ULTRASONICATION AS AN ABIOTIC ELICITOR - EFFECTS ON ANTIOXIDANT CAPACITY AND OVERALL QUALITY OF ROMAINE LETTUCE

BY

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THESIS

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ABSTRACT

Fresh produce is important for human health. Various minimal processing technologies have been developed over years to meet the growing demands of consumers for high quality and “fresh-like” fruits and vegetables. However, there have been few food processing researches emphasizing on stimulating the biological system of food itself to achieve an enhanced postharvest quality. It is hypothesized that low acoustic power density (APD) ultrasound, as a form of physical energy, could stimulate the biological stress defense response system of a plant to increase its own secondary metabolite accumulation to achieve added nutritional value and better quality retention.

The effect of ultrasound treatment at low APDs on antioxidant capacity and overall quality of Romaine lettuce was evaluated. Whole leaf lettuce was treated with ultrasound (25 kHz) at APD of 26 W/L for 1 - 3 minutes and stored at room temperature for up to 150 hours. Quality indices examined included color, texture, total phenolics, antioxidant capacity, and sensory properties. Phenylalanine ammonia-layse (PAL) activity of lettuce from different treatments was monitored during storage.

There were no differences in sample quality attributes between ultrasound treatment and control immediately after treatment. During storage, Romaine lettuce samples treated by ultrasound for 2 min and 3 min exhibited an increase in PAL activity, resulting in production of phenolic compounds as secondary metabolites and enhancement of antioxidant capacity. Ultrasonic application did not cause sample deterioration, and under certain conditions it delayed
enzymatic browning and maintained better overall quality. A hypothetical model for the effect of
low APD ultrasound as an abiotic elicitor on fresh produce was proposed based on the present
study and evidence from reports on responses of cell cultures to ultrasonication.

Further studies are needed to achieve a better understanding of the physiological responses
of fresh produce to ultrasound treatment, which would help obtain value-added fruit and
vegetable products.
To My Family
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CHAPTER 1
INTRODUCTION

There has been increasing interest in the consumption of fresh fruits and vegetables in the U.S. during the past several years (Cisneros-Zevallos, 2003; Cook 2013). Regular intake of fruits and vegetables, which provide vitamins, minerals and phytochemicals, was reported to be related to a reduced incidence of cancer and other degenerative diseases (Heber, 2004). As the largest category of phytochemicals in plant foods, phenolic compounds are highly associated with the antioxidant capacity of fruits and vegetables (Liu, 2003). The health benefits mainly are attributed to the ability of phenolic compounds to relieve oxidative stress in human cells. Phenolic compounds are classified as plant secondary metabolites. It is possible to stimulate production and accumulation of phenolic compounds by biotic and abiotic elicitors through plant defense responses during post-harvest processing to obtain better nutritional quality (Bartwal, 2012). Meanwhile, fresh produce items are perishable during distribution and storage due to growth of quality degradation organisms such as bacteria, yeast and molds and losses in sensory and nutritional quality (Beuchat et al., 1998). Many processing technologies have been proposed and tested for potential application in the produce industry to enhance the safety and quality of fresh produce.

High intensity ultrasound or power ultrasound with frequency ranging from 20-100 kHz is one of the advanced technologies applicable in food processing and preservation (Feng, 2011). It has been applied in processes such as extraction, emulsification, crystallization, heat transfer enhancement, surface decontamination, and microbial and enzyme inactivation (Patist and Bates, 2011; Zhou, 2012). Generally, ultrasound treatments at high acoustic power density (APD) lead
to destructive results while treatments at low APD have been demonstrated to stimulate living cells and enzymes.

Not until recent years have there been studies discussing the application of ultrasound for fresh produce preservation (Xu et al., 2013). Among these studies, most of them focused on the effect of ultrasound on microbial safety after processing. When used alone or combined with sanitizers, ultrasonication was able to destroy or remove bacteria, mold and yeast on the surfaces of produce such as strawberry (Cao et al., 2010a; Cao et al., 2010b), plum (Chen and Zhu, 2011), lettuce (Seymour et al., 2002), and spinach (Zhou et al., 2009, 2012). Some studies also reported that ultrasound treatment was effective in maintaining the sensory and nutritional quality of selected produce (Cao et al., 2010a; Chen and Zhu, 2011; Alexandre et al., 2012). Meanwhile, other reports (Rudolf and Resurreccion, 2005; Potrebko and Resurreccion, 2009; Sales and Resurreccion, 2010) documented an increase of total phenolics and antioxidant capacity in sonicated produce samples. However, no study so far has provided solid explanation or evidence for the physiological mechanism of these effects on fresh produce.

On the other hand, the elicitation effect of sonication has been well-investigated in biotechnology research. Ultrasound as a form of physical energy has various physical, chemical and biological effects. Several studies conducted on algal, plant cell and animal cell cultures demonstrated the defense response induced by low APD ultrasonication. Wu and Lin (2001; 2002a; 2002b) reported that ultrasound treatment triggered cross-membrane ion fluxes, production of reactive oxygen species (ROS), and a rapid increase of phenylalanine ammonia lyase (PAL) activity, followed by increased production of polyphenols (PP) and phenolic compounds in *P. ginseng* cell suspension. Wang et al. (2006) discovered another signaling molecule of the defense response pathway, nitric oxide (NO), after ultrasonication in *Taxus* cell
culture. Induced production of phenolic secondary metabolites was also reported in *Morinda citrigolia* cell cultures (Komaraiah, 2005), hazelnut cell cultures (Rezael, 2011), and *Vitis vinifera* cell cultures (Santamaria, 2012).

We hypothesized that low APD ultrasonication could act as an abiotic elicitor for whole plants, triggering the defense response system and stimulating production of secondary metabolites, which would enhance antioxidant capacity and maintain postharvest quality of fresh produce. In the present study, we determined the impact of ultrasonication on overall quality, phenolic production, and antioxidant capacity of fresh Romaine lettuce. The mechanism of the induced production and accumulation of secondary metabolites was investigated by examining the responses of defense-related enzyme to ultrasound treatment.
CHAPTER 2
LITERATURE REVIEW

2.1 Fresh and fresh-cut produce industry

2.1.1 Fresh and fresh-cut produce production and consumption

Production of produce, generally including fruits and vegetables, has experienced a remarkable increase globally. Based on FAO Statistical Yearbook 2013, total production of fruits was estimated as 640 million tons and total production of vegetables was over 1 billion tons in 2011, with an annual increase rate of 3% over the last decade (FAO, 2013).

With the demand for foods of high-quality, freshness, and convenience, with the awareness of how diets are related to human health, with more households able to pay for high-quality and convenient products, and with the policy involvement for promoting fruits and vegetables consumption, there has been a growing trend of fresh produce consumption in the United States (Cook, 2013). According to the U.S. Department of Agriculture’s Economic Research Service (ERS), the consumption of fruits and vegetables (in both fresh and processed forms) per capita increased 8.4 percent from 1976 to 2009. Although the consumption of processed fruits and vegetables still overweighs the fresh forms, the fresh shares have grown by 12 percent for fruits and 5 percent for vegetables in the past 25 years. Additionally, fresh produce consumption was found positively correlated with income and education.

The consumer’s willingness to pay for fresh, safe, healthy and convenient plant foods also led to the growth of fresh-cut produce industry. The International Fresh-cut Produce Association (IFPA) defines “Fresh-cut produce” as any fresh fruit or vegetables or any combination thereof that has been physically altered from its original form, but remains in a fresh state. Regardless of
commodity, it has been trimmed, peeled, washed and cut into 100% usable product that is subsequently bagged or prepackaged to offer consumers high nutrition, convenience and value while still maintaining freshness (IFPA, 2002). Since fresh-cut produce meets the needs of people in this fast-pace world, the market share for ready-to-eat salads keeps increasing worldwide. The estimated market value in 2008 was about $15.5 billion in the U.S. (Cook, 2008), $4.5 billion in Europe (Palmer, 2009), followed by $3.7 billion in Japan and Korea (Kim, 2007).

2.1.2 Health benefits of fruits and vegetables

Fruits and vegetables are important in human diet. The nutritional significance of fresh produce was first discovered in the early 17th century when plant food (lemon juice) was recommended to prevent scurvy during long sea voyages (Wills et al., 2007). Since then, the nutritional value of fresh produce was gradually recognized. Fruits and vegetables promote human health by providing macronutrients like water, carbohydrates, proteins, lipids, and dietary fiber as well as micronutrients including vitamins, minerals and phytochemicals. They are major sources of water-soluble vitamins especially vitamin B complex and vitamin C, and minerals like potassium, iron, calcium and magnesium. Phytochemical is another critical group of nutrients in fruits and vegetables. It is defined as the bioactive molecules in plant foods with the ability to protect against diseases, to scavenge free radicals, and to provide antimicrobial functions. Carotenoids, chlorophylls, flavonoids, isothiocyanates, and sulfur-containing compounds are among such chemicals in produce (Butt and Sultan, 2011).

A positive correlation has been established between the regular intake of fruits and vegetables and a reduced risk of cancer and other degenerative diseases (Dixon, 1995). One of the major reasons is that fruits and vegetables are antioxidant-rich foods. Antioxidants are
chemicals, when in low concentrations, that help delay or prevent oxidation of substrates (Aruoma, 1999). Antioxidants consumed through diet provide protection against oxidative stress caused by reactive oxygen species (ROS) produced during cellular metabolism in human body, and hence combat aging and chronic noncommunicable diseases (NCD) (Valko et al., 2007). Other than preventing cardiovascular diseases and certain cancers, control of diabetes mellitus, promotion of digestive health and improvement of immune system are all health benefits from fruit and vegetable consumption.

WHO and FAO recommended a minimum of 400 grams daily intake of fruits and vegetables (excluding starchy tubers) in their recent report. WHO even announced a consumption of less than five total servings (400 grams) per day for 18+ year-old adults to be an indicator of noncommunicable diseases (NCD) (WHO, 2014a). Since fruits and vegetables are so important in healthy diet, a joint program by WHO and FAO was launched in 2003 to promote fruit and vegetable consumption around the world (WHO, 2014b). In the U.S., the Dietary Guidelines for Americans, 2010 recommended Americans to eat more fruits and vegetables in diet based on recent analyses of food consumption (U.S. Department of Health and Human service, 2011). Considering all those recommendations, many states in the U.S. have started to make efforts by attempting to improve access to fruits and vegetables to communities, and to establish policies to increase consumption (CDC, 2013).

2.2 Lettuce

Lettuce (Lactuca sativa L.) is a vegetable crop grown all over the world. Lettuce contains about 95% of water, and is a good source of vitamins and minerals. In the U.S., lettuce is one of the most commonly consumed vegetables. Per capita use of lettuce was estimated as 25.8 pounds
in 2012. They are often used raw in the fresh form, particularly popular in salads. Lettuce is generally categorized into head lettuce, Romaine lettuce and leaf lettuce. Back to the 1980s, head lettuce was dominant in the market, whereas, head lettuce and Romaine & leaf types almost had similar market share in 2012 (USDA, 2013).

Like other leafy vegetables, lettuce is perishable after harvest. Meanwhile, it is sensitive to exogenous ethylene although ethylene production of its own is relatively low (Gross et al., 2004). Cold storage and low oxygen atmosphere is helpful for maintaining postharvest quality.

