ABSTRACT

Title of Thesis: EFFECTS OF MATURITY STAGES AND GROWING CONDITIONS ON PHYTOCHEMICAL PROFILE AND ANTIOXIDANT ABILITY OF BRASSICA VEGETABLES

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This thesis research was designed to evaluate the effects of maturity stages, cooking methods, and growing conditions on the phytochemical profile and antioxidant ability of *Brassica* vegetables. First, kale was used as a model vegetable species to evaluate the influence of maturity stages including microgreen, baby green and mature vegetables. In the second study, mature kale was used to examine the effects of cooking methods on availability of the selected bioactive factors and the antioxidant properties. In addition, the effects of growing conditions on the health beneficial factors were evaluated using kale, broccoli, and red cabbage microgreens. The two growing conditions were chamber and windowsill, reflecting a commercial and a home growing condition. All of testing vegetable samples were extracted with 70% methanol and evaluated for their phytochemical compositions, total phenolic contents, $ABTS^{\bullet+}$ scavenging capacities and total dietary fiber. Five, twenty-three,

twenty-six, and twenty-three polyphenols were tentatively identified in the kale microgreen, baby kale, mature kale harvested at 56th day, and mature kale harvested at 87th day using a HPLC-MS/MS method, with trisinapoyl-diglucoside, quercetin-3-sinapoly-diglucoside-7diglucoside, feruloyl-glucoside, and disinapoyl-diglucoside being the primary components in each, respectively. The aliphatic glucosinolates including sinigrin and glucoraphanin were predominant in the kale, broccoli, and red cabbage microgreens grown under both chamber and windowsill conditions. In addition, microwaved mature kale had a greater level of phytochemicals than that boiled. It was noted that the baby kale extract possessed the greatest $ABTS^{\bullet+}$ scavenging capacity. The results of this thesis may be used to improve vegetable consumption.

EFFECTS OF MATURITY STAGES AND GROWING CONDITIONS ON PHYTOCHEMICAL PROFILE AND ANTIOXIDANT ABILITY OF BRASSICA VEGETABLES

by

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DPPH: 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay

ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging capacity assay

FRAP: ferric reducing antioxidant power

CUPRAC: cupric ion reducing antioxidant capacity

TPC: Total phenolic content

GAE: Gallic acid equivalent

TE: Trolox equivalent

CA: Caffieic acid equivalent

AC: antioxidant capacity

Introduction

As people became more and more health conscious, they begin to include more vegetables that are rich in nutrients in their diet. Scientists have demonstrated the health benefit of vegetables, especially the Brassica vegetables including kale, broccoli, cauliflower, and red cabbage The goal of this thesis research was to help consumers choose vegetables with higher nutritional value and better cooking methods that could preserve more nutrients. There were three specific objectives in this thesis research, including the evaluation of 1) how maturity stage, 2) food preparation methods, and 3) growing condition on their chemical compositions and antioxidant activities. In objective one, the kale was used as a model vegetable and the total phenolic content, polyphenol compositions, antioxidant activity, and total dietary fiber were tested. The second objective was accomplished by determining the potential effects of the selected cooking methods including boil and microwave on the total phenolic content, polyphenol compositions, and antioxidant activity using mature kale. The third objective was accomplished using the microgreens of kale, broccoli, and red cabbage to examine the two growing conditions (chamber and windowsill) on the chemical compositions and antioxidant properties of the three vegetables. These microgreens were also tested for their total fiber contents. The results might lead to a greater consumption of the kale microgreens which are being used simply as an addition to soup and salads. Moreover, consuming the microgreens instead of the mature counterparts could conserve more resources including water, soil and electricity which may promote the sustainable agricultural and food system development.

Chapter I: Literature Review

Cruciferous Vegetable

Cruciferous plants, formerly known as *Brassica* vegetables, encompass many species of economic and nutritional importance worldwide. This group includes vegetables such as cabbage, broccoli, and cauliflower mustard(Abbaoui, Lucas, Riedl, Clinton, & Mortazavi, 2018). Non-communicable Chronic diseases (NCDs) are found to be a serious public health problem. Research have demonstrated the role of vegetables in prevention of NCDs(Noce, Romani, & Bernini, 2021). Also, cruciferous species have become important vegetables for global consumption due to their higher nutrient density and widely recognized functional properties(Aires, 2015). These characteristics are directly related to its phytochemical composition and represent the most prominent characteristics of this plant family. Cruciferous phytochemicals are divided into a variety of micronutrients (amino acids, minerals and vitamins), macronutrients (high protein and dietary fiber content, low carbohydrate content) and secondary metabolites(Ramirez, Abellán-Victorio, Beretta, Camargo, & Moreno, 2020). These metabolites specially synthesized by each species are responsible for providing ordinary consumers of these crops with a unique taste, as well as many interesting and beneficial biological activities. The main phytochemical compounds include phenols, polyphenols, phenolic acids, flavonoids, carotenoids (zeaxanthin, lutein and β-carotene), alkaloids, phytosterol chlorophyll, glucosinolates ,Indoles), terpenoids and glycosides, and other less studied compounds such as phytosteroids, phytoantitoxins, terpenes and tocopherols(Argento, Melilli, & Branca, 2019; Ramirez et al., 2020).

At this point, it is worth mentioning that there is scientific evidence that shows that the biological activity of Cruciferae is related to the important contribution of treating of obesity, type 2 diabetes, cardiovascular diseases (including hypertension and stroke) and other chronic diseases(Chen, Koh, Yuan, Qin, & van Dam, 2018; Raiola, Errico, Petruk, Monti, Barone, & Rigano, 2018; Thomas-Charles & Fennell, 2019). In this regard, it is important to highlight the biological activity developed by specific compounds, especially their broad antioxidant capacity and anti-inflammatory activity(Minich & Bland, 2007).

In this research, the kale was chosen as a model vegetable to evaluate. As compared to other cruciferous vegetables, due to its tolerance to frost, this is a precious fresh vegetable in winter. The kale is characterized by high levels of various nutrients and other constituent metabolites, such as glucosinolates, flavonoids and carotenoids. Among all of Brassica vegetables, kale has been reported to contain the highest antioxidant capacity and a great variety and concentration of nutrients(Kurilich et al., 1999; Nilsson et al., 2006; Podsędek, 2007).

Kale

Kale (B. oleracea L var. acephala) plays an important role in the cooking and eating in American people. It is traditionally grown by farmers on small plots of land, mainly for household consumption, whether by human or animal food. The latest trend and promotion of kale as a "super food" has made kale appear on the menus of many restaurants in the United States, especially those that focus on healthy food. The young leaves of kale are for human consumption, while the old leaves are more suitable as feed for animals(Cartea, Picoaga, Soengas, & Ordás, 2003). Kale leaves are usually eaten fresh in salads and kale leaf juices, and are added to various soups. In Europe, kale is usually eaten with bacon as a healthy side. Some plant parts are occasionally made into kimchi. Recently, dried kale or so-called "kale flakes"

have become very popular, although drying can significantly reduce its nutritional and phytochemical composition(Oliveira, Ramos, Brandão, & Silva, 2015). In addition to leaves, kale seeds can also be used as a crude supplement for bread and cakes(Ayaz et al., 2006). In the development of new functional foods, it has been reported on beverages based on apple juice, adding frozen and freeze-dried kale leaves rich in minerals and healthy phytochemicals(Biegańska-Marecik, Radziejewska-Kubzdela, & Marecik, 2017). Although heady Brassica crops, such as cabbage, are often used as fermented vegetables (sauerkraut), it is reported that kale is not traditionally prepared and eaten when fermented. However, it has been reported that someone tried to produce kale juice fermented with *Lactobacillus* strains with great nutrients(S. Y. Kim, 2017). The author recommends fermented vegetable juice as a healthy beverage with essential nutrients, but the acceptability of fermented cabbage juice for consumers needs to be investigated.

The use of kale in pharmaceutical area is very similar, such as other Brassica crops that have been used in traditional medicine for centuries, mainly for the treatment of gastritis and gastric ulcers(Leonti, 2013). In addition to alleviating the symptoms of gastric ulcers, kale is reported to be used to treat diabetes, cancer, liver disease, anemia, obesity, etc(Manchali, Murthy, & Patil, 2012).

The nutrient content of kale has been studied for a long time. Kale possesses free sugars, organic acids, amino acids, essential oils, and minerals, which are natural compounds that play an important role in providing nutrition in the human diet. The predominant sugar of kale is fructose at 2011 mg/100 g dw, followed by glucose (1056 mg/100 g, dw) and sucrose (894 mg/100 g, dw). The most abundant amino acid in kale leave is glutamic acid (Glu, 3320 mg/100 g, dw), followed by Aspartic acid (Asp, 2760 mg/100 g, dw)(Ayaz et al., 2006). In addition to

the basic nutritional compounds, kale contains antioxidants, such as polyphenols, carotenoids, glucosinolates, and vitamins C and E. It is generally believed that foods rich in antioxidants may protect against free radicals and reactive oxygen species (ROS), thereby preventing human chronic diseases. A previous study has used a variety of methods to measure the antioxidant capacity of 38 commonly used vegetables, and reported that kale, spinach, broccoli, and rhubarb are the vegetables with the highest antioxidant activity(Zhou & Yu, 2006). In addition, Sikora et al(Sikora, Cieślik, Leszczyńska, Filipiak-Florkiewicz, & Pisulewski, 2008) measured the antioxidant activity of kale, broccoli, Brussels sprouts, and green and white cauliflower, and found that the antioxidant activity of kale was much higher than that of other vegetables analyzed. Sikora has used the ABTS assay to measure the antioxidant ability of fresh kale, and the result was 33.2 μmol TE/g(Sikora & Bodziarczyk, 2012). Kale extract also showed in vitro protection against oxidation of low density lipoproteins (LDL), which may indicate a potential protective effect against cardiovascular disease(Kural, Küçük, Yücesan, & Örem, 2011). In recent decades, the health-benefit of kale was associated with the presence of some phytochemicals, such as polyphenols and glucosinolates. These bioactive compounds have been demonstrated to have free radical scavenging properties, and are also reported to affect the inflammatory pathways(Choe et al., 2018).

The flavonoids are a group of secondary plant metabolites, which are the most abundant polyphenols in human diet. There are thousands of flavonoids that have been identified to date(Andersen & Markham, 2005). The biosynthesis of polyphenolic compounds in plant tissue could be affected by multiple factors such as biotic factors, growing conditions, climate, genotype, and agricultural factors(Podsędek, 2007; Winkel-Shirley, 2002). Olsen evaluated the polyphenols content in a red variety of curly kale, the result showed a total content of flavonoids,

anthocyanins, 284 mg CGE/100 g of fw; and hydroxycinnamic acid, 163 mg CAE/100 g of fw(Olsen, Aaby, & Borge, 2010). Another important group of phytochemicals is glucosinolates (GSLs). There are around 120 different GSLs that have been identified in sixteen families of dicotyledonous angiosperms(Fahey, Zalcmann, & Talalay, 2001). The concentration of GSLs could be different among genotypes and with different postharvest treatments. It has been reported that the GSLs' breakdown products are associated with the anticarcinogenic properties(Verhoeven, Verhagen, Goldbohm, van den Brandt, & van Poppel, 1997). Velasco(Velasco, Cartea, González, Vilar, & Ordás, 2007) found that the level of insect damage could affect the concentration of total GSLs, there was a significantly decrease in the damaged kale leaves (25.8 μmol/g) as compared with the undamaged leaves (41 μmol/g). The pest-attack could cause the tissue disruption and therefore allow the myrosinase to contact with the GSLs, and resulting in the production of the toxic degradation compounds(Barth & Jander, 2006). The relationship between the environmental factors and the nutrient profile of kale remains a lot of research to be researched.

Cooking influence

Thermo-treatment alters the nutrient profile of vegetables in ways that could impact the human health; these include the increased digestibility of starch and protein, deactivation of pathogens, and partially detoxification(Carmody & Wrangham, 2009). However, cooking could also deactivate antioxidant and antimicrobial compounds present naturally in vegetables(Cowan, 1999). Several different methods can be used to process kales, the most common of which are boiling, steaming, pressure-cooking, frying, and microwaving(Armesto, Gómez-Limia, Carballo, & Martínez, 2019). Murador et.al used a meta-analysis to evaluate the impact of cooking method on carotenoid and anthocyanin content in vegetables include kale. The results showed that there

is not a significant difference between boiling, steaming, and stir-frying(Murador, da Cunha, & de Rosso, 2014). Then they designed a study to evaluate the effect of home cooking techniques on kale and red cabbage, determining the levels of carotenoids, phenolic compounds, and ascorbic acid by HPLC-DAD-MS^n. The antioxidant activity was tested by different assays. The carotenoids content was decreased in all of the three cooking techniques, with the boiled kale showing the lowest concentration of carotenoids (35.47 μg/g dw). Steaming resulted in significant increases (~1.5X) in total phenolic content in kale (91.49 mg GAE/100 g dw) and boiling resulted in significant increases in antioxidant activity using an ABTS assay in red cabbage (raw: 6.85 μmol TE/g; boiling: 9.01 μmol TE/g dw). But the ORAC assay showed an opposite result in red cabbage result (raw: 53.97 μmol TE/g; boiling: 35.40 μmol TE/g dw)(Murador, Mercadante, & de Rosso, 2016).

Several studies have demonstrated that cooking influences the physical characteristics, chemical content, sensory properties, and the nutrient bioavailability of vegetable (Table 1.1). The changes depend on the type and quality of raw vegetables and on the type and conditions of the cooking method, and they can be either beneficial or detrimental to the quality of cooked vegetables. More and more studies have demonstrated that the steaming is the best method for preserving the antioxidant capacity and bioactive contents of kale, while boiling is the worst method which have a 76.4-77.9% losses of total phenolics. However, steaming has obtained the lowest sensory scores in the study. Compared with those two methods, microwaving could relatively protect the antioxidant compound from degrading, but it caused the highest loss of soluble solids (80.8-88.2%, fw)(Armesto et al., 2019; Armesto, Gómez‐Limia, Carballo, & Martínez, 2016). Overall, among varieties of cooking techniques, the steaming is the best choice

although it may decrease the sensory properties and the boiling is usually the worst method with loss of nutrients into solution.

Table 1.1. Effects of cooking methods on nutritional characteristics or bioavailability of selected vegetables

	Cooking method	Polyphenols (mg/100 g)	Glucosinolates (μmol/g, dw)	Antioxidant	Reference
Carrots	Boiled	Not detected	Not detected	TEAC: 1.53 mmol TE/100 g, dw	(Miglio, Chiavaro, Visconti, Fogliano, & Pellegrini, 2008)
Carrots	Steamed	39.6 dw	Not detected	TEAC: 1.40 mmol TE/100 g, dw	(Miglio et al., 2008)
Carrots	fried	48.0 dw	Not detected	TEAC: 1.64 mmol TE/100 g, dw	(Miglio et al., 2008)
Broccoli	Steamed	Not detected	28.6	Ascorbic acid: 117.3 mg/100 g, dw	(Vallejo, Tomás-Barberán, & García-Viguera, 2002)
Broccoli	Microwaved	Not detected	0.2	Ascorbic acid: 63.6 mg/100 g, dw	(Vallejo et al., 2002)
Broccoli	Raw	Not detected	26.6	Ascorbic acid: 117.7 mg/100 g, dw	(Vallejo et al., 2002)
Broccoli	Boiled	27.0 dw	29.3	TEAC: 2.17 mmol TE/100 g, dw	(Miglio et al., 2008)
Broccoli floret	Steamed	61.8 dw	93.4	TEAC: 3.51 mmol TE/100 g, dw	(Miglio et al., 2008)
Broccoli floret	Fried	40.3 dw	11.4	TEAC: 2.88 mmol TE/100 g, dw	(Miglio et al., 2008)
Broccoli floret	Raw	99.8 dw	71.4	TEAC: 1.10 mmol TE/100 g, dw	(Miglio et al., 2008)
Broccoli floret	Microwaved	9.8 fw	Not detected	Ascorbic acid: 35.5 mg/100 g, fw	(D. Zhang & Hamauzu, 2004)

Microgreen

Microgreens are an emerging novel produce known as “vegetable confetti”, which are harvested between 7 and 14 days from seedling emergence(Turner, Luo, & Buchanan, 2020). Microgreens are first appeared on chefs’ menus in the late 1980’s San Francisco area. Microgreens are generally described as immature plants older than the sprout but younger than baby greens and generally used as an ingredient and garnish to improve the flavor, color and texture of salads and soups(Treadwell, Hochmuth, Landrum, & Laughlin, 2010). There are three main parts of microgreens, including the central stem, cotyledonary leaf, and a pair of true leaves(Choe et al., 2018). The major difference between microgreens and the other two stages is the time of harvest. Sprouts are harvested earlier than microgreens and include the roots. In the case of baby greens, the harvest time is between 15-40 days. The different maturity of microgreens provides distinctive colors, textures, aromas, and flavors.

Microgreens are becoming more and more popular due to their abundant nutrients and shorter growing cycles. Moreover, the microgreens have been selected as the part of the global movement towards controlled environmental agriculture (CEA), also known as indoor farming. The CEA has been driven by the population increase, decreasing arable areas and the need for guaranteed food security(Goodman & Minner, 2019). Microgreens contain a variety of sustainable characteristics, including being chemical free, year-round, fertilizer free and using less resource, which make them great CEA crops(Riggio, Wang, Kniel, & Gibson, 2019; Treadwell et al., 2010). Scientists have demonstrated that the microgreens have a higher nutrient density as compared with their mature counterparts(Huang et al., 2016; Pinto, Almeida, Aguiar, & Ferreira, 2015; Jianghao Sun et al., 2013). Nevertheless, the nutrient profile could vary with

different species and growing conditions. More research is needed to evaluate the in vivo bioavailability of microgreens. Because Microgreens are generally consumed in raw type to maintain their taste and nutrients. Moreover, they are more vulnerable to pathogen colonization than their mature counterparts and therefore, it is important to ensure the microgreen safety(Warriner, Ibrahim, Dickinson, Wright, & Waites, 2003).

