THE POTENTIAL ANTIOXIDANT AND ANTICANCER PROPERTIES OF POLYAMINES AND THEIR RESPONSE TO HIGH-INTENSITY ULTRASONICATION

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Food Science and Human Nutrition
with a concentration in Food Science
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

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ABSTRACT

Polyamines are widespread in living cells and play several important roles in both plants and animals. The polyamines spermine, spermidine and putrescine have been of primary interest, as they participate in cell growth and proliferation and thus are involved in health, disease and aging. Polyamines are also reported as secondary metabolites in stress response in plants. However, many of these functions have not been scientifically demonstrated. Thus, the objective of this research was to: (1) compare the antioxidant and anti-carcinogenic capacity of polyamines through various *in vitro* assays; (2) study the effect of ultrasound on secondary metabolite synthesis (polyamines) and maintenance of lettuce quality.

Antioxidant capacity of polyamines was compared using two commonly used antioxidant capacity assays. The inhibitory effect of polyamines on cancer cell growth was investigated using murine breast cancer 4T1 cells and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to monitor cell growth. Tyramine had the highest antioxidant capacity, followed by tryptamine, spermine, putrescine, and spermidine. Agmatine showed no antioxidant capacity. Spermine and spermidine inhibited the growth of 4T1 cells at IC_{50} of 6.0 µg/mL and 21.5 µg/mL, respectively. No anti-carcinogenic effects were observed in this cell system with putrescine. Abiotic stress application was conducted using sonication. Gas chromatographic analysis revealed that immediately after sonication, polyamine concentrations were not significantly different between unsonicated and sonicated lettuce samples. After three days recovery, the unsonicated lettuce leaves displayed significantly higher concentrations of putrescine and spermidine as compared to sonicated leaf tissue. This was also linked with a much higher quality of the lettuce leaves from sonicated tissue.

This represents the first report of comparison of different polyamines for antioxidant capacity by two popular methods (ORAC and DPPH). For anti-carcinogenic activity, data were generated from one cell line indicating that the polyamines, but not putrescine, may prevent cell growth. Ultrasonication was found to be efficient in maintaining the quality of lettuce during storage, correspondingly, the polyamine levels in sonicated lettuce were decreased after 72 hours storage compared to control lettuce. Further studies are needed to ascertain the role of polyamines in cancer utilizing other cell lines. Microbial populations in sonicated and unsonicated lettuce after 72 hours recovery can also be tested to strengthen the impact of quality maintenance related to ultrasound treatment.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my academic advisor, Dr. Nicki Engeseth, for her guidance in my education and life in graduate school. She inspired me with new ideas in academic research, and she also gives me solutions for the problems in daily life. Her support and suggestion will have a life-long impact on me.

I would like to thank Dr. Maria Beatriz Abreu Gloria, she is a visiting scholar from Brazil in our laboratory. She introduced polyamine project and raised my interest in this project. Her hardworking and determination in scientific research influence me a lot.

I would like to thank my committee members, Dr. Hao Feng and Dr. Bill Helferich, for their time and willingness to be my committee members and for their guidance during my master's study and research.

I would like to take this chance to thank my friend, Cheng Chen, and all my lab mates, Ru Shen, Alice Moon, Ziyi Zhan, Dennis Humberto Pinto Padilla, and Marlon Fernando Ac Pangan. Their friendship and encouragement give me a precious memory in UIUC.

Finally, the thesis should be dedicated to my parents. They supported me for my two and a half years education in UIUC. Although they are far away from me during these years, they always teach me to become a hardworking, independent and confident person. My parents delivered their love and guidance to me selflessly, which made me feel lucky and proud to be the beloved daughter of them. Thanks for everything in the past 24 years.

Contents

CHAPTER 1: INTRODUCTION

With the development of society, there has been growing public concern over human health. Increasing consumption of healthy foods, such as vegetables and fruits, has been linked to inhibition of diseases such as inflammation, cardiovascular disease, cancer, and aging-related disorders (Willett, 2001). Dietary antioxidants, which include polyphenols, vitamins E and C, carotenoids and others, are believed to be the active agents in fruits and vegetables to prevent oxidative stress-related diseases (Ames et al., 1995; Kaur & Kapoor, 2001). Unlike other antioxidants which are fairly well known and accepted by people, polyamines, commonly known as putrescine, spermidine, and spermine, are seldom known by people for their high antioxidant capacity although they widely present in all living cells.

Polyamines are biological compounds of low molecular weight with two or more aliphatic nitrogen groups (Valero, Martínez-Romero, & Serrano, 2002). They are formed by the derivation of ammonia, in which hydrogen atoms are replaced by alkyl moieties (Smith, 1981). Spermine and spermidine have been reported as efficient antioxidants in previous studies (Drolet et al., 1986; Løvaas, 1991); however, there are no literature reports of comparison of the antioxidant capacity of different polyamines. There is also little knowledge about antioxidant capacity of polyamines by different antioxidant assays, such as Oxygen Radical Absorbance Capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), which have been commonly utilized in antioxidant analysis.

Polyamines are considered to associate with stress responses both in animals and plants (Grimsb et al., 1997; Liu et al., 2015). In animal studies, polyamines have been reported to play an essential role in modulating various cellular functions (Park & Igarashi, 2013). Several studies

indicated that the concentration of polyamines has increased in tissues and organs of cancer patients (Zamoyska, 1994; Shike et al., 2002; Milovic & Turchanowa, 2003). Although nutritional analysis has demonstrated that polyamines have a correlation with cancer cells in both animals and humans, few studies have investigated the inhibitory effect of polyamines towards cancer cells directly. It is necessary to study the role of polyamines in cancer cell growth, whether as a promoter, or as a stress response excreted by human bodies.

In plant studies, polyamines have become a well-established example of metabolites, which are involved in stress responses or stress tolerance (Liu et al., 2015). High intensity ultrasound or power ultrasound with frequency ranging from 20-100 kHz was utilized as abiotic stress to freshly harvested plants (vegetables and fruits), which could significantly decrease the microorganisms levels on the surface of plants and maintain the postharvest quality of fresh produce (Cao et al., 2009; Yu et al., 2016).

In this study, polyamines were studied as a new focus of our laboratory. The antioxidant activity of six polyamines (spermine, spermidine, sutrescine, agmatine, tyramine, and tryptamine) was explored, in comparison with other well-recognized antioxidants, by different antioxidant assays. The inhibition effect of polyamines (spermine, spermidine, and putrescine) on murine breast cancer 4T1 cell line was tested by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The purpose was to gain some perspective on these widely suggested potential activities of polyamines. The hypothesis of this research was that ultrasound can be utilized as a physical elicitor to induce polyamine synthesis in plants, and ultrasound can be applied as a pretreatment to preserve the quality of lettuce during storage. The objectives of this study were to: (1) study the antioxidant and anticancer capacity of polyamines; (2) study the effect

of ultrasound in secondary metabolite synthesis (polyamines) of lettuce; and (3) study the effect of ultrasound in maintenance of lettuce quality during postharvest storage.

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CHAPTER 2: LITERATURE REVIEW

2.1 Introduction of bioactive amines:

Bioactive amines are biological compounds of low molecular weight with nitrogen groups (Valero, Martínez-Romero, & Serrano, 2002). They are formed by the derivation of ammonia, in which hydrogen atoms are replaced by alkyl moieties (Smith, 1981). The names of some amines are associated with their characteristics, e.g., decomposition and putrefaction are characteristics of cadaverine and putrescine; however, most polyamines are named based on their precursor amino acids, e.g., tryptamine originates from tryptophan, histamine originates from histidine, tyramine from tyrosine (Flores, Protacio, & Signs, 1989).

Different numbers of amine groups, chemical structure and biosynthetic pathways can be utilized to classify bioactive amines (Silla Santos, 1996) (Figure 2.1, Glória, 2006). Based on the number of amine groups, they can be divided into monoamines (i.e., tyramine, phenylethylamine), diamines (i.e., histamine, serotonin, tryptamine, putrescine, cadaverine) and polyamines (i.e., spermine, spermidine, agmatine). According to chemical structure, amines can be divided into aliphatic (i.e., putrescine, cadaverine, spermine, spermidine, agmatine), aromatic (i.e., tyramine, phenylethylamine) and heterocyclic (i.e., histamine, tryptamine, serotonin). Based upon biosynthetic pathways, classification includes natural amines (i.e., spermine, spermidine, putrescine, histamine) and biogenic amines (i.e., tyramine, tryptamine, octopamine). Histamine can be either natural (stored in mast cells or basophils) or biogenic.

Among all bioactive amines, putrescine (PUT, butan-1,4-diamine), spermidine (SPD, [N-(3-aminopropyl) butane-1,4-diamine]) and spermine (SPM, [NN'-bis-(3-aminopropyl) butane-1,4-diamine]) are the most commonly occurring amines in animals and plants (Smith, 1981).

Spermine and spermidine play important roles in cell growth and differentiation and cell membrane stabilization. Putrescine is an obligatory intermediate of spermidine and spermine. Bioactive amines have been known for centuries; however, their mechanisms in plants and animals remain unclear. Still today, the mechanism behind bioactive amines attracts considerable research interest related to food, biomedical, and environmental science.

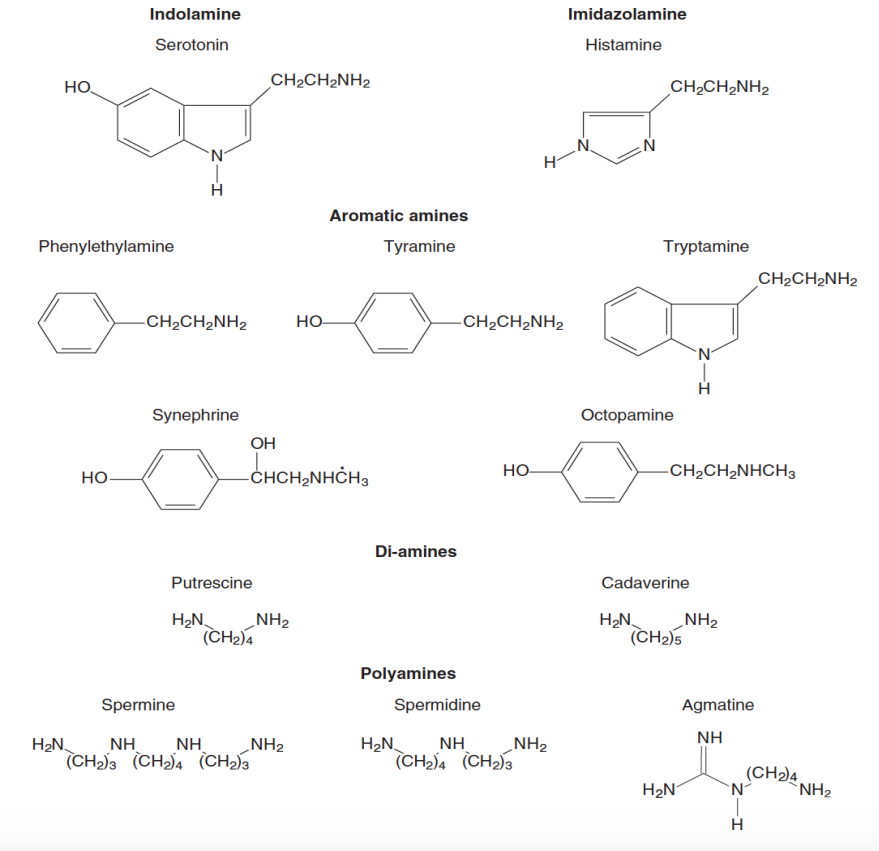

FIGURE 2.1. Classification and chemical structure of some bioactive amines (adapted from Glória, 2006).

