EFFECT OF TRANS-RESVERATROL ON SHELF-LIFE AND BIOACTIVE

COMPOUNDS IN SATSUMA MANDARIN

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EFFECT OF TRANS-RESVERATROL ON SHELF-LIFE AND BIOACTIVE COMPOUNDS IN SATSUMA MANDARIN

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EFFECT OF TRANS-RESVERATROL ON SHELF-LIFE AND BIOACTIVE

COMPOUNDS IN SATSUMA MANDARIN

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THESIS ABSTRACT

EFFECT OF TRANS-RESVERATROL ON SHELF-LIFE AND BIOACTIVE COMPOUNDS IN SATSUMA MANDARIN

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Satsuma mandarin (*Citrus unshiu* Marc.) is an important citrus commodity grown in the Gulf Coast area of Alabama. Mandarins are regarded as a valued part of a nutritious diet and health benefits are attributed to vitamin C, folate, dietary fiber, minerals as well as many phytochemicals including flavonoids, carotenoids and phenolic acids. However, mandarin fruit are highly perishable with a potential storage life of 2-4 weeks. Currently, little is known about the optimal postharvest practices for these fruit and there is an extensive need to develop advanced postharvest treatments to maintain high quality fruit during the storage and marketing period. Despite numerous advances achieved in postharvest biotechnology in ameliorating commodity losses associated with

postharvest decay, much of the nutritional value as well as the sensory quality of mandarin fruit can be lost as a result of current postharvest handling practices, which were developed primarily to maintain acceptable visual appearance with less regard to other quality aspects such as flavor and nutritional value. Although synthetic fungicides are effective in suppressing postharvest pathogen decay, public concerns regarding fungicide residue and development of pathogen resistance have stimulated the need for reduction of postharvest chemical use and for developing alternative non-chemical technologies. This led to the identification of *trans*-resveratrol (3,4',5-trihydroxystilbene), which is responsible for resistance of grapevines to fungal diseases, as a possible candidate in this regard.

In order to identify the most efficient means of providing nutritious and visually appealing fruit, a postharvest study was conducted to determine optimal dosage of *trans*-resveratrol ($1.6x10^{-3}$M, $1.6x10^{-4}$M and $1.6x10^{-5}$M) and to compare its efficacy with a mixture of wax and Imazalil or tap water. Studies were conducted on external color attributes, total phenolics, total flavonoids, vitamin C, total carotenoids and free radical scavenging properties. Results from this study indicated that resveratrol treatment not only improved shelf life of fruit but also enhanced nutritional quality. Resveratrol ($1.6x10^{-5}$M) had a significant effect on the total phenolic, ascorbic acid and total carotenoid content of fruit. A high correlation was also found between vitamin C content and antioxidant capacity determined by ABTS. Therefore, use of resveratrol as a natural fungicide offers a new simple and safe method to improve the shelf life and postharvest quality of mandarin fruit.

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I. INTRODUCTION

Due to changing lifestyles, the typical Western diet is low in phyto-antioxidants and high in saturated fats, increasing the risk of cardiovascular disease and cancer. Consumption of natural dietary antioxidants therefore may be important in inhibiting excessive free radical production associated with these pathological diseases. Satsuma mandarin is an important citrus commodity currently grown in the Gulf Coast area of Alabama. One of the major problems during storage is the postharvest fruit losses due to pathogen attack and natural senescence. Increased public awareness regarding the use of toxic fungicides has stimulated the need for reducing post harvest chemical use and for developing alternative non-chemical technologies such as the use of *trans*-resveratrol. This project evaluated and compared the stability and availability of antioxidants in Satsuma mandarin fruit treated with *trans*-resveratrol.

The specific objectives were:

1. To determine *trans*-resveratrol dosage for optimizing storage life and nutritional qualities of Satsuma mandarin during cold storage.

2. To investigate the potential effects of postharvest treatments on the antioxidant availability of mandarin fruit.

II. LITERATURE REVIEW

ROLE OF ANTIOXIDANTS IN HEALTH

Current epidemiologic studies suggest that increased consumption of fruits and vegetables rich in natural antioxidants increases the antioxidant capacity of the plasma and reduces the risk of chronic diseases related to aging such as cardiovascular disease (CVD), coronary heart disease (CHD), stroke, cancer, and neurodegenerative disease (Kris-Etherton et al., 2002). An antioxidant is defined as 'any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate' (Halliwell et al., 1995). Antioxidants may decrease the availability of oxygen, suppress the formation of free radicals and quench the existing radicals in biological systems to deter oxidative damage of DNA, proteins and lipids (Block et al., 1992). Large portions of the human diet are based on fruit and vegetables, as antioxidants cannot be generated by the human body and therefore have to be consumed in the diet. Numerous studies have been performed to elucidate their roles in health promotion and disease prevention.

Cardiovascular disease

Atherosclerosis is a leading cause of mortality in the United States and worldwide (Stocker et al., 2004). The term "cardiovascular disease" refers to any

disease that affects the cardiovascular system but it usually refers to those related to atherosclerosis. Atherosclerosis results from the accumulation of cholesterol and the proliferation of cellular elements like endothelial cells, smooth muscle cells, platelets, and leucocytes in the arterial wall (Tribble et al., 1994). It has been shown that oxidation accelerates the ability of low density lipoproteins (LDLs) to promote cellular alterations that lead to atherosclerosis including endothelial damage, uptake of LDL by foam cells, monocyte/macrophage recruitment, alteration in vascular tone, induction of growth factors, and production of antibodies (Gaziano et al., 1992). When the lumen of the vessel gradually narrows, it impedes blood flow and a stroke or myocardial infarction occurs. Atherosclerosis is marked by arterial lesions known as plaques that have been characterized into six major types of lesions that manifest the early, developing, and mature stages of the disease (Stocker et al., 2004). *In-vitro* experiments demonstrated that oxidized LDL causes endothelial cells to signal monocytes into the arterial wall (Willcox et al., 2004). The monocytes are then transformed into macrophages that engulf the oxidatively modified LDL and accumulate lipid to form nodular areas of lipid deposition that are also known as "fatty streaks" (Stocker et al., 2004), and thereby transform into lipid laden foam cells (Willcox et al., 2004). Such lesions then mature and are prone to rupture and, as a result, can trigger heart attack and stroke.

Dietary antioxidants like vitamin E (comprising tocopherols and tocotrienols, of which α-tocopherol is the predominant and most active form), β-carotene and vitamin C (ascorbic acid), may be involved in the prevention of CVD through different mechanisms. Epidemiologic studies have revealed an inverse association between consumption of fresh fruits and vegetables rich in dietary antioxidants and risk for coronary heart disease

(Joshipura et al., 2001). Vitamin E can inhibit oxidation of LDL cholesterol in plasma (Steinberg et al., 1989). In addition, Vitamin E supplementation was shown to improve endothelium-dependent vasodilation and decreased plasma TBARS concentrations (Kugiyama et al., 1999). On the other hand, β-carotene may inhibit endothelial damage by decreasing LDL cholesterol uptake into cells (Keaney et al., 1993). Vitamin C present in fruits and vegetables may have a favorable effect on vascular dilation as it may increase endothelial nitric oxide (NO) by protecting it from oxidation and increasing its synthesis (Padayatty et al., 2003).