In 1980s, the most consumed microgreens were very limited including arugula, basil, beets, kale and cilantro(Choe et al., 2018). Nowadays, there are more than 50 commercially grown microgreens, the focus is on the 34 plants listed in Table 1.

Table 1.2. Forty commercially grown microgreens (Choe et al., 2018; Di Gioia, Renna, & Santamaria, 2017; Mir, Shah, & Mir, 2017; Turner et al., 2020)

Scientific name		
Commercial name	Family	Plant color
Arugula	Brassicaceae	green
Bull's blood beet	Chenopodiaceae	Reddish-green
Celery	Apiaceae	green
chives	Alliaceae	Green
scallions	Alliaceae	Green
shallots	Alliaceae	green
China rose radish	Brassicaceae	Purplish green
cilantro	Apiaceae	green
Garnet amaranth	Amaranthaceae	red
Golden pea tendrils	Fabaceae	yellow
Green basil	Lamiaceae	green
Green daikon radish	Brassicaceae	green
Magenta spinach	Chenopodiaceae	red
cucumber	Cucurbitaceae	green
mint	Lamiaceae	green
alfalfa	Fabaceae	green
mizuna	Brassicaceae	green
Opal basil	Lamiaceae	Greenish purple
Opal radish	Brassicaceae	Greenish purple
Pea tendrils	Fabaceae	green
peppercress	Poaceae	green
Popcorn shoots	Poaceae	yellow
Nutrient purple kohlrabi	Brassicaceae	Purplish green
Purple mustard	Brassicaceae	Purplish green
Red beet	Chenopodiaceae	Reddish-green
Red cabbage	Brassicaceae	Purplish green
kale	Brassicaceae	green
Red mustard	Brassicaceae	Purplish green
Red orach	Chenopodiaceae	Reddish-green
Red sorrel	Polygonacceae	Reddish-green
sorrel	Polygonaceae	green
wasabi	Brassicaceae	green
lemongrass	Poaceae	yellow
buckwheat	Polygonaceae	green

Among all kinds of microgreens, the *Brassica* family has attracted more and more attentions in recent years as the family including broccoli, kale and red cabbage may contain the phytochemicals potentially capable of protecting against inflammation, cancer and obesity(Islam, Park, Kang, & Lee, 2020; Li, Tian, Wang, Liu, Wang, & Lu, 2021; López-García, Máñez, Alegría, Barberá, & Cilla, 2020). Other families like *Apiaceae* and *Lamiaceae* are also health beneficial to health, with antimicrobial compounds. The growing condition of microgreens are not so strict, they can grow in flats containing potting mixes, peat-based mixes, hydroponic

growth medium, or even with recycled textile fiber mats. Some novel cultivating methods, like soil-less culture (hydroponic and aeroponic systems) are also available to grow microgreens(Lakhiar, Gao, Syed, Chandio, & Buttar, 2018).

Microgreen production

Microgreens are not a specific vegetable; they are a maturity stage of vegetables between baby greens and mature greens, beginning with the appearance of the first pair of true leaves. Interestingly, not all microgreens contain better nutrition than their mature counterparts. Microgreens grown from varied vegetables have distinctive characteristics, such as color, taste, texture, and nutrient content (Xiao et al., 2019). They may also be affected by environmental factors like water, light, temperature, and biotic factors, etc (Mir et al., 2017).

Seed sowing rate is important for microgreen growth because it will affect the source availability of water and nutrients. The fresh weight is used as the indicator to evaluate the best sowing rate. The commercial seeding rate is 201 g/m^2. Anjana and Akarapon (Junpatiw & Sangpituk, 2019) tested 4 different sowing rates: 56, 93, 130, 167, and 204 g/m^2 using chia microgreens. The results showed that the seed sowing rate of 204 g/m^2 gave the highest fresh weight. Murphy and Pill (Murphy & Pill, 2010) tested four different sowing rates of 50.25, 100.50, 150.75 and 201 g/m^2 using arugula microgreens, and a linear relationship between sowing rate and fresh weight was found.

The growing conditions include soil-based and soil-less culture, in which soil-based culture is mainly influenced by fertilizers added, and soil-less cultures are mainly affected by the composition of the media. Although the microgreens could be grown without chemicals and fertilizers, the adding of fertilizers could provide essential nutrients to microgreens for their growth. Bulgari (Roberta Bulgari, Negri, Santoro, & Ferrante, 2021) has evaluated the quality of

microgreens cultivated on three different substrates, including, vermiculite, coconut fiber, and jute fabric. The fresh yield was significantly increased where jute fabric has the highest yield rate compared with the other two substrates. In addition to fertilizers in increasing the yield of microgreens, dietary micro-nutrients can be added to improve the nutritional level of products. Francesco (Di Gioia, Petropoulos, Ozores-Hampton, Morgan, & Rosskopf, 2019) has determined that the application of Zn at 5 and 10 ml/L resulted in an increase in Zn concentration from 75% to 281% as compared with the untreated control microgreens, while the adding of Fe at 10 and 20 ml/L also leads to an increase from 64% to 278%. Moreover, the basic soil condition is also important for microgreen growth. A previous study concluded that neutral pH, loose, black soil is the best condition for cultivating microgreens and results in a 1.89 and 2.6-fold higher return in terms of stem length, and 1.5 and 2.13-fold higher in terms of fresh weight than other soil conditions (Lau, Tang, & Kansedo, 2019).

As compared with soil-based conditions, the soil-less systems have contributed to improving water use efficiency. This nowadays contributes to a significant portion of indoor vegetable production in Europe, Canada, and the United states (Tavan, Wee, Brodie, Fuentes, Pang, & Gupta, 2021). It was found that the microgreens grown in hydroponic systems resulted in lower yield, low dry matter percentage, but higher shoot/root ratio (Roberta Bulgari, Baldi, Ferrante, & Lenzi, 2017). As a result, the soil-less culture of microgreens still has a lot of optimization to be done (Rajan, Lada, & MacDonald, 2019).

Another important factor is the light quality and quantity. It could affect the color, flavor, metabolism, and morphology of microgreens (M.-J. Kim, Mikš-Krajnik, Kumar, & Yuk, 2016). The intensity of light could affect their phytochemical profiles. It has been demonstrated that the red, blue, and combined red plus blue light are more effective than white light and other

wavelengths for prompting photosynthesis and regulating microgreen metabolism (Turner et al., 2020). The previous study has found that exposure to 463 μmol photons/m2/s for a short period of time resulted in a 50% increase of antheraxanthin and a 133% increase of zeaxanthin from mustard microgreens (Kopsell, Pantanizopoulos, Sams, & Kopsell, 2012). Another study, using broccoli microgreens' exposure to blue light found that the blue light led to increases in carotenoids, total glucosinolates, and minerals (Kopsell & Sams, 2013). The combination of red and blue light (15% blue and 85% red light-emitting diode (LED)) had higher yields compared with cool white fluorescent light (Ying, Kong, & Zheng, 2020). However, the influence of light is associated with multiple factors including the genotype, microgreen color, exposure time, etc. Lobiuc et al(Lobiuc et al., 2017) evaluated the effect of different LED light colors and found that blue light promotes fresh weight and anthocyanin content of both red and green microgreens. Samuoiene et al (Sirtautas et al., 2012) demonstrated different results between species: the antioxidant content did not significantly increase in amaranth, broccoli, and pea compared with most of the other species. Moreover, the antioxidant level even decreases in beet microgreens with supplemental wavelengths. Therefore the importance of environmental factors on the nutrient contents of microgreens has been demonstrated.

Nutrient Content

Over the past decades, the area of food and agriculture has developed a lot, however, at the same time, different kinds of problems have been shown, including excessive water usage, soil occupation, fertilizer, pesticide, and food waste (Weber, 2017). Therefore, to complete the sustainable development goals of the United States, the food and agricultural area has been faced with a major challenge: to provide abundant nutrition for global food requirements while minimizing negative effects on the environment (Aschemann-Witzel & Peschel, 2019). As a

result, the functional foods and nutraceuticals are obtaining more and more attention, since these foods are of health benefit and could protect people from chronic diseases. Microgreens are one of the main functional foods that contain high amounts of phytochemicals such as vitamins, polyphenols, and glucosinolates. Moreover, the microgreens need less water and room to grow as compared with their mature counterparts. Particularly, the issue of food systems, including food nutrition, food safety, and food production should be fixed in the area experiencing the coronavirus (Covid-19) pandemic. The bioactive compounds in health foods have been demonstrated to enhance the consumers' immune systems (Galanakis, 2020).

Vitamins are one group of the most important nutrients for humans. Some of vitamins function as enzyme cofactors (vitamins A, K, and C; vitamin B_6; folate and vitamin B_{12}), some vitamins function as biological antioxidants (vitamins C and E), and several function as cofactors in metabolic oxidation-reduction reactions (vitamins C, K, and E). Lipid-soluble vitamins have some specific functions, such as hormones (vitamin D), and photoreceptive cofactors in vision (vitamin A) (Combs Jr & McClung, 2016).

Vitamin C, also known as ascorbic acid, is a main antioxidant. Previous study found that the total ascorbic acid content varied from species, ranging from 20.4 to 147.0 mg/100 g. Fresh Vitamin C is a major antioxidant. Previous study found that the total ascorbic acid content varied from species, ranging from 20.4 to 147.0 mg/100 g fresh weight. (I think you just repeated the same information twice.) Among the samples, red cabbage microgreens had the highest concentration of ascorbic acid at 147 mg per 100 g fresh weight, which was 6-fold higher than that recorded recorded in mature red cabbage (Xiao, Lester, Luo, & Wang, 2012).

Vitamin K, also known as phylloquinone, is important to help with blood coagulation. It has been demonstrated that dark-green vegetables, such as kale, broccoli, and spinach are rich in

phylloquinone (Bolton-Smith, Price, Fenton, Harrington, & Shearer, 2000). Among 25 microgreens, the phylloquinone content ranged from 0.6 to 4.1 μg/g freight (do you mean fresh?) weight. Garnet amaranth had the highest concentration of phylloquinone content at 4.1 μg/g (Xiao et al., 2012).

Tocopherols and tocotrienols belong to the vitamin E family, which functions as an antioxidant similar to vitamin C. There are four isomer forms for each tocopherol and tocotrienol: α, β, γ, and δ. Green daikon radish had the greatest tocopherol content in both α (87.4 mg/100 g FW) and γ (39.4 mg/100 g FW), which were much higher than that of mature spinach leaves (2.0 and 0.2 mg/100 g FW) (Choe et al., 2018).

β-Carotene is a red-orange colored pigment found in fruits and vegetables, which acts as an antioxidant in vivo. The β-Carotene content ranged from 0.6 to 12.1 mg/100 g FW, the highest one (12.1 mg/100 g FW in red sorrel) was 20-fold higher than the lowest one (0.6 mg/100 g FW in popcorn shoots) mainly due to the relationship between β-Carotene and vegetables' color. Xiao et al(Zxa et al., 2019) found that the kale microgreens accumulated lower carotenoid content than mature kale; on the contrary, the broccoli and cauliflower microgreens contain higher contents than their mature counterparts. Some scientists have evaluated the carotenoid contents among microgreens, baby greens and mature greens, and the results showed that the microgreens grown in hydroponic systems contain lower concentrations than the other two stages (R. Bulgari, Baldi, Fe Rr Ante, & Lenzi, 2016).

Minerals are important nutrients for humans, and mineral deficiency is a common problem today. It is estimated that over 60% of the world's 7 billion people are Fe deficient. Vegetables are a good source of minerals and, as compared with their mature counterparts, microgreens contain higher concentrations of minerals. Broccoli microgreens have been

demonstrated to have around 2 times more minerals than mature broccoli. Edgar has reported that the mineral profile differed between lettuce microgreens and mature lettuce. The mature lettuce was higher in N, P, and K, and all the other elements (Ca, Fe, Mn, Se, Zn and Mo) were significantly higher in lettuce microgreens (Pinto et al., 2015). Another study has evaluated the difference between growing stages of kale. The results showed that the baby leaf contained more minerals than microgreens and mature kale. However, there was no difference in mineral content between kale microgreens and mature kale.

The polyphenols and glucosinolates are bioactive compounds that have been demonstrated to have potential health benefits in several human chronic diseases. The polyphenol profile in five Brassica species' microgreens have been analyzed: a total of 164 phenolic compounds including 30 anthocyanins, 105 flavonol glycosides, and 29 hydroxycinnamic acid and hydroxybenzoic acid derivatives were identified (J. Sun et al., 2013). This study established a great footprint for further research. Huang evaluated the concentration of polyphenols and glucosinolates in red cabbage. The result showed that red cabbage microgreen (71.s01 μmol/g) had a higher concentration of polyphenols than mature red cabbage (50.58 μmol/g). Also the glucosinolate content is ~2-fold higher than the mature red cabbage. Marios have investigated four different genotype microgreens (amaranth, cress, mizuna and purslane), and the result revealed that the most abundant phenolics was chlorogenic acid (5503 μg/g dw). Hydroxycinnamic acids account for 79% of the mean total phenolic content across species; the lowest compound was flavone glycosides (0.3%).

Beyond those bioactive compounds, microgreens also contain other types of compounds like phytosterols, organosulfur compounds, and polyamines. Phytosterols are a type of plant sterols with 28 or 29 carbon atoms as the main skeletal structure. They are natural biologically

active ingredients, which are ubiquitously present in the lipid bilayers of all plant cell membranes (MS et al., 2018). They have attracted much attention in reducing serum cholesterol levels and the risk of human heart disease, as well as having other biological activities including anti-cancer, anti-inflammatory and immunomodulatory effects (Naumoska & Vovk, 2015). There are more than 200 kinds of phytosterols, usually in the form of free sterols, which are combinations of fatty acid esters or acetylated glycosides. According to previous publications, mustard, and *Brassica napus* (rapeseed) are both rich sources of phytosterols (Ramirez et al., 2020). In addition, it has been reported that Chinese cabbage, peas, broccoli, cauliflower, and romaine contain relatively high contents among the tested vegetables (H. Wang et al., 2012). Although the content of phytosterols in new characteristic microgreens has not been studied in detail in recent publications, it is worth noting that, in tomato, Arabidopsis and tobacco, phytosterols were detected in seedlings (~14 to 21 days), indicating that phytosterols should also be present in microgreens (Liao, Chen, Wang, Bach, & Chye, 2018).

Moreover, since sprouting usually leads to the accumulation of polyamines, some beneficial polyamines, such as spermine and spermidine, have been found in microgreens, which have neuroprotective and cardioprotective benefits, and have alleviating effects on central nervous system diseases. Among the bean sprouts and four selected microgreens, including alfalfa, radish, fenugreek and lentils, spermine (922 mg/kg), spermidine (579 mg/kg), and agmatine (5392 mg/kg) are found in fenugreek, lentils and alfalfa. Compared with the corresponding bean sprouts, the content of cadaverine is lower. However, the polyamine content of white radish sprouts is better than that of the greenish vegetables (Kralj Cigić, Rupnik, Rijavec, Poklar Ulrih, & Cigić, 2020).

In summary, microgreens seem to have better nutrient density than their mature counterparts. However, there are various possible factors, such as growing conditions, species, even harvest time that can affect the chemical compositions of microgreens. As a result, further studies are needed to fully evaluate the nutrient content of microgreens and their in vivo bioavailability.

Beneficial Health Properties

Chronic diseases are a major health problem worldwide. Due to the toxicity and side effects associated with drugs, the role of dietary bioactive ingredients has been emphasized in health regulation (Y.-M. Lee, Yoon, Yoon, Park, Song, & Yeum, 2017). Daily consumption of vegetables and fruits is related to reducing the risk of many diseases (Choe et al., 2018). For microgreens, preliminary research mainly focused on the evaluation of micronutrients and bioactive components and proved that some commonly used microgreens have similar or even higher amounts of micronutrients that promote health than their mature counterparts(Gan et al., 2017). So far, the beneficial effects of a small number of microgreens have been directly confirmed in cells and animal models, but not in human clinical trials.

Obesity, cardiovascular disease, and type 2 diabetes are major chronic diseases in the United States, as well as all around the world, and generally associated with the consumption of high-calorie, high-fat, and low-fruit and vegetable diets (Cordain et al., 2005). It has been demonstrated the consumption of a diet rich in fruit and vegetables is often recommended for prevention of these diseases (Hung et al., 2004). The development and progress of these chronic diseases could be prevented through the modulation of chronic inflammation.

It has been reported that several phytochemicals found in microgreens, such as I3C and retinoic acid (RA), can suppress adipogenesis (Choi, Um, & Park, 2013). It has been reported

that RA protects mice from diet-induced obesity (DIO) through activating nuclear RA receptors (RARs) and peroxisome proliferator activated receptor (PPAR) β/δ. In addition, some other natural bioactive compounds were shown to prevent pre-adipocyte proliferation and prompt apoptosis (Rayalam, Della-Fera, & Baile, 2008). Meanwhile, a previous study found that I3C is directly associated with silent mating type information regulation 2 homologue 1 (SIRT1) to inhibit adipocyte differentiation. It has been demonstrated that the activation of SIRT1 could enhance the inhibition of inflammatory cytokines and thermogenesis regulation (Murata, Ide, & Hara, 1997; Yeung et al., 2004; J. Zhang, 2007). Some other flavonoids, including rutin, resveratrol, and genistein, were also reported to inhibit pre-adipocyte proliferation (Choe et al., 2018). As a result, a microgreen-diet could attenuate obesity and obesity-related chronic diseases through the regulation of adipogenesis and lipid metabolism.

