6 replicates per cultivar) produced values of L* (lightness), a* (redness), and b* (yellowness). The values for L* range from 0-100, representing black to perfect white, respectively. Values for a* and b* were either positive or negative where positive

for a* represents red color and negative represents green hues. Positive b* values represent yellow and blue when negative. Sample hue angle and chroma, which are three dimensional calculations in standard color space, were found using the formulas hue angle = tan-1(b*/a*) (Little, 1975) and chroma = (a*2+b*2)1/2 (Han et al., 2004).

Analysis of Textural Properties

Variations in the physical characteristics and vascular tissue orientation in assorted leafy greens required that two texture tests be administered using different probes and procedures. The firmness characteristic of the fresh cultivars were evaluated using a 13-blade multipuncture probe (A/MPP) and a knife-edge probe (HDP/BS) was employed to evaluate the structural integrity of whole single leaves, both fresh and cooked. These experiments were carried out using a TA-XT2 Texture Analyzer (Texture Technologies, Scarsdale, NY).

Multipuncture 13-Blade Probe. The multipuncture 13-blade probe test methods used were described by Fouladkhah et al. (2011), which were adapted from Wei et al. (2007), Akbas and Olmez (2007), Rico et al. (2006), and Baur et al. (2005). Six instrumental replicates were used to test the textural characteristics of each sample. All portions of the greens including vascular and photosynthetic material, as well as inner and outer leaves were used for testing. Thirty grams of whole leaves were lightly packed into the cylindrical cell of the instrument. The output of testing each sample was expressed as the maximum force (grams) to press the sample a total of 32 mm with a 13-blade multipuncture probe. Test speed, pre-speed, post-speed, distance, return distance, and cell load of this test were modified to 1.7 mm/sec, 5.0 mm/sec, 8.3 mm/sec, 32 mm, 140 mm, and 5kg, respectively.

Knife-Edge Probe. This procedure was adopted from research by Martin-Diana et al. (2006) and Tay and Perera (2004). As with the 13-blade multipuncture probe test, 6 replicates

were chosen from each sample of leafy green. The sample replicate consisted of a single leaf representative of the average size of samples for that cultivar. Both fresh and cooked samples were subjected to this test. The sample leaves were placed on the instrument platform and held down by hand so the instrument could shear the sample. Care was taken to only apply downward pressure so the instrument output would reflect the force from the knife and not user error. The instrument reported the maximum force for rupture of the leaves (grams). Test speed, pre-speed, post-speed, distance, return distance, and cell load of this test were modified to 4.5 mm/sec, 5.0 mm/sec, 8.3 mm/sec, 32 mm, 140 mm, and 5kg, respectively.

Sensory Analysis. Greens used for sensory analysis were harvested and stored in a 4°C refrigerator overnight until testing. A 9-point hedonic scale was created (Page 39) which allowed subjects to rate 4 aspects (appearance, flavor, texture, and overall acceptability) of the greens based upon whether the sample was highly acceptable, highly unacceptable, or somewhere in between. Subjects were also asked to rank the samples in order of which they preferred most to least, where 1 was most preferred. This sensory score sheet was labeled with randomly generated, 3-digit numbers to represent the different cultivars. A sample score sheet is included in the Appendix. Sensory tests were conducted on 4 fresh (uncooked) greens: arugula, cherokee lettuce, mache, and spinach. Greens cooked for sensory analysis were prepared by steaming each cultivar for 2 minutes. The cooked greens were pac choi, red kale, and spinach. One to 2 fresh leaves and 3 to 4 cooked leaves, depending on size, were placed in labeled sample cups in randomized order corresponding to the order of samples on the score sheet. Untrained panelists were then asked to evaluate the first sample on their left, analyze it, write down their score, cleanse their palate with an unsalted cracker and distilled water, and repeat these steps for

the remaining samples. A total of 50 subjects evaluated the fresh uncooked greens and 51 subjects evaluated the cooked greens as a consumer panel.

Statistical Analyses

Statistical analyses were performed on the data obtained for general reporting and exploring associations for significance. For general reporting such as means, standard deviations, and graph creation, Microsoft Excel 2010 (Redmond, WA) was used. For higher functions such as ANOVA analyses, the data were evaluated by using SAS 9.2 (SAS Institute, Inc., Cary, NC). Mean separation of output from SAS was evaluated using a least significant difference (LSD)-based approach to compare multiple pairs of means.

Total Phenolics and DPPH. One of the primary objectives of this study was to determine if differences existed between fresh and cooked samples through total phenolic content and DPPH assessments. Total phenolic content and DPPH were tested for in all greens, including those cultivars that were not cooked (arugula, cherokee lettuce, and mache). Statistical analysis of the fresh greens included 3 trials, all 6 taxa, and 3 test reps per sample (n=54) for total phenolics based on fresh weight and dry weight, and DPPH based on dry weight. Fresh versus cooked comparisons utilized 3 cultivars (pac choi, red kale, and spinach), 3 trial sets, 4 different cooking methods (fresh, steamed, boiled, microwaved), and 3 test replications for each sample. A total of 108 observations were possible; however, due to decreased yield of spinach in the first and second trials, and a lack of samples for boiled pac choi and red kale in the first, and first and second trials, respectively, only 81 observations were evaluated. Split-plot ANOVA tests on the fresh and fresh versus cooked comparison were performed using SAS.