Besides traditional whole fresh lettuce for bulk sale, fresh-cut lettuce and those combined in pre-packed mixed salads become very popular nowadays. A typical processing procedure to prepare fresh-cut lettuce includes trimming, size reduction, washing, and packaging. Since cutting leads to tissue damage, biochemical and microbiological degradations appear in fresh-cut lettuce. Enzymatic browning, tissue softening, and microbial growth are major problems that should be controlled during processing.

2.3 Advancement of postharvest processing technologies

2.3.1 Primary goal of food processing

One of the primary goals of food processing is to preserve food quality, including wholesomeness, nutritional value, sensory quality and microbial safety (Karl et al., 1975).

Globally, each year, the postharvest loss of food reaches 1.3 billion tons (Hubert et al., 2010). As for fruits and vegetables, about 40% of the total amount is lost annually during storage due to deterioration and loss of quality (Lacroix and Ouattara, 2000). While most produce are grown locally, agricultural trades take place both nationally and globally to fill the gap between production and demands. From 2000 to 2010, the export of fruits and vegetables has almost
doubled at the global level. On the other hand, in the U.S., there are very few local sales of fruits and vegetables. In 2010, this value was estimated as $3 billion out of $122.8 billion total final value of fruits and vegetables (Low and Stephen, 2011). The majority of produce are grown in distant locations and then distributed nationwide to supermarkets, other retail outlets or food service establishments by shippers. In 2010, California produced 49% of the value of fresh vegetables and 53% of fresh fruits, followed by Florida (14% of vegetables and 8% of fruits), Arizona (8% of vegetables) and Washington (21% of fruits) (Cook, 2011). The long distribution chain requires reliable preservation techniques. Meanwhile, there are growing demands for produce beyond their growing seasons and regions. Thus, improvement of postharvest preservation technologies for fruits and vegetables is essential.

2.3.2 Postharvest processing and handling for produce

Efforts have been made to develop processing technologies and handling system to better retain postharvest quality and extend shelf life of fresh produce. Several postharvest physiological properties of fruits and vegetables are taken into consideration, especially respiration rate, storage temperature, relative humidity, and gas concentration (e.g. O₂, CO₂ and ethylene) (Raju et al., 2011).

After harvest, fresh produce is graded before further handling. Sanitation procedure ensures food safety by removing external microorganisms, pests and pesticides. Water washing with 100 ppm chlorine is the most commonly used sanitation method for fresh produce (Pirovani et al., 2001), while hydrogen peroxide and ozone are also effective in sanitation (Sapers et al., 1999; Akbs and Ozdemir 2006). Precooling and cold storage slow down respiration and microbial growth, and help reduce quality degradation. Storage temperature should be carefully selected
for each produce to prevent chilling and freezing injuries. For bulk-stored fresh produce, controlled-atmosphere storage can be applied by modifying the gas composition in the environment. Reduced O₂ (3-5%) and increased CO₂ (up to 10%) can retard respiration of fruits and vegetables as well as inactive microorganisms (Ohlsson, 1994). For individually-packed products, modified-atmosphere packaging (MAP) technology significantly prolongs shelf life of fresh produce (Kader AA et al., 1989). Similar to controlled-atmosphere storage, MAP also works on optimizing O₂, CO₂ and ethylene concentrations. Packaging materials for MAP maintain ideal gas concentration in the package by controlling gaseous exchange between the interior package atmosphere and exterior ambient atmosphere.

2.3.3 Development of non-thermal and advanced thermal food processing technologies

Processing of fruits and vegetables aims to improve shelf life and quality, enhance palatability and digestibility, and increase nutrient availability by inactivation of nutritional inhibitors (Butt and Sultan, 2011).

Conventional processing technologies are mainly based on the effect of thermal energy to eliminate spoilage microorganisms and foodborne pathogens and to inactivate enzymes. However, these traditional thermal processing technologies, such as canning, drying, and freezing, bring negative influence on sensory properties and nutritional qualities of processed produce. Loss of Vitamin C (ascorbic acid) ranges from 8% to 90% during canning (Howard et al., 1999; Jiratanan and Liu, 2004) and 10% to 80% during freezing (preparation by blanching) (Fennema, 1982). Total phenolics also decline during thermal processing because they are water-soluble, thus susceptible to leaching (Richman et al., 2007).

The increasing demands for high-quality and fresh-like produce promote the development
of emerging minimal processing technologies. Non-thermal processing technologies include high pressure processing (HPP), irradiation, ultraviolet light, ultrasound, pulse electric field (PEF), etc. Advanced thermal processing technologies include ohmic heating (OH), RF heating, microwaving, etc (Ahmed and Alam, 2011). They have been proved effective in produce preservation. Similar to traditional thermal technologies, the basic concept is to prolong shelf life by rupturing or eliminating microorganisms and inactivating quality degradation. Besides, minimal processing technologies are superior to traditional methods because they maintain fresh quality and sensory attributes of fruits and vegetables by escaping excessive heat (Ohlsson, 1994; Ohlsson, 2002).

However, for the processing procedures mentioned above, food materials to be processed only play a passive role. In other words, although processing helps to retard quality degradation, after processing or during storage, physical quality and nutritional value of fruits and vegetables will not exceed the initial status.

2.4 Plant responses against environmental stresses

2.4.1 Environmental stress to plants

As living organisms, plants are exposed to various stresses during their growth. In the subject of plant physiology, “stress” is considered as a significant deviation from optimal living conditions for life, and eliciting changes and responses at all functional levels of the organism which are reversible at first, but may also become permanent (Larcher, 2003). Because stress conditions are potentially lethal to plants life, plants have developed stress response reactions to protect themselves to a normal state. According to Lichtenthaler (1996), stress response is dose-dependent. A mild stress can be favorable for plants by activating cell metabolism and
physiological activity without damaging plants even if at a long duration. However, high doses are likely to cause damaging effect or even lead to death if the stress lasts for long.

Stress factors (stressors) can be divided into two categories: biotic factors and abiotic factors. Biotic stressors, such as infection, herbivory and competition, come from interactions with other organisms. Abiotic stressors include physical stresses like temperature (heat or cold), water (drought or flooding/hypoxia), radiation (light, UV and ionizing radiation), wind, soil movement, submergence, electrical fields and magnetic fields, and chemical stresses like mineral salts (deficiency or over-supply), pollutants (heavy metals and pesticides), and gaseous toxins (Schulze et al., 2005). Although different stress factors (stressors) stimulate defense response with specific mechanisms, most stress responses follow a similar phase model (Figure 2.1). The beginning of stress response is the alarm phase, where a disturbance occurs on standard phase and results in a reduced vitality of plants. Then, if the stress intensity remains the same, restitution by repair processes such as synthesis of protein or other protective substances leads to an increase of stress resistance. The end phase appears with a prolonged stress or increased intensity, leading to the stage of exhaustion. A 4th phase, regeneration phase, may generate when stress is removed and the damage caused during stress responses is not irreversible.

2.4.2 Plant elicitors and elicitation mechanisms

Based on plant defense response, an “elicitor” can be defined as a substance which, when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds (Namdeo, 2007). Elicitor has been proved to be useful to enhance plant-secondary-metabolites synthesis by the elicitation effect on secondary-metabolism-related enzymes, oxidative burst, and signal transduction (Radman et al., 2003).
Similar to stress factors, plant elicitors can be classified as physical or chemical, biotic or abiotic and complex or defined depending on the origin (Radman et al., 2003). Most studies in this field discussed about complex biotic elicitors such as yeast cell wall and fungal spores. Some defined biotic elicitors have also been discovered including carbohydrates (polysaccharides, oligosaccharides), proteins (peptides, proteins), lipids, glycoproteins and volatiles. Abiotic elicitors are those non-biological factors from different origins. Injury is a typical physical elicitor to plants. Other abiotic elicitors include UV light, windfall, denatured proteins (RNase), heavy metals, some other non-biological-origin chemicals, etc (Namdeo, 2007).

Studies about the mechanisms of elicitation effect on plants and plant cells mainly focus on biotic elicitors. The general mechanism is based on elicitor-receptor interaction, followed by a series of biochemical reactions during defense response. It starts with the binding of elicitor to a plasma membrane receptor, which causes changes in ion fluxes across the cell membrane. Then, the induced synthesis of secondary messengers mediates Ca$^{2+}$ release, nitric oxide and octadecanoid signaling pathway. Another important step is the production of reactive oxygen specious (ROS) such as superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$), which might act as secondary signal or directly inactive microorganisms. Through signal transduction pathways, corresponding defense-response genes are activated which induce production of defense compounds such as tannins and phytoalexins. Finally, systemic resistance for certain elicitor can be acquired by plants or plant cells (Radman et al., 2003).

Mechanisms of elicitation of abiotic elicitors might be a little different from pathogen attack, especially the role of ROS plays during stress response. In respond to abiotic stress, ROS scavenging enzymes are activated to reduce ROS concentration in plants (Apel and Hirt, 2004). Since ROS, if excessively accumulated, have detrimental effects on cell vitality, certain
defense-related enzymes, for example superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), are activated to detoxify ROS (Fric, 1976). Non-enzymatic antioxidants, such as ascorbate and phenolic compounds, also involve in ROS scavenging as antioxidants during defense response.

2.4.3 Phenylalanine ammonia-lyase (PAL)

Phenylalanine ammonia-lyase (PAL; E.C.4.3.1.5; 270-330 kDa) is one of the most important enzymes during plant stress defense response. It can be found in high plants, some fungi, yeast and a single prokaryote, but is absent in true bacteria and animals (MacDonald and D`Cunha, 2007). It was discovered and isolated in 1961 (Koukol and Conn, 1961). By now, the use of PAL has extended into different industrial and medical applications.

PAL is the first and key enzyme in phenyl propanoid pathway which directly relates to secondary metabolites production. Phenyl propanoid pathway is the starting point of lignin biosynthesis, as well as other small molecules such as flavonoids, coumarins, hydroxycinnamic acid conjugates, and lignans (Fraser and Chapple, 2011). The first three reactions in the phenyl propanoid pathway are shown in Figure 2.2. PAL catalyzes the first step which transforms phenylalanine to trans-cinnamic acid and ammonia (Koukol and Conn, 1961). Phenolic compounds biosynthesis occurs subsequently in this pathway. Thus, PAL serves as a key regulatory enzyme and an indicator of plant defense system and secondary metabolism.

2.4.4 Plant secondary metabolites and phenolic compounds

Plants consist of both primary and secondary metabolites. While primary metabolites present in all plant cells and are essential for plants’ survival and reproduction, secondary
metabolites are not required directly for plants to grow (Barwal et al., 2012). However, secondary metabolites are not waste of primary metabolism because they perform specialized functions (Bennett and Wallsgrove, 1994). They determine color of plants, attract pollinators and seed-dispersing animals, and most importantly, protect plants under stress conditions (Seiger 1998; Crozier et al., 2006). Terpenoids, phenolics, alkaloids, and sulfur-containing compounds are considered to be main secondary metabolites in plants.

Phenolic compounds are one large group of plant secondary metabolites, with more than 8,000 currently identified chemically different molecules (Bravo, 1998). They diverse in structures but all consist of an aromatic ring with at least one hydroxyl group or its functional derivatives attached to it. Based on chemical structures, phenolic compounds can be categorized into flavonoids, phenolic acid, and other phenolics such as phenolic amides, resveratrol and lignans. Phenolic acids and flavonoids are the most abundant phenolic compounds in plants. Phenolic acids can be further divided into two main types: benzoic acid derivatives (C1-C6 backbone) and cinnamic acid derivatives (C3-C6 backbone) (Tsao, 2010). Flavonoids comprise of two aromatic rings, connected by an oxygen containing 3-carbon bridge. Based on degree of oxidation of the 3-carbon bridge, this group can be divided into four types: flavones, flavonols, isoflavones and anthocyanidins (Sinha, 2003).