Cancer

Cancer is a disease characterized by disorderly division of cells, combined with malignant behavior of these cells. Biological oxidative damage to DNA, lipids and proteins in the human body is generally considered to be one of the significant factors in carcinogenesis (Block et al., 1992). Oncogenic stimulation may be related to the redox imbalance found in cancer cells, induced by oxidative stress. Reactive oxygen species (ROS) play a role in all steps of cancer development; consequently, dietary antioxidants may have potential benefits at different stages of carcinogenesis (Cozzi et al., 1997). ROS-induced DNA damage involves single or double stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications and DNA cross-links (Valkoa et al., 2007). DNA damage can result in either arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, all of which are associated with carcinogenesis (Valkoa et al., 2007). Occupational exposure to asbestos increases risk to asbestosis-the second most important cause of lung cancer (Valko et al.,

2005). Asbestos is composed of iron by as much as 30% by weight. Iron-induced oxidative stress is considered to be a principal determinant of human colorectal cancer (Valko et al., 2001). It has been reported that cadmium might also be associated in the pathogenesis of human pancreatic cancer and renal carcinoma as it can cause activation of cellular protein kinases, which result in enhanced phosphorylation of transcription factors and subsequently lead to the transcriptional activation of target gene expression (Valko et al., 2005). Epidemiological studies have demonstrated a positive correlation between cancer-protective effects and high intakes of vegetables, fruits and whole grains (Cozzi et al., 1997).

Neurological disorders

The central nervous system (CNS) has high concentrations of polyunsaturated fatty acids (PUFAs) in cell membranes and a high level of transition metals that make it particularly vulnerable to attack by ROS (Zatta et al., 2002). The neurodegenerative diseases include Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis (ALS) and Huntington's disease. Even though genetic defects are assumed to be the main reason for these diseases, evidence reveals that oxidative stress including increased brain iron content, decline of superoxide dismutase (SOD) and glutathione (GSH), oxidative damage to lipids, proteins and DNA are important causes (Knight, 1997). The brain of patients with Alzheimer's disease (AD) show extensive oxidative damage associated with accumulation of amyloid-β peptide (Aβ), the main constituent of senile plaques in brain, as well as deposition of neurofibrillary tangles and neurophil threads (Butterfield et al., 2002).

Several studies investigated the effect of oxidative stress in the cascade of events leading to dopamine cell degeneration in Parkinson's disease (PD) (Sayre et al., 2001). PD involves a selective loss of neurons in an area of the midbrain called the substantia nigra (Tretter et al., 2004). The cells of the substantia nigra use dopamine (a neurotransmitter-chemical messenger between brain and nerve cells) to communicate with the cells in another region of the brain called the stratium. Consequently, a reduction in nigral dopamine levels causes a decline in stratial dopamine that is believed to cause PD symptoms (Jenner, 2003).

Several clinical studies assessed the role of antioxidants in delaying the onset or treatment of these diseases and results from these studies have revealed that dietary antioxidants may offer health benefits for neurodegenerative diseases.

PHYSIOLOGY OF REACTIVE OXYGEN SPECIES

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell et al., 1995). According to Gershman's free radical theory of oxygen toxicity, the toxicity of oxygen is due to partially reduced forms of oxygen (Gerschman et al., 1954). Oxygen free radicals or ROS, as well as reactive nitrogen species (RNS), are generated during the process of cellular physiological imbalance. They are unstable molecules that react quickly with adjacent molecules via a variety of reactions including: hydrogen abstraction (capturing), electron donation and electron sharing (McCord, 2000). They play a dual role as both harmful and beneficial species to living systems (Valko et al., 2006). Beneficial effects of ROS occur at low to moderate concentrations and are involved in a number of cellular

signaling systems and in the induction of a mitogenic response (Valkoa et al., 2007). The harmful effect of free radicals on biological systems is termed oxidative stress and occurs when there is an overproduction of ROS on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other (Kovacic et al., 2001; Valko et al., 2001). The excess ROS can destroy cellular lipids, proteins or DNA hindering their normal function.

ROS maybe produced endogenously or from exogenous sources. Significant sources of endogenous free radicals consist of prooxidative enzyme systems (e.g. lipoxygenase), drugs and their metabolites, pollutants, and other chemicals and toxins (Halliwell, 1996; Spiteller, 2001). While some of these are directly toxic, many others generate free radical fluxes via the very metabolic processes that the body uses to detoxify them. External sources such as sunlight and other forms of radiation can generate endogenous ROS, which can cause a number of diseases (Halliwell, 1996; Stief, 2003). ROS can also be formed in food through lipid oxidation and exposure to light (Fang et al., 2002).

Classification of ROS

Mitochondria are also the main source of ROS and free radicals in a cell. Approximately 1-5 % of the oxygen consumed by mitochondria is reduced and converted to these reactive oxygen species (Ames et al., 1993; Halliwell, 1991). Examples of possible ROS are illustrated in Table 1. ROS can be classified into oxygen-centered radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$) and oxygen-centered non-radical derivatives such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Other common reactive species are nitrogen

species such as nitric oxide ($NO^{\bullet}$), nitric dioxide ($NO_2^{\bullet}$), and peroxynitrite ($OONO^-$) (Rohrdanz et al., 2001).

Superoxide anion ($O_2^{\cdot-}$)

Superoxide radicals ($O_2^{\cdot-}$) are derived mostly within the mitochondria of a cell. At some stage in energy transduction, a small number of electrons "leak" to oxygen prematurely, forming the oxygen free radical superoxide, which has been associated in the pathophysiology of a variety of diseases (Kovacic et al., 2005). Measurements on submitochondrial particles suggest an upper limit of 1–3% of all electrons in the transport chain "leaking" to generate superoxide instead of contributing to the reduction of oxygen to water (Valkoa et al., 2007). When a singlet electron is added to one of the antibonding π orbitals, the parallel spin is destroyed leaving one unpaired and one coupled π orbital. A superoxide radical is formed when triplet oxygen, the most stable and abundant form of oxygen, carries one unpaired electron at its π orbital (Kao, 2006). Superoxide anion reacts with protons in water to form hydrogen peroxide (equations 1-3), which can serve as a substrate for the generation of hydroxyl radicals and singlet (Cross et al., 1987)

$$2\ O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad k\sim 0 \qquad \text{(equation 1)}$$

$$HO_2^{\bullet} + O_2^{-\bullet} + H^+ \rightarrow H_2O_2 + O_2 \quad k \sim 10^{\,8}\ M^{-1}\ s^{-1} \qquad \text{(equation 2)}$$

$$2HO_2^{\bullet} \rightarrow H_2O_2 + O_2 \quad k \sim 10^{\,6}\ M^{-1}\ s^{-1} \qquad \text{(equation 3)}$$

Superoxide anions can generate powerful reactive species from biological species through Fenton and Haber-Weiss reactions (Hosseinian, 2006).

$$O_2^{-\bullet} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad \text{(equation 4)}$$

$$H_2O_2 + O_2^{-\bullet} \rightarrow O_2 + OH^- + OH^{\bullet} \quad \text{(equation 5)}$$

Hydroxyl radical ($^{\bullet}OH$)

Hydroxyl radical, is the neutral form of the hydroxide ion. It is highly electrophilic and can extract electrons from proteins and polyunsaturated fatty acids resulting in the formation of carbon centered free radicals ($R^{\bullet}$) (equation 6) (Ashok et al., 1999). The carbon centered free radical can undergo further reactions, such as reaction with oxygen, to give peroxyl radical ($ROO^{\bullet}$) and alkoxy radical ($RO^{\bullet}$) (equation 7-9).

$$RH + {}^{\bullet}OH \rightarrow R^{\bullet} + H_2O \quad \text{(equation 6)}$$

$$RO^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad \text{(equation 7)}$$

$$ROOH + Fe^{3+} \rightarrow ROO^{\bullet} + Fe^{2+} + H \quad \text{(equation 8)}$$

$$ROOH + Fe^{2+} \rightarrow RO^{\bullet} + {}^{-}OH + Fe^{3+} \quad \text{(equation 9)}$$

Hydroxyl radicals can damage cell membranes and lipoproteins by lipid peroxidation (Gutteridge et al., 2000) which causes oxidation of cell membranes. Proteins may also be damaged by ROS, leading to structural changes and loss of enzyme activity and oxidative damage to DNA (Hosseinian, 2006).