Color Assessment. Color analysis data were also compared by separating fresh sample data, then comparing fresh sample data with cooked sample data. Ninety-three observations

were used in the fresh only analysis containing all 6 cultivars, but without designating different trials. The L*, a*, and b* values were compared separately between cultivars using a one-way ANOVA test. A total of 122 observations were included in the fresh versus cooked comparison, utilizing the 3 cooked cultivars and 4 states (fresh and following three cooking methods). A one-way ANOVA test using SAS was also performed on this data to explore the possibility of significance between four states and six cultivars.

Texture Analysis. Data for texture analysis testing were divided by cultivar (all 6 were evaluated), state (fresh, steamed, boiled, and microwaved), and probe used (13-pin and knife-edge probe). The 13-pin probe data consisted of 36 observations; 6 values collected from each of the 6 cultivars. A one-way ANOVA test was used to determine significant differences between the fresh cultivars for this probe. The knife-edge probe data contained 90 observations that included all 4 states. These values were evaluated for significant differences between cultivar and state using a two-way ANOVA test performed in SAS.

Sensory Analysis. Data for sensory analysis were split between fresh and cooked greens. The fresh greens evaluated were arugula, cherokee lettuce, mache and spinach. Fifty test subjects participated and from this data only 1 observation could not be used because it was missing from a subject's sheet. The cooked greens were pac choi, red kale, and spinach. Though spinach appeared in both fresh and cooked sensory evaluations, a comparison was not made due to different subjects testing one state or the other, not both at the same time. Additionally, all greens for cooked analysis were steamed so a comparison between states (steamed, boiled or microwaved) could not be made. Sensory data for cooked greens included 51 test subjects who evaluated 4 different attributes of the greens. One-way ANOVA tests were

performed separately for the fresh and cooked greens using SAS. Data collected on ranking of the greens by each test subject were compiled and computed using Microsoft Excel 2010.

CHAPTER IV

RESULTS AND DISCUSSION

Chemical, quality, and sensory assessment were conducted on a selection of 6 specialty leafy greens (arugula, cherokee lettuce, mache, pac choi, red kale and spinach). Total phenolic (TP) content, radical scavenging capacity (DPPH), color measurement, instrumental texture characteristics, and sensory attributes of raw versus cooked (boiled, microwaved, or steamed) samples of the select taxa were analyzed and evaluated. All 6 varieties were cultivated in a greenhouse using organic planting medium. Samples of the greens were freeze-dried as raw or post-heat treatment, and then sample extracts were analyzed for total phenolics compared to gallic acid standards and radical scavenging using DPPH compared to trolox standards. Fresh and cooked comparisons were tested for color differences using a HunterLab ColorFlex spectrophotometer and changes in texture utilizing a TA-XT2 texture analyzer. Consumer (n=50; n=51) sensory analysis was administered using a 9-point hedonic scale.

Antioxidants. Antioxidant testing, including fresh and dry weight total phenolics, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Table 4.1) revealed that mache had higher levels than the other five raw, uncooked cultivars ($p<0.05$). Compared to mache, arugula, pac choi, red kale and spinach had lower levels of total phenolics ($p<0.05$) based on fresh weight and antioxidant capacity as determined by DPPH. Cherokee lettuce was found to have lower total phenolic content based on fresh weight than mache, but more than the other greens sampled ($p<0.05$).

Levels of antioxidants in cooked samples (Table 4.2) varied without any significant relationships within fresh and boiled greens among the three cultivars and test parameters

($p<0.05$). Microwaved samples exhibited significant differences among types of greens, revealing red kale as having the most antioxidant potential, which was also true for steamed greens. Boiling tended to reduce ($p<0.05$) the total phenolic and DPPH content for pac choi and red kale compared to fresh determinations, with an exception of the boiled red kale DPPH comparison. Microwaving and steaming methods increased the total phenolic content based on dry weight for red kale as compared to fresh samples. These two cooking methods conveyed similar comparisons for each cooked cultivar by either decreasing or in some cases increasing the amount of antioxidants determined versus the uncooked greens. Roy et al. (2009) theorized that antioxidant potential of post-heat treatment samples is varied and depends on the type of product being tested. Yamaguchi et al. (2001) showed that inactivation of oxidative enzymes by boiling contributed to the suppression of antioxidant potential in some vegetables. However, Pellegrini et al. (2010) demonstrated that the TEAC of cooked broccoli samples increased with boiling and steaming, but microwave oven cooking led to a significant decrease. It has also been shown that heat treatment can shrink products, exuding water and concentrating the subsequent dry matter (Lisiewska et al., 2011), which can lead to increased total phenolics and antioxidant levels. In our study, analysis of spinach for total phenolics and DPPH did not reveal any differences between cooking methods ($p>0.05$). Conversely, Turkmen et al. (2005) found that DPPH values significantly increased for all samples subjected to boiling, microwaving and steaming. Also, Lisiewska et al. (2011) demonstrated that total amino acids of spinach samples increased for cooked and blanched samples as compared to fresh spinach. The lack of significance and large standard deviation among spinach samples could have been due to small sample sizes ($n=9$) resulting from decreased yield of this crop.