Phenolic compounds provide protection against pathogens, herbivores, UV radiation and more. Figure 2.3 briefly summarizes the role of phenolics in both biotic and abiotic plant defense response. The most significant biological function of phenolics is antioxidant activity due to their ability to donate hydrogen from hydroxyl group to terminate oxidation by ROS (Foti et al., 1994). Meanwhile, the stimulation effect of phenolic compounds on antioxidant enzymes also contributes to eliminating ROS. Phenolic compounds play important roles both in food
preservation and diseases prevention. Bioactive phenolic compounds have been proved to improve storage quality and increase shelf life of fruits, vegetables, legumes, and grains. Moreover, phenolic compounds are nutrients which can protect cells from oxidative stress generated by human cellular metabolism, as thus promising to combat noncommunicable chronic diseases (Sarkar and Shetty, 2014).

2.5 Postharvest treatments that increase secondary metabolites production and accumulation

Studies have been carried out on both physical elicitors and chemical elicitors. Wounding is the most well-investigated processing-based elicitor for fruits and vegetables. Reyes et al. (2007) evaluated the effect of wounding on PAL activity, total soluble phenolics and antioxidant capacity in different fruits and vegetables including zucchini, white and red cabbage, iceberg lettuce, celery, carrot, parsnips, red radish, sweet potato and potatoes. They found different tissues responded differently where PAL activity increased 6.0-73.3 fold, phenolic changed from a 26% decrease to a 191% increase, and antioxidant capacity ranged from a 51% decrease to a 442% increase from initial value among different produce. Figure 2.4 shows biochemical pathways for the production and accumulation of antioxidant phenolic compounds during wounding-healing process proposed by Jacobo-Velazquez and Cisneros-Zevallos (2012). This was a typical defense response triggered by abiotic stressor. Besides, processing technologies with the application of other physical elicitors such as electron-beam ionizing radiation (Reyes and Cisneros-Zevallos, 2007), UV light (González-Aguilar et al., 2007; Erkan et al., 2008), hyperoxia (Jacobo-Velazquez et al., 2011) and heat shock (Alegria et al., 2012) were found to induce phenolics accumulation and antioxidant capacity increase in a variety of fresh fruits and
vegetables. Exogenous application of chemical elicitors like ethylene and methyl jasmonate slightly affected phenolic synthesis of carrot. However, a synergistic effect to promote phenolics accumulation was observed when these chemicals were combined with wounding stress (Heredia and Cisneros-Zevallos, 2009).

### 2.6 Effect of ultrasound treatment on postharvest quality of fresh produce

Ultrasound is a type of sound wave whose frequency is above 20 kHz. The ultrasonic spectrum can be divided into two types: high-intensity ultrasound (power ultrasound) with frequency ranging from 20 kHz to around 1MHz, and low-intensity ultrasound (diagnostic ultrasound) with frequency exceeding 1 MHz (Feng, 2011).

In food industry, power ultrasound is the most commonly used type, based on its physical and chemical effects on foods or related materials. “Cavitation” is the term describing activities of microbubbles from formation to collapse caused by ultrasonication. Ultrasound treatment results in an increase in temperature and pressure in the vicinity of cavitating bubbles, generation of streaming and shear force, along with some chemical effects especially the induced production of free radicals (H• and OH•) (Ashokkumar et al., 2008). Ultrasound treatments with different properties can lead to opposite impacts. For example, ultrasound has been proved to both improve and to weaken enzyme activities in separate studies (Sakakiabra et al., 1996; Vercet et al., 2002). In general, ultrasound with high power density is destructive while mild ultrasound can stimulate biological activities (Miller et al., 1996).

Power ultrasound has been applied into a variety of areas in food processing. It is indeed promising in minimal processing of fresh produce because ultrasound treatment can be easily combined into the washing system already utilized in the industry. The effect of ultrasound on
postharvest preservation of fresh fruits and vegetables has also been investigated recently. Ultrasound treatment, if applied under appropriate conditions, can improve overall postharvest quality of fresh produce in all microbiological, physical and nutritional aspects (Xu et al., 2013). Ultrasonication enhances microbial safety by directly removing or destroying spoilage and pathogenic microorganisms through cavitation when used alone (Joyce et al., 2003), or by facilitating chemical penetration when combined with sanitizers or other chemicals. A beneficial effect of ultrasound on physical quality, especially maintaining color and delay of texture softening, has also been reported (Xu et al., 2013). The mechanisms of preservation were hypothesized to be inactivation of deterioration-related enzymes such as pectin methylesterase (PME), polygalacturonase (PG) (Cao et al., 2010), polyphenol oxidase (PPO) and peroxidase (POD) (Chen et al., 2012), along with reduced respiration rate. Meanwhile, studies also explored better nutrients retention of fruits and vegetables with ultrasound application. Some researchers attributed this phenomenon to the inhibition of decay incidence and microbial population (Cao et al., 2010) while others proposed that the reduced respiration might be the major reason for the nutritional quality retention (Xu et al., 2013). However, no evidence has been documented so far on the molecular basis for all hypotheses mentioned above.


2.7 Figures and Tables

**STRESS SYNDROME RESPONSES OF PLANTS**

<table>
<thead>
<tr>
<th>Phase without stress</th>
<th>Alarm phase</th>
<th>Stage of resistance</th>
<th>Stage of exhaustion</th>
<th>Regeneration phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>stress response</td>
<td>resistance</td>
<td>hardening</td>
<td>removal of the stressor</td>
<td>new standards</td>
</tr>
<tr>
<td>standard</td>
<td>maximum</td>
<td>acute damage</td>
<td>chronic damage, cell death</td>
<td></td>
</tr>
<tr>
<td>resistance minimum</td>
<td></td>
<td>restitution</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.1** General concept of the phase sequences and responses induced in plants by stress exposure.

（Lichtenthaler, 1996）
Figure 2.2 Phenyl propanoids pathway.

Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate: CoA ligase.

(Fraser and Chapple, 2011)
Figure 2.3 Plant’s defense mechanisms involving phenolics for biotic and abiotic stress tolerance.

(Sarkar and Shetty, 2014)
Figure 2.4 Wound-induced activation of pathways related with the biosynthesis of phenolic compounds in plants.

Abbreviations: JA = Jasmonic acid; ET = Ethylene; ROS = Reactive oxygen species; OPPP = Oxidative pentose phosphate pathway; PEP = Phosphoenolpyruvate; E-4P = Erythrose-4 phosphate; PAL = Phenylalanine ammonia-lyase; C4H = Coumarate 4-hydroxylase; 4CL = 4-Coumarate CoA ligase.

(Jacobo-Velazquez and Cisneros-Zevallos 2012)
CHAPTER 3
EFFECT OF ULTRASOUND ON ANTIOXIDANT CAPACITY AND SECONDARY METABOLITES PRODUCTION OF ROMAINE LETTUCE

3.1 Introduction

Fruit and vegetables are important in human diet due to their nutritional value. They can promote human health by providing macronutrients especially dietary fiber as well as micronutrients including vitamins, minerals and phytochemicals. A positive correlation has been established between the regular intake of fruit and vegetables and a reduced risk of cancer and other chronic diseases (Dixon, 1995). Based on their nutritional significance, WHO and FAO have launched a worldwide program aiming to promote fruit and vegetable consumption since 2003.

Fresh produce is generally considered perishable, undergoing deterioration soon after harvest. Meanwhile, the time from farm to end consumer becomes longer due to the industrialization of fresh produce production and international trade. The development of postharvest technologies to help retain quality and extend shelf life becomes crucial nowadays. Traditional thermal technologies such as canning, drying/dehydration and freezing have been widely used over years (Raju et al., 2011). Non-thermal technologies and some other innovative minimal processing technologies have also been discovered to achieve “fresh-like” food products (Ohlsson, 2002). However, most of the current food processing technologies for postharvest produce handling work under a simple concept that they help reduce or control the growth of pathogens and spoilage microorganisms, inactive enzymes, or retard plant respiration and
metabolism. Since the processed food only plays a passive role, these technologies only work for reducing quality degradation. Physical losses and nutritional quality losses are still obvious.

A few recent studies have documented the effects of selected physical and chemical treatments on the production of secondary metabolites, mainly phenolic compounds in produce. Phenolic compounds are among the stress-induced secondary metabolites categorized as antioxidants that are beneficial for human health (Heo et al., 2007). The Luis Cisneros-Zevallos’s group has published a series of articles discussing the effect of physical elicitors like wounding (Reyes et al., 2007), electron-beam ionizing radiation (Reyes and Cisneros-Zevallos, 2007), hyperoxia (Jacobo-Velazquez et al., 2011), and chemical elicitors like the exogenous application of ethylene and methyl jasmonate (Heredia and Cisneros-Zevallos, 2009). They found an increase of phenolic content and antioxidant capacity in some fruits and vegetables after wounding stress, but the increase was dependent on the type of plant tissue and wounding intensity. Electron-beam ionizing radiation and hyperoxia were proved effective to induce the production of phenolic compounds of mango fruit and carrot respectively. Ethylene and methyl jasmonate slightly affected phenolic synthesis of carrot when used alone, but induced accumulation of phenolic compounds was observed after combining wounding and hormone stresses. Studies about UV-C irradiation provided evidence for the enhanced phenolic content and antioxidant capacity in mango, strawberry and carrot (González-Aguilar et al., 2007; Erkan et al., 2008). Heat shock was also found possible to stimulate the accumulation of phenolic compounds (Alegria et al., 2012).

As a type of physical energy, ultrasound has various physical, chemical and biological effects (Miller et al., 1996). Many studies reported that mild ultrasonication (at low acoustic power density) can act as an abiotic elicitor to trigger typical defense response pathways in alga,
plant cell and animal cell cultures: an oxidative burst was stimulated, followed by the accumulation of phenolic compounds and other secondary metabolites (Wu and Lin, 2001, 2002a, 2002b; Komaraiah, 2005; Wang et al., 2006; Rezael, 2011; Santamaria, 2012). However, research for fresh produce or whole plants is still very limited. Peanuts achieved increased total phenolics and total antioxidant capacity with ultrasound treatment (Rudolf and Resurreccion, 2005; Potrebko and Resurreccion, 2009; Sales and Resurreccion, 2010). The activity of antioxidant enzymes (CAT, SOD and GR) were enhanced by ultrasonic vibration pretreatment of wheat seedling under heavy metal stress, to eliminate ROS generated by the stress (Chen et al., 2013). Meanwhile, the physiological mechanism and molecular basis for the ultrasound-induced defense response and accumulation of secondary metabolites in fresh produce are still lacking. An increased antioxidant capacity of fresh produce means an enhanced nutritional value. Therefore, a solid understanding of physiological basis is necessary in order to develop appropriate applications and optimize treatment conditions from the basis.

In this study, we proposed to utilize ultrasound as a physical elicitor to trigger the plant defense response to stress factors, thus inducing secondary metabolites synthesis. We worked on fresh Romaine lettuce to explore the effect of ultrasound treatment on the postharvest accumulation of phenolic compounds and antioxidant capacity. Defense-related enzyme (PAL) was analyzed to investigate the defense response to ultrasonication. A hypothetical physiological mechanism was also proposed based on literature and the current study.