Cancer is the second leading cause of death in the United States. Different from other diseases, the treatment and post-survival rates are still hard to control. Hence, prevention of cancer is important to improving human health. It has been reported that a vegetable based diet could prevent one-third of all cancers (Michael, 2004). Microgreens, especially those from cruciferous vegetables, are rich in a variety of phytochemicals (Bradfield & Bjeldanes, 1987). Previous studies showed that the bioactive compounds in microgreens could prevent colon cancer by increasing reactive oxygen species and decreasing glutathione (Fuente, López-García, Máñez, Alegría, Barberá, & Cilla, 2020). The regulation of xenobiotic metabolism and inflammation might also be a potential cancer prevention pathway affected by microgreens. However, the modulation of complex carcinogenic pathways remains unclear and more clinical trials are needed to evaluate the health benefit of dietary phytochemicals in cancer

prevention(Teng, Liao, & Wang, 2021). In summary, dietary microgreens may promote health by attenuating inflammation and protecting against various cancers.

Food Safety of Microgreens

Microgreens are rich in different kinds of nutrients and high in water activity. Meanwhile, as a young stage of vegetables, they share many characteristics with leafy greens and sprouts. Therefore, the microgreens' young maturity made them more vulnerable to human pathogen colonization and internalization than mature plants (Turner et al., 2020). However, they are grown in a controlled environment, thus avoiding potential safety risks during cultivation. Microgreens are consumed raw and inappropriate post-harvest processing could affect their quality, as they are harvested without roots. There could be contamination during the harvest and during storage. As a result, it is better to sell the microgreens when they are still growing in the medium(Mir et al., 2017).

During seed germination, the seed releases a mixture of nutrients that will attract surrounding bacteria in the rhizosphere (Andrews et al., 1982). Y Hara-Kudo et al found that *E. coli* O157:H7 could contaminate the edible parts of radish sprouts when the latter were cultivated from seeds soaked in inoculated medium (Hara-Kudo et al., 1997). The bacteria can enter the inner apoplastic space through germinating radicals or secondary roots, then persist in localized sites (Warriner et al., 2003). Microgreens have a weaker protective structure than do mature plants, enabling the entry of bacteria into xylem. This will induce further contamination to the stems and leaves during growth (Warriner et al., 2003). As a result, it is important to control the growing environment of microgreens to maintain their high nutritional value.

Because some micro-vegetables are very fragile and have a short shelf life (1-2 days at room temperature), the study of pre-harvest and post-harvest processing conditions is of great

significance (Kyriacou et al., 2016). The post-harvest washing of microgreens is the primary intervention approach to avoid surface contamination. In the pre-harvest stage, it was found that $CaCl_2$ spray treatment can improve post-harvest quality, with better overall visual quality and longer storage time (Kou, Yang, Liu, & Luo, 2015; Lu et al., 2018). Broccoli micro-vegetables, were cut and stored at 5 °C. Then, if they are sealed in a polyethylene film bag at 5 °C, and sprayed with 10 mM $CaCl_2$ for 10 days, this can improve the overall visual quality, while reducing the growth of microorganisms and significantly affecting the expression of aging-related genes such as BoCAT3, BoGPX6, BoSAG12 and BoSAG126 (Ghoora & Srividya, 2020). There are several requirements for the sprouts, including the decontamination of seeds, testing the irrigation water, testing the growing, harvesting, packaging, and storage conditions. However, there are no requirements for microgreens, a special category of sprouts called "green sprouts" which are grown in soil or substrate but harvested above soil or substrate. This type of sprout was separated from true sprouts and treated similar to microgreens (Administration, 2015; Q. Wang, 2016).

Chemical Composition and Extraction Methods

Phenolic Compounds

Brassicaceae phenolic compounds have been evaluated often. There are two main classes, including flavonoids and phenolic acids. The main flavonoids in Brassica vegetables are the kaempferol, quercetin, and isorhamnetin combined with the O-glycosides. There are varieties of glucose found in Brassica plants, include mono-, di-, tri-, and penta-glucosides. They are also commonly found to be acylated by different hydroxycinnamic acids. Anthocyanins are one of the most abundant colored flavonoids found in Brassica vegetables. They are the sugar-conjugated forms of anthocyanidins, which are nature compounds widely existing in the Brassica vegetables.

They are particularly responsible for red, blue, and purple colors in vegetable tissue. In addition, they are also found in stems, seeds, leaves, and roots. The anthocyanin is responsible for the color intensity and potential biological activity of vegetables. The most common anthocyanins include cyanidin, peonidin, petunidin, delphinidin, and malvidin. Cyanidin has been reported to be the main anthocyanin in Brassica vegetables (Cartea, Francisco, Soengas, & Velasco, 2011; Olsen, Aaby, & Borge, 2009; Romani, Vignolini, Isolani, Ieri, & Heimler, 2006). Hydroxycinnamic acids are a group of non-flavonoid phenolics composed of C6-C3 structure. These compounds are abundant in Brassica vegetables and occur freely or in polymer forms (cell wall) (Taofiq, González-Paramás, Barreiro, & Ferreira, 2017). In Brassica vegetables the most common compounds(?) are sinapic and ferulic acids, often found in conjugation with a sugar moiety.

The extraction procedures are important in analysis of phytochemicals. There are varieties of extraction methods, including decoction (DCE), maceration (MCE), Soxhlet extraction, supercritical fluid extraction (SFE), microwave assisted (MAE), ultrasound-assisted extraction (UAE), and accelerated solvent extraction (Raaman, 2006).

Maceration is a useful and inexpensive technique for the extraction of essential oils and phytochemicals from vegetables. This process is normally done by immersing the plant sample in a 5-10% solution of sodium hydroxide (NaOH) or potassium hydroxide (KOH) (David, 1973). The powdered crude plant sample undergoes grinding in a closed vessel where menstruum is added to increase extraction efficacy for proper mixing of powdered materials with the solvent. Then, the solvent is removed, followed by pressing the solid residue to recover an optimum amount of blocked solution. Both the pressed-out liquid and the removed solvent are separated from unwanted materials by filtration(Srivastava et al., 2021). The advantage of this method

includes: (1) useful for thermosensitive compounds, (2) available for various samples, solvents, and pH's, (3) simple and cheap. The main downside is the complex of parameters and time-consumption (Gallo et al., 2017; Jovanović, Petrović, Đorđević, Zdunić, Šavikin, & Bugarski, 2017).

Decoction is a traditional method of extraction by boiling herbal or plant samples to dissolve the bioactive compounds. Traditional decocting methods are generally performed by putting herbal materials in a casserole, soaking in water, heating with direct fire, and simmering. After two decoctions, the medicinal solution is separated from residue. In long-term practice, to make the drug with as many active ingredients as possible and less toxicity, a complete set of decoction methods has been gradually created. Among them, the special decoction method may include: using the lower part (such as rhubarb, mint), frying (such as plantain), soaking (such as saffron), glutinous (such as amber powder), etc. The traditional decoction method is convenient and economical, and the curative effect has been affirmed after long-term practice. With the advancement and development of science and technology, in addition to the traditional decoction methods, many novel decoction methods have appeared, such as far-infrared decoction, room-pressure decoction, high-pressure decoction, etc., which have gradually evolved into closed high-pressure decoction. Using a decocting machine, setting the time and pressure and other parameters, and after the decoction, manual squeezing or automatic squeezing, the liquid medicine is transported to an automatic machine for packaging, and bagging for later use. As compared with traditional decoction, modern decoction decreases the loss of volatile compounds and attenuates the oxidative reaction. Moreover, due to the increasing of pressure and the accurate control of the temperature, the extraction yield can be enhanced a lot (Chanda,

Amrutiya, & Rakholiya, 2013; Wan, Bai, Cai, Rao, Wang, & Wang, 2013; F. Zhang, Chen, & Yan, 1991).

Soxhlet extraction is commonly known as “solid-liquid extraction” or “leaching”. It has been used as a standard technique for over a century. In the conventional Soxhlet extraction, the sample is placed in a thimble-holder in the extractor which is gradually filled with the proper solvent. When the solvent reaches the overflow level, a siphon aspirates the solute from the thimble-holder and delivers it back into the distillation flask, thus carrying the extracted components into the solvent. This operation is repeated until extraction is complete, and usually takes hours, even overnight, to finish. Therefore, Soxhlet extraction could be a continuous–discrete technique (M. L. De Castro & Priego-Capote, 2010). Soxhlet extraction is a very simple technique with multiple advantages including: (1) The sample is repeatedly reacted with fresh portions of solvent as the extracted compounds will remain in the distillation flask, (2) constant heating could facilitate extracting yield, (3) equipment cost is low and filtration is not required after processing, (4) The extraction yield of the Soxhlet technique is higher than most of other methodologies (supercritical fluid extraction, microwave-assisted extraction, etc.) (M. D. L. de Castro, Valcárcel, & Tena, 2012; M. L. De Castro & Garcıa-Ayuso, 1998; Dean, Abdullah, & Zakaria, 1997; Guerin, 1999). The main drawbacks include being time-consuming, decomposition of thermosensitive compounds, environmental problems, and waste of organic solvent. Overall, the Soxhlet extraction has been used as an original method for modification to increase extracting efficacy.

Compared with those traditional methods, there are more and more novel techniques being developed, including superficial fluid extraction (SFE), microwave-assisted extraction, and ultrasonic-assisted extraction. Superficial fluid extraction is a special method, requiring a special

extraction system, in particular carbon dioxide. The carbon dioxide in superficial phase works as a non-polar solvent and can be used to extract non-polar compounds from the matrix (Gallo et al., 2017). The superficial carbon oxide can be obtained by providing pressure and temperature above the critical point of carbon oxide. In addition to the preparation of superficial fluid, there are a variety of parameters needed to be investigated and manipulated, including: temperature, pressure, solvent flow rate, and ratio of co-solvent (Sovilj, Nikolovski, & Spasojević, 2011). The advantage of this technique is that superficial carbon dioxide could be removed in the form of gas, therefore it could be more environmentally friendly compared with other methods. Moreover, due to the removal of solvent, a concentrated residue could be obtained. The drawbacks of this method include complexity, high-cost, and nonuniversal application.

Another two novel methods are modified from Soxhlet extraction are called ultrasonic-assisted extraction and microwave-assisted extraction. Microwaves possess electric and magnetic fields which are perpendicular to each other. The electric filed generates heat via dipolar rotation and ionic conduction. Unlike the classical methods, microwave assisted extraction heats the whole sample simultaneously. During the extraction, heat disrupts weak hydrogen bonds due to the dipole rotation of molecules, and the migration of dissolved ions increases the penetration of solvent into the sample or matrix (Kaufmann & Christen, 2002). This is an advanced technique, which has the capability of extracting many bioactive compounds within a shorter extraction time. The main advantage of this technique is the increased penetration of solvent into the matrix due to disruption of cell walls, produced by acoustical cavitation. This is achieved at low temperatures and, hence, it is more suitable for extraction of thermally unstable compounds (Maran, Manikandan, Nivetha, & Dinesh, 2017). In this study, the ultrasonic-assisted solid-to-liquid extraction was used for polyphenols and total phenolic content extraction. This is a

combination of a conventional technique and a modern technique. It is more efficient and faster than the conventional solvent extraction methods.

Table 1.3. Total Phenolic Contents (TPC) of selected *Brassica* vegetables

	TPC (mg GAE/g, dw)	Ref
Broccoli	23.6	(Jaiswal, Rajauria, Abu-Ghannam, & Gupta, 2011)
White cabbage	18.4	(Jaiswal et al., 2011)
Kale	16.3 – 18.8	(Zhou et al., 2006)
Spinach	9.3 – 13.0	(Zhou et al., 2006)
Broccoli	9.4 – 10.6	(Zhou et al., 2006)

Antioxidant Capacities Measurement

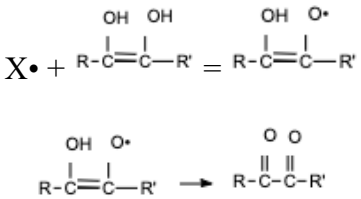
The antioxidant capacity of vegetables has been determined using varieties of techniques, including the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, oxygen radical absorbance capacity (ORAC), hydroxyl radical scavenging capacity (HOSC), etc. The basic theory of the DPPH assay is the measurement of scavenging ability of antioxidant compounds. Representing the DPPH radical by X• and the model antioxidant by RH, the initial reaction is

$$X\bullet + RH = XH + R\bullet$$

The free radical R• then reacts with another molecule through a parallel reaction

$$R\bullet + R\bullet = RS - SR$$

This result in the reduction of two DPPH molecules by the model antioxidant, which is a 1:1 stoichiometry. However, a 2:1 stoichiometry could be produced when the antioxidant compound (e.g. ascorbic acid) contains two adjacent internally connected sites for hydrogen abstraction(Kedare & Singh, 2011).



Methanol and ethanol could be good solvents for this method. Other solvent systems, such as water or acetone may result in a lower reduction value. The appropriate pH range for the assay is from 5.0 to 6.5. The useful absorbance for this method is in a range from 515 nm to 518 nm(Gómez-Alonso, Fregapane, Salvador, & Gordon, 2003; Lebeau, Furman, Bernier, Duriez, Teissier, & Cotelle, 2000; Leitão, Leitão, & Vilegas, 2002; Schwarz et al., 2001; Zhu, Hackman, Ensunsa, Holt, & Keen, 2002) and the reaction time is varied by substrates(Bondet, Brand-Williams, & Berset, 1997). The general interpretation of the assay is the percentage reduction of DPPH, Q (Refered as “quenching” or “inhibition”) and it can also be expressed by the amount of sample needed to react with 50% DPPH in terms of the amount of Trolox reacted(Suda, 2000).

The ABTS assay is similar to the DPPH assay, and is also based on the scavenging capacity of antioxidants but different radical molecules. The ABTS• is commonly generated by reacting with a robust oxidizing compound such as potassium permanganate or potassium persulfate(Opitz, Smrke, Goodman, & Yeretzian, 2014). The initial ABTS• absorbance should be controlled at 734 nm(Dorsey & Jones, 2017). The ABTS• is terminated by the antioxidant addition followed with the adsorption decreasing. This assay is comparable to the results of the DPPH method as both could be interpreted by the Trolox equivalent antioxidant capacity. Floegel et al(Floegel, Kim, Chung, Koo, & Chun, 2011) compared the usefulness of ABTS versus DPPH assays using 50 of the most popular antioxidant-rich vegetables, fruits and beverages in the US diet. The result showed that the antioxidant capacity determined by ABTS assay was positively correlated with the ORAC assay from the USDA database and significantly

higher for samples compared with DPPH assay. This could be caused by a better detection of high-pigment and hydrophilic antioxidants by the ABTS assay. These data suggested that the ABTS assay may be better than DPPH assay for evaluating antioxidant capacity in a variety of vegetables and fruits.

In addition to the DPPH and ABTS assays, there are two other methods (ORAC and HOSC) that are considered to be of biological relevance and which could express the antioxidant effectiveness of a sample. The ORAC assay is based on the reaction of peroxyl radicals and a fluorescent probe resulting in the loss of fluorescence (Zhong & Shahidi, 2015). The generation of peroxyl radicals is shown as below:

The peroxyl radical scavenging ability of the antioxidants is calculated by the decay curves of fluorescence. Similarly, the Trolox can be used as the standard compound for the ORAC assay(Malta & Liu, 2014). However, not all solvent systems work with this method. It has been reported that the acetone works well with the ORAC assay, but the methanol showed an inhibition of the loss of fluorescence(Yalcin & Sogut, 2014).

The HOSC is based on the reaction of antioxidants and pure hydroxyl radical generated by Fenton-like Fe^{3+}/H_2O_2 reaction under physiological pH. The interpretation is similar as ORAC assay, using the fluorescence decay curve and expressed as Trolox equivalents per unit of the antioxidant(Moore, Yin, & Yu, 2006).

Radical scavenging capacity of vegetable antioxidants was evaluated against $ABTS^{+\bullet}$ generated by the chemical method according to a previously reported protocol(Miller & Rice-Evans, 1997). ABTS assay has been used a lot in the determination of antioxidant activity. Zhou and You extracted the kale sample by 50% acetone solution and using ABTS assay to test the

antioxidant activity(Soengas Fernández, Sotelo Pérez, Velasco Pazos, & Cartea González, 2011). A 5 ml of vegetable antioxidants in 70% methanol was diluted with 95 ml of 70% methanol to obtain the testing samples. ABTS+ was prepared by oxidizing 5 mmol/l aqueous solution of ABTS, 2,20-azinobis (3-ethylben- zothiazoline-6-sulfonic acid diammonium salt, with manganese dioxide at ambient temperature for 30 min. The ABTS+ antioxidant reaction mixture contained 1.0 ml of ABTS+ with an absorbance of 0.7 at 734 nm. The absorbance at 734 nm was measured at 1 min of the reaction, and the TE was calculated using a standard curve prepared with Trolox under the same experimental conditions. This study is trying to provide the customer a guideline for choosing and processing of vegetables that result in a higher nutrient consumption. There are three main objectives in current study: 1. Evaluation of polyphenols' profile, antioxidant capacity, and total dietary fiber of kale in different maturity stages; 2. Determination of phytochemical (polyphenols and glucosinolates) profile, antioxidant capacity, and total dietary fiber of microgreens under two growing conditions (commercial growth and home growth); 3. Evaluation of protective effects of two cooking methods (boil and microwave)

Chapter II: Effects of Maturity Stage on Phytochemical Profiles and Antioxidant Abilities (Free Radical Scavenging Capacities) of Kale

Abstract

Kale microgreen, baby kale and the mature kale harvested on two different days (56^{th} day and 87^{th} day) were extracted with 70% methanol and evaluated for their polyphenol compositions, free radical scavenging capacities, and total dietary fiber analysis. A total of five, twenty-three, twenty-six and twenty-three polyphenols were detected in the kale microgreens, baby kale and the two harvests of the mature kale, with trisinapoyl-diglucoside (10.61 μmol/g dw), quercetin 3-sinapoly-diglucoside-7-diglucoside (6.30 μmol/g dw), feruloyl-glucoside (2.99 μmol/g dw) and disinapoyl-diglucoside (2.57 μmol/g dw) as the primary components of each. The total polyphenol concentration of the two mature kales were similar and significantly lower than the kale microgreens (20.89 μmol/g dw) and baby kale (35.29 μmol/g dw). The baby kale extracts had the greatest $ABTS^{\bullet+}$ scavenging capacity of 96.13 μmol trolox equivalents (TE)/g dw. The results provide a good theoretical support in enhancing the growing and consumption of kale microgreen and baby kale.