Color Analysis. Comparison of color for fresh greens (Figure 4.1 and Table 4.3) revealed varying differences among cultivars. The range of means for the L* parameter for

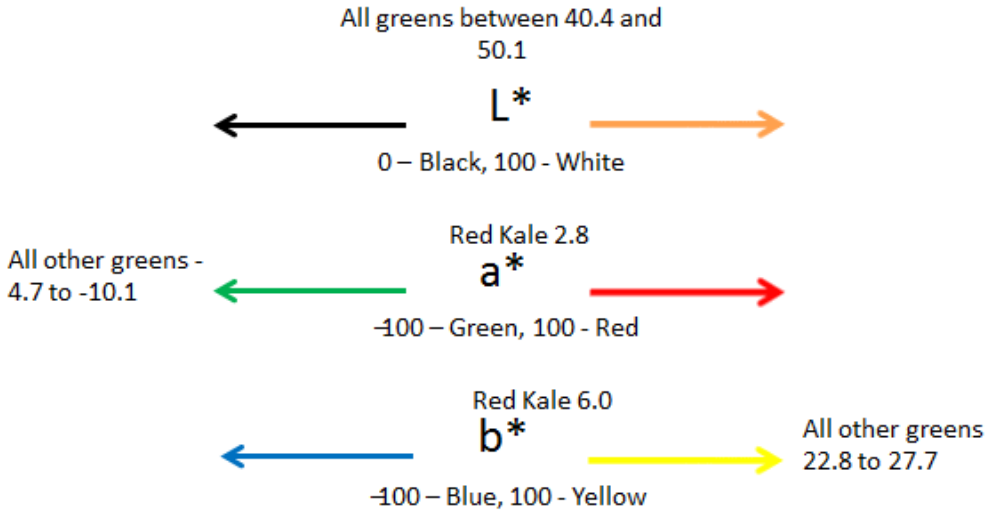

FIGURE 4.1. VISUAL REPRESENTATION OF FRESH COLOR RESULTS

lightness extended from 40.4 for red kale to 50.1 (lighter) for pac choi. A significant difference between red kale and other greens was found for determinant a*, where red kale had a slight red hue and other cultivars exhibited green values. Mache, pac choi, and spinach were found to have the highest green values ($p<0.05$) from the 6 cultivars tested, which follows visual observations of the red pigmentation of arugula, cherokee lettuce, and red kale. Testing of b* showed that only red kale was significantly different, with a lower level of yellow hue than other cultivars. No significant differences were found among the b* values of the other 5 greens.

Color analysis of cooked versus uncooked greens (Table 4.4) revealed fresh samples were higher in L* values (or lighter), which was not the result of testing reported by Mazzeo et al. (2011) where spinach samples increased in L* values when subjected to boiling and steaming compared to fresh samples. Pac choi was significantly lighter than other greens for fresh and all cooking methods. Values of a* showed significant differences in green coloration between cooked and uncooked samples within cultivars, and interestingly, steaming pac choi brought out

a slightly red hue, or loss of green hue (positive value), in the samples tested. Pellegrini et al. (2010) tested different *Brassica* samples (broccoli, Brussels sprouts, and cauliflower) pre- and post-cooking treatments and found mixed results for a* values between cooking methods. For most data, the values between cooked and raw samples for coordinate b* were significantly decreased; however, cooked red kale samples increased yellow hue in all three cooking methods (boiling, microwaving, steaming) as compared to fresh red kale. Spinach decreased b* values for all cooking methods tested, which follows results of Mazzeo et al. (2011). For cultivars evaluated for coordinate b*, all red kale samples were significantly lower than both pac choi and spinach.

Texture. Texture differences between fresh samples (Table 4.5) tested with the 13-pin probe showed that spinach required the most force ($p<0.05$) to puncture the samples a distance of 32 mm compared to the other five cultivars. The means of force between spinach and the second highest value, pac choi, were 184.52g and 133.58g, respectively. Samples with lower puncture values had less resistance to puncture than spinach. Analysis of texture using the knife-edge probe revealed a significant difference for rupture testing between pac choi and the other greens, even with a large standard deviation. Values of arugula, lettuce, and pac choi were similar to the results found by Fouladkhah et al. (2011) using the same equipment and test parameters. Pac choi required much greater force to rupture than the other greens evaluated. No differences among most other greens were observed ($p>0.05$), though mache and arugula were determined to have the lowest rupture values (more tender) than the other greens using this particular test method.