Hydrogen Peroxide (H_2O_2)

Although H_2O_2 is the least reactive molecule among ROS it is very damaging because it can be converted to hydroxyl radical (Hawkins et al., 2001). H_2O_2 may be generated either through a dismutation reaction from superoxide anion by superoxide dismutase or from superoxide anion by monomeric oxidases such as amino acid oxidase and xanthine oxidase (Evans et al., 1999). As soon as H_2O_2 is generated, it is metabolized by catalase or glutathione peroxidase to generate water and oxygen (Evans et al., 1999). In the presence of metals such as Fe^{2+} or Cu^{2+}, H_2O_2 can generate hydroxyl radical through Fenton type reactions in which Fe^{2+} is oxidized to Fe^{3+} and H_2O_2 is converted to $OH^{\bullet}$ and OH^- (equation 10).

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^- + Fe^{3+} \qquad \text{(equation 10)}$$

Singlet oxygen ($^1O_2$)

Singlet oxygen, a non radical oxygen species may be generated from H_2O_2 during interaction with hypochlorite (OCl^-) in cells and tissues (equation 11) (Hawkins et al., 2001; Stief, 2003). Singlet oxygen has been known to be associated with oxidation of PUFA during the initiation step of lipid peroxidation, which involves an oxygen radical attacking a methylene group ($-CH_2-$) of a PUFA to abstract a hydrogen atom and an electron as a result splitting the double bonds (Kao, 2006; Spiteller, 2001).

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \longrightarrow H_2O_2 + {}_1O^2 \qquad \text{(equation 11)}$$

$$^-OCl + H_2O_2 \longrightarrow H_2O + Cl^- + {}_1O^2 \qquad \text{(equation 12)}$$

$$ONOO^- + H_2O_2 \rightarrow NO_2 + H_2O + {}^1O_2 \quad \text{(equation 13)}$$

$$O_2{}^{-\bullet} + H_2O_2 \rightarrow {}^{\bullet}OH + {}^-OH + {}^1O_2 \quad \text{(equation 14)}$$

Peroxy and alkoxy radicals

Peroxyl radicals ($ROO^{\bullet}$) are formed by a direct reaction of oxygen with alkyl radicals ($R^{\bullet}$), for example, the reaction between lipid radicals and oxygen (Hosseinian, 2006). Decomposition of alkyl peroxides (ROOH) also results in peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals. They are both good oxidizing agents that are involved in the propagation stage of lipid peroxidation. They create a chain reaction by abstracting another hydrogen atom, and so free radicals are continuously being propagated (Kao, 2006).

Lipid Peroxidation in humans

Lipid peroxidation is a harmful self-propagation reaction that leads to generation of lipid radicals and lipid peroxides (Halliwell et al., 1993). During lipid oxidation, malonaldehyde (product of oxidation) can react with the free amino group of proteins, phospholipids, and nucleic acids damaging their structures and functions (Hosseinian, 2006). It is the initiation step of cardiovascular diseases, neurodegenerative diseases and diabetes (Willcox et al., 2004).

Lipid peroxidation in food

Flavor of food is greatly affected by ROS generated during storage and processing and makes food products less acceptable to consumers by lowering the quality of foods (Choe et al., 2006). Lipid peroxidation can cause rancidity of food products such as fats and oils, which affects odor, taste and nutritional value (Shahidi, 2000). The major purpose of using antioxidants in foods is to retard, delay or prevent autoxidation and therefore to extend shelf life and minimize nutritional losses (Shahidi, 2000). Lipid peroxidation involves hydrogen extraction to form a lipid radical ($L^{\bullet}$) (equation 15), which can react with oxygen to form a lipid peroxyl radical (equation 16). The peroxyl radical propagates the chain reaction by abstracting hydrogen from another lipid (equation 17), usually the rate-limiting step in lipid peroxidation (Shahidi, 2000). Antioxidants may work by blocking the propagation step (equation 3) through hydrogen atom donation. Antioxidants can also interfere with the oxidation process by chelating catalytic metals and acting as free radical scavengers.

$$R^{\bullet} + LH \rightarrow RH + L^{\bullet} \qquad \text{(equation 15)}$$

$$L^{\bullet} + O2 \rightarrow LOO^{\bullet} \qquad \text{(equation 16)}$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \qquad \text{(equation 17)}$$

DIETARY ANTIOXIDANTS

Antioxidants help protect the human body against damage by reactive oxygen species. A compound might exert antioxidant activity *in vivo* or in food by inhibiting generation of ROS, or by directly scavenging free radicals (Halliwell et al., 1995).

Vitamin C

Vitamin C or L-ascorbate is a water soluble vitamin, found widely in plants and is regarded to be an important vitamin in fruits and vegetables for human nutrition. According to Lee and Kader (2000) more than 90% of the vitamin C consumed by humans comes from fruits and vegetables. Vitamin C is required for the maintenance of healthy skin, gums and blood vessels and its deficiency causes scurvy. Vitamin C is also known to have many biological functions in collagen formation, absorption of inorganic iron, reduction of plasma cholesterol level, inhibition of nitrosamine formation, enhancement of the immune system, and reaction with singlet oxygen and other free radicals (Lee et al., 2000).

Vitamin C includes L-ascorbic acid (AA) and dehydroascorbic acid (DHAA). AA is the most biologically active form. However, DHAA can be readily converted into a usable form, so both forms need to be measured to get an accurate representation of vitamin C content (Lee et al., 2000). Vitamin C is an electron donor and acts as an antioxidant or reducing agent by donating two electrons from the C2-C3 double bond, forming the intermediate ascorbate free radical, which is unstable and is reversibly reduced to ascorbate. Vitamin C protects against oxidative damage by scavenging reactive oxygen species including superoxide and aqueous peroxyl radicals, singlet oxygen, and ozone (Halliwell, 1996). When ascorbate reacts with a hydroxyl radical, it results in the formation of semidehydroascorbate radical and a water molecule. Two semidehydroascorbate radicals combine to generate a molecule of ascorbate and a molecule of dehydroascorbate. Dehydroascorbate breaks down into further products (Halliwell et al., 1995).

Ascorabte + $OH^{\cdot}$ → Semidehydroascorbate radical + H_2O

2 Semidehydroascorbate → Ascorbate + Dehydroascorbate

Dehydroascorbate → Oxalate, threonate, etc.

The function of vitamin C in disease prevention is thought to be from its ability to scavenge free radicals through the ascorbate-glutathione cycle in chloroplast of plants and mitochondria of animals. The dietary benefits of vitamin C include its preventive effects against cancer, heart disease, cataracts and the common cold. In addition to scavenging ROS, vitamin C is also capable of regenerating tocopherol from a tocopheroxyl radical which is formed by the inhibition of lipid peroxidation by vitamin E (Kao, 2006). The recommended dietary allowances (RDA) for adult men and women are 90mg and 75 mg respectively. Smokers are recommended to consume an additional 35 mg of ascorbate daily (Gropper et al., 2005).