2.2 Biosynthesis of bioactive amines in organisms

Food intake, cellular synthesis and microbial synthesis in the gut represent the three main sources for bioactive amines in organisms (Minois, Carmona-Gutierrez, & Madeo, 2011). Most

bioactive amines are formed through decarboxylation of precursor amino acids (Hui, 2006). There are two pathways which lead to the synthesis of bioactive amines in organisms (Galston & Kaur-Sawhney, 1990; Kumar, Altabella, Taylor, & Tiburcio, 1997; Tiburcio, Altabella, Borrell, & Masgrau, 1997; Walden, Cordeiro, & Tiburcio, 1997). One is transformation from arginine to ornithine, activated by the enzyme and then, ornithine can be converted to putrescine in organisms as catalyzed by ornithine decarboxylase (ODC). The other pathway involves the decomposition of arginine by arginine decarboxylase (ADC). Agmatine forms after decarboxylation of arginine, then agmatine will convert into N-carbamoylputrescine catalyzed by agamtine iminohydrolase. N-carbamoylputrescine will finally transfer to putrescine by N-carbamoylputrescine amidohydrolase.

Two alternative routes (ADC/ODC) work together to regulate amines in organisms (Valero et al., 2002) (Figure 2.2). To stabilize amine concentrations in organisms, reversible inhibitors dl-adifluoromethylarginine (DFMA) and difluoromethylornithine (DFMO) can respectively inhibit the functions of ADC and ODC. Putrescine is the initiator in the synthesis of spermine and spermidine. In parallel to putrescine production, S-adenosylmethionine (SAM) which will form decarboxylate S-adenosylmethionine (DCSAM), catalyzed by the enzyme SAM decarboxylase (SAM-DC). DCSAM is used as an aminopropyl donor in the conversion of putrescine to spermidine and of spermidine to spermine, catalyzed by two separate and distinct enzymes, spermidine synthase and spermine synthase, respectively (Valero et al., 2002 & Minois et al., 2011). Besides the synthesis of amines, several other processes including degradation, conjugation, and transport can also regulate the intracellular free polyamine pool in organisms. Overall, amine concentrations are tightly regulated in organisms.

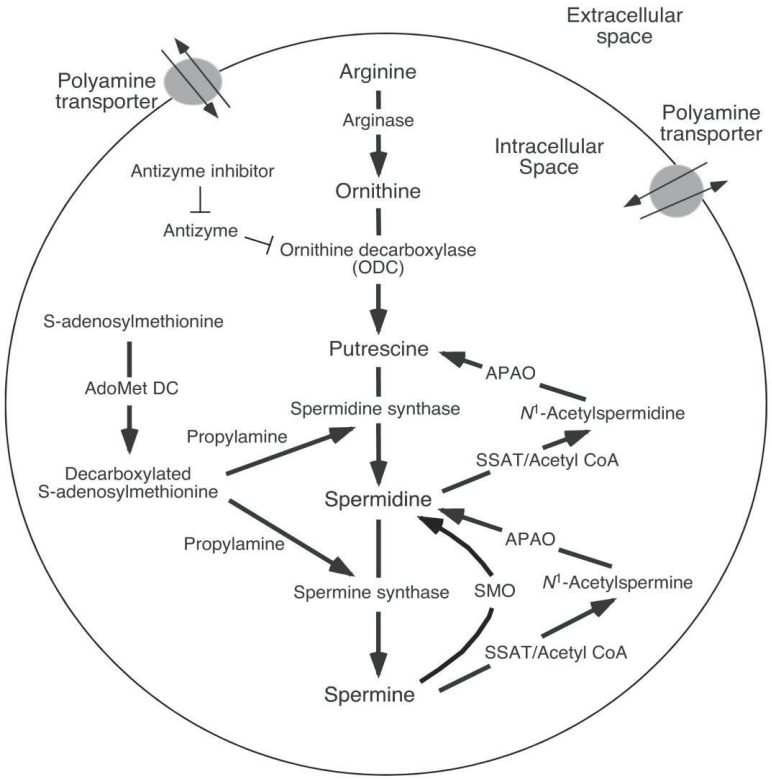

Figure 2.2. Pathways for the synthesis of polyamines (Adapted from Valero et al., 2002)

2.3 Bioactive amines in plants

Bioactive amines are widely spread in plants and have been considered as a new category of plant growth regulator (Bouchereau, Guénot, & Larher, 2000). Many important processes of plant growth, e.g., embryogenesis, cell division, morphogenesis, and development or stress tolerance, may also be associated with polyamines in plants (Flores et al., 1989). As common chemicals existing in daily life, the amount and category of amines in foods depends on the nature and origin of the commodity. Production methods, processing, fermentation, storage and hygienic conditions may also change the levels of polyamines in foods, leading to the concept that

polyamines might reflect the freshness, quality, and processing of plant products (Hui, 2006). Researchers start to realize that polyamines in plants is an area deserving future investigation.

Previous studies demonstrated that bioactive amines are widely spread in food plants (Adapted from Nishibori, Fujihara and Akatuki, 2005) (Table 2.1).

2.4 Ultrasound

Ultrasound is an application of sound waves with frequencies higher than the upper audible limit of human hearing, which can be divided into high-intensity ultrasound with frequency ranging from 20kHz to 1MHz and low-intensity ultrasound with frequency excessing 1 MHz (Yu et al, 2016).

Ultrasound technology is known to cause chemical and physical changes in biological structures (in a liquid medium) due to the high-energy chemistry it produced during the process. The high-energy chemistry effect of ultrasound is a result of acoustic cavitation in a liquid instead of the direct interaction with molecular species (Suslick, Grinstaff, Kolbeck, & Wong, 1994). "Cavitation" is the activities of the formation, growth, and implosive collapse of bubbles in liquid caused by the ultrasound, which results in a concentrating of the diffuse energy of sound (Suslick, 1990). When the bubble induced by cavitation collapse, the intense local heating and high pressure can be produced, however, the lifetimes for these bubbles are very short. These hot spots, which contain high energy, can reach the temperatures of roughly 5000°C, pressures of about 500 atmospheres, and heating and cooling rates are greater than 109 K/s (Suslick, Hammerton, & Cline, 1986). Ultrasound treatment with different intensity cam lead to opposite impacts (Yu et al, 2016), in general, the energy released from high power density ultrasound is capable of destroying cells, ejecting particles from the solid surface and increasing mass transfer to the surface by disruption

of the interfacial boundary layer (Zhou, 2011), while mild ultrasound can stimulate biological activities (Miller et al., 1996).

Ultrasound treatment has been applied in food processing within a variety of areas. When ultrasound is applied to fruits or vegetables, the temperature increases and high pressure is released directly towards their surface, upon collapse of the bubbles. The effect of ultrasound was demonstrated in litchi fruit postharvest. The activities of oxidase and peroxidase were inhibited by 120 W ultrasound treatment for 10 min; this significantly delays the pericarp browning of litchi fruits (Chen et al., 2011). Ultrasound treatment (40 kHz) was also applied to freshly harvested strawberry; decay and microorganisms were significantly decreased, while the color and firmness of strawberries were steadily maintained by ultrasonic treatment (Cao et al., 2009). Ultrasound treatment has been shown to control enzymatic browning, tissue softening, and microbial growth during processing, which is considered to be a new trend in the pretreatment of postharvest fruits and vegetables (Xu et al., 2013).

2.5 Amines and stress

2.5.1 Abiotic stress in plants

Plants require energy (light), carbon, water, and minerals for growth (Cramer, Urano, Delrot, Pezzotti, & Shinozaki, 2011). As sensitive organisms, there will be a variety of environmental stresses throughout the lifecycle of plants. Most environmental stresses, which are referred to as abiotic stresses, are defined as environmental conditions that negatively affect the optimal growing conditions for plants, such as salt, cold, drought, and UV light. Boyer (1982) indicated that crop production could be limited by as much as 70% under abiotic stress conditions. A report from FAO in 2007 illustrated that only 3.5% of the global land area is not affected by

some environmental constraint (Haugen, 2011). Plant growth and yield can be decreased as a result of abiotic stress, while the responses to abiotic stresses in plants are dynamic and complex; yet these responses remain unclear (Skirycz & Inzé, 2010 & Cramer, 2010). Since abiotic stress can negatively affect plant development and productivity (Tuteja & Sopory, 2008), there is increasing concern about plants' responses to abiotic stresses. Interaction and crosstalk with many molecular pathways are involved in the plant molecular responses to abiotic stresses. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved early in the response signal, which can regulate gene expression and modify enzyme activity. In addition, hormones are also important regulators of plant responses to abiotic stress. The two most important are abscisic acid (ABA) and ethylene (Goda et al., 2008).

2.5.2 Production of polyamines as a stress response in plants

The low-molecular-weight aliphatic polyamines, including putrescine, spermidine, and spermine, played a critical role in stress responses in plants (Liu et al., 2015). Several evidences have demonstrated the polyamine responses in plants. First, stress can induce the activities of the polyamine biosynthetic enzymes and the transcript levels of corresponding genes in plants (Liu, Inoue & Moriguchi, 2008); secondly, the exogenous supply of polyamines, or overexpression of polyamine biosynthetic genes, results in enhanced stress tolerance, which may maintain the quality and extend the shelf life of plants even under the stress environment (Yang, Wang, & Rao, 2016); and thirdly, on the contrary, a decrease in endogenous polyamines may accompany with the reduced tolerance to stress in plants.

There is increasing evidence indicating that the role of polyamines in stress tolerance is mainly associated with antioxidant system modulating (Shi et al., 2010). A large amount of

reactive oxygen species (ROS) were produced under abiotic stresses, which may have a direct, or indirect roles in regulating antioxidant systems or suppressing ROS production. Polyamines can modulate the homeostasis of these reactive oxygen species (ROS) as antioxidants in response to these stress (Liu et al., 2015). Meanwhile, future perspectives on polyamine research are also suggested.