After cooking treatments, the force required to rupture leaves of red kale using the knife-edge probe actually increased (Table 4.6), though only significantly ($p<0.05$) for steaming,

compared to uncooked kale. These results are most likely due to the increase in pliability of the greens once cooked, leading to a stretching which increased the force required to shear the greens. The force required to rupture cooked spinach samples decreased by two-fold compared to the uncooked reference. Between cultivars, all mean values of fresh and cooked spinach compared to pac choi were significantly lower in this analysis. For microwaved and steamed trials, all three cultivars were significantly different from each other. This testing also revealed large variations (standard deviation) among results. This most likely occurred from the small sample size (n=6), which made determining significance among cooking methods difficult. There is a lack of research comparing cooked versus fresh greens using instrumental texture analysis, which also makes comparison to other studies difficult. However, several studies have been conducted on various microbial and storage treatments where length of storage time on texture were tested. Some, such as a study by Gomes et al. (2008) researching electron beam irradiation on spinach quality, found mixed results on texture values throughout the 15-day testing period. One study by Martin-Diana et al. (2005a) demonstrated that a loss of turgor, or moisture in the cells, increased elasticity of samples and led to the maximum load force increasing as temperature of treatments and storage days increased.

Sensory. Sensory testing was based on a 9-point hedonic scale, where 1 was the 'least acceptable' and 9 was the 'most acceptable' value given to each of four characteristics: flavor, appearance, texture and overall acceptability. Fresh samples included arugula, Cherokee lettuce, mache, and spinach. Cooked samples included only pac choi, red kale, and spinach. Fresh sample testing revealed a few significant ($p<0.05$) findings including lettuce being the least accepted visually and arugula being least accepted for flavor, texture, and overall acceptability traits. Similarly to research by Fouladkhah (2011), panelists noted a spicy flavor corresponding

to the arugula sample, which seemed to contribute to the deciding factor of overall acceptability. Using cooked samples, sensory testing showed pac choi as most acceptable ($p<0.05$) for appearance, texture, and overall acceptability, and significantly more acceptable than spinach in flavor. This result, according to panelists' comments, is most likely because pac choi maintained its crunchy characteristic even after cooking, whereas spinach becomes soft/mushy and kale samples had less crunch and were noted as having little to no flavor by most panelists. The least acceptable values for each characteristic were always associated with either red kale or spinach.

Conclusions. Comparisons of fresh greens indicated mache had higher ($p<0.05$) total phenolics and radical scavenging ability than all other cultivars. Cooked samples revealed that spinach values for each test did not differ ($p>0.05$) from fresh samples and red kale contained more total phenolics in microwaved and steamed samples, but less ($p<0.05$) in boiled samples, than the fresh comparisons. Other significant findings included reductions ($p<0.05$) in lightness of cooked samples and cooked spinach samples in all heating treatments were observed to be more tender than fresh samples. This research helps to fill information gaps which exist in leafy greens research. Many studies focus on one cultivar using one testing method, but little research has been conducted on these types of greens using several test methods to obtain comparable data.

CHAPTER V

RECOMMENDATIONS FOR FUTURE STUDIES

This study focused on determining differences between fresh and cooked leafy greens in terms of antioxidant capacity, total phenolics, and other quantitative and qualitative testing. One limitation of this research was that only three greens were included in the cooked analysis. It would be more beneficial to have a single control cultivar and more greens used for cooked comparisons. Also, allowing the greens to grow to maturity instead of a 'microgreen' state or purchasing the greens directly from a grocery store where most consumers obtain their greens would better reflect as-consumed nutrition, although shelf-life and taxa factores would be impossible to control. Future studies using this research could elaborate on the total phenolics and antioxidant capacity by identifying specific compounds and antioxidants that are directly affected by cooking methods.

With the introduction of the Food Safety Modernization Act (FSMA) by the US Food and Drug Administration, important work can be done developing food safety plans for areas such as the greenhouses on campus and the agricultural experiment station. In time the FDA will require such plans for all food producers, including farms, and such work will help maintain the safety of the food coming from CSU.

TABLE 4.1: MEAN VALUES* FOR TOTAL PHENOLICS AND DPPH OF 6 TYPES OF FRESH LEAFY GREENS

Leafy Green	Total Phenolics (mg GAE/100g fresh weight)	Total Phenolics (mg GAE/g dry weight)	DPPH (µmole TEAC/g dry weight)
Arugula	$29.7 \pm 1.7_{C}$	$458.8 \pm 133.0_{AB}$	$50.9 \pm 12.8_{B}$
Cherokee Lettuce	$46.9 \pm 8.6_{B}$	$311.7 \pm 68.2_{B}$	$46.8 \pm 11.2_{B}$
Mache	$62.1 \pm 7.9_{A}$	$702.7 \pm 203.9_{A}$	$71.7 \pm 4.0_{A}$
Pac Choi	$24.0 \pm 3.1_{C}$	$248.9 \pm 120.3_{B}$	$56.5 \pm 17.3_{B}$
Red Kale	$27.2 \pm 2.1_{C}$	$344.9 \pm 102.0_{B}$	$46.8 \pm 9.1_{B}$
Spinach	$19.8 \pm 1.5_{C}$	$260.1 \pm 41.4_{B}$	$26.3 \pm 6.0_{C}$

*Each value within a column is a mean of 27 determinations and when followed by different letters is different ($p<0.05$).