KEYWORD: kale, maturity stage, UHPLC-MS, antioxidant

Introduction

Vegetables constitute an essential part of the human diet since they provide vitamins, dietary fiber, and phytochemicals. Each vegetable group contains a unique combination and concentration of these nutrients, which distinguishes them from other vegetables within their own group (Dias, 2012). In recent decades, increasing attention has been paid to the nutritional

value and health benefits of Brassica vegetables. Brassica vegetables belong to the cruciferous family and include a variety of the genus of broccoli, kale, and cabbage. Several studies have reported that Brassica vegetables contain a high concentration of dietary antioxidants, minerals, and dietary fiber(Podsędek, 2007). It has been demonstrated that the beneficial effects of Brassica vegetables are partly related to these antioxidant compounds. It is well-established that antioxidants act together to scavenge free radicals, reduce lipoperoxidation, and enhance the endogenous enzymatic defenses. (Niki, 2010; Santos-Sánchez, Salas-Coronado, Villanueva-Cañongo, & Hernández-Carlos, 2019). Clinical trials and epidemiological studies have demonstrated that antioxidants found ubiquitously in vegetables could prevent chronic diseases including cancer, cardiovascular disease, type-2 diabetes, etc (Bennett, Rojas, & Seefeldt, 2012; Blomhoff, 2005; Hantikainen et al., 2021; Kaluza, Harris, Linden, & Wolk, 2018; Rahimi, Nikfar, Larijani, & Abdollahi, 2005; Tang, Meng, Li, Zhao, Liu, & Li, 2017; Z. Zhang, Bergan, Shannon, Slatore, Bobe, & Takata, 2018). There are a variety of antioxidants, such as ascorbic acid, tocopherols, carotenoids, and polyphenols. Polyphenols are the most abundant antioxidants in a plant-based diet. The polyphenol profile of Brassica vegetables has been identified by HPLC-PDA-ESI/HRMS^n, and may include simple phenolic compounds, anthocyanins, flavonol glycosides, and hydroxycinnamic acid (Olsen et al., 2010). In addition to the dietary antioxidants, Brassica vegetables contain high levels of dietary fiber, which according to Yang et al. can attenuate and prevent the infectious diseases related to gut microbiota (Yang, Sun, Cai, Chen, & Gu, 2020). Dietary fibers are a specific group of plant polysaccharides, such as cellulose and hemicellulose, that are resistant to the hydrolysis in the human gastrointestinal tract. Dietary fiber has been demonstrated to lower the risk of several chronic human diseases, including diabetes, coronary heart disease, obesity, and gastrointestinal disease. The

recommended dietary fiber intake for children and adults is 14 g/1000 cal per day (Anderson et al., 2009). It can be fermented by the bacteria in the gut to produce short chain fatty acids (SCFAs). In previous reports, butyrate, one specific SCFA, showed an important immunomodulatory function through the activation of signaling cascades (Parada Venegas et al., 2019). Taken together, these previous studies revealed the potentially beneficial health effects and compounds in the Brassica vegetables and suggest a valuable utilization of these brassica vegetables in nutraceuticals and functional foods.

Kale is becoming considered a "super food" in recent years due to its beneficial health effects. It is a rich source of many natural antioxidants, including ascorbic acid, polyphenols, carotenoids and tocopherols, and it has been reported to have a great antioxidant capacity (de Azevedo & Rodriguez‐Amaya, 2005; M. J. Kim, Chiu, & Ku, 2017; Zietz et al., 2010). Commercial kale is normally harvested in 50-70 days after sprouting, at which time the terminal bud on the kale could continue to grow. Several studies have investigated the influence of maturity stage on the total phenolic content and antioxidant capacity of cauliflower and broccoli sprouts (Patras, Stoleru, Filimon, Padureanu, Chelariu, & Biliaderis, 2017). However, the polyphenol profile of kale at different maturity stages remains unclear.

This study was conducted to evaluate the polyphenol compositions, antioxidant capacity, and dietary fiber contents of kale in different maturity stages. This was the first time that kale microgreens, baby kale, and two stages of mature kale (56^{th} days & 87^{th} days) were compared for their polyphenol composition and radical scavenging capacities. The results from this study can serve as a scientific foundation for further studies on kale and other Brassica vegetables.

Materials and Methods

Materials

Kale microgreen, baby kale and mature kale were grew and harvested by USDA collaborators, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), 2,2'-azinobis (3-ethylbenzothia-zoline-6-sulfonic) diammonium salt (ABTS), Folin-Ciocalteu (FC) reagent, Gallic acid, Sodium carbonate (Na_2CO_3), Sulfatase (from Helix pomatia), glucotropaeolin potassium salt (≥ 90.0%), DEAE Sepharose CL-6 B Suspension, Imidazole formate and rutin hydrate (≥ 94%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid, Ethanol, acetone, HPLC grade methanol, acetonitrile and water were obtained from Fisher Scientific (Waltham, MA, USA). Total dietary fiber assay kit was purchased from Megazyme (Brey, Iredland).

Kale Cultivars

Kale was grown by the collaborators at USDA under chamber condition and harvested at different maturities (kale microgreen, baby kale, 56^{th} days mature kale, and 87^{th} days mature kale). The growing procedure was shown below: 1. Stack one tray with holes on top of another tray without holes; 2. Pour 2700 mL of soil in top tray and spread out evenly; 3. Pour 2500 mL of tap water onto soil; 4. Pat down soil and confirm all of the soil is wet and the water is spread out evenly in the soil; 5. Wait 20 minutes for water to soak through soil; 6. Scatter 7 grams of seeds evenly and directly on top of the soil; 8. Stack another tray without holes on top of the soil and seeds; 9. Allow seeds to germinate for a few days (4 days for kale); 10. After germination, remove the top tray and place the top with soil under the light; 11. Check the water level of the tray and rotate the tray 180 degrees every day; 12. Harvest the kale in different maturities with clean scissors

Sample Extraction and Preparation

For polyphenols extraction, dried sample powder (100 mg) was added with 5 mL methanol-water (7:3, v/v), and then sonicated for 1 h at room temperature. The slurry was centrifuged at 5000 g (IEC Clinical Centrifuge, Damon/IEC Division, Needham, MA, USA) and then filtered with 0.45 μm syringe filter (VWR Scientific, Seattle, WA, USA). The samples (2 μL) were injected into liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) system. In this study, extraction of glucosinolates (GSL) was carried out following the ISO9167-1 method with slight modification [22]. The kale powder (200 mg) and glucotropaeolin (Internal standard, 100 μL, 5 mM) were transferred to the tube. The samples were extracted with boiling methanol (5 mL) for 20 mins. The slurry was centrifuged at 5000 g (IEC Clinical Centrifuge, Damon/IEC Division, Needham, MA, USA), and then the supernatant (1 mL) was transferred to the prepared ion exchange columns (DEAE Sepharose CL-6 B Suspension, 500 μL). Two 1 mL portions of the sodium acetate buffer (pH 4) were gently added to the columns, allowing the buffer to drain after each addition. Purified sulfatase solution (100 μL) was added to each column, leaving to act overnight at room temperature. The desulfo-glucosinolate was eluted and collected with two 1 mL portions of water. The mixture was filtered with 0.45 μm syringe filter (VWR Scientific, Seattle, WA, USA), and then 2 μL was injected into UHPLC-HRMS system.

ABTS•+ Scavenging Capacity

Radical scavenging capacity of kale antioxidant was evaluated against $ABTS^{\bullet+}$ generated by the chemical method according to a previously reported protocol(Miller et al., 1997). A 5 mL of vegetable antioxidants in 70% methanol was diluted with 95 mL of 70% methanol to obtain the testing samples. $ABTS^{\bullet+}$ was prepared by oxidizing 5 mmol/l aqueous solution of ABTS, 2,20 -

azinobis (3-ethylben- zothiazoline-6-sulfonic acid diammonium salt, with manganese dioxide at ambient temperature for 30 min. The antioxidant reaction mixture contained 1.0 mL of $ABTS^{\bullet+}$ with an absorbance of 0.7 at 734 nm. The absorbance at 734 nm was measured at 1 min of the reaction, and the TE was calculated using a standard curve prepared with Trolox under the same experimental conditions.

Total Phenolic Content

The total phenolic content of kale sample was evaluated by measuring its reducing capacity with the Folin-Ciocalteu (FC) reagent using a spectrophotometer(Stevanato, Fabris, & Momo, 2004). The reaction mixture contained 50 μL of vegetable extracts, 250 μL of the Folin–Ciocalteu reagent freshly prepared in our laboratory, 750 μL of 20%（w/v）sodium carbonate, and 3 mL of pure water. After 2 h of reaction at ambient temperature, the absorbance at 765nm was measured and used to calculate the phenolic contents using gallic acid as a standard.

Total Dietary Fiber Determination

The total dietary fiber was determined using the Megazyme TDF Test kit. Weighting duplicate 1 g dried kale sample in the beakers (accurate to 0.1 mg). Adding 50 mL phosphate buffer (pH 6.0) to each beaker and adjust pH to 6.0 ± 0.1. A control group without sample was processed to remove the effects of solutions. Firstly, samples were incubated at 98 - 100 °C for 15 mins with 50 μL heat-stable α-amylase solution (to give gelatinization, hydrolysis and depolymerization of starch). Cooling down to room temperature and adjust to pH 7.5 ± 0.1 by adding 10 mL 0.275 N NaOH solution. Then, samples were incubated at 60 °C for 30 mins with 100 μL of protease solution under pH 7.5 ± 0.1 (to solubilize and depolymerize protein); Cooling down to room temperature and adjust pH to 4.5 ± 0.2 by adding 10 mL 0.325 N HCl solution. After that,

samples were incubated at 60 °C for 30 mins with 200 µL amyloglucosidase at pH 4.5 ± 0.2 (to hydrolyze starch fragments to glucose). After incubation, samples were treated with 280 mL 95% EtOH pre-heated to 60 °C to precipitate fiber. Weighting crucible containing Celite to nearest 0.1 mg, then wet and distribute with 78% EtOH from wash bottle. Applying suction to draw Celite onto fritted glass as even mat. The residue was filtered and washed successively with 20 mL portions of 78% ethanol, two 10 mL portions of 95% ethanol and two 10 mL portions of acetone. Then the residue was dried overnight in a 70 °C vacuum oven and weighted. One of the duplicate samples was analyzed for protein by rapid N exceed (fast N-analyzer machine, elementar, Ronkonkoma, NY), using N × 6.25 as a conversion factor. Theother sample was ashed at 525 °C for 5 hours. The total dietary fiber was calculated by using the Megazyme **Mega-Calc**^{TM}, downloaded from the Megazyme web site (www.megazyme.com).

Identification and quantification (UHPLC-PDA-ESI/HRMSn)

The Ultra-High-Performance Liquid Chromatography photo diode array high-resolution multi-stage mass spectrometry (UHPLC-HRMS) consisted of a Vanquish UHPLC and Orbitrap Fusion ID-X Tribrid mass spectrometer. The separation was carried out on a Thermo Hypersil Gold AQ RP- C_{18} UHPLC column (200 mm × 2.1 mm i.d., 1.9 µm) (ThermoFisher Scientific, Waltham, MA, USA) with an UltraShield pre-column filter (Analytical Scientific Instruments, Richmond, CA, USA) at a flow rate of 0.3 mL/min. The mobile phase consisted of a combination of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v). For polyphenols quantitation, the linear gradient was from 2% to 10% B (v/v) at 10 min, to 40% B at 25 min, to 75% B at 30 min, to 90% B at 50 min and maintained 90% B till 60 min. The photodiode array (PDA) recorded spectra from 200-500 nm was used to monitor the peaks. A full mass range was set at m/z 100-1600 with a resolution of 60,000. Data-dependent MS^2 acquisition method was

constructed for the top 3 intense ions. AGC values were set to 2×10^5 for MS and 10^4 for MS/MS. The mass injection time was set at 60 mins and 30 mins for polyphenols and GSLs, respectively. H-ESI ion source was used under negative mode with a spray voltage of 2500 V. Sheath gas, aux gas and sweep gas were set at 40, 10, and 5 (arbitrary unit), respectively. Ion transfer tube temp. was set at 300 °C and vaporizer temp. was set at 275 °C. Quantification of total polyphenols was done based on a standard curve of rutin, and linearity of the calibration curve was achieved between 10 to 500 µg/mL ($r^2 = 0.9994$). For GSLs quantitation, the linear gradient was from 1% to 3% B (v/v) at 5 min, to 20% B at 15 min and to 75% B at 30 min. The UV wavelength was set at 229 nm to record the peaks. Relative response factors were applied to correct for differences in UV absorbance between glucotropaeolin (Internal standard) and desulfoglucosinolates

LC-MS Data Pre-treatment and Handling

Raw files from UHPLC-HRMS were converted into mzXML format using Proteowizard 3.0.20210 (http://proteowizard.sourceforge.net/), and XCMS online was selected for advanced data processing [23]. The step included ion feature extraction, peak picking and alignment. Parameter settings for XCMS processing were as follows: centWave for feature detection (Δm/z = 2.5 ppm, minimum peak width = 10 s, and maximum peak width = 60 s); obiwarp settings for retention-time correction (profStep = 1); and parameters for chromatogram alignment, including mzwid = 0.015, minfrac = 0.5, and bw = 5. The relative quantification of ion features was based on extracted ion chromatogram (EIC) areas. The ion feature list was downloaded and entities at retention time 0.5–25.0 min for polyphenols and 0.5-20.0 min for GSLs were used for further statistical analysis. Then, the entities were exported into a two-dimensional (samples × mass peaks) matrix in Excel (Microsoft Corporation, Redmond, WA, USA).

Statistical analysis

All experiments were carried out in triplicate. Data are expressed as mean ± standard deviation. Statistical analysis was determined by a one-way analysis of variation (ANOVA), followed by a post hoc test (Tukey test) using Prism 9 (Graphpad, San Diego, CA, USA). P values less than 0.05 were statistically significant.

Results and Discussion

To evaluate their beneficial effects and nutritional value, the kale microgreen, baby kale, mature M-56 kale (harvested at 56^{th} days from seedling), and mature M-87 kale (harvested at 87^{th} days from seedling) were extracted and examined for polyphenol compositions using UHPLC-HRMS, free radical scavenging capacity against $ABTS^{\bullet+}$, and total dietary fiber using a commercial kit (AOAC method 985.29).

Total Phenolic Content

The total phenolic content of the kale extracts is shown in Fig.1 Among all the kale maturity stages, baby kale had the highest phenolic content of 27.35 mg GAE/g dw, followed by kale microgreen (25.22 mg GAE/g dw), mature M-87 kale (16.61 mg GAE/g dw), and mature M-56 kale (15.84 mg GAE/g dw). Phenolic compounds are considered as important antioxidant constituents in plants(Aryal, Baniya, Danekhu, Kunwar, Gurung, & Koirala, 2019).

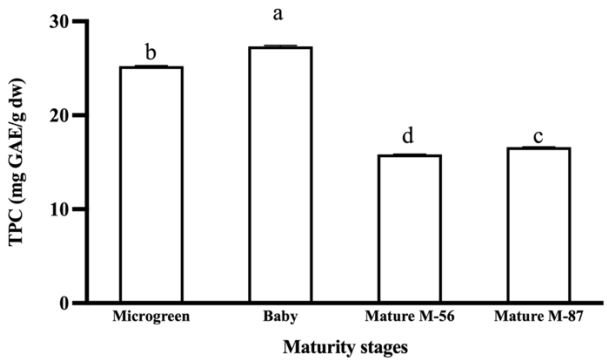


Figure 2.1. The total phenolic content of kale in different maturity stages (Mature M-56 kale: harvested at 56th days from seedling; Mature M-87 kale: harvested at 87th days from seedling, n = 3, $p < 0.05$)

Polyphenoll Composition of the Kale Microgreen, Baby Kale, and Mature Kale

Polyphenol is an important secondary metabolite for plant defense systems. Some of them have been demonstrated to have health benefits in the human body. There are varieties of polyphenols in fruits and vegetables, including flavonols, chalcones, and anthocyanidins etc(S.-O. Lee, Lee, Yu, Im, & Lee, 2005). The polyphenols are functional as antioxidants at relatively low concentrations; however, they could behave as pro-oxidants at higher concentrations.

A total of five chemical compounds including sinapoyl-glucoside, querectin-3-sinapoyl-diglucoside-7-diglucoside, kaempferol-3-sinapoyl-triglucoside-7-diglucoside, disnapoyl-diglucoside, trisinapoyl-diglucoside were tentatively identified in the kale microgreen, with trisinapoyl-diglucoside as the primary component (10.34 μmol/g dw; Table 1). Tomas et al identified the polyphenol compounds in kale microgreen using UHPLC-QTOF, and the result showed that the phenolic acids were the most abundant compounds (343.8 mg/100 g dw)(Tomas, Zhang, Zengin, Rocchetti, Capanoglu, & Lucini, 2021). Comparing with the kale microgreen, the baby kale and mature kale (two harvest points) contained more types of polyphenols. Twenty-three phenolic compounds were identified in baby kale with quercetin as the primary component (19.29 μmol/g dw). Twenty-three and twenty-six phenolic compounds were identified in mature

M-56 kale and mature M-87 kale with kaempferol as the primary component (3.681 μmol/g dw and 3.379 μmol/g dw). The baby kale and mature kale had a similar polyphenol profile.