2.6 Amines and cancer

2.6.1 Polyamines in the body

Polyamines, i.e., putrescine, spermidine, and spermine are present in all living cells and are essential for eukaryotic cell growth. Polyamines modulate various cellular functions, including macromolecular synthesis and interact with negatively charged molecules such as DNA, RNA, acidic proteins and phospholipids (Park & Igarashi, 2013).

Several in *vitro* studies demonstrated that cells could absorb polyamines from their surroundings (Bogdan, 2001 & Holán, Krulová, Zajícová, & Pindjáková, 2002), and orally administered radiolabeled polyamines have been shown to be immediately distributed to almost all organs and tissues (Boshra, Li, & Sunyer, 2006). Blood cells contain a majority of polyamines, which have the function to modify polyamine concentration in human bodies (Janeway, 1992). Compared to healthy people, the concentration of polyamines has increased in tissues and organs of cancer patients (Zamoyska, 1994). Milovic & Turchanowa (2003) found that polyamine levels in colon mucosa from cancer patients increased 3-4 times compared to the normal colon mucosa from patients without cancer, which indicated that polyamines can be considered as markers of neoplastic proliferation in the colon. Some other studies were unable to detect these differences. Polyamine concentrations in the blood vary considerably among healthy individuals such that

concentrations are not necessarily higher in cancer patients than in otherwise normal subjects (Shike et al., 2002), and this wide variation precludes the use of polyamine concentration as a tumor marker as well as making detection of differences in polyamine concentrations between normal tissues of cancer patients and normal subjects difficult.

2.6.2 Mechanisms of polyamine upregulation in cancer

The potential mechanisms associated with the link between polyamines and cancer became clearer when the association between cancer risk factors and polyamine metabolism was studied (Gerner & Meyskens, 2004). Individuals with familial adenomatous polyposis (FAP), whose tumor suppressor adenomatous polyposis coli (APC) is mutated or lost in the germline (Groden et al., 1991), upregulated ODC (Ornithine Decarboxylase) in their intestinal mucosa which can increase the expression and activity of ODC (Giardiello et al., 1997). The loss of APC in colon tumor cells can lead to increased expression of the MYC oncogene, extra expression of which is associated with the development of Burkitt's lymphoma and several epithelial cancers, in addition to colorectal cancer (T. C. He et al., 1998). The proliferation of some normal cells requires a transcription factor encoded by MYC, but the overexpression of MYC can lead to uncontrolled growth and cancer (Hermeking, 2003). In an animal study, the expression of ODC gene is increased in intestinal tissue of ApcMin/+ mice while a specific inhibitor of ODC suppressed intestinal carcinogenesis in this model, which confirms that ODC is a direct transcriptional target of MYC (Erdman et al., 1999). The studies showed that conditional expression of wild-type APC suppresses ODC gene expression in an MYC-dependent manner in human colon tumor cells supported the hypothesis that ODC is a modifier of APC-dependent tumorigenesis (Fultz & Gerner, 2002). The transcriptional regulation of ODC involves other mechanisms in addition to the

pathway involving APC and MYC. ODC is not the only polyamine metabolic gene that is regulated by oncogenes and tumor suppressor genes (Gerner & Meyskens, 2004). The ODC regulator antizyme (OAZ), is also regulated by APC in the ApcMin/+ mouse model (Erdman et al., 1999).

2.6.3 Polyamine as a marker for cancer

Many diseases including cancer (Gerner & Meyskens, 2004), inflammation (Babbar, Murray-Stewart, & Casero, 2007), stroke (Yoshida et al., 2010), renal failure (Igarashi, Ueda, Yoshida, & Kashiwagi, 2006) and diabetes (D. L. Kramer et al., 2008) can be caused by dysregulation of the polyamine pathway. Polyamine concentrations in human urine and plasma have been utilized as a diagnostic marker for cancer. Among all amines, putrescine (PUT), spermidine (SPD), and spermine (SPM) are considered promising tumor markers, since their excretion has been detected in patients with various types of cancers (Fu, Xiao, Zhao, & Yu, 2012).

Normal cell and cancer cell growth can be regulated by cellular polyamine concentrations in humans. Studies showed that polyamines play an important role in carcinogenesis, by studying transgenic mice which overexpress polyamine biosynthetic enzymes. This indicated that tumor growth is associated with an increase in polyamines and polyamine synthetic enzymes (Park & Igarashi, 2013).

Higher polyamine concentrations in blood or urine will not only indicate some advanced diseases such as cancer, but also may accelerate tumor invasion and metastasis in humans (Soda, 2011). Results from animal studies indicated that tumor growth and degree of metastasis can be reduced by inhibition of polyamine synthesis through difluoromethylornithine (DFMO) and/or methylglyoxalbisguanylhydrazone (MGBG). Therefore, the effect of polyamines on the metastatic potential of cancer cells, the host's anti-tumor immunity should be taken into consideration.

2.7 Amine analysis

Thin-layer chromatography (TLC), High performance liquid chromatography (HPLC), and Gas chromatograph (GC) with several detection systems were applied in detection of polyamines in food system. To obtain an optimal condition of analysis, extraction, concentration and derivatization procedures were used before analysis.

2.7.1 Extraction

Although the extraction of polyamines from foods is usually a simple process; it depends on the type of food sample (Leo & Fidel, 2015). For solid foods, extraction procedures are more complicated than liquid foods (Hui, 2006). Solid foods need to be treated by grinding, crushing, homogenization, or degreasing before extractions. Acids were commonly used in amine extraction, while water and organic solvents can also be used for the extraction of amines from solid foods (Custódio, Tavares, & Glória, 2007). The acids used for the extraction of amines from the food matrix include mineral (hydrochloric acid, HCl) and organic acids (trichloroacetic acid, TCA and sulfosalicylic acid—SSA). 1M hydrochloric acid has been widely used for amine extraction from some fruits, cheese, and other dairy products. TCA (5%) is the most widely used extraction acid as it provides good recovery of amines from meat, fish, fruits, and vegetables (Bandeira, Evangelista, & Gloria, 2012; Gloria & Adão, 2013). Clean supernatants can be obtained for later analysis after agitation, centrifugation, and filtration. It is usually simple for the amine extraction of liquid samples such as juice, wine and beer; liquid will be injected into HPLC directly after homogenization and filtering through a 0.45 μm pore membrane filter. An optimized method for amine analysis in soy sauce was conducted by diluting in acid solution (trichloroacetic acid, 5% TCA), agitation and centrifugation prior to HPLC analysis (Lr & Mb, 2012). Not all amines in liquid foods can be extracted effectively by TCA, milk was better extracted with sulfosalicylic

acid (1.5% SSA) and centrifugation at 4°C to facilitate fat separation from the sample prior the analysis (Rigueira, Rodrigues, & Gloria, 2011).

The types of amines may also affect the selection of extraction agents. Spermine, spermidine and putrescine were found to be more effectively extracted by HCl; the aromatic amines (tyramine, phenylethylamine) and the indolamines (tryptamine, serotonin) have a better recoverability with organic solvents (methanol and ethanol). However, in a multiamine analyses method, 1 M HCl was chosen as it provided good recoveries for most of the amines (Hui, 2006).

2.7.2 Analysis by GC

Compared to HPLC, GC method is not widely used in amine analysis in foods. Some of amines such as putrescine, cadaverine, and aliphatic amines, can be easily and directly analyzed by GC due to their volatility (Kataoka, Shindoh, & Makita, 1995). However, some free amines are not suitable for amine analysis on GC owing to the high-water solubility, and to the adsorption and decomposition on the column which results in peak tailing and losses. To solve these problems, a derivatization step is included to make amines volatile for GC separation (Kataoka, 1996). Besides the increased volatility, derivatization can also improve the detection sensitivity, and promote the extraction rate from food samples.

Acylation, silylation, and the formation of carbamate, sulfonamide, and phosphoamide are five main derivatization methods for amine analysis, and acylation is the most popular derivatization reaction for primary and secondary amines among all of these (Smith, 1981). In previous studies, acetic anhydride (Baker et al., 1994), trifluoroacetic anhydride (Jiang, 1990; Khuhawar et al., 1999), trifluoroacetylacetone—TFAA (Khuhawar et al., 1999; Awan et al., 2008a), pentafluoropropionic anhydride—PFPA (Rattenbury et al., 1979; Baker et al., 1994), and

heptafluorobutiric chloride (Chia and Huang, 2005) have been used as acylation agents. Compared to other derivatization, these compounds have the advantage which can react with amines under mild conditions.

For silylation derivatization, N-methyl-bis-trimethylsilyltrifluoroacetamide was commonly used before as derivatization agent in GC analysis; however, it was replaced by some new derivatization such as hexamethyldisilazane (HMDS) and N-methyl-bisheptafluorobutyramide (MBHFBA). Compared to the old one, new generated reagents can react specifically with amine groups and prevent the formation of side products.

For carbamate formation, isobutyl chloroformate was used in the analysis of amines in beer, wine, and grape by GC-MS. Isobutyl choloroformate was used as derivatization in amine analysis in 22 beer samples (Almeida, Fernandes, & Cunha, 2012). By using dispersive liquid-liquid microextraction (DLLME) gas chromatography mass-spectrometry (GC-MS) method, fourteen of the eighteen biogenic amines analyzed were found in most of the beers, which including putrescine, tyramine, dimethylamine, cadaverine, pyrrolidine and 1,3-diaminopropane. However, isobutyl choloroformate is not a suitable for all the amines, especially for spermine (Mh, Kr, & Bc, 2000); because when the multi-isoBOC groups react with amines, the newly formed derivatives become less volatile and the remaining polar active hydrogen atoms in amino groups cause peak tailing.

Table 2.1 Polyamine contents of foods (cont.)