TABLE 4.2: MEAN VALUES* FOR TOTAL PHENOLICS AND DPPH OF 6 TYPES OF FRESH OR COOKED LEAFY GREENS

Leafy Green	**Total Phenolics** (mg GAE/100g fresh weight)			
	Fresh	Boiled	Microwaved	Steamed
Pac Choi	$24.0 \pm 3.1_{A,X}$	$14.8 \pm 0.7_{A,Y}$	$21.8 \pm 0.9_{B,X}$	$20.4 \pm 2.7_{B,XY}$
Red Kale	$27.2 \pm 2.1_{A,X}$	$13.7 \pm 0.3_{A,Y}$	$30.0 \pm 4.2_{A,X}$	$28.7 \pm 3.6_{A,X}$
Spinach	$19.8 \pm 1.5_{A,X}$	$23.0 \pm 2.0_{A,X}$	$24.0 \pm 0.7_{AB,X}$	$23.7 \pm 0.8_{AB,X}$
Leafy Green	**Total Phenolics** (mg GAE/g dry weight)			
	Fresh	Boiled	Microwaved	Steamed
Pac Choi	$248.9 \pm 120.3_{A,X}$	$86.4 \pm 9.6_{A,Y}$	$185.6 \pm 13.2_{B,XY}$	$154.1 \pm 31.3_{B,XY}$
Red Kale	$344.9 \pm 102.0_{A,Y}$	$164.8 \pm 3.6_{A,Z}$	$491.04 \pm 71.3_{A,X}$	$460.3 \pm 92.9_{A,XY}$
Spinach	$260.1 \pm 41.4_{A,X}$	$217.7 \pm 19.1_{A,X}$	$228.1 \pm 6.9_{B,X}$	$238.7 \pm 7.6_{AB,X}$
Leafy Green	**DPPH** (µmole TEAC/g dry weight)			
	Fresh	Boiled	Microwaved	Steamed
Pac Choi	$56.5 \pm 17.3_{A,X}$	$24.2 \pm 3.81_{A,Y}$	$56.1 \pm 11.99_{AB,X}$	$49.3 \pm 10.4_{AB,X}$
Red Kale	$46.8 \pm 9.1_{A,XY}$	$21.3 \pm 3.8_{A,Y}$	$65.8 \pm 4.2_{A,X}$	$64.0 \pm 6.5_{A,X}$
Spinach	$26.3 \pm 6.0_{B,X}$	$20.7 \pm 3.3_{A,X}$	$27.7 \pm 1.5_{B,X}$	$23.4 \pm 1.9_{B,X}$

*Each value within a row is a mean of 27 determinations with the exception of boiled pac choi (n=18), boiled red kale (n=9), and all cooked spinach values (n=9) due to low yield for these greens. Values in columns (a,b,c) and rows (x,y,z) are different ($p<0.05$) when the corresponding letters are different.

TABLE 4.3: MEAN COLOR VALUES* OF 6 TYPES OF FRESH LEAFY GREENS

Leafy Green	L* - value	a* - value	b* - value
Arugula	$43.4 \pm 5.5_{B}$	$-6.8 \pm 2.6_{B}$	$22.8 \pm 3.1_{A}$
Cherokee Lettuce	$47.3 \pm 6.3_{A}$	$-4.7 \pm 3.4_{C}$	$24.9 \pm 5.2_{A}$
Mache	$44.1 \pm 5.6_{B}$	$-10.1 \pm 0.8_{A}$	$25.0 \pm 1.3_{A}$
Pac Choi	$50.1 \pm 5.0_{A}$	$-9.8 \pm 1.4_{A}$	$27.1 \pm 2.1_{A}$
Red Kale	$40.4 \pm 5.6_{B}$	$2.8 \pm 5.8_{D}$	$6.0 \pm 4.4_{B}$
Spinach	$46.4 \pm 4.5_{AB}$	$-9.9 \pm 0.7_{A}$	$27.7 \pm 2.0_{A}$

*Each value is a mean of 18 determinations, except for mache, which is a mean of 9 determinations. Values (mean ± standard deviation) within a column in each data set followed by different letters are different ($p<0.05$).

TABLE 4.4: MEAN COLOR VALUES* OF 6 TYPES OF FRESH OR COOKED LEAFY GREENS

Leafy Green	**L* - value**			
	Fresh	Boiled	MW	Steamed
Pac Choi	$50.1 \pm 5.0_{A,X}$	$29.8 \pm 2.0_{A,Y}$	$29.0 \pm 3.1_{A,Y}$	$28.9 \pm 2.3_{A,Y}$
Red Kale	$40.4 \pm 5.6_{C,X}$	$24.7 \pm 0.6_{B,Y}$	$25.5 \pm 2.1_{B,Y}$	$22.8 \pm 1.5_{B,Y}$
Spinach	$46.4 \pm 4.5_{B,X}$	$23.2 \pm 1.5_{B,Y}$	$24.1 \pm 2.1_{B,Y}$	$24.1 \pm 1.7_{B,Y}$
Leafy Green	**a* - value**			
	Fresh	Boiled	MW	Steamed
Pac Choi	$-9.8 \pm 1.4_{A,X}$	$-8.3 \pm 0.7_{B,X}$	$-9.7 \pm 1.6_{A,X}$	$4.9 \pm 0.9_{C,Y}$
Red Kale	$2.8 \pm 5.8_{B,Z}$	$-7.3 \pm 1.2_{B,X}$	$-3.7 \pm 2.6_{B,Y}$	$-1.9 \pm 1.4_{B,Y}$
Spinach	$-9.9 \pm 0.7_{A,Y}$	$-12.9 \pm 0.4_{A,X}$	$-12.4 \pm 1.0_{A,XY}$	$-12.6 \pm 0.9_{A,X}$
Leafy Green	**b* - value**			
	Fresh	Boiled	MW	Steamed
Pac Choi	$27.1 \pm 2.1_{A,X}$	$23.1 \pm 3.3_{A,Y}$	$22.6 \pm 3.4_{A,Y}$	$19.9 \pm 1.9_{A,Z}$
Red Kale	$6.0 \pm 4.4_{B,Z}$	$17.1 \pm 2.8_{B,W}$	$12.6 \pm 1.6_{B,X}$	$9.5 \pm 2.6_{B,Y}$
Spinach	$27.7 \pm 2.0_{A,X}$	$23.5 \pm 0.8_{A,Y}$	$23.1 \pm 1.8_{A,Y}$	$22.2 \pm 1.8_{A,Y}$