Carotenoids

Carotenoids are a large group of compounds which consist of α-carotene, β-carotene, lycopene, β-cryptoxanthin, zeaxanthin, lutein, canthaxanthin and astaxanthin. The yellow-orange color of carotenoids is an attractive index for consumers and in addition influences the health effects of foods (Nishiyama et al., 2005). Biological functions of carotenoids include antioxidant activity, intercellular communication, cell differentiation, immuno enhancement, and inhibition of mutagenesis and transformation (Lan, 2006). Many epidemiological studies have supported an inverse relationship between dietary intake or blood levels of carotenoids and risk of numerous chronic

diseases, such as cancer, heart disease, cataracts, diabetes and macular degeneration and Alzheimer's disease (Lan, 2006). Other antioxidant functions of carotenoids involve prevention of free radical induced damage to cellular DNA and other molecules. The antioxidant capacity of carotenoids is primarily due to conjugated double bonds, a structural feature that permits carotenoids to quench or inactivate several highly reactive molecules. Several reports have shown that high intakes of tomatoes and tomato products as well as high blood levels of lycopene are associated with a decreased risk of prostate cancer. There is also strong evidence that supports the protective role of lutein and zeaxanthin against age-related eye diseases because they accumulate in the pigment of the macular region and filter blue light, which is particularly damaging to photoreceptors and to the retinal pigment epithelium color (Nishiyama et al., 2005). The intake of vitamin A is expressed as retinol activity equivalents (RAE). The RDA of vitamin A for men and women are 900 and 700 μg RAE respectively (Gropper et al., 2005).

Polyphenols and Flavonoids

Polyphenols and flavonoids are secondary plant metabolites that belong to the group phytochemicals which are defined as chemicals derived from plant material with potentially beneficial effects in human health (Cadenas et al., 1996). Dietary phenolics include phenolic acids, flavonoids namely flavanones, flavonols, flavones, isoflavonoids, flavanols, anthocyanidins), tannins, stilbenes and coumarins (Cadenas et al., 1996). Over 4000 different flavonoids have been identified and their molecular structures consist of an aromatic ring A, condensed to heterocyclic ring C attached to a second aromatic ring B and they may contain several phenolic hydroxyl groups attached to the aromatic rings,

which confer their potent antioxidant activity (Lan, 2006). The principal mode of antioxidant activity is through radical scavenging via hydrogen donation. Other radical quenching mechanisms are through singlet oxygen quenching and electron donation. Antioxidant activity of flavonoids is related directly to their degree of hydroxylation, and they are effective scavengers of superoxide anion, hydroxyl, and peroxyl radicals (Lan, 2006). Flavonoids have a wide range of biological effects, such as inhibition of key enzymes in mitochondrial respiration, protection against coronary heart disease and anti-inflammatory, antitumour, and antimicrobial activities (Wang et al., 2007). The total dietary intake of polyphenols is about 1 g/day and is 10 times higher than that of vitamin C and 100 times higher than those of vitamin E and carotenoids (Kao, 2006).

CITRUS AS A DIETARY COMPONENT

Citrus is the most important tree fruit crop in the world, with world production far exceeding that of other deciduous tree fruits such as apples, pears, peaches and plums (Saunt, 1990). During the 2003-04 crop year, the total citrus production in the USA was 16.4 million tons on 983,600 acres. Approximately 75% of US citrus fruit were processed as juice and 25% were sold as fresh fruit (Zhang, 2006). The production of grapefruit and oranges in the USA ranks the first and second in the world, respectively (Zhang, 2006)

Citrus fruits have been long regarded as part of a healthy diet and provide an array of essential nutrients like vitamin C, vitamin A, folate, dietary fiber, and minerals as well as many phytochemicals including flavonoids, carotenoids, glucarates, coumarins, monoterpenes, limonoids, triterpenes and phenolic acids (Economos et al., 1998).

Energy and macronutrients

Total calories for 100 grams of oranges, grapefruit (white), grapefruit (pink and red) and tangerines are 47, 33, 42 and 53, respectively (Zhang, 2006). Protein content ranges from 0.69 to 0.94 grams per 100 grams of oranges, grapefruit and tangerines (Table 2). Citrus fruits are low in fat ranging from 0.1 to 0.31 grams per 100 grams of fresh oranges, grapefruit and tangerines (Table 2). They are also rich sources of dietary fiber ranging from 1.1 to 2.4 grams per 100 grams of edible portion (Table 2). Carbohydrate content ranges from 6.9 to 10.6 grams per 100 grams fresh weight of oranges (Table 2).

Minerals

Citrus fruit contain a wide range of minerals including Ca, Fe, Mg, P, K, Zn, Cu, Mn, and Se. Potassium (K) and calcium (Ca) content ranges from 135 to 181 and 12 to 40 mg/100 grams of edible portion respectively (Table 3). Sodium content in citrus is very low, ranging from 0 to 2 mg/100 grams of edible portion (Zhang, 2006). Potassium is an essential mineral needed for the normal physiological functioning of the body and in combination with sodium and calcium, maintains normal heart rhythm, controls the body's water balance, and is responsible for the conduction of nerve impulses and the contraction of muscles (Zhang, 2006). An increased potassium intake and limited sodium intake may reduce hypertension (He et al., 2005). Many studies have shown that on average, the higher salt (sodium chloride) intake, the higher the blood pressure (Meneton et al., 2005). Nearly all Americans consume substantially more salt than they need, and

decreasing salt intake is advisable to reduce the risk of elevated blood pressure as it is one of the risk factors for coronary heart diseases (Zhang, 2006).

The iron (Fe) content and magnesium (Mg) of citrus fruits ranges from 0.06 to 0.15 and 9 to 12 mg/100 grams of edible portion, respectively (Table 3). Even though citrus fruits are relatively low in Fe content, the high vitamin C content of the fruits helps release Fe from other food and therefore citrus fruits play an important role in maintaining iron status (USDA Dietary Guidelines, 2005). Citrus fruit also contain zinc, copper and manganese (Table 3).

Dietary fiber

Consumers will obtain more dietary fiber with the consumption of citrus fruit in contrast to drinking a glass of citrus juice. Diets rich in fiber have been shown to have a number of health benefits including decreased risk of coronary heart disease and improvement in laxation (USDA Dietary Guidelines, 2005). The recommended dietary fiber intake is 14 grams per 1,000 calories consumed (USDA Dietary Guidelines, 2005). Dietary fiber is classified as soluble dietary fiber and insoluble dietary fiber. Fiber derived from fruits and vegetables, has significantly higher amount of soluble fibers such as pectin, while cereal fiber has more insoluble fibers such as cellulose and hemicellulose. Pectin, cellulose and hemicellulose, with only trace amounts of lignin are the main components of dietary fiber from most fruit including citrus (Zhang, 2006). Citrus fruit contain both soluble and insoluble fiber, mostly in the peel (albedo), membrane and juice sac (Zhang, 2006). Diets poor in fiber promote gastrointestinal problems such as

constipation, diverticular disorders, colon cancer, high blood pressure, coronary heart disease and alterations in glycemic control (Zhang, 2006).

Citrus as a good source of antioxidants

Vitamin C

Vitamin C is an important health component of citrus fruit ranging from 23.6 - 83.2 mg/100g FW depending on the species (Lee et al., 2000). The recommended dietary allowances (RDA) for adult men and women are 90mg and 75 mg respectively. Smokers are recommended to consume an additional 35 mg of ascorbate daily (Gropper et al., 2005). One serving of a medium orange (154 grams) will provide 83 mg vitamin C, which is approximately equivalent to the RDA of this nutrient. According to a few reports, the contribution of vitamin C to the total antioxidant capacity (TAC) was 26.9% to 45.9% (Abeysinghe et al., 2007). This wide variation in vitamin C contribution is due to different fruit species and even different cultivars within citrus species. Vitamin C deficiency causes scurvy (Silalahi, 2002).