Food	Polyamine concentration (n mol/g or ml)						
	Putrescine		Spermidine		Spermine		n
	Mean	Range	Mean	Range	Mean	Range	
Cereals							
Bread	44	13-105	73	15-159	24	6-52	5
Flour	32	13–60	34	28–40	7	5–8	5
Macaroni	5	4–6	23	14–34	9	5–14	4
Rice	2	2–3	3	2–4	3	1 – 4	6
Wheat noodles	4	1–9	3	2–3	1	1 – 1	5
Potatoes							
Potato	82	66–119	50	30–112	14	3–23	6
Sweet potato	43	7–76	31	12 – 55	4	1–14	7
Taro	34	18–49	30	23–34	6	2–8	5
Yam	127	111–145	144	23–300	4	1–7	4
Sugar	0	0–0	0	0–0	0	0–0	6
Beans							
Adzuki bean	41	23–58	454	322–556	392	261–521	5
Cow pea	16	13–18	361	288–445	381	266–684	5
Soybean	194	73–275	728	608–865	181	150–206	5
Soybean flour	42	11–89	102	30–279	18	7–46	5
Natto (fermented soybean)	175	81–267	232	150–314	19	10–26	4
Tofu (soybean curd)	0	0–0	1	0–1	0	0–0	4
Deep-fried tofu	9	6–11	59	33–82	34	27–40	4
Nuts							
Almond	19	13–33	41	35–51	67	58–77	5
Cashew	9	3–14	32	22–36	119	91–140	5
Peanut	18	9–32	110	88–132	88	66–127	4
Pistachio	207	82–314	77	59–86	66	48–92	5
Vegetables							
Asparagus	61	37–75	120	15–222	28	1–64	5
Bean sprout	207	174–279	50	37–66	3	2–5	5
Broccoli	21	15–30	156	111–195	43	28–62	5
Burdock root	111	15–286	114	18–245	43	15–68	5
Butterbur	22	11–31	13	8–15	2	2–3	6
Cabbage	31	8–71	61	23–136	12	4–33	8
Carrot	168	91–281	42	31–53	7	6–9	5
Celery	194	97–263	98	78–130	19	8–27	5
Chinese cabbage	10	5–18	72	21–109	4	0–12	12
Cucumber	149	24–293	107	31–224	1	0–2	5
Eggplant	198	128–250	31	18–39	2	1–3	4
Field pea	23	10–55	115	80–180	28	12–42	5
Garlic	26	8–69	77	51–130	29	18–35	4
Ginger	30	7–42	26	12–55	2	0–4	6
Green peas	367	63–581	341	31–651	32	21–43	6
Green pepper	621	354–955	80	53–123	45	20–103	10
Green soybeans	147	68–218	334	240–463	20	11–34	5
Japanese hornwort	178	63–421	110	25–281	33	10–79	6
Japanese radish	13	6–24	40	5–89	2	1–5	7
Kidney French bean	24	8–54	81	28–189	29	11–54	7
Komatsuna	32	4–45	118	11–202	25	12–41	6
Lettuce	235	116–481	303	102–717	16	2–64	7
Lotus root	116	50–209	210	119–354	2	1–4	6
Maize	576	208–969	144	56–269	8	1–25	6
Onion	7	2–11	16	8–21	4	2–11	6
Parsley	99	45–148	33	21–46	9	7–11	5

Table 2.1 Polyamine contents of foods (cont.)

Pumpkin	75	37–123	42	39–68	91	33–205	5
Spinach	50	20–153	123	97–154	19	15–27	5
Tomato	114	60–235	15	10–25	1	1–2	5
Welsh onion	14	3–26	141	65–189	9	5–14	4
Pickled Japanese radish	50	34–64	78	56–98	4	0–7	5
Banana	140	127–156	40	29–57	3	0–8	5
Cherry	19	4–49	11	0–25	4	2–10	6
Chinese citron	315	234–394	4	2–7	0	0–0	4
Mandarin orange	256	118–468	10	1–16	0	0–1	5
Orange	244	73–413	4	0–10	0	0–1	7
Strawberry	11	9–14	14	11–20	2	1–3	5
Mushrooms							
Enoki mushroom	50	26–75	224	172–266	0	0–0	6
Mushroom	45	23–67	610	430–959	17	15–22	5
Shiitake mushroom	2	0–7	388	229–495	0	0–0	6
Shimeji mushroom	108	61–151	153	75–255	2	1–3	5
Seaweed							
Tangle	0	0–1	0	0–1	3	0–12	6
Wakame seaweed	5	0–16	3	0–7	0	0–1	6
Fish and shellfish							
Horse mackerel	53	3–146	21	10–37	62	33–97	5
Japanese needlefish	1	1–3	3	1–6	5	0–15	4
Mackerel	9	4–14	19	15–22	21	8–34	5
Octopus	12	8–17	25	23–28	157	130–	5
Salmon	21	16–30	26	18–34	45	32–69	5
Sardine	9	4–18	17	15–20	28	19–36	5
Sardine (the small fry, dried)	81	63–100	203	157–253	199	155–	5
Short-necked clam	27	20–33	104	73–137	208	162–	4
Shrimp	0	0–1	1	1–3	2	1–3	4
Squid	1	1–2	0	0–1	47	25–74	5
Tuna	3	2–3	14	4–22	82	36–129	5
Fish sausage	10	4–14	6	2–7	9	5–13	4
Meat							
Beef	10	4–15	16	8–28	154	132–	5
Chicken	8	5–11	45	31 -57	226	187–	4
Pork	3	1–6	8	5–12	129	96–143	5
Ham	6	2–23	10	5–26	50	10–155	7
Eggs							
Eggs (chicken)	3	0–7	1	0–2	1	0–2	4
Dairy products							
Cheese (processed)	0	0–0	30	17–46	7	2–13	4
Milk (cow)	0	0–1	0	0–0	0	0–0	5
Yoghurt	0	0–1	1	0–3	0	0–1	5
Oil							
Rape seed oil	0	0–0	0	0–0	0	0–0	5
Sesame oil	0	0–0	0	0–0	0	0–0	5
Confectionery							
Candy	0	0–1	0	0–0	0	0–0	4
Chocolate	4	0–10	17	15–17	7	4–11	5
Cookie	3	1–5	7	1–14	3	0–7	6
Potato chips	69	0–142	65	8–110	12	1–25	4
Rice cracker	18	4–49	3	0–7	3	1–4	5
Beverages							
Beer							
Sake	46	37–51	0	0–0	0	0–2	5

Table 2.1 Polyamine contents of foods (cont.)

Seasonings	2	0–3	0	0–0	0	0–0	7
Cocoa	3	0–7	3	0–7	1	0–2	5
Coffee	0	0–0	0	0–1	0	0–0	5
Tea	0	0–0	0	0–0	0	0–0	7

(Adapted from Nishibori, Fujihara and Akatuki, 2005)

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CHAPTER 3: ANTIOXIDANT AND ANTICANCER CAPACITY OF POLYAMINES

3.1 Introduction

Vegetables and fruits are considered to be healthy foods. Increasing consumption has been linked to inhibition of diseases such as inflammation, cardiovascular disease, cancer, and aging-related disorders (Willett, 2001). Dietary antioxidants are believed to be the active agents in fruits and vegetables to prevent these oxidative stress-related diseases, including polyphenols, vitamins E and C, carotenoids and others (Ames et al., 1995; Kaur & Kapoor, 2001). As defined by the Institute of Medicine, antioxidants have a broad scope in food science. "A substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans" can be called antioxidants (Institution of Medicine, 1998).

Unlike other antioxidants which are well accepted by people, polyamines, commonly known as spermidine and spermine, are seldom known by people for their high antioxidant capacity although they are widely present in all living cells. Løvaas (1991) studied the antioxidant effect of polyamines (spermine, spermidine, and putrescine) and concluded that the oxidation of polyunsaturated fatty acids, tocopherol, and carotenoid pigments can be efficiently inhibited by polyamines. Drolet et al. (1986) also confirmed that di- and polyamines are effective scavengers of free radicals both in chemical and *in vitro* enzyme systems.

In addition to antioxidant capacity, polyamines have many biological functions including macromolecular synthesis and interaction with negatively charged molecules, such as DNA, RNA, acidic proteins and phospholipids (Park & Igarashi, 2013). Polyamines have been regarded as a marker for cancer for a long time. Fu et al. (2012) considered putrescine (PUT), spermidine (SPD), and spermine (SPM) as promising tumor markers since their excretion has been detected in cancer

patients. Park & Igarashi (2013) indicated that tumor growth is associated with an increase in polyamines and polyamine synthetic enzymes, which further indicated that there was a correlation between polyamines and cancer. Soda (2011) pointed out that increased polyamine levels in the body may also accelerate tumor invasion and metastasis in humans.

Polyamines (Spermine, spermidine, and putrescine) have been reported as efficient antioxidants in several experimental biological systems. Nutritional analysis has demonstrated that polyamines have a correlation with cancer cells in both animals and human bodies; however, there is no comparison of the antioxidant capacity of different polyamines by different assays. The lack of study has also been found in cancer research, although hundreds of studies have concentrated on polyamine levels in cancer patients and normal people. However, few studies investigate the inhibitory effect of polyamines towards cancer cells. Therefore, the first aim of this chapter was to explore the antioxidant activity of six amines (spermine, spermidine, putrescine, agmatine, tyramine, and tryptamine), in comparison with other well-recognized antioxidants, by different antioxidant assays. The second aim was to investigate the inhibition effect of some amines (spermine, spermidine, and putrescine) on murine breast cancer 4T1 cell line by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The purpose was to gain some perspective on these widely suggested potential activities of polyamines.

3.2 Materials and Methods

3.2.1 Antioxidant capacity

3.2.1.1 DPPH (1,1-diphenyl-2-picryl-hydrazyl) Assay

Preparation

Amine standards (spermine, spermidine, putrescine, agmatine, tyramine, and tryptamine), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ascorbic acid and methanol were purchased from Sigma Chemical Company (St. Louis, MO, USA). Working solutions (10 mg/mL) of amines were prepared in 100% methanol. A stock solution of DPPH (3mM) was prepared in methanol, from which a working solution of DPPH was created (0.3mM). Ascorbic acid (2mM in methanol) was utilized as standard to compare the effect of polyamines. All these solutions were kept in the dark at 4°C.

Determination of antioxidant capacity

Antioxidant capacity was measured by the free radical scavenging activity using the DPPH method of Blois (1958) with slight modifications. Amines (200 µL) at 10 mg/mL were added to 1800 µL 0.3 mM DPPH solution 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 served as the blank. The antioxidant capacity was calculated by the following formula:

$$\% \text{ DPPH Inhibition} = \left(1 - \frac{Af}{Ai}\right) \times 100$$

Where *Af* is the final absorbance after 30 min incubation and *Ai* is the initial absorbance.

3.2.1.2 Oxygen Radical Absorbance Capacity (ORAC) Assay

This method was first developed by Cao et al. (1993), then further modified by Dávalos et al. (2004) and Parker et al. (2010). All assays were conducted on a Synergy H1 Hybrid Multi-Mode Reader (BioTek Instruments Inc., Winooski, VT) using Costar 96-well clear bottom, black-sided plates (Corning Inc., Corning, NY). Amine solutions were weighed and rinsed (1:10 w/v) into glass test tubes with 70:29.5:0.5 acetone: water: acetic acid (AWA). Samples were vortexed (Fisher Scientific, Bohemia, NY), sonicated at 37°C, shaken twice during sonication to suspend samples, held at room temperature, vortexed again and held again at room temperature. Tubes were then centrifuged at 1,250xg and the supernatant removed to a glass test tube. A second aliquot of AWA was added to the pellet and the extraction repeated. Combined supernatants were adjusted to a final volume of 20 mL. The final extract was dispensed into 1.5 ml Eppendorf™ tubes (Fisher Scientific, Fair Lawn, NJ) and stored at -80 ºC until analyzed.