*Each fresh value is a mean of 18 determinations; cooked values are means of 9 determinations. Values in columns (a,b,c) and rows (w,x,y,z) are significantly different ($p<0.05$) when the corresponding letters are different. MW = microwaved.

TABLE 4.5: MEAN TEXTURE ANALYSIS VALUES* OF 6 TYPES OF FRESH LEAFY GREENS USING TWO ATTACHMENTS

Leafy Green	13-Pin Probe Puncture (g)	Knife-Edge Probe Rupture (g)
Arugula	$127.43 \pm 7.17_{B}$	$291.0 \pm 107.7_{BC}$
Cherokee Lettuce	$129.55 \pm 14.44_{B}$	$447.3 \pm 62.2_{B}$
Mache	$120.47 \pm 17.12_{BC}$	$228.5 \pm 50.5_{C}$
Pac Choi	$133.58 \pm 18.13_{B}$	$951.2 \pm 353.7_{A}$
Red Kale	$107.62 \pm 16.53_{C}$	$522.3 \pm 88.3_{B}$
Spinach	$184.52 \pm 17.33_{A}$	$446.2 \pm 80.3_{B}$

*Each value is a mean of 6 determinations. Values (mean ± standard deviation) within a column in each data set followed by different letters are different ($p<0.05$).

TABLE 4.6: MEAN TEXTURE ANALYSIS VALUES* OF 6 TYPES OF FRESH OR COOKED LEAFY GREENS USING A KNIFE-EDGE PROBE

Leafy Green	**Knife-Edge Probe** Rupture, g			
	Fresh	Boiled	Microwaved	Steamed
Pac Choi	$951.2 \pm 353.7_{A,XY}$	$726.97 \pm 122.10_{A,Z}$	$1088.63 \pm 122.47_{A,X}$	$908.12 \pm 143.29_{A,Y}$
Red Kale	$522.3 \pm 88.3_{B,Y}$	$562.45 \pm 134.52_{A,XY}$	$647.67 \pm 202.99_{B,XY}$	$728.73 \pm 185.41_{B,X}$
Spinach	$446.2 \pm 80.3_{B,X}$	$222.15 \pm 51.36_{B,Y}$	$238.13 \pm 52.98_{C,Y}$	$220.48 \pm 48.65_{C,Y}$

*Each value is a mean of 6 determinations. Values in columns (a,b,c) and rows (x,y,z) are different ($p<0.05$) when the corresponding letters are different.

TABLE 4.7: "ACCEPTABLE AND/OR HIGHLY ACCEPTABLE" AND "UNACCEPTABLE AND/OR HIGHLY UNACCEPTABLE" PERCENTAGES OF SELECTED LEAFY GREENS ATTRIBUTES.

	Appearance		**Flavor**		**Texture**		**Overall Acceptability**	
	% Acceptable*	% Unacceptable**	% Acceptable	% Unacceptable	% Acceptable	% Unacceptable	% Acceptable	% Unacceptable
Arugula†	68.0	0.0	34.0	26.0	64.0	6.0	44.9	18.4
Lettuce†	44.0	4.0	42.9	0.0	64.6	0.0	60.4	0.0
Mache†	79.6	0.0	56.0	0.0	64.0	0.0	69.4	2.0
Spinach†	86.0	0.0	58.0	0.0	74.0	0.0	61.2	0.0
Spinach‡	64.7	2.0	36.0	4.0	39.2	58.8	45.1	3.9
Pac Choi‡	92.2	0.0	51.0	3.9	64.7	0.0	66.6	2.0
Red Kale‡	35.3	5.9	35.3	2.0	43.1	7.8	37.3	2.0

* Percent of ratings from panelists as acceptable or highly acceptable in a 9-point hedonic test.

** Percent of ratings from panelists as unacceptable or highly unacceptable in a 9-point hedonic test.

† Greens were administered in the sensory test as fresh/uncooked.

‡ Greens were administered in the sensory test as cooked.