Carotenoids

Carotenoids are responsible for the orange/yellow color of citrus fruits. Over 600 carotenoids have been reported but those found most commonly in citrus peels include (9*Z*)-violaxanthin (8–33%), β-citraurin (11–28%), β-cryptoxanthin (3–23%), and lutein (4–8%) (Agocs et al., 2007). 30% of carotenoids found in pulp include β-cryptoxanthin followed by lutein and (9*Z*)-violaxanthin (Agocs et al., 2007). The protective effects of β-

carotene may be associated with antioxidant protection and enhancement of immune response (Silalahi, 2002).

Polyphenols and flavonoids

In addition to vitamin C and carotenoids, a variety of bioactive phenolic compounds, namely flavanone glycosides and hydroxyl-cinnamic acids, are present in citrus fruits (Abeysinghe et al., 2007). Naringin and hesperidin are the two major flavanone glycosides present in citrus fruits (Abeysinghe et al., 2007). The content of total phenolics and flavonoids varies significantly among the different species and cultivars of citrus. The contribution of hesperidin to TAC ranged from 5.9% to 54% and was much higher than naringin. It may be due to the differences in both the number and position of hydroxyl groups present in their chemical structures (Abeysinghe et al., 2007).

SATSUMA MANDARIN

'Satsuma' mandarin (Citrus unshiu Marc.) is an important citrus commodity currently grown in the gulf coast area of Alabama. Satsuma mandarin is a native of China and is abundantly grown in China, Japan, India and the East Indies (Morton, 1987). The commercial cultivation of mandarin oranges in the United States has developed mostly in California, Louisiana, Florida, Alabama and Mississippi and to a lesser extent in Texas and Georgia. The 'Owari' Satsuma, selected in Japan centuries ago, is the most commonly grown variety in Alabama. Mandarin fruit are almost seedless and very easy to peel. They are highly perishable and susceptible to postharvest decay. Reduced shelf life and quality are often related to inadequate sanitation and improper storage. The two

major economically important postharvest diseases of citrus are caused by *Penicillium digitatum* (green mold) and *Penicillium itallicum* (blue mold). Stem-end rot caused by *Diplodia natalensis* and *Phomopsis citri* are also problematic for Satsuma producers during postharvest handling and storage (Campbell, 2005). Satsuma fruit develop stem end rot prior to harvest and the infection travels through the core of the fruit where it develops unevenly throughout the fruit (Campbell, 2005). Green and blue mold are initiated through open wounds made during harvest or during the processing of the fruit. Infected fruits are unsuitable for fresh market purposes. Among the various effective postharvest pathogen control measures, synthetic fungicides such as Thiabendazole (TBZ) and Imazalil (IMZ) are considered standard postharvest chemical treatments (Eckert, 1987). Applying postharvest dip of either TBZ or IMZ or in combination with wax before Satsumas are packed and shipped will decrease the incidence of *Diplodia* sp., *Phomopsis* sp., *Anthracnose* sp., and *Penicillium* sp., (Campbell, 2005). It is also known that much of the nutritional value as well as the sensory quality of mandarins can be lost as a result of current postharvest handling practices, which were developed primarily to maintain acceptable visual appearance with less regard for other quality aspects such as flavor and nutritional value. Although synthetic fungicides are effective in suppressing postharvest pathogen decay, public concerns regarding fungicide residue and development of pathogen resistance have stimulated the need for reduction of postharvest chemical use. Prolonged over-use of synthetic fungicides to control fungal decay in citrus has resulted in the appearance and proliferation of pathogen resistant strains of *P. digitatum* isolates and *Alternaria* sp. (Kinay et al., 2007). Several alternative non-chemical methods to control postharvest disease and decay of fruit through "natural host

exploitation" are particularly attractive. However many of non-chemical methods such as postharvest heat treatments (Lurie et al., 2004), biocontrol (Conway et al., 2004), biofumigation (Schnabel et al., 2006), and application of shortwave UV light for beneficial hormetic responses (Shama, 2007) are most effective when used in combination. Among the non-chemical technologies reported to be effective in suppressing postharvest diseases is the use of the "inductive natural plant defense" method (Gonzalez et al., 2003). This led to the identification of *trans*-resveratrol (3, 4', 5-trihydroxystilbene) which is responsible for resistance of grapevines to fungal diseases (Gonzalez Urena et al., 2003). This strategy may offer significant advantages over synthetic fungicides in pathogen control, maintenance of fruit quality, nutrition and safety.

POSTHARVEST TRANS –RESVERATROL APPLICATION ON SATSUMA MANDARINS

Resveratrol is a naturally occurring phytochemical produced by some higher plants. It belongs to the class of phytochemicals known as stilbenes, and to the subclass phytoalexins. It was first isolated from an extract of the Peruvian legume *Cassia quinquangulata* in 1974. One of the richest sources is the weed *Polygonum cuspidatum*, root extracts of which have played a vital role in Japanese and Chinese folk-medicines (Tomera et al., 1999). It is commonly found in grapes, its highest concentration is in grape skins (Burns et al., 2002), wine, peanuts (Sanders et al., 2000) and berries of *Vaccinum* species including blueberries, bilberries and cranberries (Rimando et al., 2004).

Resveratrol is fat-soluble and occurs in a *trans* and a *cis* configuration. Since *trans* isomer is more commonly found in plants and widely studied, the term resveratrol refers to 3, 5, 4'-*trans*-hydroxystilbene. Both *cis* and *trans*-resveratrol also occur as

glucosides (bound to a glucose molecule) and resveratrol-3-*O*-beta-glucoside is also called piceid (Goldberg et al., 2003). Resveratrol accumulates substantially in tissues in response to certain biotic and environmental abiotic stresses such as pathogenic attack or irradiation with UV light (Benett et al., 1994).

Resveratrol biosynthesis is catalysed by stilbene synthase and consists of repetitive decarboxylative condensation of *p*-coumaroyl-CoA with three C_2 units from malonyl-CoA. Resveratrol is susceptible to oxidative degradation, while the glycosylated piceid form is resistant. Glycosylated resveratrol maintains its biological activity, is more stable and soluble and is therefore more readily absorbed by the human intestine (Regev-Shoshani G. et al., 2003).

Interest in resveratrol was caused by the existence of the "French Paradox" which confirms that in certain parts of France, the death rate caused by coronary artery diseases is lower despite relatively high fat consumption in the human diet. This has been attributed to the high consumption of wine (Renauld et al., 1992). This fact led to an assumption that the enjoyment of wine could act against the effect of high-fat-diet and therefore limit incidence and extent of coronary artery diseases (Filip et al., 2003). Resveratrol has also been associated with reduced chronic disease such as artherosclerosis, cancer, inflammation and cardiovascular disease. These beneficial properties are associated with the consumption of red wine which contains resveratrol (=1.5-3.0 mg/L of red wine) (Renauld et al., 1992). The biological properties of resveratrol include antioxidizing effect, anti-artherosclerotic effect, effect on cardiovascular system, anti-mutagenic effect and chemopreventive advantage against cancer proliferation (Filipa et al., 2003).