3.2 Materials and Methods

3.2.1 Sample preparation

Romaine lettuce (L. sativa, var longifolia) was purchased from a local supermarket
(Champaign, IL) and stored in cold room at 34°F until used. Samples were pre-selected based on size, color, and visual quality to ensure the initial consistency. Before processing, the outer three leaves, as well as any other leaves with visible damage were discarded. The next 4 or 8 undamaged leaves were carefully removed, and the bottom part of the stem was cut with a clean sharp stainless steel knife to make each leaf a 10 g sample. They were then randomly divided into different treatment groups for at least 3 replications.

3.2.2 Ultrasound treatment

A specially designed ultrasound treatment chamber was utilized in this study (Figure 3.1). The system consisted of an ultrasound generator and a stainless steel water tank (400 mm × 660 mm × 460 mm). Two transducer boxes (400 mm × 400 mm × 50 mm) were vertically attached to the inner walls of the tank face to face, with a space of 280 mm between the boxes. The pair of transducer boxes was at the frequency of 25 kHz with 2 kW nominal power. Unlike traditional ultrasound washing baths or probe units that generate non-uniform sound intensity distribution in the medium (Ando and Kagwa, 1989; Klima et al., 2006), the acoustic field distribution of the ultrasound treatment chamber utilized here was nearly uniform, as determined by an aluminum foil test (Zhou, 2012). A uniform exposure of samples to ultrasonication was important because we wanted to make sure samples placed at different locations of the tank received almost the same ultrasonic energy. A single layer of Romaine lettuce leaves were placed with no overlapping in a sample holder (400 mm × 400 mm × 30 mm) made by a wooden frame and two pieces of stretchable polyethylene mesh to fix samples without blocking sound waves.

Before the test, the water tank was filled with 115 L room temperature tap water. In each test run, Romaine lettuce samples (60 g, 6 leaves) were properly fixed in the holder which was
submerged in the water tank parallel to the transducer boxes. Ultrasound at 25 kHz frequency and 100% power output from the generator was applied to samples through the water medium for 1, 2 or 3 min in each treatment. Samples washed with tap water for 3 min were used as the control.

Whole leaf Romaine lettuce was used for all analyses to eliminate the influence of wounding by cutting (Reyes, 2007). Treated whole leaf lettuce samples were air-dried (30 min) and stored in plastic gallon zipper storage bags (GLAD, Oakland, CA). All samples were sealed in bags, covered with thick brown paper to prevent light exposure, and stored at room temperature up to 150 hrs. Each group had at least 3 replicates.

3.2.3 Acoustic power density determination

Three power values should be considered in a sonication based food processing system: $P_E$ which is the output power by the generator, $P_T$ which is the power supplied to the transducer, and $P_{diss}$ which is the power dissipated in the medium. In this study, $P_{diss}$ was determined to represent the acoustic power density in the system.

A calorimetric method as described by Manas et al. (2000) was used. The estimation of acoustic power density was based on the temperature increase in medium because ultrasonic waves dissipate as heat. $P_{diss}$ was calculated with the following equation:

$$P_{diss} = mc_p \left( \frac{dT}{dt} \right)$$

where $m$ is the mass of the liquid (kg), $c_p$ is the specific heat capacity of the liquid (J/ (kg K)), and $\left( \frac{dT}{dt} \right)$ is the temperature rise in the liquid by time (K/s).

In the measurement, ultrasound at 25 kHz frequency and 100% power output was applied to the chamber filled with 115 L room temperature water for 10 minutes. Temperature of the water
medium was recorded every second by thermometer. The specific heat capacity of water (4181.3 J/ (kg K)) was used in calculation. The acoustic power density (APD) of system mentioned above was calculated as 26 W/L.

### 3.2.4 Determination of total phenolics and antioxidant capacity

#### 3.2.4.1 Sample extraction

Whole leaf Romaine lettuce samples (10 g) taken 0, 30, 60, 90 hrs after storage were homogenized in 50 mL methanol at high speed for 1 min in a kitchen blender. The homogenate was transferred into a 250 mL covered plastic bottle, and incubated at 40°C for 1 hr. After incubation, the homogenate was filtered under vacuum (Whatman No.1 filter paper) and the filtrate was collected as the sample extract and stored at -18°C for later analysis. Three sample extractions were prepared for each treatment condition.

#### 3.2.4.2 Determination of total phenolics

Total phenolic content was determined by the two methods described by Kang and Salveit (2002) with slight modifications.

##### 3.2.4.2.1 Absorbance at 320 nm

The absorbance (320 nm) of each sample extract prepared as described above was directly read by a UV/Vis/NIR spectrophotometer (Perkin Elmer Lamda 950). Pure methanol was set as the blank.

##### 3.2.4.2.2 Folin-Ciocalteu Reaction
Total phenolics were also determined by the Folin-Ciocalteu method commonly used for phenolics measurement and expressed as gallic acid equivalents (GAE). Folin-Ciocalteu reagent (1:10, v:v) was prepared by combining 10 mL of the original Folin & Ciocalteu’s phenol reagent and 90 mL of distilled water, sodium carbonate solution (7.5%, w/v) was prepared by dissolving 7.5 grams of sodium carbonate in 100 mL distilled water and gallic acid stock solution (1mg/1mL) was prepared by dissolving 0.1 grams of gallic acid in 100 mL methanol. For each test, 0.2 mL sample extract was mixed with 1.0 mL of Folin-Ciocalteu Reagent 1:10 (v:v), kept at room temperature for 3 min before 0.8 mL of sodium carbonate solution (7.5%, w/v) was added. After 2 hrs incubation (~23 °C), the absorbance (765 nm) was recorded. Samples were compared to a standard curve prepared with gallic acid (7.81, 15.63, 31.25, 62.5, 125, 250, 500, and 1000 mg/L). Blank contained methanol in place of sample extract.

3.2.4.3 Determination of antioxidant capacity by DPPH method

The antioxidant capacity of sample extracts was measured by the free radical scavenging activity using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method (Blois, 1958) with slight modifications. Sample extract (0.4 mL) was added to 3.6 mL freshly prepared DPPH solution (0.024 g DPPH in 100 mL methanol) and shaken well. Initial absorbance (515 nm) was immediately recorded prior to room temperature incubation (30 min). Then the final absorbance at 515 nm was recorded. Pure methanol was set as the blank. The antioxidant capacity was calculated by the following formula:

\[
\% \text{ DPPH Inhibition} = (1 - \frac{A_f}{A_i}) \times 100
\]

where \( A_f \) is the final absorbance after 30 min incubation and \( A_i \) is the initial absorbance.
3.2.5 Determination of phenylalanine ammonia-lyase (PAL) activity

3.2.5.1 Sample extraction

Sample extraction was conducted according to the procedure of Yu et al. (2012). One leaf of Romaine lettuce (~10g) was homogenized in 50 mL sodium borate buffer (20 mM, pH 8.7) containing 20 mM β-mercaptoethanol at high speed for 1 min in a kitchen blender. The homogenate was filtered through four layers of cheesecloth and centrifuged (10,000 g, 30 min, 4 °C). The supernatant was used to determine protein content and PAL enzymatic activity. Extractions were prepared at least in triplicate for each group.

3.2.5.2 Determination of protein content

The Bio-Rad Protein Assay was used for protein content determination. The dye reagent (1:4, v:v) was prepared by diluting 30 mL of Dye Reagent Concentrate by 120 mL of distilled water, and filtering through Whatman No.1 filter paper. The filtrate was collected, sealed, stored at room temperature, and used within 2 weeks. Samples were compared to standard curves generated using bovine serum albumin (BSA) (0.2, 0.4, 0.6, and 0.8 mg/mL). Based on preliminary experiments, enzyme extracts were diluted (1:1, v:v) with sodium borate buffer (20 mM, pH 8.7) containing 20 mM β-mercaptoethanol to fit into the linear range of the BSA standard curve. Diluted enzyme extract 0.1 mL or protein standard was combined with 5.0 mL of dye reagent, vortexed (5 s), and incubated at room temperature (15 min) prior to measuring absorbance (595 nm). The dye reagent (1:4, v:v) was set as the blank.

3.2.5.3 PAL activity analysis

PAL activity was determined as described by Qin et al. (2003). Enzyme extract (1 mL), 2.0
mL of sodium borate buffer (20 mM, pH 8.7) containing 20 mM β-mercaptoethanol, and 0.5 mL of L-phenylalanine (20 mM) were mixed together in a test tube. After incubation at 40 °C (1 hr), the enzymatic reaction was stopped by adding 0.1 mL HCl (6 M) and vortexed (5 s). Measurement of the absorbance at 290 nm was taken. Blanks were prepared for each enzyme extract respectively: 1.0 mL of enzyme extract, 2.0 mL of sodium borate buffer (20 mM, pH 8.7) containing 20 mM β-mercaptoethanol, 0.5 mL of L-phenylalanine (20 mM) and 0.1 mL of HCl (6 M) were all combined at once to serve as blank.

3.2.6 Statistical analysis

All treatments were replicated at least 3 times. Data were compiled by Microsoft Excel (Microsoft Corporation, Redmond, WA) and statistical analysis was performed using SAS software (SAS Institute, Cary, NC). At each storage time, treatments were compared by ANOVA (Analysis of Variance) to determine if there was at least one treatment significantly different from others (p < 0.05). LSD (Fisher’s Least Significant Difference) test was conducted to further analyze the difference among treatments (p < 0.05) when ANOVA gave a significant result. ANOVA and LSD tests were also applied to the analysis of the difference of the same treatment during different storage times (p < 0.05).

3.3 Results and discussion

3.3.1 Effect of ultrasound on total phenolic content

The phenolic content measured by Abs320 indicated that ultrasonication induced an increase in the phenolic content in whole leaf Romaine lettuce under certain conditions (Figure 3.2.A). After room temperature storage for 60 hours, Romaine lettuce treated by ultrasound for 1 minute
and 2 minutes showed significantly higher phenolic content (35.28% and 26.74%) than the control. The samples treated with ultrasound for 3 minutes also exhibited a higher phenolic content than the control, though the result was not significant, probably due to variations among biological samples. Similar results were obtained by the Folin-Ciocalteu Method. After 60 hours storage samples treated using ultrasound for 1, 2 and 3 minutes had phenolic content 22.50%, 16.25%, and 17.92% higher than the controls, respectively (Figure 3.2.B). There was no significant difference among ultrasound-treated group and the control immediately right after the treatment, indicating that the generation and accumulation of phenolic compounds in response to treatment takes time.

After room temperature storage for 30 hours, samples treated with ultrasound for 1 minute had significantly lower phenolic content than the control (Figure 3.2.A). This decrease in phenolic content might be attributed to consumption of phenolics to overcome the oxidative stress from reactive oxygen species (ROS) caused by ultrasound treatment (Adyanthaya et al., 2009). The total phenolic content in plant is determined by the interplay between factors that promote the production of phenolic compounds and those that consume them. A reduction in phenolic content thus indicated that the consumption of phenolics may have surpassed the generation and accumulation. After 90 hours storage there was no difference in phenolic content between the ultrasound-treated samples and the control. This indicates the need to study when to apply stimulation, when the triggered phenolics production and accumulation will surpass the consumption, and if multiple stimulations at selected time frame will produce a significant and long-lasting increase in phenolics and thus antioxidant capacity of produce.