Baby kale contained the highest average total polyphenol content (35.29 μmol/g dw) which was ~2X more than the mature M-56 kale (18.43 μmol/g dw). The total polyphenol content of kale microgreen and mature M-87 kale was 20.89 μmol/g dw and 16.71 μmol/g dw. Trisinapoyl-diglucoside accounts for around half of the total concentration of polyphenols in kale microgreens. The composition of total polyphenols was varied by maturity stages, there are only five compounds identified from kale microgreens comparing with twenty-three, twenty-six, and twenty-three compounds identified from baby kale, mature kale and mature M-87 kale. The overlapped phenolic compounds were shown in Table 1, the kale microgreen contained the highest sinapoyl-glucoside, disinapol-diglucoside and trisinapoyl-diglucoside. The trisinapoyl-diglucoside of kale microgreen (10.34 μmol/g dw) was ~50X higher than the mature kale (0.278 μmol/g dw). Moreover, most of discrimitive compositions were assigned as sinapoyl esters such as sinapoyl-glucoside, Quercetin 3- sinapoyl-diglucoside-7-diglucoside, Disinapol-diglucoside, and Trisinapoyl-diglucoside, which are considered to exert antioxidant and anticancer activity(Peyrot, Mention, Brunissen, & Allais, 2020). The variations between different maturity stages are affected by multiple factors, including temperature, light, humidity, and insect attacks. As reported, the extracts of young Melia azedarach (L.) leaves demonstrated the greater total phenol content (85.4 mg GAE/g dw) compared to old leaves (59.1 mg GAE/g dw)(M'rabet et al., 2017).

Milic et al(Milić, Djilas, & Čanadanović-Brunet, 1998) have reported that the alkoxyl radical scavenging ability in a lipid peroxidation system, gallic, caffeic, and chlorogenic acids had a relative higher scavenging activity at a lower concentration (0.5 mmol); At higher

concentration (2.0 mmol), the gallic acid had the highest scavenging activity (%RI of the PBN-alkoxyl adduct was 8.42%). Previous studies have found that kale leaves had maximum lutein concentrations after the full expansion of leaves, typically at 1-3 weeks old which is just in the range of microgreen and baby maturity stage(Walsh, Bartlett, & Eperjesi, 2015). This is consistent with the trend of polyphenols concentration in kale. In the quantitative determination of the polyphenols, results showed that the trisinapoyl-diglucoside was present at the greatest concentration of phenolic compounds in microgreen maturity stages, representing 49% of the total amount of polyphenols in the kale microgreen. Moreover, the sinapic derivatives were much higher in kale microgreens than in mature kale. The sinapic acid and its derivatives have been demonstrated to show antimicrobial, anti-inflammatory, and anti-cancer activities(Nićiforović & Abramovič, 2014). This could be potential evidence to support that kale microgreens may have a better health benefit than their mature counterparts where further biological studies are needed.

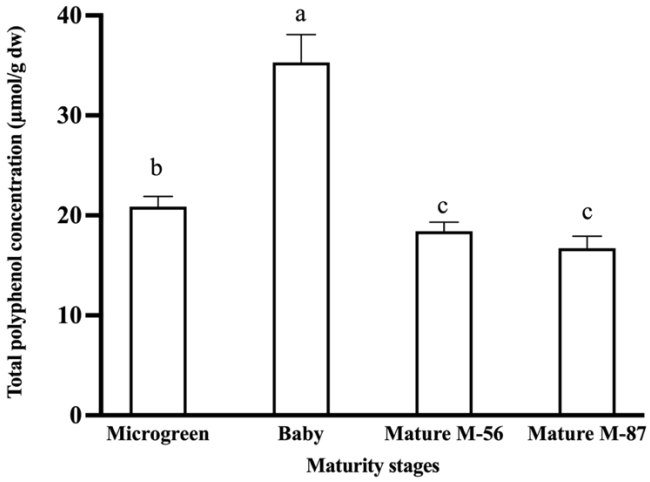


Figure 2.2. The total polyphenol concentration of kale in different maturity stages (Mature M-56 kale: harvested at 56th days from seedling; Mature M-87 kale: harvested at 87th days from seedling, n = 3, $p < 0.05$)

Table 2.1. Characterization of compounds present in kale at different maturity stages

	theor. [M – H]$^-$	exptl. [M – H]$^-$	Chemical formula	Kale microgreen	Baby kale	Mature M-56 kale	Mature M-87 kale
				Concentration (µmol/g)			
Chlorogenic acid	360.6109	353.0880	C16H18O9	N/A	$2.790_{a} \pm 0.282$	$2.450_{a} \pm 0.411$	$1.970_{b} \pm 0.154$
Sinapoyl-glucoside	390.8526	385.1144	C17H22O10	$4.980_{a} \pm 0.010$	ND	$1.790_{b} \pm 0.224$	$1.260_{c} \pm 0.117$
Quercetin 3-sinapoyl-diglucoside-7-diglucoside	1329.3544	1317.7582	C56H70O36	$0.961_{a} \pm 0.142$	$6.300_{b} \pm 0.630$	$0.903_{a} \pm 0.103$	$0.586_{c} \pm 0.054$
Kaempferol-3-sinapoyl-triglucoside-7-diglucoside	1305.2658	1301.3617	C56H70O35	$0.543_{a} \pm 0.024$	$1.730_{b} \pm 0.086$	$0.543_{a} \pm 0.030$	$0.761_{c} \pm 0.191$
Disinapol-diglucoside	770.0968	753.2246	C34H42O19	$4.070_{a} \pm 0.136$	$0.515_{b} \pm 0.053$	$0.418_{c} \pm 0.096$	$1.770_{d} \pm 0.170$
Trisinapoyl-diglucoside	966.7662	959.2838	C45H52O23	$10.300_{a} \pm 0.798$	$0.650_{b} \pm 0.030$	$0.278_{c} \pm 0.035$	$0.625_{b} \pm 0.086$

(thero. [M – H]- and exptl. [M – H]- were theoretical and experimental *m/z* of molecular ions; Data are shown as mean±SD, n = 3 for each stage, ND means not detected. $p < 0.05$)

Overall, the UHPLC-HRMS analysis revealed a wide distribution of flavonols and phenolic acids in different maturity stages of kale. Flavonoids are a type of secondary metabolites abundant in Brassicaceae vegetables. They have varieties of functions including scavenging of free radicals, protection against pathogens, and inhibition of inflammatory factors. In this regard, phenolic acids were more abundant in kale microgreens and flavonols were more abundant in baby kale (O-glycosylated form of quercetin) and mature kale (O-glycosylated form of kaempferol). Our findings were consistent with Sun et al(Jianghao Sun et al., 2013), who profiled the polyphenols in five Brassica species microgreens.

ABTS$^{\bullet+}$ Scavenging Capacity

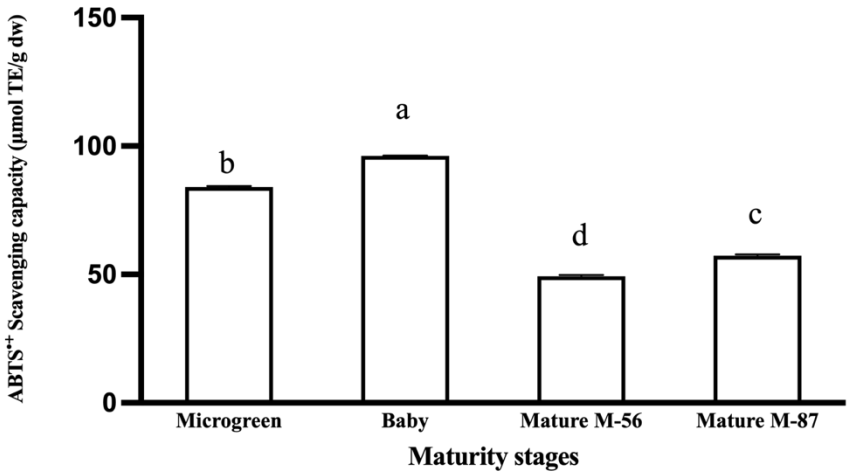


Figure 2.3. The ABTS$^{\bullet+}$ scavenging capacity of kale in different maturity stages (Mature M-56 kale: harvested at 56th days; Mature M-87 kale: harvested at 87th days, n = 3, $p < 0.05$)

Antioxidant activities of kale extracts are shown in Fig 2.3. For all kale maturity stages, the baby kale had the greatest antioxidant activity (96.13 µmol TE/g dw), followed by kale microgreen (84.08 µmol TE/g dw), mature M-87 kale (57.28

μmol TE/g dw), and mature m-56 kale (49.34 μmol TE/g dw). These values are comparable to the $ABTS^{\bullet+}$ scavenging capacities of vegetables previously reported. Cao et al has evaluated the antioxidant capacity in 22 different vegetable types and showed a range from 0.5-19.4 μmol TE/g fw. Among all 22 vegetables, the kale contained the greatest antioxidant capacity(Cao, Sofic, & Prior, 1996). Korus et al determined the antioxidant activity of three varieties of kale in different maturity stages (Maturity stages: I. 70 days after planting seedlings in the field, II. 98 days after planting seedlings in the field, III. 126 days after planting seedlings in the field). All three maturity stages are mature kale, the result showed that an average of 26% higher capacity was found between stage I and stage III (Korus, 2011). Wojdylo et al compared the $ABTS^{\bullet+}$ scavenging capacities between sprout and microgreen of radish, the result showed that the $ABTS^{\bullet+}$ scavenging capacities of radish sprout was three times more than the radish microgreen. This is the first comparison of $ABTS^{\bullet+}$ scavenging capacities for the kale in the different maturity stages. The antioxidant capacity is related to the profiling of phenolic compounds,

Several studies have evaluated the relationship between total phenolic content and antioxidant capacity. Velioglu et al(Velioglu, Mazza, Gao, & Oomah, 1998) found a strong correlation between total phenolic content and antioxidant capacity in selected vegetable products. The total phenolic content of plant materials varied from 1.69 to 105.50 mg/g of dry product. The red onion scale contained the highest total phenolic content while the fibrotein MK43 had the lowest TPC value. There was a significant correlation between TPC and antioxidant capacity for all the plant materials ($p < 0.001$). However, there was a lower correlation between TPC and

antioxidant capacity in anthocyanin-containing plant materials, including blue berries, cherries, and red onion scales. In this study, there was a correlation between TPC and antioxidant capacity among different maturity stages of kale. For example, baby kale had the highest total phenolic content, and the antioxidant capacity of baby kale was also the highest one (Figure 3).

Total Dietary Fiber Content

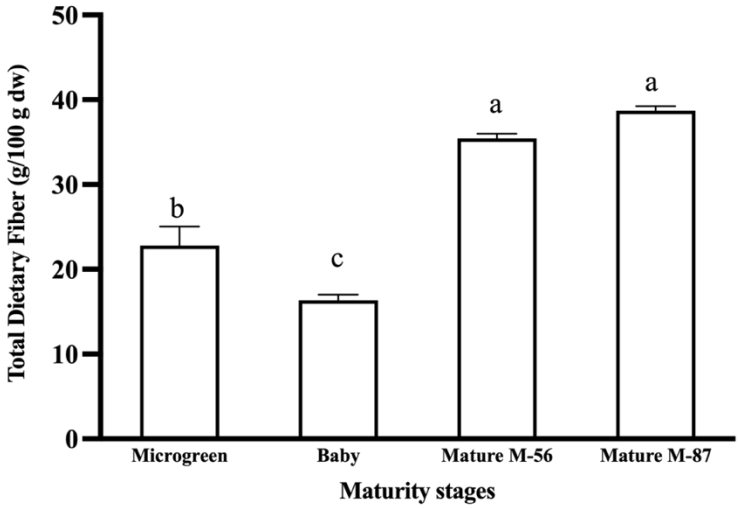


Figure 2.4. Total dietary fiber content of kale in different maturity stages (Mature M-56 kale: harvested at 56th days; Mature M-87 kale: harvested at 87th days, n = 3, $p < 0.05$)

The content of total dietary fiber in foods can be evaluated in several ways, including biochemical analysis, enzymatic method, and chemical method. The biochemical method is the most complex and inaccurate due to the ignoration of indigestible protein, fat, and other plant cell wall related components. The enzymatic method is the most accurate method, however, it includes multiple hydrolysis and adjustment of pH value between each step(Schaller, 1978). In this study, the enzymatic method (AOAC method 985.29) was used to determine the total dietary

content of kale. Both mature kales contain higher fiber content than kale microgreen and baby kale (mature M-56 kale: 35.44 g/100 g dw, mature M-87 kale: 38.73 g/100 g dw, baby kale: 16.33 g/100 g dw, kale microgreen: 22.82 g/100 g dw). The higher content in the mature stage is reasonable as fiber in vegetables was mainly functioned as structural components, the elder the plant, the more plant cell wall could be produced. Punna et al evaluated the change of total, soluble and insoluble fiber in different maturity stages of leafy greens. The result showed there was a significant increase of total dietary fiber and insoluble dietary fiber from tender to mature and to the coarse stage; meanwhile, the soluble dietary fiber only increase from tender to mature, but there was no further increase from mature to coarse stage(Punna & Rao Paruchuri, 2004).

Chapter III: Effects of Cooking Method on the Phytochemical Profile and Antioxidant Abilities (Free radical scavenging capacities) of Mature Kale

Abstract

Cooking is a critical determinant of nutrients in vegetables. This study showed that the mature kale processed by microwaving and boiling significantly enhanced the releasable levels of phytochemicals as compared to the uncooked fresh mature kales. Furthermore, the microwaved kale samples maintained the highest polyphenols (24.68 μmol/g dw**)** and glucosinolates (7.87 μmol/g dw) under the experimental conditions. The observation may be partially explained by the deactivation of related enzymes that may degrade the phytochemicals during the processing. In addition to the cooking effect, the result showed that the mature kale from different sources varied in their responses to food preparation methods.

KEYWORD: cooking, kale, polyphenol, glucosinolate

Introduction

Cooking is an important technique during the consumption of vegetables. It is usually used to soften the vegetable tissue, inactivate anti-nutritional compounds and forming texture and enhancing color. However, the effect of cooking on ??? is still obscured. Some studies reported an increase in bioactive compounds levels and antioxidant capacity after cooking whereas other research reported decreases in???. For example,

Imsic et al(Imsic, Winkler, Tomkins, & Jones, 2010) reported that the 15 min boiling resulted in a significantly decrease in the concentration of β-carotene in carrot. Turkmen et al found an increase of phenolic content after boiling, streaming and microwaving(Turkmen, Sari, & Velioglu, 2005). Several different methods can be used to process kales, the most common of which are boiling, steaming, pressure-cooking, frying, and microwaving(Armesto et al., 2019). Murador et.al used a meta-analysis to evaluate the impact of cooking method on carotenoid and anthocyanin content in vegetables. The result showed that there are no significant difference among boiling, steaming and stir-frying(Murador et al., 2014). They further designed a study to evaluate the effect of home cooking techniques on kale and red cabbage, determining the levels of carotenoids, phenolic compounds, and ascorbic acid by HPLC-DAD-MS^n, wheareas the antioxidant activity was tested using different assays. The carotenoids content has decreased in all cooked kale samples. The boiling process resulted in the lowest concentration of carotenoids (35.47 μg/g) in the cooked samples. The steaming resulted in significant increases (~1.5 X) in total phenolic content in kale (91.49 mg GAE/100 g) and boiling resulted in significant increases in using ABTS radical cation scavenging activity in the red cabbage (uncooked: 6.85 μmol TE/g; boiling: 9.0 μmol TE/g). But the ORAC values showed an opposite result in red cabbage result, which streaming resulted in the highest antioxidant activity in red cabbage and kale (red cabbage: raw 53.97 μmol TE/g, boiling 35.40 μmol TE/g, streaming 62.89 μmol TE/g; kale: raw 46.6 μmol TE/g, boiling 26.53 μmol TE/g, streaming 70.45 μmol TE/g)(Murador et al., 2016).

Several studies have demonstrated that cooking influence the physical characteristics, chemical content, sensory properties, and the nutrient bioavailability of vegetable(Miglio et al., 2008; Vallejo et al., 2002; D. Zhang et al., 2004). The changes depend on the type and quality of raw vegetables and on the type and conditions of the cooking method, and they can be either beneficial or detrimental to the quality of cooked vegetables. More and more studies have demonstrated that the steaming is the best method of preserving the antioxidant capacity and bioactive contents of kale, and the boiling is the worst method which have a 76.4-77.9% losses of total phenolics. However, steaming has obtained the lowest sensory scores in the study. Comparing with those two methods, microwave could relatively protect the antioxidant compound from degrading, but it caused the highest loss of soluble solids (80.8-88.2% dw)(Armesto et al., 2019; Armesto et al., 2016). Overall, among varieties of cooking techniques, the steaming is the best choice although it may decrease the sensory properties.

This study was conducted to evaluate the polyphenol compositions and antioxidant capacity of mature kale under different cooking methods (boil and microwave). The results from this study can serve as a scientific foundation for further studies on kale and other Brassica vegetables.

Materials and Methods

Materials

Mature kale, mature broccoli, and mature red cabbage were purchased from market, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), 2,2'-azinobis (3-ethylbenzothia-zoline-6-

sulfonic) diammonium salt (ABTS), Folin-Ciocalteu (FC) reagent, Gallic acid, Sodium carbonate (Na_2CO_3), Sulfatase (from Helix pomatia), glucotropaeolin potassium salt (≥ 90.0%), DEAE Sepharose CL-6 B Suspension, Imidazole formate and rutin hydrate (≥ 94%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid, Ethanol, acetone, HPLC grade methanol, acetonitrile and water were obtained from Fisher Scientific (Waltham, MA, USA). Total dietary fiber assay kit was purchased from Megazyme (Brey, Iredland).

Cooking Method

There are two different cooking methods: 1. Boiling, first boiling the water and then put mature kales into the pot with boiling water at the ratio of 1:10 (w/v) for 2 mins; 2. Microwave, mix the fresh and intact raw mature kale with boiling water at the ratio of 1:10 (w/v) in a microwavable container, then put the container with sample into the microwave (household microwave oven, SHARP) for 2 mins under default mode (800 W).