Resveratrol has also been shown to have broad antifungal activity and it can be fungitoxic against the phytopathogenic fungus *Botrytis cinerea* which is a major cause of postharvest rot of perishable plant products in a huge variety of crops (Jimenez et al., 2005). Due to its antioxidant properties, resveratrol can also have positive effects on fruit conservation during storage (Jimenez et al., 2005). Prior investigations have revealed that exogenous application of *trans*-resveratrol ($1.6x10^{-4}M$) to apples, grapes and tomatoes maintained their postharvest quality, sensory and nutritional value when compared to control treated fruit (Jimenez et al., 2005). Therefore, resveratrol can be used as a natural antibiotic to reduce microbial contamination in fruit during the time of postharvest up to delivery to the consumers. Such post harvest treatments also reduced water loss without affecting nutritional content. The maintenance of water content and fruit firmness after resveratrol treatment was attributed to two distinct modes of action as explained by Jimenez (2005). One mechanism was hypothesized to be due to the action of resveratrol as a natural pesticide, thus avoiding mechanical damage to the skin by microbial growth and penetration into the fruit. As a result, treated fruit preserved the integrity of the epidermis to a higher extent and consequently, the water loss would be lower as compared with control treated fruit. A second mechanism is that resveratrol may act as an impermeabilizer, which produces a coat that surrounds the fruit (Jimenez et al., 2005). Resveratrol distributes a thin coat around the fruit and reduces water vapor pressure and consequently, preserves water content and fruit firmness (Jimenez et al., 2005). Additionally, the beneficial effect of resveratrol in extending the shelf life of food commodities has also been observed in the meat industry (Bekhit et al., 2004).

SPECIFIC OBJECTIVES AND SIGNIFICANCE OF THIS RESEARCH

Resveratrol application to Satsuma mandarin would be beneficial not only as a potential postharvest conservation method in reducing the need for dependence of synthetic fungicides during postharvest handling and storage of citrus, but also in regards to health promotive properties. The objective of this research was to compare effectiveness of various concentrations of resveratrol to fruit treated with a mixture of wax and Imazalil (IMZ) or water and further analyze availability of antioxidants in treated fruit during storage.

III. EFFECT OF TRANS RESVERATROL ON COLOR RETENTION OF SATSUMA MANDARIN FRUIT

Abstract

Satsuma mandarins (*Citrus unshiu* Marc., cv. Owari) were treated with either aqueous solution of *trans*-resveratrol at $1.6x10^{-4}$M and $1.6x10^{-5}$M, mixture of wax and Imazalil or tap water and stored at 10^{o}C for 12 weeks to investigate the effect of *trans*-resveratrol treatment on fruit color as a potential alternative to toxic fungicides to maintain mandarin quality during storage. Trans-resveratrol at $1.6x10^{-5}$M had positive effects on b*, chroma, total carotenoid content in both peels and segments at the end of storage. Resveratrol also had no adverse effects on fruit quality. The development of fungal infection was lower in resveratrol treated fruit than in control. The results confirmed that resveratrol treatment could be applied to Satsuma mandarin as an effective method to maintain postharvest quality during storage.

Keywords: *trans*- Resveratrol, Satsuma mandarin, color, carotenoids.

Introduction

Satsuma mandarin is an important citrus crop for gulf coat area of Alabama. During maturation, fruit and vegetables change their color due to chlorophyll degradation and increase in other pigments such as carotenoids or polyphenols (Olmo

et al., 2000). Fruit color is considered to be one of the most important external factors of fruit quality that influences consumer preference (Campbell, 2005). The relationship between color and degree of maturation has been widely studied in tomatoes, peaches and nectarines (Singh and Reddy, 2006). In citrus, Jiménez-Cuesta (1981) proposed the use of the formula 1000a*/(L*b*) as a "Color Index" for recording the process of degreening in citrus. The rind color of citrus fruit is dependent largely on climatic conditions during fruit maturation. The most favorable temperature combination for chlorophyll degradation and carotenoid biosynthesis leading to a bright orange rind is 20/5^{o}C, cool night air temperatures and cool soil temperatures (Barry and van Wyk, 2006). High quality mandarins have bright reddish orange peel.

Satsuma mandarins are regarded as part of a healthy and nutritious diet as they provide an array of essential nutrients like vitamin C, vitamin A, folate, dietary fiber, minerals as well as many phytochemicals including flavonoids, carotenoids, glucarates, coumarins, monoterpenes, limonoids, triterpenes and phenolic acids (Economos and Clay, 1998).

Postharvest decay represents a major loss for most fruits and vegetables. Despite numerous advances achieved in postharvest biotechnology in ameliorating commodity losses associated with postharvest decay, this aspect perhaps is the most important feature which defines commodity quality, value and consumer demand. It is also known that much of the nutritional value as well as the sensory quality of mandarin fruit can be lost as a result of current postharvest handling practices, which were developed primarily to maintain acceptable visual appearance with less regard to other quality aspects such as flavor and nutritional value. Increased public awareness regarding the use of toxic fungicides has stimulated the need for developing

alternative non-chemical technologies reported to be effective in suppression of postharvest decay (Jimenez et al., 2005). Prior investigations have revealed that exogenous application of *trans*-resveratrol at $1.6x10^{-4}$M to apples, grapes and tomatoes maintained their postharvest quality, sensory and nutritional value when compared to control fruit (Jimenez et al., 2005).

The objective of the present study was to determine the effects of exogenous application of *trans*-Resveratrol on fruit color and carotenoid content of Satsuma mandarin during storage.

Materials and Methods

Fruit Source & Treatment

Satsuma mandarin fruit were harvested from fully mature mandarin trees budded onto *Poncirus trifoliate* rootstock and grown at Gulf Coast Research Extension Center in Fairhope, AL. Satsumas were harvested after the fruit reached 10:1 soluble solids to titratable acidity ratio. The optimum (10:1) ratio was reached in mid-November and harvest followed, which is the typical harvest period for this cultivar in this region (Campbell, 2005). Fruit were randomly harvested and samples were washed, graded and damaged fruit were discarded. Selected fruit were randomly grouped into four treatment lots of 10 boxes each (each containing 50 fruit). Fruit were treated with either varying concentrations of *trans*-Resveratrol ($1.6x10^{-4}$M and $1.6x10^{-5}$M) (Sigma Chemical Co., St. Louis, MO), tap water or mixture of wax (StaFresh 4201, FMC technologies, Riverside, CA) and 1% Imazalil (IMZ) (Freshgard 700, FMC technologies, Riverside, CA) by submerging fruit for 5 seconds and stored for a duration of 12 weeks at 10^{o}C and 95% RH. 100 fruit from each treatment were randomly sampled, peeled and separated at the end of 0, 4, 8, and 12

weeks of storage. Fruit segments and peels were stored separately at -80^{o}C for further analysis.

Chemicals

Diethyl Dithio-Carbamate (DDC), butylated hydroxytolulene (BHT) and other chemical reagents, solvents or standards were either purchased from Sigma Chemical Co. (St. Louis, MO) or Fischer Scientific (Fischer Scientific, Raleigh, NC) and were either high-performance liquid chromatography (HPLC) or analytical grade quality. Ultrapure Milli-Q water was used throughout this study and had electrical conductivity of 18.2 M Ωcm^2 obtained through a Millipore Direct-Q$^{TM.}$ 5 filter system (Millipore Corp., Bedford, MA).

Rind color analyses

Peel color was measured at four equally spaced sites of the marked fruit around the equator using a colorimeter (CR-200, Minolta Co., Japan). Color changes of fruit samples were measured in terms of CIE-$L^*C^*h^*a^*b^*$ to describe a three dimensional color space and interpreted as follows: L^* indicates lightness read from 0 (completely opaque or "black") to 100 (completely transparent or "white") (Goncalves et al., 2007). A positive a^* value indicates redness (-a^* is greenness) and a positive b^* value yellowness (-b^* is blueness) on the hue-circle (Voss, 1992). Hue angle (°) expresses the color nuance (Voss, 1992) and values are defined as follows: red-purple: 0°, yellow: 90°, bluish-green: 180°, and blue: 270° (Goncalves et al., 2007). The chroma, obtained as $(a^{*2} + b^{*2})^{1/2}$, is measure of chromaticity (C^*), which denotes the purity or saturation of the color (Voss, 1992) C^* the chroma, h^* is the hue

angle '*a**' (redness and greenness) and '*b**' (yellowness and blueness). Color index (CI) was calculated using the following formula (Jiménez-Cuesta et al., 1981).

$$CI = \frac{1000 \cdot a^*}{L^* b^*}$$

Before measurements, the instrument was calibrated with a standard white plate ($Y = 93.4$, $x = 0.3129$, $y = 0.3189$). Samples of 25 fruit from each treatment were marked for color measurements by placing over the 8 mm aperture of sample measurement port of the colorimeter. Change in color was measured up to 12 weeks at intervals of 4 weeks.