### 3.3.2 Effect of ultrasound on antioxidant capacity
Antioxidant capacity of whole leaf Romaine lettuce was expressed by percentage DPPH inhibition (Figure 3.3), with higher percentage inhibition indicating higher antioxidant capacity. After 60 hours storage, DPPH inhibition of samples sonicated for 1, 2, and 3 minutes was 97.84%, 75.22%, and 75.87% higher than the control, respectively. These differences were statistically significant. After 90 hours storage, only the antioxidant capacity of samples sonicated for 2 minutes remained significantly higher than controls. Similar to total phenolic content, no significant difference was found in antioxidant capacity immediately after sonication.

Lettuce treated by ultrasonication for 1 and 2 minutes experienced a sharp decrease in antioxidant capacity by 50.87% or 64.24%, respectively during the first 30 hours, followed by a significant increase in antioxidant capacity by 97.07% or 83.67% during the next 30 hours. No such pattern of change was observed in the 3 minute ultrasound-treated group or the control group. As an abiotic elicitor, ultrasound can trigger oxidative bursts in vivo (Wu and Lin, 2002a), leading to defense responses of plant cells. The oxidative stress caused by ultrasonication was harmful to the cells, so the natural antioxidants already present in the plants were directly used to combat reactive oxygen species at the beginning. During storage, additional antioxidants were continuously synthesized by lettuce cells joining force with the natural antioxidants to further eliminate the adverse effects of the oxidative burst (Reyes, 2007). This ultrasound-induced production of antioxidants is likely responsible for increased antioxidant capacity during storage.

Samples without ultrasound treatment also showed a significant decrease in antioxidant capacity during the first 30 hours, but the decline was less than that of 1 and 2 minute ultrasound-treated groups and continued declining afterwards. Since a small amount of ROS has been shown to occur even with normal cellular metabolism of postharvest leafy vegetables (Toivonen, 2004), antioxidants previously stored in cells were likely used to regulate production
of ROS leading to the slow decrease of antioxidant capacity during postharvest storage.

A positive correlation was observed between total phenolic content and antioxidant capacity of Romaine lettuce (Figure 3.4). Since phenolic compounds have been proved to be highly associated with the antioxidant capacity of fruits and vegetables, this linear trend can explain the change of antioxidant capacity during storage: the consumption and the ultrasound-induced production of phenolic compounds appeared to be a major reason.

3.3.3 Effect of ultrasound on phenylalanine ammonia-lyase (PAL) activity

Phenylalanine ammonia-lyase (PAL) is one of the most important antioxidant enzymes in plants and is directly related to secondary metabolite production. PAL catalyzes the first step in phenylpropanoid metabolism which is the synthetic pathway of phenolic compounds in plant cells. As a key regulatory enzyme in plant defense and secondary metabolite synthesis, PAL serves as an indicator of the defense response triggered by environmental stresses (Dixon and Paiva, 1995). No significant difference in PAL activity was found among different groups in the first 30 hours (Figure 3.5). Afterwards, the PAL activity increased at 60 hours in all groups. Noticeably, the 2 and 3 minute ultrasound-treated groups expressed significantly higher PAL activity than the control. With reference to post-treatment time, a bell-shaped curve of PAL activity was observed in all three ultrasound-treated groups, similar to the wound-induced pattern reported by Choi et al. (2005). These changes in PAL activity as well as the increase of antioxidant capacity after ultrasound treatment might be explained by the similar physiological defense responses triggered by wounding. However, the peaks occurred much later than wound-induced plant defense responses (Ritenour et al., 1995; López-Gálvez et al., 1996). No data specifically discussed about PAL activity of Romaine lettuce stored at room temperature.
For iceberg lettuce, at 15°C storage, the maximum activity of PAL was reached within 6-16 h depending on the wounding level. At 5°C storage, the peak occurred later in all wounded samples, generally within 12-72 h. For Romaine lettuce processed and stored under same cold storage conditions with iceberg lettuce, PAL activity followed a similar pattern. Meanwhile, PAL activity of whole leaf iceberg lettuce was induced by 10 ppm ethylene in the air, with peaks showed up after 4 and 2 days under 15°C and 20°C storage, which were slower than wound-induced responses but similar to our results. So we assumed the pattern observed in our study was probably due to the fact that the stimulation of ultrasound was not as strong as wounding by cutting or processing.

3.3.4 Hypothetical model for the effect of low-APD ultrasound on fresh produce

The data reported in this study demonstrate that ultrasound treatment at low APD acts as an abiotic elicitor to trigger physiological responses in plant defense systems of selected fresh produce, similar to common abiotic stresses such as wounding. Although more complicated, whole plants share similar physiological foundations with plant cell cultures. Based on this present study on ultrasound treatment of whole produce and previous literature about plant cell culture, a hypothesized mechanism for the effect of low APD ultrasound on whole fresh produce was proposed, as shown in Figure 3.6. As a typical elicitor-induced plant defense response, ultrasound may first trigger the oxidative burst including the increase of cross-membrane ion fluxes ($\text{Ca}^{2+}$ and $\text{K}^+ / \text{H}^+$) and the production of reactive oxygen species (ROS) which are two early and important steps (Wu and Lin, 2002a). The induced production of nitric oxide (NO) may also be involved in oxidative burst by the activation of nitric oxide synthase (NOS) (Wang et al., 2006). Acting as signaling molecules, hydrogen peroxide ($\text{H}_2\text{O}_2$) and nitric oxide (NO) may
activate other signal pathways such as increasing the biosynthesis of jasmonic acid (JA) and its derivatives (Wu and Ge, 2004). These signal pathways work together as a network for activating the subsequent defense responses including antioxidant enzyme activation, defense-related gene expression, and secondary metabolite production (Chen et al., 2007; Safari et al., 2013). Production of phenolic compounds may be increased with an elevated level of phenylalanine ammonia-lyase (PAL) activity via phenylpropanoid pathway induced by ultrasound elicitor (Wu and Lin, 2002b), which ultimately achieves an elevated antioxidant capacity and nutrition quality improvement of fresh produce.

3.4 Conclusion

The elicitation effect of low-APD ultrasound as an abiotic elicitor to stimulate the plant defense response was confirmed in fresh Romaine lettuce. Lettuce had different responses to different ultrasound treatment. Generally, ultrasound did not affect the nutritional quality immediately after treatments. At 60 hours storage under room temperature, all ultrasound-treated groups showed a higher total phenolic content along with a higher antioxidant capacity than the water washing control. The rapid decrease of antioxidant capacity observed in 1 min and 2 min ultrasound-treated groups during the first 30 hours might be the result of the consumption of antioxidants, which were previously stored in plant cells, to eliminate ultrasound-generated reactive oxygen species (ROS). The activity of phenylalanine ammonia-lyase (PAL) of ultrasound-treated samples illustrated a “bell-shaped” curve during storage which indicated activation of PAL by ultrasound-induced defense response, followed by production of phenolic compounds and thus increased the antioxidant capacity of fresh Romaine lettuce.

We proposed a hypothetical model on the physiological mechanism underlying the
responses of whole fresh produce to ultrasound treatment. The model was built mainly based on the knowledge obtained in cell culture studies. Further studies are necessary for a better understanding of the physiological responses of fresh produce to ultrasound treatment. This knowledge will be of great help for modifying the defense system to obtain an improved nutritional quality.
3.5 Figures and Tables

Figure 3.1 Ultrasound treatment equipment.
Figure 3.2 Effect of ultrasound treatment on total phenolic content of Romaine lettuce treated at 25 kHz determined by (A) absorbance at 320 nm and (B) Folin-Ciocalteu Reaction.

a-b means within time results with different letters are different at α 0.05.
x-z means within treatments results with different letters are different at α 0.05.
Figure 3.3 Effect of ultrasound treatment on antioxidant capacity of Romaine lettuce treated at 25 kHz determined by DPPH method.

a-b means within time results with different letters are different at α 0.05.
x-z means within treatments results with different letters are different at α 0.05.
Figure 3.4 Correlation of antioxidant capacity versus total phenolic content of Romaine lettuce determined by (A) absorbance at 320 nm and (B) Folin-Ciocalteu Reaction.
**Figure 3.5** Effect of ultrasound treatment on phenylalanine ammonia-lyase (PAL) activity of Romaine lettuce treated at 25 kHz.

*a-b* means within time results with different letters are different at α 0.05.

*x-z* means within treatments results with different letters are different at α 0.05.
Figure 3.6 Hypothetical mechanism of an ultrasound stimulated secondary metabolism response in fresh produce.
CHAPTER 4

EFFECT OF ULTRASOUND ON QUALITY RETENTION OF ROMAINE LETTUCE

4.1 Introduction

Fruits and vegetables are rich sources of various nutrients that are beneficial to human health. Fresh-cut vegetables are those ready-to-use, minimal processed, trimmed and/or peeled, and/or cut parts of vegetables (Krasae koopt and Bhandari, 2011). They provide a convenient alternative to conventional fresh produce. The market share for minimal processed produce keeps increasing due to customers’ demand for quick and convenient plant foods with fresh appearance, natural color, flavor and texture, retentive of nutritional value, and fewer food additives (Jongen, 2002). Generally, vegetables are selected, pre-washed, prepared by peeling/trimming/cutting/shredding, washed, and packaged before distributed to vendors.

The problem occurs that fresh produce degrades both biochemically and microbiologically during postharvest handling and storage. Processing procedures used to prepare fresh-cut vegetables further accelerate quality degradation (O’Beirne and Francis, 2003) as the result of wounding-induced ethylene production (Kader, 1985; Saltveit et al., 2005), increased respiration rate (Cantwell and Suslow, 2002), discoloration, texture deterioration, water loss and nutrients degradation. The growth of microorganisms and the susceptibility of pathogens are also promoted because of the cell breakdown on the cutting edge. The perishable characteristic and the processing damage result in a limited shelf-life of fresh produce. Since the “fresh-like” quality attributes are always the most important criterion when customers make purchase of fresh-cut produce, various preservation methods have been developed over years. Examples include chemical-based washing treatments e.g. chlorine-based solutions, organic acid, hydrogen
peroxide, calcium-based solutions, ozone, electrolyzed water and natural preservatives, and physical treatments e.g. modified atmosphere packaging, heat-shock, irradiation, UV light, and hurdle technology (Barry-Ryan et al., 2007).

Power ultrasound is an innovative preservation technology which has been proved effective in surface decontamination. Recent studies started to investigate its application in minimal processed fresh produce (Seymour et al., 2002). The effect of ultrasound treatment to reduce spoilage and pathogenic microorganisms on fresh produce is based on cavitation. The elimination of bacteria, yeasts and molds directly improves microbial safety, retards produce physical and nutritional quality decay, and thus extends produce shelf life. The sanitation could be enhanced when ultrasound is applied with sanitizers in the washing system. Exposure to ultrasound also helps maintain quality parameters with its ability to reduce physiological activities of fresh fruit and vegetables (Xu et al., 2013). In addition, ultrasound treatment equipment can be easily combined into the washing procedure which has already been widely used for fresh-cut produce processing. So, ultrasonication is a promising postharvest processing technology in minimal processed fresh produce industry.