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APPENDIX I: IRB DOCUMENTS

Colorado State University

Institutional Review Board

REQUEST FOR EXEMPTION
for the Use of Human Subjects in Research

Research involving surveys, interviews, the use of existing data, taste and food quality evaluation and standard educational research generally fall within the exempt category. Projects that are considered exempt must be less than minimal risk to the participants.

An IRB Administrator or IRB member must review the application and determine that the project is exempt from expedite or full review. Once a protocol has been determined to be exempt, the protocol will not be monitored by the IRB on an ongoing basis. If the research qualifies for exemption, a notification will be forwarded to the PI. Please keep the notification for documentation that the project is considered exempt and does not need continuing review by the IRB. The PI must notify the IRB Administrator if any proposed changes to the research will be made. At that time, an IRB Administrator or IRB member will determine whether the status of the research has changed. Any complaints that may have been received during the course of the research must also be reported.

A determination that research is exempt does not absolve the investigators from ensuring that the welfare of human subjects participating in research activities is protected, and that methods used and information provided to gain subject consent are appropriate to the activity.

> Data collection may not begin until the PI has been notified that the project has been determined to be exempt.

The six exempt categories can be found at: http://web.research.colostate.edu/ricro/hrc/forms.aspx
Below are exceptions that are NOT considered exempt.

Exceptions:
Exemptions will not be granted for the following circumstances:

- Research involving prisoners. All prisoner research is reviewed by the full IRB.
- Research that includes both exempt and non-exempt activities cannot be determined to be exempt and should be submitted for expedite or full review.

■ Research involving coercion, undue influence, deception, risks or discomforts greater than encountered in daily life.

■ Research conducted outside of the United States.

NOTE: If the project is determined not to be Exempt, the protocol will need to be entered into eProtocol and sent out for Expedite or Full review electronically.

Colorado State University

Institutional Review Board (IRB)

REQUEST FOR EXEMPTION

(Administrative Review) for the Use of Human Subjects in Research

APPLICATION INSTRUCTIONS: Complete the 2 parts below, submit to address at the end of this form.
NOTE: The form is protected for your convenience to tab through the form. If you need to unprotect the document, please contact Janell.Barker@Research.colostate.edu.

PART I: GENERAL INFORMATION

Title of Project: Increasing the Availability, Safety, and Acceptance of Diverse Leafy Green Vegetables

Principal Investigator (PI): Marisa Bunning **email:** mbunning@cahs.colostate.edu
Department: Food Science & Human Nutrition
Campus mail code: 1571 **phone:** (970) 491-7180

Co- Principal Investigator (Co-PI): Martha Stone **email:** stone@cahs.colostate.edu
Department: Food Science & Human Nutrition
Campus mail code: 1571 **phone:** (970) 491-6772

Source of funding: Colorado Agricultural Experiment Station

If externally funded, include PASS number if known: enter pass number here
Please provide a copy of the grant proposal, if applicable.

Indicate the anticipated start and ending date for this project.
Start: July 1, 2010 **End:** June 30, 2012

Rank of PI: ☒ Faculty ☐ PhD student ☐ Masters Student ☐ Undergraduate ☐ Other: describe 'other' here

Rank of Co-PI: ☒ Faculty ☐ PhD student ☐ Masters Student ☐ Undergraduate ☐ Other: describe 'other' here

PART II: PROJECT DESCRIPTION

thesis/dissertation methods section if applicable.

This project focuses on expanding the production and promoting the safe consumption of underutilized leafy greens in Colorado. It includes investigating cultivation, nutritional, sensory, and culinary characteristics of six selected leafy vegetables. The outreach plan involves the development of child-friendly leafy vegetable preparation methods targeted at parents of pre-schoolers as well as recommended preservation methods aimed at community supported agriculture participants. Participants will be recruited to assess possible differences in flavor, appearance, texture, aroma, and overall acceptability of leafy greens prepared and preserved in different ways. Parents of preschool children will be recruited to participate in a series of culinary workshops that involve preparing dishes that contain various types of leafy green vegetables.Members of Community Supported Agriculture (CSA) programs will be recruited to participate in food preservation workshops.

2. **Describe the participant population, including age range and inclusion/exclusion criteria. State how many will be recruited.**

   A maximum of 200 participants will be recruited to take part in 3 separate activities. To evaluate the sensory attributes of 6 types of leafy green vegetables, 40 participants will be recruited from CSU students, faculty and staff. Eighty parents of preschool aged children will be recruited to participate in culinary workshops. For food preservation workshops, 80 members of local CSAs will be recruited. Since there will be workshops on different types of food preservation (freezing, dehydration) some partipants may attend more than one workshop.

3. **Describe how potential participants will be approached about the research and how informed consent will be obtained. Alternatively, provide an explanation of why informed consent or documented informed consent will not be obtained. Please attach a copy of the consent document, if applicable.**

   To recruit participants for the sensory study, flyers will be posted on CSU campus and emailed to the Department of Food Science and Human Nutrition and Department of Horticulture and Landscape Architecture. To recruit parents of preschool aged children, flyers will be distributed to local preschools, daycare centers, and an announcement will be posted on CSU Today webpage. To recruit CSA members to partipate in food preservation workshops, emails will be sent to managers of local CSAs.