Kale Extraction and Preparation

For polyphenols extraction, sample powder (100 mg) was added with 5 mL methanol-water (7:3, v/v), and then sonicate for 1 h. The slurry was centrifuged at 5000 g (IEC Clinical Centrifuge, Damon/IEC Division, Needham, MA, USA) and then filtered with 0.45 μm syringe filter (VWR Scientific, Seattle, WA, USA). The samples (2 μL) were injected into liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) system. In this study, extraction of glucosinolates (GSL) was carried out following the ISO9167-1 method with slight modification [22]. The kale powder (200 mg) and glucotropaeolin (Internal standard, 100 μL, 5 mM) were

transferred to the tube. The samples were extracted with boiling methanol (5 mL) for 20 mins. The slurry was centrifuged at 5000 g (IEC Clinical Centrifuge, Damon/IEC Division, Needham, MA, USA), and then the supernatant (1 mL) was transferred to the prepared Ion exchange columns (DEAE Sepharose CL-6 B Suspension, 500 µL). Two 1 mL portions of the sodium acetate buffer (pH = 4) were gently added to the columns, allowing the buffer to drain after each addition. Purified sulfatase solution (100 µL) was added to each column, leaving to act overnight at room temperature. The desulfo-glucosinolate was eluted and collected with two 1 mL portions of water. The mixture was filtered with 0.45 µm syringe filter (VWR Scientific, Seattle, WA, USA), and then 2 µL was injected into UHPLC-HRMS system.

ABTS•+ Scavenging Capacity

Radical scavenging capacity of kale antioxidant was evaluated against $ABTS^{\bullet+}$ generated by the chemical method according to a previously reported protocol(Miller et al., 1997). A 5 mL of vegetable antioxidants in 70% methanol was diluted with 95 mL of 70% methanol to obtain the testing samples. $ABTS^{\bullet+}$ was prepared by oxidizing 5 mmol/l aqueous solution of $ABTS^{\bullet+}$, 2,20 -azinobis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, with manganese dioxide at ambient temperature for 30 min. The $ABTS^{\bullet+}$–antioxidant reaction mixture contained 1.0 mL of $ABTS^{\bullet+}$ with an absorbance of 0.7 at 734 nm. The absorbance at 734 nm was measured at 1 min of the reaction, and the TE was calculated using a standard curve prepared with Trolox under the same experimental conditions.

Total Phenolic Content

The total phenolic content of kale sample was evaluated by measuring its reducing capacity with the Folin-Ciocalteu (FC) reagent using a spectrophotometer(Stevanato et al., 2004). The reaction mixture contained 50 µL of vegetable extracts, 250 µL of the Folin–Ciocalteu reagent freshly prepared in our laboratory, 750 µL of 20% (w/v) sodium carbonate, and 3 mL of pure water. After 2 h of reaction at ambient temperature, the absorbance at 765nm was measured and used to calculate the phenolic contents using gallic acid as a standard.

Statistical Analysis

All experiments were carried out in triplicate. Data are expressed as mean ± standard deviation. Statistical analysis was determined by a one-way analysis of variation (ANOVA), followed by a post hoc test (Tukey test) using Prism 9 (Graphpad, San Diego, CA, USA). P values less than 0.05 were statistically significant.

Results and Discussion

Total Phenolic Content

The total phenolic content of the kale extracts is shown in Fig 3.1. Among all the samples, the microwaved kale contained the highest total phenolic content (26.57 mg GAE/g dw), followed by boiled kale (16.86 mg GAE/g dw), and raw kale (15.84 mg GAE/g dw). Previous study showed that the boiled kale showed the lowest concentration of carotenoids (35.47 µg/g dw). The steaming resulted in significant increasing (~1.5X) in total phenolic content in kale (91.49 mg GAE/100 g dw)(Murador et al., 2016).

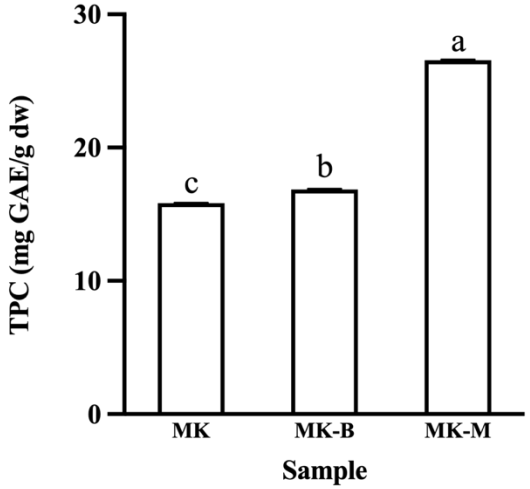


Figure 3.1. The total phenolic content of uncooked and cooked vegetables using different home food preparation methods (MK: Commercialized Mature kale; MK-B: Boiled mature kale MK-M: Microwaved mature kale, n = 3, *$p < 0.05$)

Polyphenol Composition of the Uncooked Mature Kale, Boiled Mature Kale, and Microwaved Mature Kale

The polyphenol concentration was significantly different. The microwave processing (24.690 μmol/g dw) contained the highest total polyphenol content, followed with boil technique (15.240 μmol/g dw). There were some polyphenol compounds that increased in microwave processing but decreased or no change in boil processing compared with raw kale including chlorogenic acid, quercetin 3-diglucoside-7-diglucoside, quercetin 3-sinapoyl-diglucoside-7-glucoside, kaempferol-3-disinapoyl-triglucoside-7-diglucoside.etc. Kaempferol-3-sinapoyl-diglucoside-7-diglucoside (3.317 μmol/g dw) and sinapoyl-feruloyl-diglucoside (3.435 μmol/g dw) in microwaved kale were ~3 times more than in the uncooked kale (1.139 μmol/g and 1.375 μmol/g dw).

During the study, the kale grown in a chamber with constant temperature and light duration had a different phytochemical profile comparing with the kale from the

local market. Thus, we designed a study to investigate the phytochemical profile of kale from different sources. The polyphenol profile was shown in table 3.1.

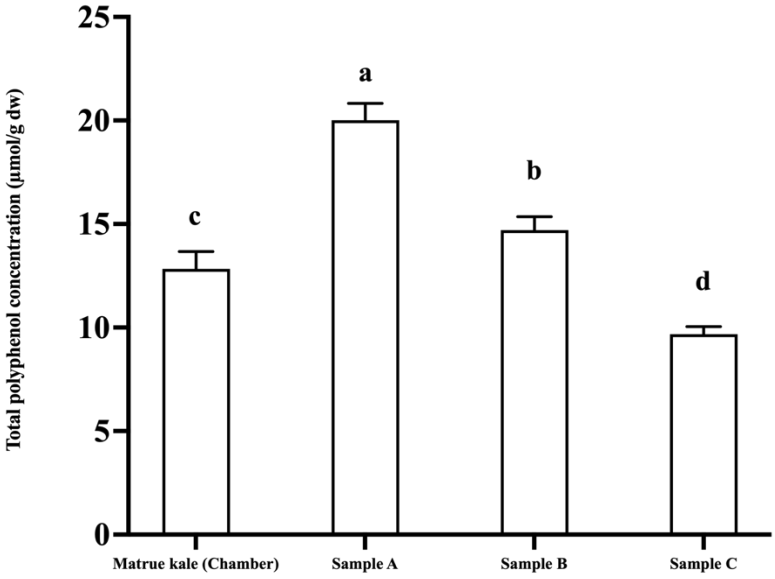


Figure 3.2. Total polyphenol content of mature kale from different sources (n = 3, *$p < 0.05$)

Table 3.1. Characterization of compounds present in mature kale under different cooking methods

	theor. [M – H]⁻	exptl. [M – H]⁻	Chemical formula	Raw mature kale	Boil mature kale	Microwave mature kale
				Concentration (µmol/g)		
rhamnosyl-ellagic acid Chlorogenic acid or its isomer	448.33	447.0544	C20H16O12	$1.893_{b} \pm 0.049$	$1.674_{c} \pm 0.028$	$3.523_{a} \pm 0.21$
Quercetin 3-sinapoyl-diglucoside-7-glucoside	1156.99	1155.3052	C50H60O31	$0.771_{b} \pm 0.022$	$0.467_{c} \pm 0.013$	$2.154_{a} \pm 0.021$
Kaempferol-3-sinapoyl-triglucoside-7-glucoside	1140.99	1139.3194	C50H60O30	$1.139_{c} \pm 0.019$	$1.990_{b} \pm 0.011$	$3.317_{a} \pm 0.019$
Kaempferol-3-feruloyl-diglucoside-7-diglucoside;	1110.97	1109.2997	C49H58O29	$1.105_{b} \pm 0.020$	$0.417_{c} \pm 0.010$	$3.278_{a} \pm 0.022$
Qn-3- disinapoyl-triglucoside-7-diglucoside	1525.32	1523.4413	C67H80O40	$0.183_{c} \pm 0.020$	$0.863_{a} \pm 0.010$	$0.439_{b} \pm 0.018$
Kaempferol-3-disinapoyl-triglucoside-7-diglucoside	1509.33	1507.4213	C67H80O39	$0.507_{b} \pm 0.020$	$0.135_{c} \pm 0.015$	$1.303_{a} \pm 0.023$
sinapoyl-feruloyl-diglucoside	724.66	723.2147	C33H40O18	$1.375_{c} \pm 0.041$	$1.997_{b} \pm 0.033$	$3.435_{a} \pm 0.023$
trisinapoyl-diglucoside	960.88	959.2838	C45H52O23	$0.259_{b} \pm 0.031$	$0.241_{b} \pm 0.012$	$0.392_{a} \pm 0.021$

(thero. [M – H]- and exptl. [M – H]- were theoretical and experimental *m/z* of molecular ions; Data are shown as mean ± SD, n = 3 for each stage, ND means not detected. $p < 0.05$)

Glucosinolates Composition of the Uncooked Mature Kale, Boiled Mature Kale, and Microwaved Mature Kale

The main reason of glucosinolates decreasing is related to the presence of myrosinase. Three varieties of glucosinolates were identified in raw, boil and microwave kale. The microwave processing had a higher glucosinolates content (7.87 μmol/g dw**)**, while the boil processing did not show a significant enhancement of glucosinolates. Previous studies have demonstrated that the thermo-processing could inactivate the myrosinase instead of improving the concentration of glucosinolates. However, it has shown that some of glucosinolates include glucoraphanin and glucobrassicin could be lost during steaming(Jones, Frisina, Winkler, Imsic, & Tomkins, 2010). The possible reason is that boiling may destruct the cell wall and induce the contact of glucosinolates and myrosinase. Song et al., confirmed that the steaming, microwaving, and stir-fry did protect the loss of glucosinolates, while the boiling showed significant lower GSLs content due to the leaching of nutrient into cooking water. The microwave processing could directly induce a heating inside the cell without damage(Jones et al., 2010; Song & Thornalley, 2007). As a result, the microwave had a better preservation of glucosinolates.

Table 3.2. Glucosinolates profile of mature kale by different cooking method

	Raw mature kale	Boiled mature kale	Microwaved mature kale
	Compound (µmol/g)		
Total	$6.349_a \pm 0.196$	$6.548_a \pm 0.492$	$7.873_b \pm 1.33$
Glucobrassicin	$2.180_b \pm 0.668$	$1.540_c \pm 0.140$	$3.00_a \pm 0.827$
Methoxyglucobrassicin	$3.450_b \pm 0.509$	$2.870_b \pm 0.201$	$4.95_a \pm 0.401$
Neoglucobrassicin	$0.719_b \pm 0.346$	$2.140_a \pm 0.151$	$0.345_c \pm 0.150$

ABTS$^{\bullet+}$ scavenging Capacity of the Uncooked Mature Kale, Boiled Mature Kale, and Microwaved Mature Kale

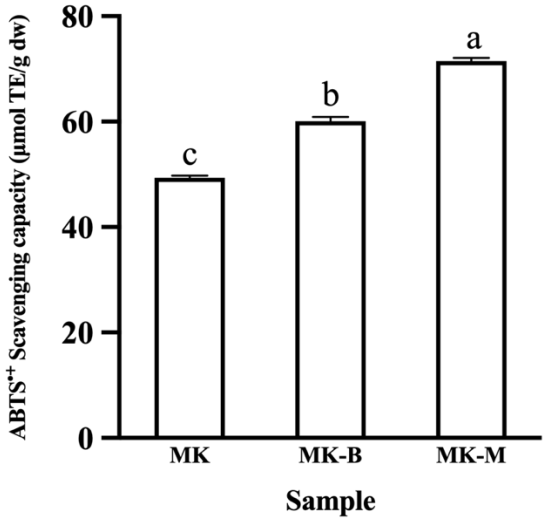


Figure 3.3. The ABTS$^{\bullet+}$ scavenging ability of mature kale in different cooking method, mature broccoli, and mature red cabbage (MK: Commercialized Mature kale, MK-B: Boiling mature kale, MK-M: Microwave mature kale, n = 3, *$p < 0.05$)

Antioxidant activities of microgreen extracts are shown in Fig 3.3. Both of cooking techniques had increased the antioxidant capacity of mature kale, the microwave (71.51 µmol TE/g dw) was the better method in enhancing antioxidant

activity than boiling (60.10 μmol TE/g dw). This could be the loss of hydrophilic antioxidant compounds during boiling.

Chapter IV: Effects of Growing Conditions on Phytochemical Profiles and Antioxidant Abilities (Free Radical Scavenging Capacities) of the Kale, Broccoli, and Red Cabbage Microgreens

Abstract

Microgreens have been demonstrated to contain many health-beneficial compounds, such as polyphenols and glucosinolates (GSLs). The phytochemical composition of microgreens may be affected by growing conditions. The purpose of the current study was to evaluate and compare the phytochemical composition, antioxidant capacity, and total dietary fiber of kale, broccoli, and red cabbage microgreens growing under two conditions (chamber and windowsill). Trisinapoyl-diglucoside was identified as the primary compound in the kale under both conditions, sinapoyl-glucoside was identified as the main compound in broccoli grown under both conditions. For red cabbage, disinapoyl-diglucoside was found at a higher concentration in the chamber sample and sinapoyl-glucoside was found at a higher level in the windowsill sample. Additionally, GSLs, seven in kale, nine in broccoli and eight in red cabbage were also determined. Comparing with the windowsill group, all the three species in the chamber group showed a higher antioxidant capacity. The results demonstrated a significant difference in phytochemical composition between the two growing conditions. This study suggested that the growing factors may have a significant effect on the phytochemical composition and antioxidant capacity in microgreen secondary metabolites.

KEYWORD: microgreen, growing condition, UHPLC-MS, antioxidant

Introduction

In Chapter II, kale microgreens were found to have a greater total polyphenol content than their mature counterpart. Moreover, kale microgreen is an economy-friendly and environment-friendly crop owing to its less requirement of natural sources (water and soil) and time to produce, and it's more suitable to grow at home than mature kale. Mature vegetables, like kale, broccoli, and red cabbage productions on industrial farms generally take 60-150 days, but growing microgreens indoor, only takes 11-14 days, depending on growing conditions(Kelley et al., 2009; Le Strange, Cahn, Koike, & Smith, 2010).

In recent decades, more and more studies have measured the nutrient content of microgreens. Several studies reported that the broccoli, cauliflower, and red cabbage microgreens had higher nutrient concentrations than the mature vegetables(Klopsch, Baldermann, Voss, Rohn, Schreiner, & Neugart, 2018; Xiao et al., 2012; Xiao et al., 2019). Previous studies have shown that broccoli microgreens were rich in anthocyanins (172.51 μg/g fw) , total ascorbic acid (124.1 μg AsA_{TOT}/g fw) and total phenolic content (3.63 μg/g fw)(Marchioni et al., 2021). Beatriz et al evaluated four genotypes of Brassica microgreens, and the result showed that the kale microgreen contained the highest concentration of total soluble polyphenols (2.42 g/100 g fw), and the mustard microgreen contained lest phenolics (1.89 g/100 g fw).

Phytochemical profiling of Brassica microgreens could be affected by pre-harvest and post-harvest factors. It has been demonstrated that the nutritional value of

microgreens could be affected by multiple factors, include genotype, temperature, light quality and quantity, and pest control. Existing studies have indicated that nutrition supplies such as sulphur and nitrite fertilization can have a remarkable impact on the content of phenolic compounds in broccoli [13]. Similarly, glucosinolates (GSLs) are also known to be affected by environmental factors, such as temperature, insect attack, and moisture, which may affect GSLs composition.

To investigate the environmental effects on Brassica microgreens, three species of microgreens (kale, broccoli, and red cabbage) were grown under chamber and windowsill conditions. Generally, the growth chamber provides a more stable environment comparing to the windowsill condition, which could be affected by the daily temperature and light. The antioxidant capacity, polyphenol composition, glucosinolate composition, and total dietary fiber were investigated since this could provide valuable information for agricultural factories.

Materials and Methods

Materials

Kale microgreen, broccoli microgreen and red cabbage microgreen were grew and harvested by USDA collaborators, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2'-azobis (2- amidinopropane) dihydrochloride (AAPH), 2,2'-azinobis (3-ethylbenzothia-zoline-6-sulfonic) diammonium salt (ABTS), Folin-Ciocalteu (FC) reagent, Gallic acid, Sodium carbonate (Na_2CO_3), Sulfatase (from Helix pomatia), glucotropaeolin potassium salt (≥ 90.0%), DEAE Sepharose CL-6 B Suspension, Imidazole formate and rutin hydrate (≥ 94%) were purchased from

Sigma-Aldrich (St. Louis, MO, USA). Formic acid, Ethanol, acetone, HPLC grade methanol, acetonitrile and water were obtained from Fisher Scientific (Waltham, MA, USA). Total dietary fiber assay kit was purchased from Megazyme (Brey, Iredland).