Extraction and determination of total carotenoid from peel

Extraction and determination of fruit pigments were performed according to Barry and van Wyk (2006). A 10 g composite rind sample (flavedo tissue) from five fruit taken from each treatment were immersed in liquid nitrogen and finely ground in a mortar and pestle. A 0.5 g sub sample of finely powdered flavedo tissue was added to 10 mL of cold 80% acetone containing butylated hydroxytolulene BHT (100 mg L^{-1}) and diethyl dithio-carbamate (DDC) (200 mg L^{-1}) to prevent carotenoid degradation. Samples were vortexed for two 1 min bursts and stored at 4 °C for 1.5 h to extract pigments. Homogenate was centrifuged (Beckman Centrifuge, model J2-21, San Antonio, TX) at 13,500 rpm for 15 min at 4^{o}C and filtered through four layers of Miracloth (Calbiochem, La Jolla, CA). The supernatant was collected and the remaining residue was re-extracted three times until the residue was colorless. The absorbance was measured at 470, 649 and 664 nm using a microplate reader (Synergy HT, BIO-TEK Instruments, Inc., Winooski, Vermont). Absorbance values were used to calculate total carotenoid concentrations (as mg/100g fresh weight) according to Lichtenthaler (Lichtenthaler, 1987).

$$C_a = (12.5 * A_{663}) - (2.79 * A_{646})$$

$$C_b = (21.5 * A_{646}) - (5.1 * A_{663})$$

$$C_{x+c} = \left(\frac{1000 \cdot A_{470} - 3.27C_a - 104 \cdot C_b}{229} \right)$$

Extraction and determination of total carotenoids in fruit segment

Total carotenoids in citrus segments were measured using the method of Talcott and Howard (1999) was used. Total carotenoids were extracted (20 g/25 mL) with a solution of acetone/ethanol (1:1) containing 200 mg L^{-1} BHT. Samples were extracted using a Virtis Shear homogenizer (Cyclone Virtishear) at a speed of 70 rpm for 1 min. The homogenate was centrifuged at 13,500 rpm for 15 min at 4^{o}C and filtered through four layers of Miracloth. The supernatant was collected and the remaining residue was re-extracted three times until the residue was colorless. Finally, all extracts were combined and brought to a final volume of 40ml using the same extraction solvent. Absorbance was measured at 470 nm using a microplate reader.

Total carotenoids were calculated using the equation

$$\frac{A \cdot V \cdot 10^6}{A^{1\%} \cdot 100 \cdot G}$$

where A is the absorbance at 470 nm, V is the total volume of extract, $A^{1\%}$ is the extinction coefficient for a mixture of solvents arbitrarily set at 2500, and G is the sample weight in grams (Talcott and Howard, 1999).

Statistical analysis

Statistical analyses were conducted using The SAS System for Windows V9.1 (SAS Institute, Inc., Cary, N.C., 2004-2005) and Stata 10 (Stata Corp., College Station, Texas). Analysis of variance and protected least significance difference (LSD) tests were performed via One-way Analysis of Variance (ANOVA) to identify differences among means, while the Pearson Correlation test was conducted to determine correlations among the means. Statistical significance was determined at $P \leq 0.05$.

Results and Discussion

Rind color

The chromatic characteristics of various treatments on Satsuma fruit are shown in Fig. 3. There were significant differences in a*, b*, chroma, total carotenoid content of peels and segments among the four treatments at the end of the storage period. The initial peel color of Satsuma mandarins treated with water was reddish-yellow with $L^*C^*h^*$ values of 63.34±0.33, 67.9±0.65 and 68.0±0.41, respectively. L* and hue angle of Satsuma fruit were higher initially and these parameters decreased during storage. The loss of lightness was indicated by a reduction of L* values. Trans-resveratrol treatments had no effect on lightness of peel color during storage (Fig. 3A). An increase in chroma was observed during the storage period (Fig. 3D). An increase in chroma corresponds to a decrease in tonality of the fruit color which could have been caused by a decrease in carotenoids (Goncalves et al., 2007). There was significant difference ($p<0.0096$) in C*/chroma values among the treatments. Resveratrol, $1.6x10^{-5}$M had the highest mean chroma value (70.82). Hue angle averaged 67.43 degrees during week 0 and decreased to 61.64 degrees at the end of

week 12. However, the treatments did not have a significant influence on hue angle values (Fig. 4A). There was an increase (p<0.0001) in Color Index (CI) during storage. However, there were no significant differences among the treatments (p<0.119) (Fig. 4B). Trans-resveratrol treatments had a significant effect on overall peel color with resveratrol at $1.6x10^{-5}$ M producing more visually appealing fruit. The results were similar or slightly higher than that of wax+IMZ. The fruit treated with water were not only dehydrated but also deteriorated and developed fungal symptoms at the end of 8 weeks of storage while resveratrol and wax + IMZ treated fruit showed deterioration at the end of 12 weeks of storage.

Total carotenoids from peel and segments

The amount of total carotenoids in peels and segments extracted from different treatments are shown in Table 4. Total carotenoid content in peels ranged from 0.85 ±0.007 to 1.039 ± 0.0125 during week 0. The carotenoid content decreased during storage and varied significantly among treatments (p<0.0001) with resveratrol $1.6x10^{-5}$ M treated fruit retaining the highest amount of carotenoids in peels (0.768 mg/ 100 g FW) at the end of storage period. The total carotenoid content in segments varied from 1.958 ± 0.0212 to 3.907 ± 0.0476 mg/ 100 g FW. The total carotenoid content was highest at the end of 4 weeks of storage and reduced significantly over time (p< 0.0091). Trans-resveratrol at 1.6 x 10^{-5} M retained the highest amount of total carotenoids throughout storage.

Conclusion

In this study, the effect of *trans*-resveratrol on Satsuma mandarin peel color of fruit cultivated in Alabama was investigated. Resveratrol at $1.6x10^{-5}$M had a positive effect on fruit color during extended storage. Resveratrol at $1.6x10^{-5}$M had a

significant effect on peel color (a*, b*, chroma and color index) throughout storage. Total carotenoid content of fruit segments and peels were also significantly affected by resveratrol treatments. The results from this study indicates that application of resveratrol $1.6x10^{-5}$M prior to storage serves as a potential postharvest conservation method in reducing the need for dependence of synthetic fungicides and enhances storage life, nutritional quality and consumer appeal of Satsuma mandarin without impairing any of the other fruit quality parameters. Further studies conducted within this laboratory have determined the influence of *trans*-resveratrol on the antioxidative properties of Satsuma mandarin fruit.