Each well of the 96-well plate contained 120 µL 70.3 nM fluorescein (prepared in 75 mM phosphate buffer, final concentration), 20 µL AWA (blank), 0.4 mM Trolox (prepared in AWA, standard curve) or sample, and 60 µL 12 mM AAPH, added immediately prior to beginning measurement. The temperature was closely monitored and maintained at 37ºC throughout plate preparation and fluorometry. For each run, one row consisted of a blank well followed by a Trolox standard curve of 1, 2, 3, and 4 µM Trolox (final concentration), repeated in reverse order and a second blank. Subsequent rows contained a similar symmetrically matched blank, 1 µm Trolox (internal standard), and samples. Each sample was measured in duplicate across each row and values were averaged to account for inconsistencies across the plate. The measurement was taken at an emission wavelength of 515 nm and an excitation wavelength of 493 nm. Readings were taken every min for 80 min and the plate was shaken for 3 s at intensity 3 before each reading. Results were expressed in µmol Trolox equivalents/g (µmol TE/g) using the Trolox standard curve

run with each group of samples. Each row was also adjusted based on the ratio of internal standard to that of the standard curve.

3.2.2 Anti-cancer capacity

3.2.2.1 Cell culture testing

Polyamine standards (putrescine, spermidine, and spermine) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Cell growth media (Dulbecco's Modification of Eagle's Medium with 4.5g/L glucose, L-glutamine & sodium pyruvate) was purchased from Mediatech (Manassas, VA, USA) and added with 10% fetal bovine serum (Atlanta® Biologicals, Flowery Branch, GA, USA) and 5% Penicillin-Streptomycin (gibco® by Life Technologies, Grand Island, NY, USA). Stock solutions of polyamines (10 mg/mL) were prepared in cell culture media and kept at 4°C. Working solutions of spermine were diluted from stock solutions to 2.5, 5.0, 10.0, 20.0 and 40.0μg/mL using the same medium, and working solutions of spermidine and putrescine were diluted by cell culture medium to 5.0, 10.0, 20.0, 40.0 and 60.0 μg /mL. Murine breast cancer 4T1 cell line was provided by Dr. Helferich's Laboratory (University of Illinois at Urbana Champaign, Urbana, IL). The cells were maintained as monolayer cultures in a 75mL flask containing 20 mL cell culture medium as described above for future cell culture testing.

Polyamine treatment for murine breast cancer 4T1 cells

For determination of tumor cell growth inhibition by some amines (spermine, spermidine, and putrescine), exponentially growing cells were seeded into 96-well microtiter plates and cell number per well originally was approximately 5000 cells by using Countess™ Automated Cell Counter (Carlsbad, CA, USA). Cell growth media was changed once the cells adhered to the 96-well microtiter plates and entered exponential growth, which took approximately 24 h. Five

concentrations of polyamines were added to the cell cultures (twelve replicate wells per concentration), and cell growth medium was used as control (twelve replicate wells). After 24 h incubation at 37°C in 5% CO_2 in the CO_2 water-jacketed incubator (Forma Scientific, Inc., Marietta, OHIO, USA), the cells were ready for final cell density determination. Concentration effect curves were constructed for comparison of polyamine effects on murine breast cancer 4T1 cells. The operations described above were carried out in Nuaire Biological Safety Cabinets (Plymouth, MN, USA) under aseptic conditions.

Determination of tumor cell growth inhibition by polyamines

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (van Meerloo et al., 2011) was utilized to test the inhibition effect of polyamines on tumor cells. Concentration effect curves were constructed for comparison of polyamine effects on murine breast cancer 4T1 cells, and IC_{50} values for each polyamine were calculated from curves.

3.2.3 Statistical analysis

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). ANOVA (Analysis of Variance) was used to determine treatment effects of different concentrations of polyamines ($p<0.1$). LSD (Fisher's Least Significant Difference) test was conducted to further analyze the difference among treatments ($p < 0.1$) 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 ($p < 0.1$).

3.3 Results and Discussion

3.3.1 Antioxidant capacity of amines

Antioxidant capacities of six amines (spermine, spermidine, putrescine, agmatine, tyramine, and tryptamine) were presented by Ascorbic Acid Equivalent (mM/g Amine) using DPPH method (Figure 3.1, Table 3.1), with higher Ascorbic Acid Equivalent indicating higher antioxidant capacity. As demonstrated by DPPH assay, tyramine (13.50±0.55 mM Ascorbic Acid/g) had the highest antioxidant capacity, followed by spermine (11.75±0.62 mM Ascorbic Acid/g), putrescine (10.32±0.92 mM Ascorbic Acid/g), tryptamine (9.48±0.12 mM Ascorbic Acid/g) and spermidine (3.80±0.12 mM Ascorbic Acid/g). Agmatine showed no antioxidant capacity in DPPH method. The antioxidant capacities of six amines showed a significant difference from each other ($p<0.1$).

Compared to the DPPH assay, the data given by ORAC assay were significantly different among different amines, however, the ranking of the antioxidant capacity of six amines was still similar to the results indicated in DPPH assay (Figure 3.2, Table 3.2). Tyramine (96511.8±5753.7 μM TE/g) had the highest antioxidant capacity again, followed by tryptamine (66085.9±570.5 μM TE/g), spermine (139.5±6.2 μM TE/g), putrescine (27.4±2.3 μM TE/g), and spermidine (4.2±0.004 μM TE/g). Agmatine also showed no antioxidant capacity in ORAC assay.

Two assays (DPPH and ORAC) were used in antioxidant capacity testing to improve the accuracy of results. ORAC assay is said to be more relevant because it utilizes a biologically relevant radical source, however, the results given by ORAC is less reproducible compared to other antioxidant determination methods such as ABTS, DPPH and FRAP (Prior et al., 2003). Therefore, DPPH assay was used as a complementary method to see the reproducibility of the antioxidant capacity of six amines. According to the results, a comparable ranking of antioxidant capacity was given by these two methods except for tryptamine. Tryptamine showed a lower

capacity in DPPH assay but a much higher capacity in ORAC assay; while tyramine has the highest antioxidant capacity in both of the assays, and agmatine is the lowest one among six amines in DPPH and ORAC assay. Since ascorbic acid and Trolox were used as different equivalents in DPPH and ORAC respectively, the data represented antioxidant capacities of six amines can be different in these two assays.

This research represents the first report of a comparison of different polyamines for antioxidant capacity by means of ORAC and DPPH. Toro-Funes et al. (2012) studied the radical-scavenging activity of spermine and spermidine by DPPH method in methanol and ethyl acetate solvent. Spermine showed a higher antioxidant capacity than spermidine in both of the solvent, which was similar to the results from this research. It is also mentioned that the radical-scavenging activity of amines may change in different solvents, which can be a new area for further exploration. Drolet et al. (1986) indicated that radical scavenging properties of polyamines appeared to be related to the number of amine groups in the amines. Spermidine and spermine, which have three and four amino groups respectively, were more effective scavengers than the diamines such as putrescine and cadaverine. However, putrescine showed a higher antioxidant capacity than spermidine in this study, which is opposite to the theory given by Drolet et al. (1986). Polyamines may react differently with the superoxide radicals and the DPPH radicals we used in this study, which may cause the difference in their radical scavenging capacity. Further studies are needed to look into the radical scavenging capacity of polyamines to different types of radicals.

3.3.2 Anticancer capacity of amines

Anticancer capacities of three amines (spermine, spermidine, and putrescine) which were focused on in this research were analyzed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using murine breast cancer 4T1 cell line. Inhibition effect of

polyamines was determined by IC_{50} value which can be found on concentration effect curves according to the cell density changing (Figure 3.3-3.5, Table 3.3-3.5), with a higher IC_{50} value indicating lower anticancer capacity. As the results showed in the figures, both spermine and spermidine inhibited the growth of cancer cells as their concentrations increased from 0 to 0.06mg/mL, while putrescine solutions showed no effect on 4T1 cells. IC_{50} values of spermine and spermidine to murine breast cancer 4T1 cell line can be directly read from figures which are 6.0 μg/mL and 21.5 μg/mL, respectively, indicating that spermine has higher anticancer capacity to murine breast cancer 4T1 cell than putrescine. No anticarcinogenic effects were observed in this cell system with putrescine. The polyamine concentrations in human blood analyzed by Soda et al. (2009) was 5.3±0.4 μmol/L, which was lower than the IC_{50} values we found in this study. However, it was also indicated that long-term oral intake of enhanced polyamines could increase blood polyamine concentrations in both mice and humans, which might directly affect the function of immune cells. Compared to the antioxidant capacity, it seems that there is relevance between amine' antioxidant and anticancer capacity. Spermidine has significantly lower antioxidant capacity than spermine, and low anticancer potential than spermine. Further research needs to explore the correlation between antioxidant and anticancer capacity.

3.4 Conclusion

The antioxidant capacity of spermine, spermidine, putrescine, agmatine, tyramine, and tryptamine have been analyzed by DPPH and ORAC assays. As demonstrated by ORAC, tyramine (96511.8±5753.7 μM TE/g) had the highest antioxidant capacity, followed by tryptamine (66085.9±570.5 μM TE/g), spermine (139.5±6.2 μM TE/g), putrescine (27.4±2.3 μM TE/g), and spermidine (4.2±0.004 μM TE/g) while agmatine showed no antioxidant capacity in ORAC assay. DPPH results were similar to ORAC values. When tested for anti-carcinogenic activity, spermine

and spermidine inhibited the growth of 4T1 cells at IC_{50} of 6.0 µg/mL and 21.5 µg/mL respectively. No anti-carcinogenic effects were observed in this cell system with putrescine.

This represents the first report of comparison of different polyamines for antioxidant capacity by means of ORAC and DPPH. ORAC and DPPH are two antioxidant assays detected by different mechanisms, ORAC is based on hydrogen atom transfer while DPPH is based on electron transfer (Huang, Ou & Prior, 2005), which explained that tryptamine behaved differently in two assays. We have also generated data from one cell line indicating that the polyamines, but not putrescine, may prevent cell growth. Further studies for other cell lines are necessary for a better understanding of the role of polyamines in cancer. This knowledge might also be of great help for understanding the correlation between antioxidant and anticancer capacity of polyamines in the future.