Microgreen Production

Microgreen growth could be affected by multiple factors include genotype, light, temperature etc. In this study, we compared the difference between chamber condition and windowsill condition. Chamber condition had a controlled temperature and light supply, on the contrary, the windowsill condition was followed with the natural weather. The detailed parameters were shown in Supplementary material Appendix 1, Fig. 1. All of three microgreens grown under windowsill had a higher total hour of light than chamber group, but the average temperature of windowsill group was lower than chamber group. Due to the difference of growing condition, the growth rate varied between windowsill and chamber. The final height between two conditions was similar for all of three microgreens. The broccoli microgreen (chamber: 10.05 cm, windowsill: 9.72 cm) had the highest final height, followed by kale (chamber: 7 cm, windowsill: 5.20 cm) and red cabbage (chamber: 6.22 cm, windowsill: 5.86 cm). Moreover, the result showed that the microgreens grown under windowsill had a more consistent growing rate, and the chamber group grew faster at the beginning and then became stable after achieving the final height.

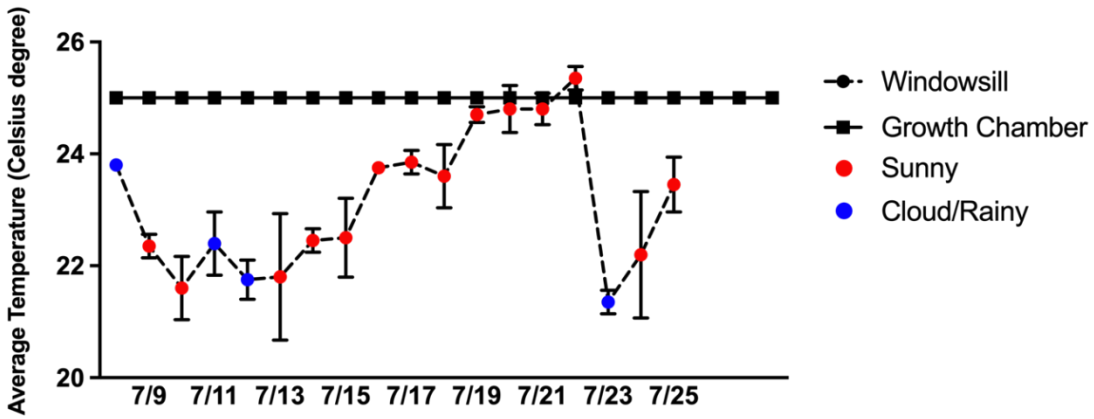


Figure 4.1. The temperature information of kale, broccoli, and red cabbage microgreens growing under different conditions

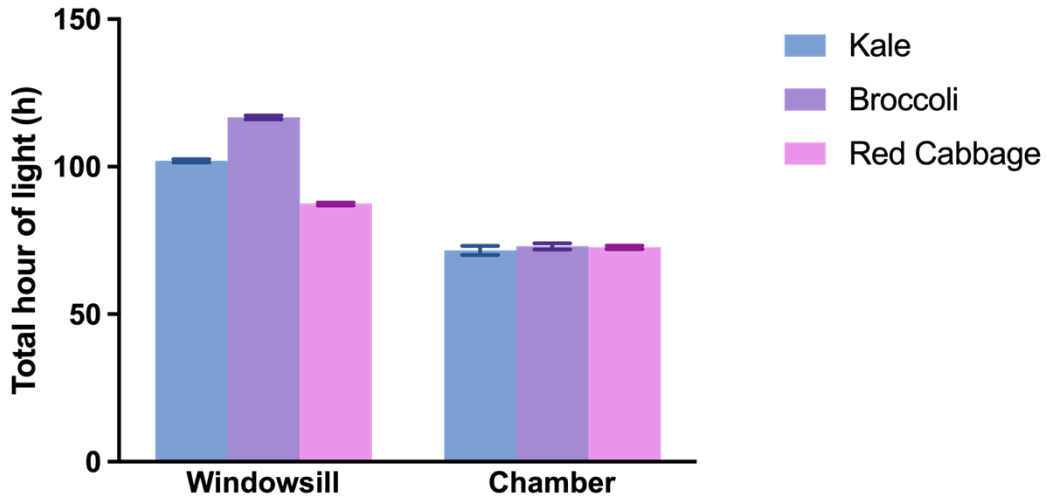


Figure 4.2. The light information of kale, broccoli, and red cabbage microgreens growing under different conditions

Sample Extraction and Preparation

For polyphenols extraction, dried sample powder (100 mg) was added with 5 mL methanol-water (7:3, v/v), and then sonicated for 1 h at room temperature. The slurry was centrifuged at 5000 g (IEC Clinical Centrifuge, Damon/IEC Division, Needham,

MA, USA) and then filtered with 0.45 μm syringe filter (VWR Scientific, Seattle, WA, USA). The samples (2 μL) were injected into liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) system. In this study, extraction of glucosinolates (GSL) was carried out following the ISO9167-1 method with slight modification [22]. The kale powder (200 mg) and glucotropaeolin (Internal standard, 100 μL, 5 mM) were transferred to the tube. The samples were extracted with boiling methanol (5 mL) for 20 mins. The slurry was centrifuged at 5000 g (IEC Clinical Centrifuge, Damon/IEC Division, Needham, MA, USA), and then the supernatant (1 mL) was transferred to the prepared ion exchange columns (DEAE Sepharose CL-6 B Suspension, 500 μL). Two 1 mL portions of the sodium acetate buffer (pH 4) were gently added to the columns, allowing the buffer to drain after each addition. Purified sulfatase solution (100 μL) was added to each column, leaving to act overnight at room temperature. The desulfo-glucosinolate was eluted and collected with two 1 mL portions of water. The mixture was filtered with 0.45 μm syringe filter (VWR Scientific, Seattle, WA, USA), and then 2 μL was injected into UHPLC-HRMS system.

ABTS$^{\bullet+}$ Scavenging Capacity

Free radical scavenging capacity of kale antioxidant was evaluated against ABTS$^{\bullet+}$ generated by the chemical method according to a previously reported protocol(Miller et al., 1997). A 5 mL of vegetable antioxidants in 70% methanol was diluted with 95 mL of 70% methanol to obtain the testing samples. ABTS$^{\bullet+}$ was prepared by oxidizing 5 mmol/l aqueous solution of ABTS, 2,20 -azinobis (3-ethylben-

zothiazoline-6-sulfonic acid diammonium salt, with manganese dioxide at ambient temperature for 30 min. The antioxidant reaction mixture contained 1.0 mL of $ABTS^{\bullet+}$ with an absorbance of 0.7 at 734 nm. The absorbance at 734 nm was measured at 1 min of the reaction, and the TE was calculated using a standard curve prepared with Trolox under the same experimental conditions.

Total Phenolic Content

The total phenolic content of kale sample was evaluated by measuring its reducing capacity with the Folin-Ciocalteu (FC) reagent using a spectrophotometer(Stevanato et al., 2004). The reaction mixture contained 50 μL of vegetable extracts, 250 μL of the Folin–Ciocalteu reagent freshly prepared in our laboratory, 750 μL of 20% (w/v) sodium carbonate, and 3 mL of pure water. After 2 h of reaction at ambient temperature, the absorbance at 765nm was measured and used to calculate the phenolic contents using gallic acid as a standard.

Total Dietary Fiber Determination

The total dietary fiber was determined using the Megazyme TDF Test kit. Weighting duplicate 1 g dried kale sample in the beakers (accurate to 0.1 mg). Adding 50 mL phosphate buffer (pH 6.0) to each beaker and adjust pH to 6.0 ± 0.1. A blanking group without sample was processed to remove the effects of solutions. Firstly, samples were incubated at 98 - 100 °C for 15 mins with 50 μL heat-stable α-amylase solution (to give gelatinization, hydrolysis and depolymerization of starch). Cooling down to room temperature and adjust to pH 7.5 ± 0.1 by adding 10 mL 0.275 N NaOH solution. Then, samples were incubated at 60 °C for 30 mins with 100 μL of

protease solution under pH 7.5 ± 0.1 (to solubilize and depolymerize protein); Cooling down to room temperature and adjust pH to 4.5 ± 0.2 by adding 10 mL 0.325 N HCl solution. After that, samples were incubated at 60 °C for 30 mins with 200 µL amyloglucosidase at pH 4.5 ± 0.2 (to hydrolyze starch fragments to glucose). After incubation, samples were treated with 280 mL 95% EtOH pre-heated to 60 °C to precipitate fiber. Weighting crucible containing Celite to nearest 0.1 mg, then wet and distribute with 78% EtOH from wash bottle. Applying suction to draw Celite onto fritted glass as even mat. The residue was filtered and washed successively with 20 mL portions of 78% ethanol, two 10 mL portions of 95% ethanol and two 10 mL portions of acetone. Then the residue was dried overnight in a 70 °C vacuum oven and weighted. One of the duplicate samples was analyzed for protein by rapid N exceed (fast N-analyzer machine, elementar, Ronkonkoma, NY) , using N × 6.25 as a conversion factor. Theother sample was ashed at 525 °C in a for 5 hours. The total dietary fiber was calculated by using the Megazyme **Mega-Calc**^{TM}, downloaded from the Megazyme web site (www.megazyme.com).

Identification and quantification (UHPLC-PDA-ESI/HRMSn)

The UHPLC-HRMS consisted of a Vanquish UHPLC and Orbitrap Fusion ID-X Tribrid mass spectrometer. The separation was carried out on a Thermo Hypersil Gold AQ RP- C_{18} UHPLC column (200 mm × 2.1 mm i.d., 1.9 µm) (ThermoFisher Scientific, Waltham, MA, USA) with an UltraShield pre-column filter (Analytical Scientific Instruments, Richmond, CA, USA) at a flow rate of 0.3 mL/min. The mobile phase consisted of a combination of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v). For polyphenols quantitation, the linear

gradient was from 2% to 10% B (v/v) at 10 min, to 40% B at 25 min, to 75% B at 30 min, to 90% B at 50 min and maintained 90% B till 60 min. The photodiode array (PDA) recorded spectra from 200-500 nm was used to monitor the peaks. A full mass range was set at m/z 100-1600 with a resolution of 60,000. Data-dependent MS^2 acquisition method was constructed for the top 3 intense ions. AGC values were set to 2×10^5 for MS and 10^4 for MS/MS. The mass injection time was set at 60 mins and 30 mins for polyphenols and GSLs, respectively. H-ESI ion source was used under negative mode with a spray voltage of 2500 V. Sheath gas, aux gas and sweep gas were set at 40, 10, and 5 (arbitrary unit), respectively. Ion transfer tube temp. was set at 300 °C and vaporizer temp. was set at 275 °C. Quantification of total polyphenols was done based on a standard curve of rutin, and linearity of the calibration curve was achieved between 10 to 500 µg/mL ($r^2 = 0.9994$). For GSLs quantitation, the linear gradient was from 1% to 3% B (v/v) at 5 min, to 20% B at 15 min and to 75% B at 30 min. The UV wavelength was set at 229 nm to record the peaks. Relative response factors were applied to correct for differences in UV absorbance between glucotropaeolin (Internal standard) and desulfoglucosinolates

LC-MS Data Pre-treatment and Handling

Raw files from UHPLC-HRMS were converted into mzXML format using Proteowizard 3.0.20210 (http://proteowizard.sourceforge.net/), and XCMS online was selected for advanced data processing [23]. The step included ion feature extraction, peak picking and alignment. Parameter settings for XCMS processing were as follows: centWave for feature detection ($\Delta m/z = 2.5$ ppm, minimum peak width = 10 s, and maximum peak width = 60 s); obiwarp settings for retention-time correction

(profStep = 1); and parameters for chromatogram alignment, including mzwid = 0.015, minfrac = 0.5, and bw = 5. The relative quantification of ion features was based on extracted ion chromatogram (EIC) areas. The ion feature list was downloaded and entities at retention time 0.5–25.0 min for polyphenols and 0.5-20.0 min for GSLs were used for further statistical analysis. Then, the entities were exported into a two-dimensional (samples × mass peaks) matrix in Excel (Microsoft Corporation, Redmond, WA, USA).

Statistical analysis

All experiments were carried out in triplicate. Data are expressed as mean ± standard deviation. Statistical analysis was determined by a one-way analysis of variation (ANOVA), followed by a post hoc test (Tukey test) using Prism 9 (Graphpad, San Diego, CA, USA). P values less than 0.05 were statistically significant.

Results and Discussion

Total Phenolic Content

Phenolic compounds are a group of secondary metabolites that naturally found in microgreens and showed great antioxidant capacities(Ho, 1992). The total phenolic content of the kale extracts is shown in Fig 4.1. The pattern of TPC was similar as antioxidant capacity that the chamber group was higher than the windowsill group. Among all the materials, red cabbage grown under chamber had the highest phenolic content (36.01 mg GAE/g, dw), followed by chamber kale microgreen (25.22 mg GAE/g dw), chamber broccoli microgreen (24.73 mg GAE/g dw). The red cabbage microgreen showed a much higher TPC value which is almost three times higher than

the windowsill group (13.01 mg GAE/g dw). Previous study has evaluated the TPC value of thirty commercial *Brassicaceae* microgreens. The result showed that the ruby radish microgreen contained the highest phenolic content (8.11 mg GAE/g, fw) and the cress microgreen had the lowest phenolic content (0.89 mg GAE/g, fw). The evaluation of total phenolic content could be affected by the source of samples and sample preparation method(Podsędek, 2007). As a result, the data reported by different studies are sometimes hard to compare.

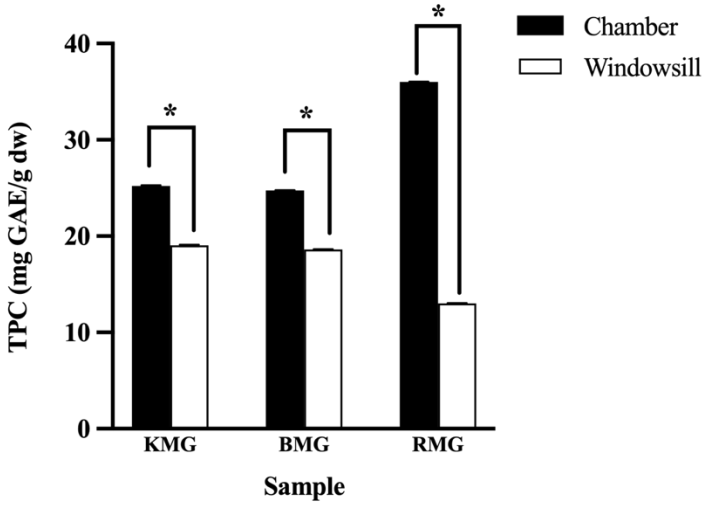


Figure 4.3. The total phenolic content of microgreens under different growing conditions (KMG: kale microgreen, BMG: broccoli microgreen, RMG: red cabbage microgreen, n = 3, *$p < 0.05$)

Polyphenol Composition of kale, broccoli, and red cabbage microgreens under different growing conditions

UHPLC-HRMS analysis of the methanolic extracts of microgreens revealed their phenolic composition influenced by environmental conditions. There was no significant difference for total phenolic concentrations between two growing conditions in kale microgreen and broccoli microgreen. However, the red cabbage microgreen grown under windowsill (34.04 μmol/g, dw) had a greater total

polyphenol concentration than red cabbage grown under chamber (23.01 μmol/g, dw). All samples contained some phenolic acids (mainly sinapic, chlorogenic, ferulic and their derivatives) and flavonols (mainly quercetin, kaempferol, isorhamnetin). Dominant microgreen flavonols include derivatives of quercetin (e.g., quercetin 3-sinapoyl-diglucoside-7-diglucoside) and kaempferol (kaempferol 3-sinapoyl-triglucoside-7-diglucoside).

Phenolic acids and their derivatives were a dominant group of polyphenols in microgreens of kale, broccoli and red cabbage growing under growth chamber (88.3%, 64.5%, 76.1% of total phenolic compounds, respectively) and windowsill condition (83.0%, 88.5%, 85.1% of total phenolic compounds, respectively). The trisinapoyl-diglucoside was the most abundant compounds in kale microgreen both from the chamber (10.34 μmol/g dw) and from the windowsill (7.75 μmol/g dw). The Sinapoylglucoside was the highest compound in broccoli microgreen grown under windowsill (12.66 μmol/g, dw) which was ~2X more than kale microgreen under windowsill condition (6.73 μmol/g, dw). The chlorogenic acid was only identified in red cabbage microgreen growing under windowsill, while the sinapic acid and its derivatives were found in three microgreen species growing under both conditions. Interestingly, all of three microgreens under two conditions had scant amounts of flavonols and their glycosides that are common in mature Brassica vegetables. It has been demonstrated that the temperature could be the main variable that affect the levels of phenolic compounds(Rezende, Borges, Santos, Alves, & Paula, 2015). In chapter 2, flavonols and their derivatives were the dominant group of the baby kale and mature kale (82.7% and 50% of total phenolic compounds, respectively). This

was comparable with previous studies(Baenas, Gómez-Jodar, Moreno, García-Viguera, & Periago, 2017; Jianghao Sun et al., 2013). Microgreens have been demonstrated to be a great source of polyphenols. Sun et al reported the polyphenol profile of fiver Brassica microgreens species using ultrahigh performance chromatography photodiode array high resolution multistage mass spectrometry. A total of 164 polyphenols including 105 flavonol glycosides, 30 anthocyanins, and 29 hydroxycinnamic were identified. This study showed that the Brassica microgreen species contained more complex polyphenol profile than their mature counterparts(J. Sun et al., 2013).

The results demonstrated clear variation in secondary metabolism between two conditions and suggested that environment factors could greatly affect the phytochemical composition of *Brassica* microgreens, which would provide valuable information for the agricultural industries. The main factors that may affect the phytochemical composition include temperature and light dosage. The chamber condition was kept under a stable condition, whereas the turbulent temperature and light changed with the weather showed a significant influence on phenolic compounds.