Lettuce and prepared salad packs are the most common fresh-cut vegetable products available nowadays. As mentioned in chapter 3, we have successfully achieved an enhanced nutritional quality after the ultrasonication application on Romaine lettuce. However, we observed a significant increase of phenylalanine ammonia-lyase (PAL) activity in our study. A correlation was established between PAL activity and enzymatic browning of some fruits and vegetables (Saltveit, 2000), and PAL has been suggested to be a predictor to determine shelf-life for some fresh-cut produce (Couture, 1993). Whereas, PAL activity was also found to be unrelated to enzymatic browning level by some other researchers. Meanwhile, there has been
concern about the deteriorative effect of ultrasound under high power or long treatment time. Ajlouni et al. (2006) described treatment with 40 kHz ultrasonication for 20 min caused significant tissue damage and reduced Cos lettuce quality. Birmpa et al. (2013) found similar results that strawberry and Romaine lettuce expressed more severe color change during storage after 37 kHz, 30 W/L power ultrasound treatment for 45min and 60 min. It is important to determine the effect of low acoustic power density (APD) ultrasound treatment on sensory quality of Romaine lettuce because fresh produce with unaccepted sensory quality will not be purchased by consumers even with good nutritional value.

The present study aimed to examine the effect of ultrasound on the quality retention of Romaine lettuce under same conditions with which we have achieved a better nutritional value in our previous study. We hypothesized that short-time low-APD ultrasound would not induce tissue damage to Romaine lettuce and no adverse influence would generate during storage. Meanwhile, if applied in an appropriate manner, the increased antioxidant capacity may help protect produce from oxidative stress during postharvest processing and storage which in turn achieves better overall quality retention.

4.2 Materials and Methods

4.2.1 Sample preparation and ultrasound treatment

Romaine lettuce prepared for the overall quality analysis were purchased, pre-selected, cut and divided into different treatment groups as described in 3.2.1. Samples in different treatment groups were washed with tap water for 1, 2, 3 min with ultrasound (25 kHz frequency, 100% power output from the generator, which equaled to APD of 26 W/L) applied, or 3 min with no ultrasound respectively in the specially designed ultrasound treatment chamber (Figure 3.1).
After washing, fresh-cut Romaine lettuce was used for sensory evaluation to better estimate commercial product, and whole leaf Romaine lettuce was used for all instrumental quality analyses to eliminate the influence of wounding by cutting (Reyes, 2007). Treated whole leaf lettuce samples were air-dried (30 min) and stored in plastic gallon zipper storage bags (GLAD, Oakland, CA). Fresh-cut lettuce samples were prepared by carefully cutting into square pieces (∼2.54 × 2.54 cm) by a sharp stainless steel knife, and dried with a salad spinner (OXO, New York, NY) (1 min) before being transferred into plastic zipper storage bags. All samples were sealed in bags, covered with thick brown paper to prevent light exposure, and stored at room temperature up to 150 hrs. Each group had at least 3 replicates.

4.2.2 Quality evaluation

4.2.2.1 Instrumental analysis

4.2.2.1.1 Texture

Texture of Romaine lettuce samples was determined by firmness measurements utilizing a TA-XT2 Texture Analyzer with a 5-blade Kramer Shear Press (Texture Technologies Corporation, Scarsdale, NY). Before each test, samples were cut into 2.54 × 2.54 cm square pieces, and 25 g sample was weighed and evenly spread in the holder (internal dimension: 82 × 63 × 89 mm). The height of lettuce sample in the holder was about 55 mm, so the 5-blades (blade thickness: 1.5 mm each) plunger was initially set at a height of 65 mm from the bottom of sample holder, and then forced down through all samples at a test speed of 1.0 mm/s until the bottom layer of lettuce was cut broken. Data was collected by Texture Expert software, version 2.55 (Texture Technologies Corporation, Scarsdale, NY). The maximum force (N) was recorded to represent firmness (Bourne, 1997). Three replicates were measured for each treatment group at each
4.2.2.1.2 Hunter color

To represent color quality of whole leaf Romaine lettuce, leaf surface color and rib cut edge color of samples were measured at the same time, with a Minolta CR-300 Chroma Meter connected to a DP-301 Data Processor (Minolta Corporation, Ramsey, NJ). Color analysis was based on the Hunter Lab color space: briefly, it is a color-opponent three-dimension space with $L^*$ (lightness), $a^*$ (positive values indicate red and negative values indicate green), and $b^*$ (positive values indicate yellow and negative values indicate blue). Three readings were taken on the front surface from the left, middle, and right part of each leaf, and the average was used to represent sample surface color. One reading for cut edge color analysis was taken directly from the rib edge damaged by cutting before treatment. To better express sample color, hue angle and chroma (color saturation) were calculated (McGuire, 1992):

\[
\text{hue angle} = \tan^{-1}(b^*/a^*) \quad \text{chroma} = \sqrt{a^{*2} + b^{*2}}.
\]

4.2.2.2 Sensory evaluation

Descriptive sensory evaluation was performed to assay the quality change of fresh-cut Romaine lettuce due to different treatments and storage times. Sensory quality parameters including overall visual quality, surface browning/discholoration, cut edge browning, sogginess/watery, and off-odor were evaluated by a trained panel of 14 individuals (6 males and 8 females, all students or scholars in the Department of Food Science and Human Nutrition at the University of Illinois) after 0, 30, 60, 90, and 150 hrs room temperate storage. Different samples were placed on separate white plates labeled with a random 3-digit code, and randomly set on the
booth. For overall visual quality, a scale from 1-9 (1 = “poor/inedible,” 9 = “excellent and no
difference from the fresh reference”) was used and a score of 5 was set as the limit of acceptance.
For surface browning/discoloration and cut edge browning, a scale of 1-5 was applied (1 = “no
browning”, 5 = “significant browning”, 3 was the limit of acceptance). Similar scales were used
for sogginess/watery and off-odor evaluation (1 = “crispy” or “fresh odor”, 5 = “severe watery”
or “severe off-odor”, 3 was the limit of acceptance).

In order to equip the sensory panel with an appropriate understanding of descriptors for
specific attributes, panelists were trained twice through one online training session and one
onsite training.

During the online training, a sample ballot of the Romaine lettuce sensory evaluation
(Figure 4.1) was provided to panelists along with evaluation criteria for each parameter (Figure
4.2). During the onsite training, a “fresh reference” of fresh-cut Romaine lettuce sample was
presented with score 9 for overall visual quality, and score 1 for surface browning/discoloration,
cut edge browning, sogginess/watery and off-odor. Other evaluation criteria were reviewed again
before beginning the sensory evaluation. A prescreen was performed with standardized samples
to test if panelist had obtained correct understanding after training. Only those who passed the
prescreen were selected to participate in the following sensory evaluation.

4.2.3 Statistical analysis

All treatments were replicated at least 3 times. Data were compiled by Microsoft Excel
(Microsoft Corporation, Redmond, WA) and statistical analysis was performed using SAS
software (SAS Institute, Cary, NC). At each storage time, treatments were compared by ANOVA
(Analysis of Variance) to determine if there was at least one treatment significantly different
from the others (p < 0.05). LSD (Fisher’s Least Significant Difference) test was conducted to further analyze the difference among treatments (p < 0.05) when ANOVA gave a significant result. ANOVA and LSD tests were also applied to the analysis of the difference of the same treatment during different storage times (p < 0.05).

4.3 Results and discussion

4.3.1 Effect of ultrasound on texture of fresh Romaine lettuce

Texture and color are two critical physical parameters that determine postharvest quality of fresh produce. Lower maximum shear force is associated with a decrease in firmness and crispness of leaf tissue, which is a good indicator for loss of textural quality (Bourne, 1997). Immediately after sonication, texture of all treated groups decreased comparing to the untreated raw samples, with exception of the 1 minute ultrasound-treated group (Table 4.1). Loss of firmness will occur during storage due to loss of moisture in plant tissue (Ryall et al., 1972). Lettuce samples treated by ultrasound exhibited significantly higher firmness (maximum force, N) after 30 hours storage. Xu et al. (2013) also reported a delay in softening of fresh produce after ultrasound treatment, but the physiological mechanism was still unknown.

Interestingly, there was an increase of maximum shear force at the end of 90 hours storage which was out of expectation. The plant’s self-recovery system and the production of phenolic compounds could help retain tissue firmness which might contribute to the increase at the end (Wang, 2004). Similar to us, Martin-Diana et al. (2005) observed an increase of maximum puncture load of salad-cut iceberg lettuce after 10 days storage at 4°C. They observed a correlation between maximum water loss and maximum crispiness readings (maximum puncture load), so they suggested the loss of moisture in cell could dehydrate tissues and increase tissue
elasticity which reflected as an elevated maximum puncture load. According to their conclusion, an increased maximum force in the above texture analysis system could stand for both the increase and decrease of firmness or crispness. Their explanations also worked for our findings at the end of storage. They might even be used for explaining why the maximum force value in all ultrasound-treated and water-washed groups were lower than the intact samples when measured immediately after treatment: during washing, lettuce leaves could absorb water to gain a higher turgor of cell which led to a lower maximum shear force reading. Further studies are needed to find the correlation between lettuce leaf fresh weight and the maximum force reading during storage. The measurement of moisture content during storage can also help.

However, not a lot studies reported similar trends in texture change of lettuce during storage, so more experiments should be done to confirm this phenomenon. Actually, a later report from the same group found no such increase in maximum force of lettuce after long time storage, when a Kramer cell (similar to ours) was used for the texture analysis (Martin-Diana et al., 2006). Because their earlier results were obtained from a penetrometer test with a puncture cell, they might not properly describe crispness which was based on breaking characteristic responses.

Texture is a complex quality parameter of fresh produce. It is difficult to describe texture characteristics simply by one instrument measured value. Sensory evaluation conducted by a panel should also be added as a supplement to the instrumental quality analysis for a better assessment.

4.3.2 Effect of ultrasound on Hunter color of fresh Romaine lettuce

Another major problem in quality loss during processing and storage of lettuce is discoloration, mainly attributes to enzymatic browning and chlorophyll degradation. Color of
both surface and cut edge of whole leaf Romaine lettuce were measured during storage to evaluate quality change. At the end of 90 hours storage, “L”, “hue” and “chroma” values of the surface color in all groups didn’t change significantly comparing to the initial color, and no significant difference was observed among groups (Table 4.2a). When considering the color change of cut edge, the place where is most susceptible to enzymatic browning, we found “hue” value decreased, “chroma” value increased while “L” remained the same during storage (Table 4.2b). “Hue” value can be used to evaluate browning by estimating the change of color from green to red in lettuce, and a lower value indicated more severe browning (Castaner et al., 1999). At the end of storage, a significant decrease of “hue” value in all groups illustrated that enzymatic browning happened during storage regardless of different treatments. According to Cantos et al. (2001), wounding at the cut edge accelerated enzymatic browning. However, ultrasound treatment delayed browning reaction in the early time of storage: 1min and 2 min ultrasound-treated groups showed a significantly higher “hue” value than the control at 30 hours, meaning less browning and better quality. The effect of ultrasound on delaying browning might be explained by the reduction of phenolic compounds in first 30 hours after ultrasound treatment, since phenolic compounds are substrates of enzymatic browning reaction (Mai and Glomb, 2013). Meanwhile, although some early studies suggested that the phenolic compound accumulation via an elevated level of PAL activity was correlated with an increase susceptibility of plant tissue enzymatic browning (Peiser et al., 1998; Cantos, 2001; Pereyra, 2005), no difference of browning was observed among different treatments after 60 hours storage even with an increased PAL activity and a higher phenolic content in ultrasound-treated groups at the same time as described in Chapter 3.
4.3.3 Effect of ultrasound on sensory quality of fresh-cut Romaine lettuce

Fresh-cut Romaine lettuce samples were used in sensory evaluation to estimate the effect of ultrasound on sensory quality. The most important quality parameters of fresh-cut leafy vegetables are fresh appearance with no discoloration and no decay, crisp texture and good aroma (Cantwell, 1996). During 150 hours storage at room temperature, the loss of specific quality attributes was evaluated by a trained panel from following aspects: surface and cut-edge browning, tissue sogginess and off-odor generation (Figure 4.3). As shown in Table 4.3, generally, different times of exposure to sonication did not change sample quality at 0 hour, but the overall quality of all groups significantly declined over time. Samples treated with 1 minute ultrasonication were consistently rated higher or similar to the control during storage, and the quality of this group maintained an acceptable score (5.80) at the end of 150 hours. The samples exposed to ultrasound for 2 and 3 minutes received quality scores similar to the control. Similar to overall quality, browning on the surface or cut-edge was not detected right after treatment. Ultrasound-treated samples (1 minute) showed a significantly lower surface browning in 30 hours and a lower cut-edge browning in 60 hours than the control. No difference in the trend of browning was found between the control and either the 2 or 3 minute ultrasound-treated groups. All samples lost their crispness and thus received a higher score of sogginess over time, but no difference appeared among treatments. Neither ultrasound treatment nor water washing brought change of aroma to lettuce at 0 hour, but slight off-odor was detected at similar level in all groups at the end of 150 hours storage.