3.5 Figures and Tables

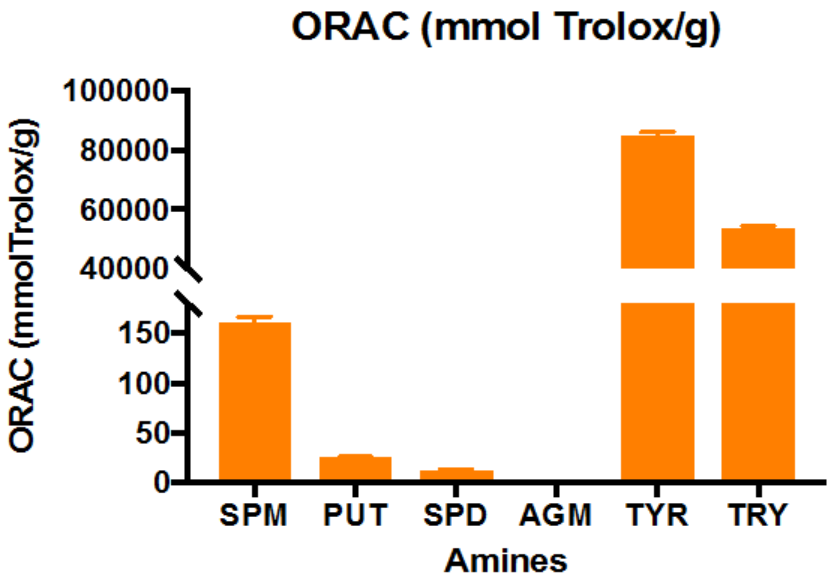

Figure 3.1 Antioxidant capacity of polyamines by ORAC assay

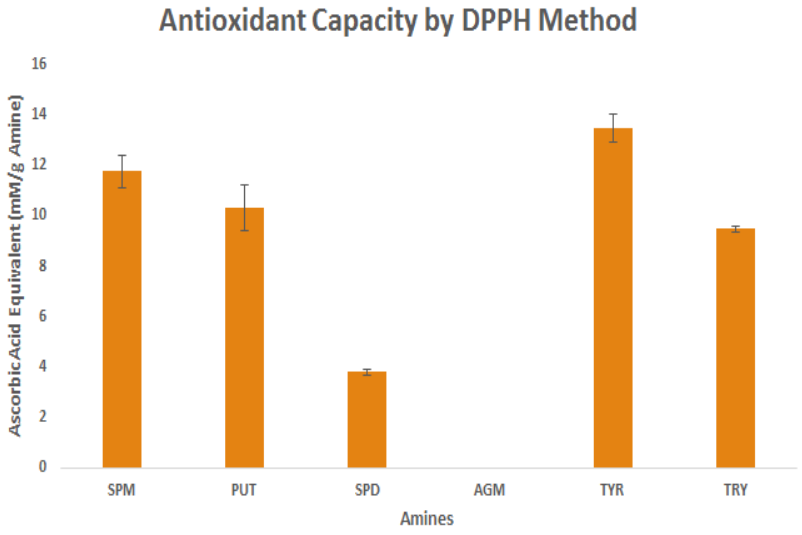

Figure 3.2 Antioxidant capacity of polyamines by DPPH assay

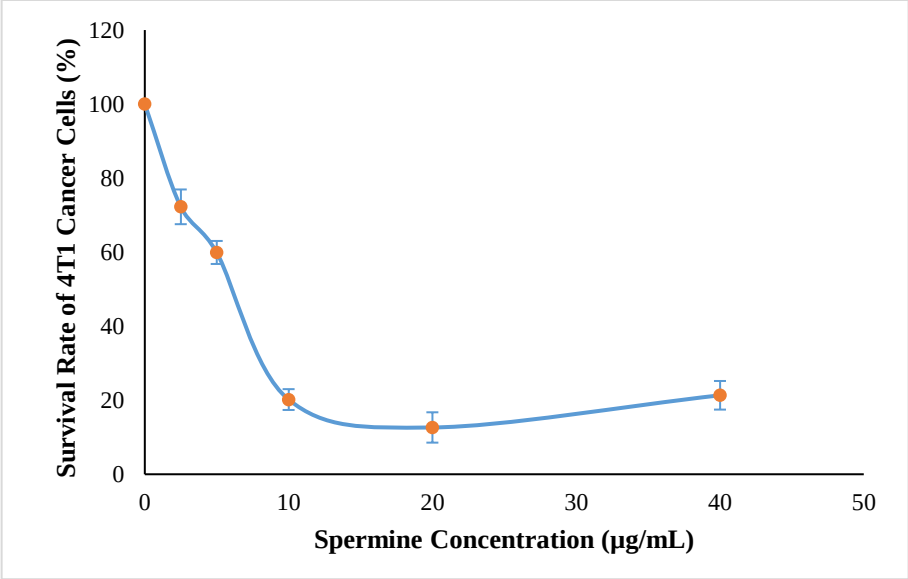

Figure 3.3 Anticancer capacity of spermine by MTT assay

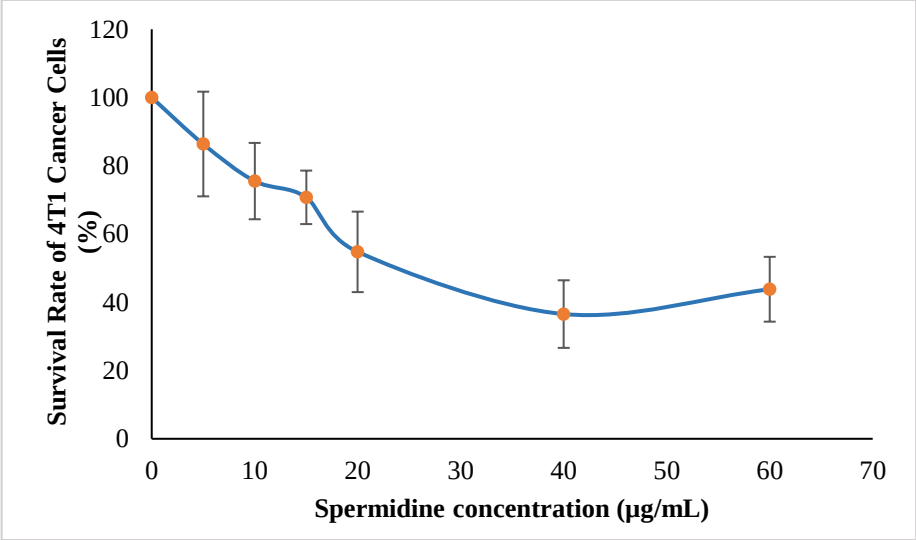

Figure 3.4 Anticancer capacity of spermidine by MTT assay

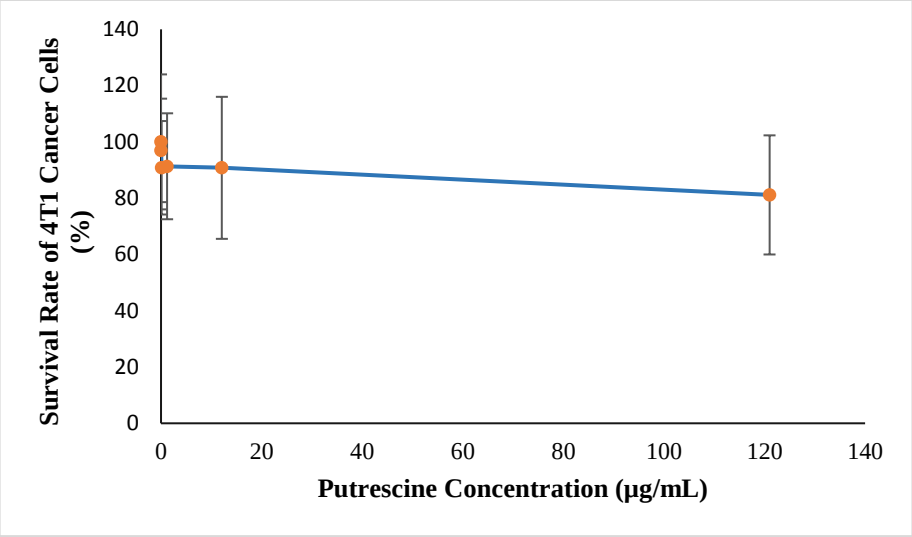

Figure 3.5 Anticancer capacity of putrescine by MTT assay

Table 3.1 Antioxidant capacity of polyamines by DPPH assay

Amine	DPPH (mmol Ascorbic Acid/g)
Agmatine	0^{a}
Spermine	11.8 ± 0.6^{b}
Spermidine	3.8 ± 0.1^{c}
Putrescine	10.3 ± 0.9^{d}
Tryptamine	9.5 ± 0.1^{e}
Tyramine	13.5 ± 0.5^{f}

a-f means within polyamines (column) results with different letters are different at α 0.1.

Table 3.2 Antioxidant capacity of polyamines by ORAC assay

Amine	ORAC(μmol Trolox/g)	ORAC(mmol Trolox/g)
Agmatine	0[a]	N/A
Spermine	139.5±6.23[b]	N/A
Spermidine	4.227±0.004[c]	N/A
Putrescine	27.40±2.283[d]	N/A
Tryptamine	66085.87±570.49[e]	66.09±0.66
Tyramine	96511.85±5753.68[f]	96.51±5.75

a-f means within polyamines (column) results with different letters are different at α 0.1.

Table 3.3 Inhibitory effect of spermine on 4T1 breast cancer cells

	Concentration of Spermine in Cell Culture Media					
Conc (μg/mL)	0	2.5	5.0	10.0	20.0	40.0
Survival Rate (%)	100	71.96±8.28	59.51±3.01	20.07±3.08	12.74±4.79	21.37±4.97

Table 3.4 Inhibitory effect of spermidine on 4T1 breast cancer cells

	Concentration of Spermidine in Cell Culture Media					
Conc (μg/mL)	0	5.0	10.0	20.0	40.0	60.0
Survival Rate (%)	100	86.38±15.34	75.51±11.18	54.77±11.79	36.54±9.90	43.82±9.50

Table 3.5 Inhibitory effect of putrescine on 4T1 breast cancer cells

	Concentration of Putrescine in Cell Culture Media					
Conc (μg/mL)	0	0.01	0.10	1.00	10.00	100.00
Survival Rate (%)	100	96.98±18.38	90.80±16.60	91.33±18.82	90.78±25.24	81.16±21.16

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CHAPTER 4: EFFECT OF HIGH-INTENSITY ULTRASOUND ON POLYAMINE CONCENTRATIONS IN ROMAINE LETTUCE

4.1 Introduction

Lettuce is one of the most popular vegetables consumed all over the world. It is most often grown as a leafy vegetable, but sometimes its stem and seeds are used for consumption. Lettuce is commonly used in salads, although it is also utilized in other kinds of food, such as soups, sandwiches, and wraps. Lettuce is generally categorized into head lettuce, Romaine lettuce, and leaf lettuce. Head lettuce was dominant in the market in the 1980s, whereas, head lettuce, Romaine, and leaf types had similar market share in 2012 (USDA, 2013).

Lettuce is a rich source of vitamin K, vitamin A, and a moderate source of folate and iron; thus, it is an often overlooked source of vitamins and minerals. In addition, polyamines (bioactive amines) are also widespread in lettuce. Almost all plant foods contain putrescine, spermine, and spermidine. Polyamines are considered a healthy nutritional supplement for the human diet. In addition to the potential antioxidant and anticancer capacity of polyamines (Chapter 2), many important processes of plant growth, e.g., embryogenesis, cell division, morphogenesis, and development or stress tolerance, are associated with polyamines (Gill & Tuteja, 2010). Application of environmental stress during plant growth, i.e., exposure to salt, cold, drought, and ultraviolet light, can induce production of secondary metabolites, including polyamines (Boyer, 1982).