Table 4.1. Characterization of polyphenol compounds present in microgreens under different growing conditions

	theor. [M – H]⁻	exptl. [M – H]⁻	Chemical formula	KMG-C	KMG-W	BMG-C	BMG-W	RMG-C	RMG-W
				Concentration (µmol/g)					
Sinapoyl-glucoside	390.8526	385.1144	C17H22O10	4.980 ± 0.010	6.734* ± 0.457	10.482 ± 0.506	12.663* ± 0.330	5.609 ± 0.535	10.768* ± 1.876
Quercetin 3-sinapoyl-diglucoside-7-diglucoside	1329.3544	1317.7582	C56H70O36	0.961 ± 0.142	0.776 ± 0.018	ND	ND	ND	ND
Kaempferol-3-sinapoyl-triglucoside-7-diglucoside	1305.2658	1301.3617	C56H70O35	0.543* ± 0.024	0.174 ± 0.042	ND	ND	ND	ND
Disinapol-diglucoside	770.0968	753.2246	C34H42O19	4.067 ± 0.136	3.549 ± 0.136	4.403 ± 0.105	4.120 ± 0.325	8.307 ± 2.017*	7.014 ± 0.753
Trisinapoyl-diglucoside	966.7662	959.2838	C45H52O23	10.341* ± 0.798	8.506 ± 0.631	4.323* ± 0.091	3.256 ± 0.098	1.776 ± 0.387	1.439 ± 0.047

(thero. [M – H]- and exptl. [M – H]- were theoretical and experimental *m/z* of molecular ions;
Data are shown as mean±SD, n = 3 for each stage, ND means not detected. $p < 0.05$)

Glucosinolates Composition of kale, broccoli, and red cabbage microgreens under different growing conditions

Glucosinolates (GSLs) is another important phytochemical which has great health benefit in human diet. Nowadays, the most consumed and well-studied glucosinolates within our diets are those derive from methionine, such as sulforaphane and allyl-glucosinolates, and aromatic glucosinolates, such as phenethyl- and benzyl-glucosinolate(Traka, 2016). Glucosinolates themselves are not biologically active compound, but they could hydrolysis in some specific situations and generate several biologically active molecules(Bones & Rossiter, 1996). One of the main compounds produced from the hydrolysis of glucosinolates is isothiocyanate (ITC). The in vivo metabolism of ITC has been studied a lot, after exporting to the extracellular matrix, ITC metabolites moved to the liver through the hepatic portal vein, where they are metabolized through mercapturic acid pathway(Brüsewitz et al., 1977). Several studies have measured the glucosinolates content in varieties of vegetables. The average content of GSLs for broccoli sprouts ranged from ~160 to >250 mg/100 g, the cauliflower was ~40 to 80 mg/100 g, and ~30 to 95 mg/100 g for red cabbage(Ciska, Martyniak-Przybyszewska, & Kozlowska, 2000). In this study, there were 8 main glucosinolates have been identified including Glucoiberin (GIB), Sinigrin (SIN), 4-hydroxyglucobrassicin (4-HGBS), Glucoibervirin (GER), Glucobrassicin (GBS), Methoxyglucobrassicin (MGBS), and Neoglucobrassicin (NGBS). Overall, the chamber group contained a higher glucosinolates content than

windowsill group. Kale microgreen (33.12 μmol/g dw) grown under chamber had the highest concentration of glucosinolates and red cabbage (12.51 μmol/g dw) grown under windowsill contained the lowest concentration of glucosinolates. Interestingly, the main compound is varied from species, sinigrin is the highest GSLs in kale (chamber: 18.73 μmol/g dw; windowsill: 17.06 μmol/g dw), glucoraphanin is the highest GSLs in broccoli (chamber: 10.56 μmol/g dw; windowsill: 10.55 μmol/g dw), and progoitrin is the most abundant GSLs in red cabbage (chamber: 7.16 μmol/g dw; windowsill: 5.79 μmol/g dw). The potential reason of lower concentration in windowsill group could be the degradation of glucosinolates during growth. It has been demonstrated that when the plant faced with negative issues such as insect attack, the glucosinolates will convert to the toxic isothiocyanates to reduce the outside attack(Hopkins, van Dam, & van Loon, 2009).

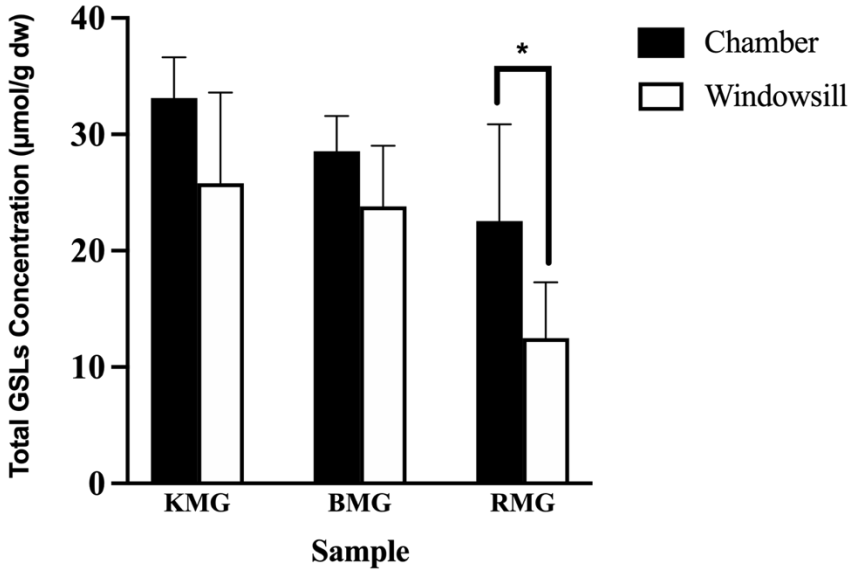
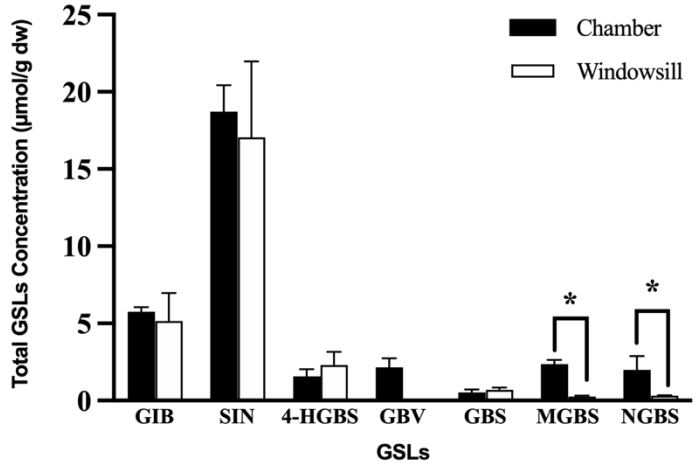
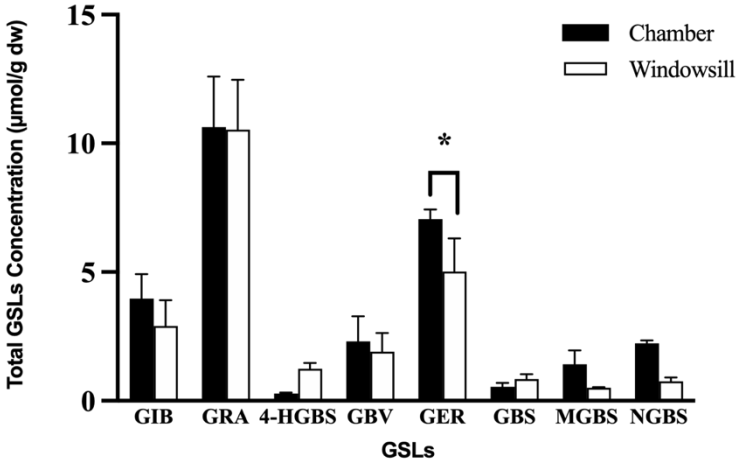


Figure 4.4. Total Concentrations of the GSLs from different types of microgreens identified via UHPLC-HRMS under chamber and windowsill growing conditions

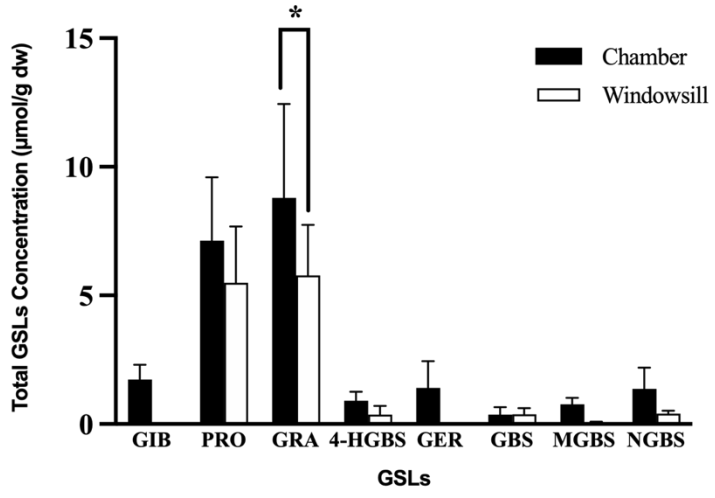
(KMG: kale microgreen, BMG: broccoli microgreen, RMG: red cabbage microgreen, n = 3, *$p < 0.05$*).



A



B



C

Figure 4.5. Concentrations of the GSLs from different types of microgreens identified via UHPLC-HRMS under chamber and windowsill growing conditions
(A: Kale, B: Broccoli, C: Red cabbage, Glucoiberin (GIB), Sinigrin (SIN), 4-hydroxyglucobrassicin (4-HGBS), Glucoibervirin (GER), Glucobrassicin (GBS), Methoxyglucobrassicin (MGBS), and Neoglucobrassicin (NGBS), n = 3, *$p < 0.05$)

ABTS$^{\bullet+}$ scavenging Capacity of the kale, broccoli, and red cabbage microgreens under different growing conditions

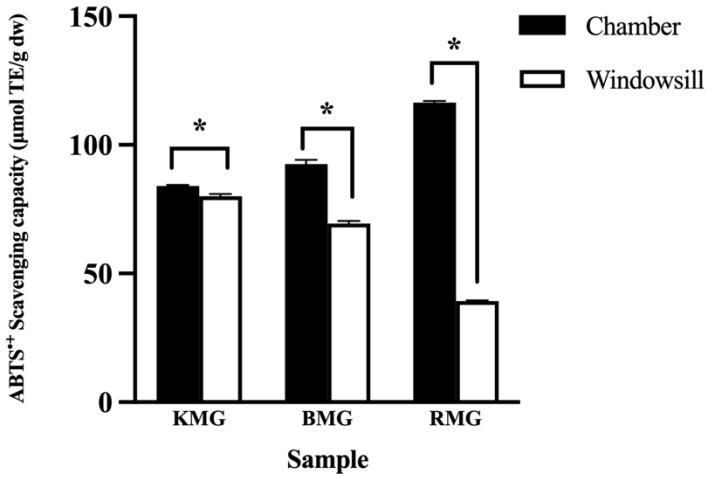


Figure 4.6. The ABTS cation radical scavenging ability of microgreens under different growing conditions
(KMG: kale microgreen, BMG: broccoli microgreen, RMG: red cabbage microgreen, n = 3, *$p < 0.05$)

Antioxidant activities of microgreen extracts are shown in Fig 4. For all microgreen materials, the red cabbage microgreen grown under chamber condition had much higher antioxidant activity (116.38 µmol TE/g dw), which was ~3X more than red cabbage microgreen grown under windowsill (39.33 µmol TE/g dw). Other two chamber groups, broccoli microgreen (892.54 µmol TE/g dw), and kale

microgreen (84.08 μmol TE/g dw) also had a higher concentration than their windowsill groups. Tan has measured the antioxidant capacity and total phenolic concentration of broccoli microgreens from different source (commercially versus local farm). No significantly difference in antioxidant capacity and total phenolic concentration between two sources was found. But the one from a local farm had a higher chlorophyll concentration(Tan et al., 2020).

Total Dietary Fiber Content of kale, broccoli, and red cabbage microgreens under different growing conditions

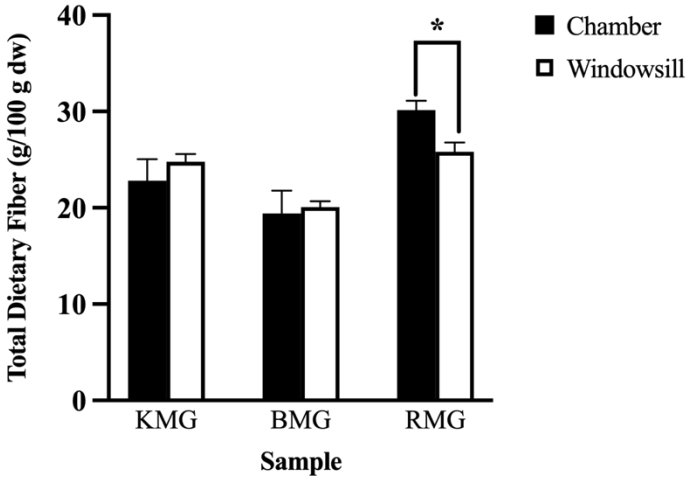


Figure 4.7. Total dietary fiber content of microgreen in different growing conditions
(KMG: kale microgreen, BMG: broccoli microgreen, RMG: red cabbage microgreen; n = 3, *$p < 0.05$)

No significant difference was found in kale microgreen and broccoli microgreen. Red cabbage microgreen grown under chamber (30.16 g/100 g dw) had a higher fiber content than windowsill red cabbage microgreen (25.83 g/100 g dw). And the red cabbage microgreen grown under chamber condition also contained the

highest concentration of total dietary fiber. It has been found that the dietary fiber content was high in chicory genotypes ranging from 0.19-0.8 g/100 g, fw. In addition, both red cabbage samples contained higher total dietary fibers than other two species. The dietary fiber plays a different role in human health when compared with phytochemicals.

Appendices

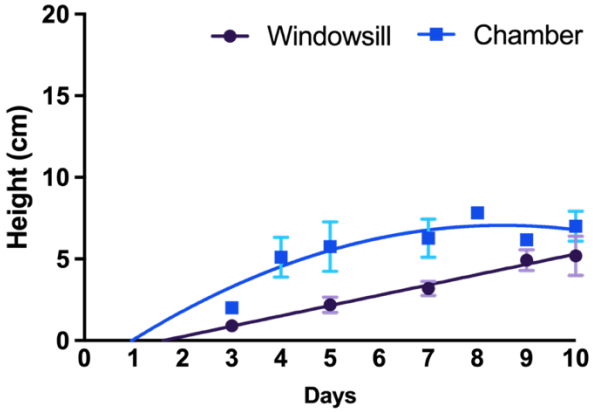


Figure S1. The germination rate of kale microgreen growing under different conditions

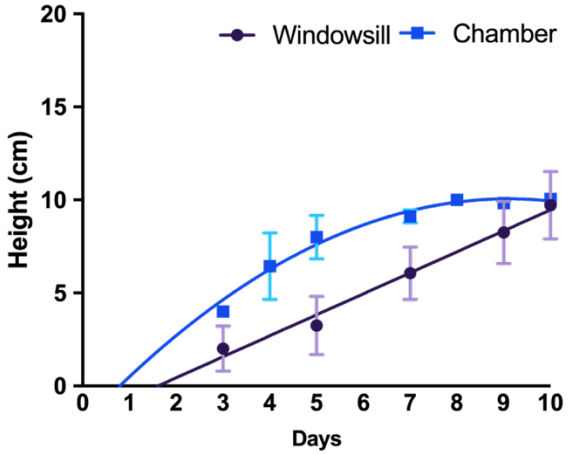


Figure S2. The germination rate of broccoli microgreen growing under different

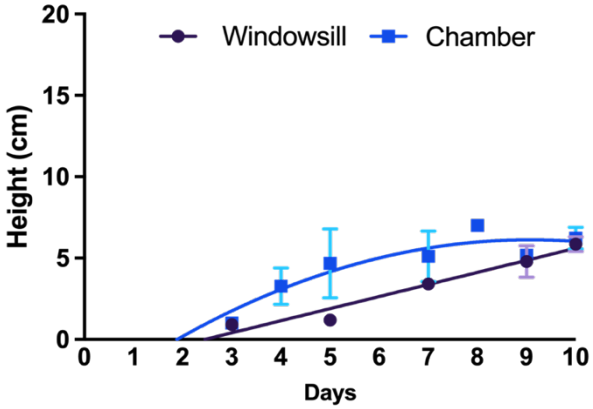


Figure S3. The germination rate of red cabbage microgreen growing under different

	theor. $[M-H]^-$	exptl. $[M-H]^-$	Chemical formula	Kale microgreen	Baby kale	Mature M-56 kale	Mature M-87 kale
				Concentration (µmol/g)			
Chlorogenic acid	360.6109	353.0880	C16H18O9	N/A	2.790 [a] ± 0.282	2.450 [a] ± 0.411	1.970 [b] ± 0.154
Sinapoyl-glucoside	390.8526	385.1144	C17H22O10	4.980 [a] ± 0.010	ND	1.790 [b] ± 0.224	1.260 [c] ± 0.117
Quercetin 3-sinapoyl-diglucoside-7-diglucoside	1329.3544	1317.7582	C56H70O36	0.961[a] ± 0.142	6.300 [b] ± 0.630	0.903 [a] ± 0.103	0.586 [c] ± 0.054
Kaempferol-3-sinapoyl-triglucoside-7-diglucoside	1305.2658	1301.3617	C56H70O35	0.543 [a] ± 0.024	1.730 [b] ± 0.086	0.543 [a] ± 0.030	0.761 [c] ± 0.191
Disinapol-diglucoside	770.0968	753.2246	C34H42O19	4.070 [a] ± 0.136	0.515 [b] ± 0.053	0.418 [c] ± 0.096	1.770 [d] ± 0.170
Trisinapoyl-diglucoside	966.7662	959.2838	C45H52O23	10.300 [a] ± 0.798	0.650 [b] ± 0.030	0.278 [c] ± 0.035	0.625 [b] ± 0.086

Table S1. Characterization of Compounds Present in kale at different maturity stages

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