To notice, the results from sensory evaluation might not exactly correspond to the results found by instrumental color analysis. Lettuce leaves were cut before storage which had introduced wounding as another elicitor which may have some impacts on PAL activity, total
phenolins, antioxidant capacity (Reyes et al., 2007) as well as overall quality (Cantos et al., 2001). However, the sensory evaluation experiment design we used here was still valid based on the assumption that the effect of wounding was at a similar level for all treatment groups. Thus, this interferential factor can be regarded as a background noise which could be neglected in later data analysis if we only used the result to compare the difference among different treatments.

To sum up, none of the tested ultrasound treatments caused a deterioration of the sensory quality of fresh-cut lettuce. In addition, if treated in an appropriate manner, ultrasonication may help maintain a better overall quality and extend the shelf-life.

4.4 Conclusion

The ultrasound treatment we applied in this study was a low-APD (26 W/L) and short-time (up to 3 min) processing procedure. Sensory quality analyzed by both instrumental methods and sensory evaluation showed similar results that ultrasound treatment did not cause adverse effect to produce quality during storage.

Ultrasound treatment did not adversely affect Romaine lettuce texture. The loss of firmness occurred in all ultrasound-treated and control groups due to the loss of turgor in cell during storage. Ultrasonication delayed tissue softening in first 30 hrs during storage. Lettuce leaf surface color remained unchanged during 90 hours storage, while cut edge color significantly changed mainly because of enzymatic browning. “Hue” angle, which could be used as an indicator for browning, decreased over time at the cut edge in all groups, showing the browning generation. The sensory panel reported similar results as the instrumental analysis. They identified the quality degradation during storage with a declined overall visual quality score, and increased scores for leaf discoloration, cut edge browning, sogginess, and off-odor.
ultrasound-treatment samples obtained slightly longer shelf life than other groups. Generally, ultrasound-treated Romaine lettuce achieved similar quality retention, if not better, as lettuce simply washed by tap water. 1 min ultrasound treatment even delayed lettuce tissue browning and in turn maintaining a better quality and a longer shelf-life. No correlation was observed between PAL activity and leaf surface or cut edge browning in our study which proved ultrasonication to be a promising postharvest processing method for fresh Romaine lettuce quality preservation while promoting the production of phenolic compounds at the same time.

Response Surface Methodology (RSM) should be conducted in the future to optimize the treatment conditions for maximizing the antioxidant capacity and secondary metabolites production while retaining a better overall quality and an extended shelf-life.
4.5 Figures and Tables

ROMAINE LETTUCE SENSORY EVALUATION SAMPLE BALLOT

SAMPLE  999  

Q1. Overall Visual Quality:

<table>
<thead>
<tr>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
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<tr>
<td>Poor/inedible</td>
<td>Limit of acceptance</td>
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Q2. Surface Browning/Discoloration:

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<th>5</th>
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<tr>
<td>None</td>
<td>Limit of acceptance</td>
<td>Severe</td>
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<tr>
<td>(no browning/ discoloration at leaf surface)</td>
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Q3. Cut Edge Browning:

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<tr>
<td>None</td>
<td>Limit of acceptance</td>
<td>Severe</td>
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<td></td>
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<tr>
<td>(no browning at cut edge)</td>
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Q4. Sogginess/Watery

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<th>4</th>
<th>5</th>
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</thead>
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<tr>
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<td>Limit of acceptance</td>
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Q5. Off-odor

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<th>3</th>
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<tbody>
<tr>
<td>None</td>
<td>Limit of acceptance</td>
<td>Severe</td>
<td></td>
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<td>(characteristics of Romaine lettuce)</td>
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* A real lettuce sample as the reference for “Fresh Romaine Lettuce” (score “9” for Q1 and score “1” for Q2-Q5) will be shown to the panel before the sensory test starts.

**Figure 4.1** Romaine lettuce sensory evaluation sample ballot.
Figure 4.2 Evaluation criteria given in the panel training.
<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1min Ultrasonication</th>
<th>2min Ultrasonication</th>
<th>3min Ultrasonication</th>
<th>3min Water Washing</th>
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**Figure 4.3** Fresh-cut Romaine lettuce treated by ultrasound at 25 kHz, and stored at room temperature for up to 150 hours for sensory evaluation.
Table 4.1 Effect of ultrasound on texture of Romaine lettuce treated at 25 kHz.

<table>
<thead>
<tr>
<th>Storage Time (h)</th>
<th>1min Ultrasonication</th>
<th>2min Ultrasonication</th>
<th>3min Ultrasonication</th>
<th>3min Water Washing</th>
<th>No Treatment</th>
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<td>335.05±24.77&lt;sup&gt;ab(y)&lt;/sup&gt;</td>
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<td>299.29±25.49&lt;sup&gt;bc(y)&lt;/sup&gt;</td>
<td>289.01±19.08&lt;sup&gt;2(y)&lt;/sup&gt;</td>
<td>355.43±10.59&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>30</td>
<td>277.16±16.27&lt;sup&gt;ab(z)&lt;/sup&gt;</td>
<td>296.97±25.10&lt;sup&gt;a(y)&lt;/sup&gt;</td>
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-<sup>a-c</sup> means within time (row) results with different letters are different at α 0.05.
-<sup>x-z</sup> means within treatments (column) results with different letters are different at α 0.05.
Table 4.2a Effect of ultrasound on the leaf surface color (Hunter color) of Romaine lettuce treated at 25 kHz.

<table>
<thead>
<tr>
<th>Color Parameter</th>
<th>Storage Time (h)</th>
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<td></td>
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<td>Lightness</td>
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<td>50.32±3.47&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>33.40±2.40&lt;sup&gt;a(x)&lt;/sup&gt;</td>
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<td>121.75±0.91&lt;sup&gt;a(x)&lt;/sup&gt;</td>
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<td>30.39±2.60&lt;sup&gt;a(y)&lt;/sup&gt;</td>
<td>30.12±1.62&lt;sup&gt;a(y)&lt;/sup&gt;</td>
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<td>122.48±1.06&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>33.39±2.60&lt;sup&gt;a(x)&lt;/sup&gt;</td>
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<td>34.47±2.16&lt;sup&gt;a(x)&lt;/sup&gt;</td>
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<td>49.11±3.72&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>121.50±1.14&lt;sup&gt;a(x)&lt;/sup&gt;</td>
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<td>49.11±3.72&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>121.50±1.14&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>33.47±3.46&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>34.47±2.16&lt;sup&gt;a(x)&lt;/sup&gt;</td>
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<td>49.11±3.72&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>121.50±1.14&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>33.47±3.46&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>34.47±2.16&lt;sup&gt;a(x)&lt;/sup&gt;</td>
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<td>33.47±3.46&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>34.47±2.16&lt;sup&gt;a(x)&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>49.11±3.72&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>121.50±1.14&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>33.47±3.46&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>34.47±2.16&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a-b</sup> means within time (row) results with different letters are different at α 0.05.<br><sup>x-y</sup> means within treatments (column) results with different letters are different at α 0.05.
Table 4.2b Effect of ultrasound on the cut edge color (Hunter color) of Romaine lettuce treated at 25 kHz.

<table>
<thead>
<tr>
<th>Color Parameter</th>
<th>Storage Time (h)</th>
<th>Treatment</th>
<th>1min Ultrasonication</th>
<th>2min Ultrasonication</th>
<th>3min Ultrasonication</th>
<th>3min Water Washing</th>
<th>No Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lightness</td>
<td>0</td>
<td>Ultrasonication</td>
<td>55.75±3.57 a(x)</td>
<td>56.83±2.98 a(x)</td>
<td>55.48±3.24 a(xy)</td>
<td>54.36±3.14 a(x)</td>
<td>57.19±2.63 a</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Water Washing</td>
<td>52.99±3.37 a(x)</td>
<td>53.81±6.37 a(x)</td>
<td>57.60±5.03 a(x)</td>
<td>55.72±3.92 a(x)</td>
<td>54.69±3.15 a</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>Water Washing</td>
<td>54.86±4.87 a(x)</td>
<td>53.27±2.60 a(x)</td>
<td>52.13±3.52 a(y)</td>
<td>52.30±5.11 a(x)</td>
<td>54.69±3.15 a</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>Water Washing</td>
<td>56.03±6.66 a(x)</td>
<td>53.86±6.28 a(x)</td>
<td>54.40±2.92 a(xy)</td>
<td>53.72±7.38 a(x)</td>
<td>54.69±3.15 a</td>
</tr>
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<td>Hue</td>
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<td>Ultrasonication</td>
<td>114.69±1.82 a(x)</td>
<td>113.87±0.97 a(x)</td>
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<td>115.03±1.14 a</td>
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<td>104.82±5.50 a(x)</td>
<td>106.74±5.34 a(y)</td>
<td>101.56±1.93 b(y)</td>
<td>100.40±2.95 b(y)</td>
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<td>102.05±7.68 a(y)</td>
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<td>100.45±5.12 a(y)</td>
<td>101.52±4.48 a(y)</td>
<td>101.08±4.64 a</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>Water Washing</td>
<td>101.32±5.60 a(y)</td>
<td>98.03±9.18 a(z)</td>
<td>99.71±8.71 a(y)</td>
<td>101.08±4.61 a(y)</td>
<td>101.08±4.64 a</td>
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<td>10.72±0.96 a(y)</td>
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<td>9.51±1.82 a(y)</td>
<td>10.24±1.44 a(y)</td>
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</tr>
<tr>
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<td>30</td>
<td>Water Washing</td>
<td>14.25±1.91 a(x)</td>
<td>13.71±1.43 a(x)</td>
<td>14.49±1.18 a(x)</td>
<td>14.64±2.05 a(x)</td>
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<tr>
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<td>13.51±3.94 a(xy)</td>
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<tr>
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<td>Water Washing</td>
<td>14.78±1.87 a(xy)</td>
<td>14.39±2.18 a(xy)</td>
<td>15.42±4.23 a(x)</td>
<td>12.80±1.17 a(x)</td>
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</table>

a-b means within time (row) results with different letters are different at α 0.05.

x-z means within treatments (column) results with different letters are different at α 0.05.
Table 4.3 Effect of ultrasound on overall quality, surface browning, cut edge browning, sogginess and off-odor of Romaine lettuce treated at 25 kHz determined by sensory evaluation.