Similar to other leafy vegetables, lettuce is perishable after harvest, particularly when the leaves are broken. Contaminated lettuce is often a source of bacterial, viral and parasitic outbreaks in humans, including E. coli and Salmonella (Yu et al., 2016). Cold storage and synthetic chemical fungicides (such as imazalil (IMZ), thiabendazole (TBZ), sodium ortho-phenil phenate (SOPP)) have been used for a long time to maintain the quality and stability of harvested fruits and

vegetables (Palou et al., 2015). Some innovative physical methods such as heat treatment, ultraviolet light (UV-C), and ionizing radiation have also been applied in postharvest control (Stevens et al., 1997; Lurie, 1998). These newly developed processes are part of an attempt to discover non-chemical pretreatment in fruits and vegetables to control quality during storage. Recent technological advances include the application of ultrasound in the postharvest preservation of fruits and vegetables due to its shorter treatment time, enhanced product quality and reduced chemical hazard (Xu et al., 2013).

Ultrasound technology is the application of sound waves with frequencies higher than the upper audible limit of human hearing, which is known to cause chemical and physical changes in biological structures (in a liquid medium). Cavitation bubbles can be rapidly formed and collapsed during sonication (Elisabete et al., 2012). Extreme heat (several thousand Kelvin degrees), high pressure (70-100 MPa), and shear forces are generated when the bubbles collapse, and at the same time, free radicals from water are formed (Ashokkumar et al., 2008). When ultrasound is applied to fruits or vegetables, the temperature increases and high pressure is released directly towards their surface, upon collapse of the bubbles. The energy released from ultrasound is capable of destroying cells, ejecting particles from the solid surface and increasing mass transfer to the surface by disruption of the interfacial boundary layer (Zhou, 2011). Ultrasound treatment (40 kHz) was applied to freshly harvested strawberry; decay and microorganisms were significantly decreased, while the color and firmness of strawberries were steadily maintained by ultrasonic treatment (Cao et al., 2009). The effect of ultrasound was also demonstrated in litchi fruit postharvest. The activities of oxidase and peroxidase were inhibited by 120 W ultrasound treatment for 10 minutes; this significantly delayed the pericarp browning of litchi fruits (Chen et al., 2011). Ultrasound treatment has been shown to control enzymatic browning, tissue softening, and microbial growth

during processing, which is considered to be a new trend in the pretreatment of postharvest fruits and vegetables (Xu et al., 2013).

The hypothesis of this study was that ultrasound can be utilized as a physical elicitor to induce polyamine synthesis in plants, and ultrasound can also be applied as a valid pretreatment to preserve the quality of lettuce during storage. The objectives of this chapter are: (1) Study the effect of ultrasound in secondary metabolite synthesis (polyamines) of lettuce; (2) Study the effect of ultrasound in quality retention of lettuce during postharvest storage.

4.2 Materials and Methods

4.2.1 Sample preparation

Romaine Lettuce (*Lactuca sativa*) was purchased from a local supermarket and immediately utilized for ultrasound treatment. Color, size and visual quality of lettuce were pre-selected, and the leaves with visible damage were discarded before processing to ensure initial consistency of samples. Lettuce samples were then randomly divided into an ultrasound treatment group or control group, with at least 3 replications.

4.2.2 Ultrasound treatment

A specially designed ultrasound system which contains an ultrasound generator and a stainless steel water tank (400 mm*660 mm*460 mm) was utilized as ultrasound treatment chamber in this study (Figure 4.1). A pair of transducer boxes (400mm*400mm*50mm) with working frequency of 25 kHz and 2 kW nominal power were vertically attached to the inner walls of the tank, face to face, with a space of 280 mm between the boxes. Uniform sound intensity was

distributed by the transducer boxes to ensure that samples in the tank of different locations received the same ultrasonic energy.

The ultrasound treatment chamber was filled with 115 L room temperature water before each treatment. Lettuce samples were properly fixed in the holder which was submerged in the water tank parallel to the transducer boxes. Ultrasound was applied at 25 kHz frequency and 100% power output through water medium for 5 min in sonicated treatment, while control samples were fixed in the holder and submerged in water for 5 min. Whole leaf Romaine lettuce was used for all analyses to eliminate the influence of wounding by cutting (Reyes & Cisneros-Zevallos, 2007).

Four treatments were applied to four groups of an equivalent amount of lettuce. Sonicated A group was treated with 5 min sonication, and Control A group was immersed in the degassed water for 5 min. Polyamine levels in Sonicated A and Control A groups were analyzed immediately. Sonicated B group was treated with 5 min sonication, and Control B group was immersed in the degassed water for 5 min. The polyamine levels in Sonicated B and Control B groups were analyzed after 72 h recovery at room temperature. After treatment, both control and treated samples were air-dried for 60 min. Control A group and Sonicated A group were then placed into plastic gallon zipper storage bags (Ziploc, Racine, WI) for freeze-drying. Control B group and Sonicated B group were sealed in bags (Ziploc, Racine, WI), covered with 3-4 layers of brown paper towels to prevent light exposure, and stored at room temperature up to 72 h before freeze drying. Each treatment was applied to at least 3 replicates.

4.2.3 Freeze drying

Before freeze drying, lettuce samples were frozen at -20ºC for 24 h. Plastic bags which stored freezing lettuce samples were then placed into specialized glass containers for freeze drying.

After sealing with special rubber lids, freeze drying was conducted for 24 h in the freeze dryer. The moisture content of lettuce after free (check line spaving)ze drying process is 10%.

4.2.4. 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 (Yu et al., 2016).

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:

$P_{diss} = mc_p\,(dT/dt)$

Where m is the mass of the liquid (kg), c_p is the specific heat capacity of the liquid (J/ (kg* K)), and (dT/dt) 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 min. The temperature of the water medium was recorded every second by a thermometer. The specific heat capacity of water (4181.3 J/ (kg K)) was used in the calculation. The acoustic power density (APD) of the system mentioned above was calculated as 26 W/L.

4.2.5 Determination of total polyamines in lettuce

Polyamine levels in lettuce were analyzed by gas chromatography with flame ionization detection (GC-FID) to see their responses to different sonication treatments.

4.2.5.1 Preparation of standard solutions

Amine standards (putrescine, spermidine, and spermine), diethyl ether, ethyl chloroformate (ECF) and pentafluoropropionyl anhydride (PFPA) were purchased from Sigma Chemical Company (St. Louis, MO, USA). 1, 6-diaminohexane (internal standard) was obtained from Merck KGaA (Germany). Other chemicals were of guaranteed reagent grade. Stock solutions of polyamines were prepared (0.1mg/mL) in 0.1 M HCl and stored at 4˚C. Working solutions were prepared to 10 µg/mL. The internal standard (I.S.) working solution was prepared by diluting a 1, 6-diaminohexane stock solution (1.0 µg/mL) in 0.1 M HCl.

4.2.5.2 N-ethoxycarbonylation and N-pentafluoropropionylation

Prior to derivatization, 1 mL polyamine working solution (10 µg/mL) was combined with 20 µL internal standard (1 ppm). One mL of this solution was combined with 1.0 mL of diethyl ether containing 50 µL ECF and pH was adjusted to 11-12 with 2 M NaOH (0.9 mL) for the reaction of N-ethoxycarbonylation (EOC). After shaking (20 min), samples were centrifuged 5min at 3019 x g. The ether layer was separated by placing in a dry ice-acetone bath. The rest of the solution was re-extracted with diethyl ether (2mL) and the combined ether extracts were evaporated to dryness under nitrogen. Pentafluoropropionyl (PFP) derivatization was performed by adding PFPA (20 µL) and ethyl acetate (100µL) to the dried N-EOC residues. The combination was incubated at 50˚C for 30 min and dried under nitrogen. The N-EOC-N-PFP residue was stored in a vacuum desiccator over KOH for 30 min before instrumental analysis. Ethyl acetate (40 µL) was added to the dried residue for GC analysis.

4.2.5.3 Sample preparation

HCl (10 mL, 1 M) was added into 0.35 g dried lettuce powder with 50 µL I.S. (20 mg/L). The mixture was incubated in Brinkmann Incubator (Brinkmann Instruments Inc, Riverview, FL, USA) at 40ºC with agitation in Brinkmann OrbiMix (Brinkmann Instruments Inc, Riverview, FL, USA) at speed 6 for 2 h; samples were kept at 40˚C for 16 h and washed with another 5 mL double deionized water. The HCl and water extraction layers were combined, and 2M NaOH solution (~1.6 mL) was used to adjust the pH to 11-12. Then the polyamine extraction was applied with the n-EOC reaction, and followed by the PFP reaction as described above.

4.2.5.4 Instrumental Conditions

Gas Chromatography was performed with a Hewlett-Packard HP Model 5890A gas chromatograph (Hewlett-Packard, Avondale, PA, USA) equipped with a FID detector. Samples were injected into a DB-5 (SE-54 bonded phase) fused-silica capillary column (J&W Scientific, Folsom, CA, USA; dimensions: 30 m length x 0.32 mm I.D., 1 µm film thickness) in split mode (6:1) at 260˚C. Initial oven temperature was 140˚C and raised to 210˚C at 8˚C /min, held for 2 min prior to reaching a final temperature of 310˚C at 20˚C /min (for 15 min). Helium, as carrier gas, was set to a column head pressure of 13 psi (column flow: 1.0 ml/min at 140˚C).

4.2.6 Statistical analysis

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). ANOVA (Analysis of Variance) was used to determine treatment effects of different concentrations of polyamines ($p<0.1$). LSD (Fisher's Least Significant Difference) test was conducted to further analyze the difference among treatments (p

< 0.1) 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 ($p < 0.1$).

4.3 Results and Discussion

Sonicated lettuce had 0.1115±0.0196 µg putrescine/g fresh leaves and 0.1820±0.0458 µg spermidine/g fresh leaves (Table 4.1); Unsonicated lettuce had polyamine levels with 0.1324±0.0380 µg putrescine/g fresh leaves and 0.2650±0.0723 µg spermidine/g fresh leaves. Spermine was not detected in both sonicated and unsonicated lettuce without three day recovery. Both putrescine and spermidine levels were not significantly different from each other in the sonicated group and the unsonicated group, which indicated that sonication did not affect polyamine levels in lettuce immediately, and the generation and accumulation of polyamines in response to sonication occurred over time.