4. **Describe how identifying information will be recorded and associated with the data, i.e., codes. Alternatively, provide details on how study data will be collected and stored anonymously (i.e., without a code or identifiers linking the data to the participants' identity.)**

   Sensory evaluation scoresheets will be coded with numbers and names will not be attached to any of the information collected. Only first names will be used during the culinary and preservation workshops and no names will be attached to any of the information collected.

5. **Describe all study procedures, including topics that will be discussed in interviews and/or surveys. Please attach the interview questions or survey questions, if applicable.**
   The sensory evaluations will take place at the Gifford Building on the campus of Colorado State University. Participants will taste samples of leafy green vegetables prepared in a food laboratory in the Department of Food Science and Human Nutrition. Greens will be presented raw or cooked according to the traditional method used for that type of leafy green, for example if collard greens are sampled, those would be cooked. On a paper scoresheet, partipants will score qualities such as visual appearance, flavor, texture, sweetness, and overall acceptability using 9-point hedonic scales ranging from unacceptable to very acceptable. The sample testing session will not take more than 15 minutes. Participants will be given a complimentary beverage at the completion of the testing session. A sample scoresheet is attached.
   Culinary workshops will be conducted in the FSHN Nutrition Center on the first floor of the Gifford Building and food preservation workshops will be conducted in the kitchen facilities in on the second floor of the Gifford Building. For culinary and food preservation workshops, participants will be asked to complete workshop evaluation forms. A workshop evaluation is attached.

6. **Which exemption category does your study fall in? (see next page of this document for description of categories)**
   Category 6

As the principal investigator, I assure the IRB that all procedures performed under this project will be conducted exactly as outlined in this form and that any modification to this protocol will be submitted to the IRB in the form of an amendment for its approval prior to implementation.

Principal Investigator:

Marisa Bunning_____May 21, 2010
(typed/printed name) (signature, if paper copy) (date)

WHEN COMPLETE:

Email electronic version from PI's email address to: Janell.Barker@Research.Colostate.edu
Sent email will serve as electronic signature from PI.
OR
Deliver signed original copy to:
IRB Administrator, RICRO, 321 General Services Building, campus delivery 2011

Thank you! We will soon contact you regarding the status of this application.

APPENDIX II: SAMPLE SENSORY SCORE SHEET

Panelist No. 001_

SCORE SHEET FOR LEAFY VEGETABLES

Please eat the entire sample and cleanse palate with water and crackers between samples. Under the corresponding sample number, please check the box that best describes your evaluation of each sample for appearance, flavor, texture, and overall acceptability.

APPEARANCE	Sample Number			
	187	926	445	603
Highly Acceptable	☐	☐	☐	☐
Acceptable	☐	☐	☐	☐
Moderately Acceptable	☐	☐	☐	☐
Slightly Acceptable	☐	☐	☐	☐
Neither Acceptable nor Unacceptable	☐	☐	☐	☐
Slightly Unacceptable	☐	☐	☐	☐
Moderately Unacceptable	☐	☐	☐	☐
Unacceptable	☐	☐	☐	☐
Highly Unacceptable	☐	☐	☐	☐

FLAVOR	Sample Number			
	187	926	445	603
Highly Acceptable	☐	☐	☐	☐
Acceptable	☐	☐	☐	☐
Moderately Acceptable	☐	☐	☐	☐
Slightly Acceptable	☐	☐	☐	☐
Neither Acceptable nor Unacceptable	☐	☐	☐	☐
Slightly Unacceptable	☐	☐	☐	☐
Moderately Unacceptable	☐	☐	☐	☐
Unacceptable	☐	☐	☐	☐
Highly Unacceptable	☐	☐	☐	☐

TEXTURE	Sample Number			
	187	926	445	603
Highly Acceptable	☐	☐	☐	☐
Acceptable	☐	☐	☐	☐
Moderately Acceptable	☐	☐	☐	☐
Slightly Acceptable	☐	☐	☐	☐
Neither Acceptable nor Unacceptable	☐	☐	☐	☐
Slightly Unacceptable	☐	☐	☐	☐
Moderately Unacceptable	☐	☐	☐	☐
Unacceptable	☐	☐	☐	☐
Highly Unacceptable	☐	☐	☐	☐

OVERALL ACCEPTABILITY	Sample Number			
	187	926	445	603
Highly Acceptable	☐	☐	☐	☐
Acceptable	☐	☐	☐	☐
Moderately Acceptable	☐	☐	☐	☐
Slightly Acceptable	☐	☐	☐	☐
Neither Acceptable nor Unacceptable	☐	☐	☐	☐
Slightly Unacceptable	☐	☐	☐	☐
Moderately Unacceptable	☐	☐	☐	☐
Unacceptable	☐	☐	☐	☐
Highly Unacceptable	☐	☐	☐	☐

Please write in the sample number in the space provided by ranking the samples in order of your preference (1 = Liked most; 4 = Liked least):

1) _____ 2) _____ 3) _____ 4) _____

Please provide specific comments for each sample (i.e. sweetness, crispness, mouthfeel, what you liked or did not like)

187_____ **926**_____

445_____ **603**_____