<table>
<thead>
<tr>
<th>Quality Parameter</th>
<th>Storage Time (h)</th>
<th>1min Ultrasonication</th>
<th>2min Ultrasonication</th>
<th>3min Ultrasonication</th>
<th>3min Water Washing</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Overall Quality</td>
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<td></td>
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<td></td>
<td></td>
<td>0</td>
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<tr>
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<td></td>
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<td>5.10±2.28&lt;sup&gt;ab(y)&lt;/sup&gt;</td>
<td>4.10±1.73&lt;sup&gt;bz&lt;/sup&gt;</td>
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<td></td>
<td>150</td>
<td>5.80±1.75&lt;sup&gt;aw&lt;/sup&gt;</td>
<td>5.10±1.45&lt;sup&gt;abzy&lt;/sup&gt;</td>
<td>3.40±1.71&lt;sup&gt;c(y)&lt;/sup&gt;</td>
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<td></td>
<td>Surface Browning</td>
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<td>1.33±0.50&lt;sup&gt;az&lt;/sup&gt;</td>
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<tr>
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<td>30</td>
<td>1.11±0.33&lt;sup&gt;b(x)&lt;/sup&gt;</td>
<td>2.00±0.50&lt;sup&gt;axy&lt;/sup&gt;</td>
</tr>
<tr>
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<td></td>
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<td>60</td>
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<td>2.44±1.24&lt;sup&gt;ab(wx)&lt;/sup&gt;</td>
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<tr>
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<td></td>
<td></td>
<td>90</td>
<td>2.56±1.01&lt;sup&gt;ab(w)&lt;/sup&gt;</td>
<td>2.89±1.27&lt;sup&gt;a(w)&lt;/sup&gt;</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>150</td>
<td>2.44±0.53&lt;sup&gt;b(w)&lt;/sup&gt;</td>
<td>2.56±0.73&lt;sup&gt;bwx&lt;/sup&gt;</td>
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<td>Cut-edge Browning</td>
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<td>1.00±0.00&lt;sup&gt;a(z)&lt;/sup&gt;</td>
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<tr>
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<td></td>
<td>30</td>
<td>1.56±0.53&lt;sup&gt;b(x)&lt;/sup&gt;</td>
<td>1.78±0.67&lt;sup&gt;bxy&lt;/sup&gt;</td>
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<tr>
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<td>60</td>
<td>1.44±0.53&lt;sup&gt;b(x)&lt;/sup&gt;</td>
<td>2.22±0.67&lt;sup&gt;ab(x)&lt;/sup&gt;</td>
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<td>2.22±0.97&lt;sup&gt;b(w)&lt;/sup&gt;</td>
<td>3.56±1.13&lt;sup&gt;a(w)&lt;/sup&gt;</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>150</td>
<td>2.33±0.87&lt;sup&gt;b(w)&lt;/sup&gt;</td>
<td>2.44±0.88&lt;sup&gt;b(x)&lt;/sup&gt;</td>
</tr>
<tr>
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<td>Sogginess</td>
<td>0</td>
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<td>1.33±0.71&lt;sup&gt;axy&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>1.56±0.53&lt;sup&gt;a(x)&lt;/sup&gt;</td>
<td>1.67±0.71&lt;sup&gt;a(x)&lt;/sup&gt;</td>
</tr>
<tr>
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<td>1.67±1.12&lt;sup&gt;a(xy)&lt;/sup&gt;</td>
<td>1.78±0.67&lt;sup&gt;a(x)&lt;/sup&gt;</td>
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<td>2.89±0.93&lt;sup&gt;a(wx)&lt;/sup&gt;</td>
<td>2.56±1.33&lt;sup&gt;a(wx)&lt;/sup&gt;</td>
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<tr>
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<td></td>
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<td>150</td>
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<td>2.22±1.09&lt;sup&gt;a(wx)&lt;/sup&gt;</td>
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Table 4.3. Effect of ultrasound on overall quality, surface browning, cut edge browning, sogginess and off-odor (E) of Romaine lettuce treated at 25 kHz determined by sensory evaluation. (cont.)

<table>
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<tr>
<th>Quality Parameter</th>
<th>Storage Time (h)</th>
<th>1min Ultrasonication</th>
<th>2min Ultrasonication</th>
<th>3min Ultrasonication</th>
<th>3min Water Washing</th>
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<tr>
<td>Off-odor</td>
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<td>1.11±0.33  $^{a(x)}$</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.44±1.01  $^{a(x)}$</td>
<td>1.56±1.01  $^{a(wx)}$</td>
<td>1.11±0.33  $^{a(x)}$</td>
<td>1.67±1.00  $^{a(wx)}$</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.78±0.83  $^{a(wx)}$</td>
<td>2.11±1.36  $^{a(wx)}$</td>
<td>2.78±1.64  $^{a(w)}$</td>
<td>1.89±0.93  $^{a(wx)}$</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>2.44±1.13  $^{a(w)}$</td>
<td>2.00±1.12  $^{a(wx)}$</td>
<td>2.00±1.00  $^{a(wx)}$</td>
<td>1.89±0.93  $^{a(wx)}$</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>1.56±0.73  $^{b(x)}$</td>
<td>2.33±0.87  $^{ab(w)}$</td>
<td>2.22±0.83  $^{ab(w)}$</td>
<td>2.56±1.24  $^{a(w)}$</td>
</tr>
</tbody>
</table>

$a$-$b$ means within time (row) results with different letters are different at $\alpha$ 0.05.

$w$-$z$ means within treatments (column) results with different letters are different at $\alpha$ 0.05.
CHAPTER 5
FUTURE WORK

In current study, we investigated the response of fresh Romaine lettuce to different low APD ultrasound treatments. Generally, ultrasonic treatment did not affect the sensory or nutritional quality immediately. Activation of phenylalanine ammonia-lya (PAL) and production of phenolic compounds became apparent during storage and thus the antioxidant capacity of lettuce after 60 hours storage at room temperature was higher than that of the control. The sensory quality analyzed by both instrumental methods and human panelists indicated that ultrasound treatment did not adversely affect lettuce quality during storage. Exposure to ultrasound for 1 minute delayed lettuce tissue browning and in turn maintained better quality and a longer shelf-life.

All operational parameters, such as frequency, duration and acoustic power density, may alter the effect of ultrasound treatment on fresh produce quality. Only a series of treatment times was reported in the current study. In preliminary experiments, no difference was observed among strawberry fruits treated under frequencies ranging from 25 kHz to 75 kHz (Figure A.1). However, ultrasonication with increased acoustic power density or longer duration was found adversely affecting spinach quality due to tissue damage (data not shown). Response Surface Methodology (RSM) should be conducted in the future to optimize the treatment conditions for maximizing the antioxidant capacity and secondary metabolites production while retaining a better overall quality and an extended shelf-life. Meanwhile, we observed that different produce showed different responses to ultrasonication (Figure A.2, Figure B.1, and Figure C.1). Separate studies might be required for different fruits and vegetables.
Further studies are also needed for a better understanding of the physiological responses of fresh produce to ultrasound treatment. Defense-related enzymes are important indicators for plant defense response. Thus, the analysis of oxidative enzymes (e.g. SOD, POD, and CAT) and secondary-metabolism-related enzymes (e.g. PAL and PPO) should be conducted. Apart from total phenolic content and total antioxidant capacity, more detailed researches about the change of phenolic profiles and specific phenolic compounds would not only benefit the comprehension of defense response, but also provide knowledge to achieve controlled production of ideal secondary metabolites. Moreover, investigation for the production of ROS, secondary signaling molecules, and gene expression due to ultrasound treatment should be considered since they are helpful to figure out the accurate biochemical pathways during response. This knowledge will be useful to regulate the defense system of produce to obtain improved nutritional quality.

An exploratory research has been done to examine the effect of ultrasound treatment on growing plants. Difference between ultrasound-treated spinach seedlings and controls can be observed both in plant growth and biochemical properties like antioxidant capacity (Figure D.1 and Figure D.2). However, due to limited sample and replications, no conclusion can be drawn yet. Therefore, more experiments with a large scale are necessary in the future for this topic.
CHAPTER 6
REFERENCES


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Reyes LF, Villarreal JE, Cisneros-Zevallos L. 2007. The increase in antioxidant capacity after wounding depends on the type of fruit or vegetable tissue. Food Chem. 101: 1254-1262.


Science and Technology. 37(5): 547-557.


APPENDIX A

EFFECT OF ULTRASOUND ON VISUAL QUALITY AND ANTIOXIDANT CAPACITY OF STRAWBERRY

Figure A.1 Effect of ultrasound on (A) total color difference, (B) firmness, and (C) antioxidant capacity of strawberry fruit stored at 4 °C after ultrasonication at 25 kHz, 40 kHz and 75 kHz for 10 min or water washing for 10 min.
Figure A.2 Effect of ultrasound on (A) total color difference, (B) firmness, and (C) antioxidant capacity of strawberry fruit stored at room temperature after ultrasonication at 25 kHz, 40 kHz and 75 kHz for 10 min or water washing for 10 min.
Conclusions:

- At room temperature storage, sonicated strawberries (100% power output, 25 kHz, 26 W/L APD, for 10 min) exhibited higher antioxidant activities than water-washed samples at Day 2 (Figure A.2.C).

- During 4°C storage, no frequency effect on antioxidant capacity of strawberry fruit was detected (Figure A.1.C).

- No significant differences in color and firmness were observed between strawberry samples treated with or without ultrasound under both storage conditions.
APPENDIX B

EFFECT OF ULTRASOUND ON TISSUE DAMAGE AND ANTIOXIDANT CAPACITY
OF SPINACH AND ROMAINE LETTUCE

Figure B.1 (A) Conductivity and (B) antioxidant capacity of Romaine lettuce and spinach stored for up to 7 days at 4°C storage after ultrasonication at frequency of 25 kHz for 2 min or water washing for 2 min.
Conclusions:

- For spinach stored at 4 °C, sonicated spinach (100% power output, 25 kHz, 26W/L APD, for 2 min) exhibited a higher antioxidant capacity than water-washed ones (Figure B.1.B) and also these samples showed a faster tissue recovery (Figure B.1.A).

- During cold storage for up to 7 days, ultrasound did not show any effect on antioxidant capacity or tissue recovery of Romaine lettuce under the treatment condition tested.
APPENDIX C

EFFECT OF ULTRASOUND ON PAL ACTIVITY OF SPINACH AND BABY CARROT

Figure C.1 Effect of ultrasound treatment on phenylalanine ammonia-lyase (PAL) activity of (A) spinach and (B) baby carrot treated at 25 kHz after 48 hour storage under room temperature.
Conclusions:

No significant difference of spinach or baby carrot PAL activity was found between ultrasound-treated groups (at 100% power output, 25 kHz) and the water-washing control after 48 hours storage at room temperature.
Figure D.1 Spinach seedlings treated by ultrasound at 25 kHz. Plants (A) before the treatment, (B) right after the treatment, and (C) 20 days after the treatment.
Figure D.2. Antioxidant capacity of spinach seedlings growing for up to 10 days after ultrasonication at frequency of 25 kHz, 20% power output for (A) 30 seconds and (B) 10 seconds and 20 seconds, comparing to the control.