Allowing for three day recovery (Sonicated B and Control B groups), sonicated lettuce had 0.0943±0.0364 µg putrescine/g fresh leaves and 0.3551±0.2943µg spermidine/g fresh leaves; Unsonicated lettuce had higher polyamine levels with 0.1429±0.0213µg putrescine/g fresh leaves. Spermidine concentration is 0.6198±0. 4386µg /g fresh leaves with no significant difference. Spermine is not detectable in both sonicated and unsonicated lettuce with three day recovery. The putrescine levels in the sonicated group were significant lower the unsonicated group ($p<0.1$), which indicated that sonication did not induce polyamine accumulation in lettuce. On the contrary, sonication inhibited the generation and accumulation of putrescine in lettuce during storage, while the levels of spermidine remain the same in both sonicated and unsonicated lettuce.

Previous research data from HPLC analysis (Cipolla *et al.*, 2007) indicated that lettuce had 7.9mg putrescine/kg fresh leaves and 13.9mg spermidine/kg fresh leaves, which is dramatically

higher that the values obtained for this research. Two reasons may cause the difference in polyamine levels analysis. First, extraction processing may cause this difference. In the HPLC method, the fresh lettuce was mashed and extracted with acid. In this research, freeze dried lettuce powder was extracted with acid. Acid extraction rates can be higher in liquid-liquid extractions than in solid-liquid extractions (Takhar, 2015 personal communication), which may subsequently cause the difference in results. Secondly, GC analysis has an additional extraction step that may account for a loss in polyamines.

Groups C and D reflected the greatest impact of sonication on polyamines (Table 4.1, Figure 4.2). After three days storage at room temperature (Group A and B, 26℃, no light exposure), the appearance of sonicated lettuce remain the same as fresh lettuce, while the outside layers of unsonicated lettuce were mainly decayed (Figure 4.3). Although the decayed leaves were discarded before freeze-drying to ensure all the samples used in the research were intact, we can still see from the data that putrescine levels in the unsonicated lettuce were significantly higher than in the sonicated lettuce.

Compared to sonicated lettuce with three-day recovery (Group C), increased putrescine levels and decayed out layer in unsonicated lettuce (Group D) indicated that sonication can maintain the freshness of plant leaves and prevent plants from further decay during post-harvest transportation and storage. Killing microbes on the surface of lettuce leaves by ultrasonication can be one of the possible explanations that cause this difference. It has been demonstrated that ultrasonication enhances food microbial safety by directly removing or destroying spoilage and pathogenic microorganisms through cavitation (Joyce et al., 2003). Putrescine, can be produced either by microbes or by plant tissues. If produced mainly by microorganisms, it would be prominent in decayed plant and animal tissues. Reducing the microbial load after sonication could

preserve the quality of plants and extend the storage time. The other hypothesis to explain these results can be that sonication induces 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). Other research also proposed that sonication can reduce the respiration rate during storage, which might be another reason for the nutritional quality retention. Appropriate application of ultrasonication to fruits and vegetables can improve overall postharvest quality of fresh produce in all microbiological, physical and nutritional aspects (Xu et al., 2013), which has been widely used in a variety of areas in food processing.

4.4 Conclusion

Ultrasound was utilized as a physical elicitor to induce polyamine levels in Romaine lettuce. Different ultrasound treatments resulted in different polyamine responses in lettuce. The polyamine levels in lettuce were not affected by ultrasound immediately after treatment, however, after 72 hours storage under room temperature in the dark, the sonicated group showed a significant lower putrescine level than in the control group; while the spermidine level remains no difference in two groups. The reduction of putrescine level observed in the sonicated group after 72-hour-recovery might be due to the two hypotheses. One is the consumption of polyamines as antioxidants to eliminate ultrasound-generated reactive oxygen species (ROS), however, this hypothesis is hard to explain the stable level of spermidine. The other one can be that ultrasound treatment reduced the microbial population and inactived deterioration-related enzymes, which inhibit the synthesis of putrescine in the lettuce, as putrescine is always considered as the signal chemical in decayed animals and plants.

4.5 Figures and Tables

Ultrasound water tank with transducer boxes

Ultrasound generator

Figure 4.1 Ultrasound treatment equipment

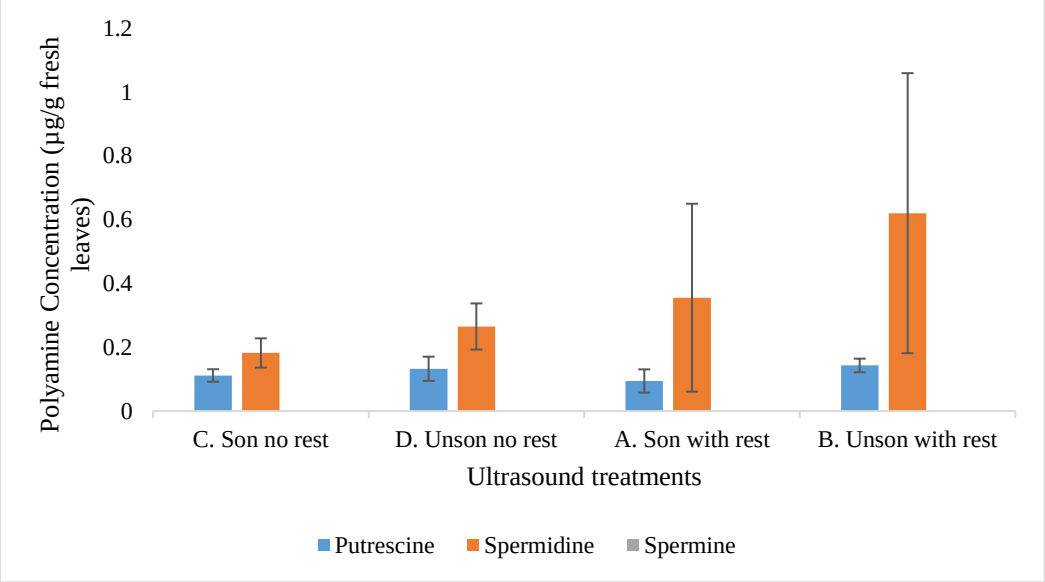

Figure 4.2 Effect of ultrasound on polyamine levels in Romaine lettuce at 25 KHz

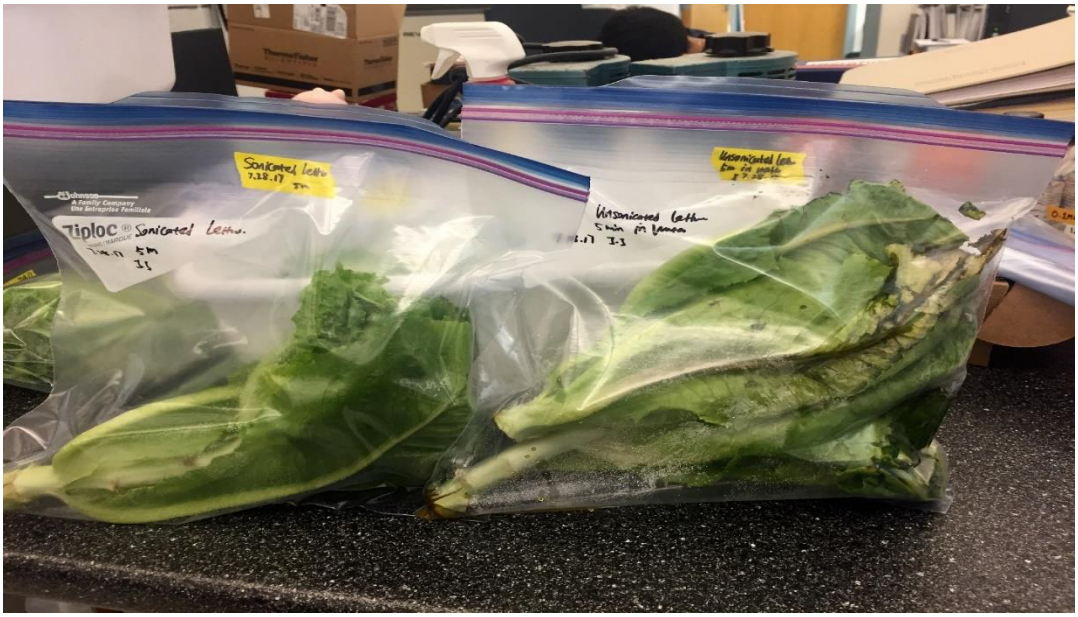

Figure 4.3 Impact of the sonication on lettuce (polyamines) after three-day storage

Table 4.1 Effect of ultrasound on polyamine levels in Romaine lettuce at 25 KHz

	Treatment			
Polyamines	Sonication no rest (C)	Unsonication no rest (D)	Sonication with rest (A)	Unsonication with rest (B)
Putrescine (µg/g fresh leaves)	0.1115±0.0196^{ab}	0.1324±0.0380^{ab}	0.0943±0.0364^{b}	0.1429±0.0213^{a}
Spermidine (µg/g fresh leaves)	0.1820±0.0458^{b}	0.2650±0.0723^{ab}	0.3551±0.2943^{ab}	0.6198±0.4386^{a}
Spermine (µg/g fresh leaves)	N/A	N/A	N/A	N/A

a, b mean within treatment (row) results with different lettuce are different at 0.1.

4.6 References

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CHAPTER 5: FUTURE WORK

This study represents the first comparison of different polyamines for their antioxidant and anticancer capacity, although the review articles comment about these properties without documentation. ORAC and DPPH assays were utilized to compare the antioxidant capacity of polyamines (spermine, spermidine, putrescine, agmatine, tyramine, and tryptamine), while the anticancer capacity of three polyamines (spermine, spermidine and putrescine) against 4T1 cells was first represented through MTT method. The polyamine levels in fresh Romaine lettuce in response to ultrasound was also investigated in this research.

As demonstrated by DPPH assay, tyramine had the highest antioxidant capacity, followed by spermine, putrescine, tryptamine, and spermidine. The ranking of the antioxidant capacity of six amines was similar in both antioxidant assays. Agmatine showed no antioxidant capacity by either assay. Response to abiotic stress revealed that concentrations of polyamines were not affected by ultrasound treatment immediately, while after a three-day recovery period, polyamine concentrations were different between sonicated and unsonicated lettuce. Ultrasound treatment also showed potential to maintain lettuce quality postharvest. This is exciting – as a first demonstration of such a significant improvement in quality with little treatment.

For future antioxidant capacity studies it may be important to include additional assays such as ABTS, and Ferric Reducing Antioxidant Power (FRAP), included to demonstrate potential differences by multiple assays.

It is crucial that more cancer cell lines be tested to determine the anticancer potential of polyamines in different cancer cells. *In vivo* studies with mouse models can also be utilized as a complementary study to further investigate the effect of polyamines on cancer; this study has only scratched the surface.

Hydroponic lettuce will be utilized in future abiotic stress studies due to the ability to tightly control the growing conditions and to analyze the effect of ultrasound as a stress in living lettuce (preharvest). Microbial concentrations in sonicated and unsonicated lettuce after three-day recovery can also be tested to strengthen the quality maintaining effect of ultrasound treatment. The decayed tissues which is expected to find in the unsonicated lettuce after three-day recovery will also be included in the microbial concentrations testing materials.