IV.INFLUENCE OF TRANS- RESVERATROL TREATMENT ON ASCORBIC ACID, PHENOLICS, FLAVONOIDS AND CAROTENOIDS DURING STORAGE OF SATSUMA MANDARIN

Abstract

Satsuma mandarins (*Citrus unshiu* Marc., cv. Owari) were treated with *trans*-resveratrol, tap water or mixture of wax and Imazalil and were subsequently stored at 10^{o}C and 95% RH for up to 12 weeks. They were analyzed to determine the effect of resveratrol on total phenolic content, total flavonoid content, ascorbic acid content and total carotenoid content during storage. Treating mandarin fruit with resveratrol prior to storage reduced storage losses and improved the nutritional quality of fruit. Fruit treated with *trans*-resveratrol at 1.6x10^{-5}M had higher total phenolic, ascorbic acid and total carotenoid content at the end of storage.

Keywords: *trans*-Resveratrol, Satsuma, mandarin, ascorbic acid, phenolics, flavonoids, carotenoids.

Introduction

Current epidemiologic studies suggest that increased consumption of fruit and vegetables rich in natural antioxidants increases the antioxidant capacity of the plasma and reduces the risk of chronic diseases related to aging such as cardiovascular disease (CVD), coronary heart disease (CHD), stroke, cancer, and neurodegenerative disease (Kris-Etherton et al., 2002). Fresh fruit are especially rich in natural

antioxidants and these compounds can reduce oxidative damage *in vivo*, which would otherwise increase the risk of chronic diseases (Liu et al., 2000). Alkaloids, phenolics, carotenoids, and several nitrogenous compounds, in fruit and vegetables, are reported to account for a range of biological roles, e.g. antioxidant, antiproliferation, antifungal, antibacterial and antiviral activities (Abeysinghe et al., 2007).

Satsuma mandarin (*Citrus unshiu* Marc.) is an important citrus commodity, consumed mostly as fresh produce because of its nutritional value and flavor. The health benefits of Satsuma fruit have mainly been attributed to the presence of bioactive compounds such as phenolics, ascorbic acid and carotenoids (Xu et al., 2007). However, mandarin fruit are highly perishable with a potential storage life of 2-4 weeks (Kader, 2002) and there is an extensive need to develop advanced postharvest treatments to maintain high quality fruit during the storage and marketing period. This need has led to the development of alternative non chemical methods to control postharvest disease and decay.

Among the non-chemical technologies reported to be effective in suppressing postharvest diseases is the use of the "inductive natural plant defense" method such as *trans*-resveratrol (3, 4', 5-trihydroxystilbene) which is responsible for resistance of grapevines to fungal diseases (Gonzalez et al., 2003). Postharvest resveratrol application has attracted recent interest as a promising alternative to replace or reduce the use of toxic chemicals during storage (Jimenez et al., 2005). Prior investigations have revealed that exogenous application of *trans*-resveratrol ($1.6x10^{-4}$M) to apples, grapes and tomatoes maintained the postharvest quality, sensory and nutritional value of those fruit when compared to non-treated control fruit (Jimenez et al., 2005). The use of resveratrol as a natural antibiotic to reduce microbial contamination in fruit may be very advantageous, as it could also be used during postharvest, up to delivery

to the consumers, without risks to human health, but with potential benefits as added value to the treated food (Jimenez et al., 2005).

Maintaining fruit quality and nutrition is critical to Satsuma mandarin for extended periods of time. However, information is scarce regarding the beneficial effects of resveratrol treatment on citrus as an efficient postharvest treatment to improve its storage stability. The objective of this study was to determine the effect of *trans*-resveratrol application on total phenolics, total flavonoids, ascorbic acid and carotenoid content of Satsuma mandarin during postharvest storage.

Materials and Methods

Fruit source & Treatment

Satsuma mandarin fruit were harvested from fully mature mandarin trees budded onto *Poncirus trifoliate* rootstock and grown at Gulf Coast Research Extension Center in Fairhope, AL. When samples reached 10:1 soluble solids to titratable acidity ratio, fruit were randomly harvested and samples were washed, graded and damaged fruit were discarded. Selected fruit were randomly grouped into five treatment lots of 10 boxes each (each containing 50 fruit). Fruit were treated with either varying concentrations of *trans*-Resveratrol ($1.6x10^{-3}$M, $1.6x10^{-4}$M and $1.6x10^{-5}$M) (Sigma Chemical Co., St. Louis, MO), tap water or mixture of wax (StaFresh 4201, FMC technologies, Riverside, CA) and 1% Imazalil (IMZ) (Freshgard 700, FMC technologies, Riverside, CA) by submerging fruit for 5 seconds and stored for a duration of 12 weeks at 10^{o}C and 95% RH. 100 fruit from each treatment were randomly sampled, peeled and separated at the end of 0, 4, 8, and 12 weeks of storage. Fruit segments and peels were stored separately at -80^{o}C for further analysis.

Chemicals

All chemical reagents, solvents or standards were either purchased from Sigma Chemical Co. (St. Louis, MO) or Fischer Scientific (Fischer Scientific, Raleigh, NC) and were either of high performance liquid chromatography (HPLC) or analytical grade quality. Solvents included methanol, acetone, o-phosphoric acid, acetic acid and hydrochloric acid. For assays to determine total phenolics and total flavonoids, Folin–Ciocalteu's Reagent (FCR), sodium carbonate, sodium nitrite, aluminum chloride as well as standards, gallic acid and catechin were bought from Sigma Chemical Co. Sodium hydroxide (A.C.S. certified grade) was purchased from Fisher Scientific. For determination of ascorbic acid, dithiothreitol (DTT) N-ethylmaleimide, α,α'-dipyridyl and Trichloro acetic acid (TCA) were purchased from Sigma Chemical Co. For carotenoid analysis, Diethyl Dithio-Carbamate (DDC) and Butylated hydroxytolulene (BHT) were purchased from Sigma-Aldrich. Ultrapure or Milli-Q water (18.2 MΩ cm^2) was used throughout this study and was obtained through a Millipore Direct -$Q^{TM.}$ 5 filter system (Millipore Corp., Bedford, MA).

Extraction preparation

All sample operations were performed under amber fluorescent lighting conditions (GE F40/ G0, 40W, General Electric Co.) and care was taken to exclude any direct exposure to sunlight. The phenolic compounds were extracted according to the method by Abeysinghe et al. (2007) with slight modifications. 30 g of frozen fruit tissue was homogenized in 20 ml of extraction solvent (1.2 M HCl in 80% methanol/water) in a Virtis Shear homogenizer (Cyclone Virtishear) at a speed of 70 for 1 min. The homogenate was transferred into a 50 ml Oak Ridge Centrifuge tube (Nalge Nunc International Corporation, Rochester, NY). The samples were sonicated

for 15 min (Branson, model 5510 Branson Ultrasonic Corporation, Danbury, CT). Sonicated samples were centrifuged (Beckman Centrifuge, model J2-21, San Antonio, TX) at 13,000 rpm for 15 min at 4^{o}C, then filtered with Miracloth (Calbiochem, La Jolla, CA) and diluted to a final volume of 40 ml. Samples were stored in a -80^{o}C freezer until analyzed.

Determination of total phenolics (TP)

Total phenolic content of fruit extracts was determined spectrophotometrically by the Folin–Ciocalteu method (Singleton and Rossi, 1965). Appropriately diluted methanolic extracts (200µl) or gallic acid standard solutions were mixed with 2.6 ml of Milli-Q water. A standard curve was generated by constructing four different concentrations of gallic acid (30, 60, 90 and 120 mg/L). A blank was prepared using Milli-Q water instead of sample. Subsequently, 200 µl of Folin–Ciocalteu's Reagent (FCR-1:10 dilution with Milli-Q water) was mixed with methanolic sample, standard or blank. The reaction mixture was allowed to stand at room temperature for 6 min to permit FCR to react completely with oxidizable substrates. Following incubation, 2.0 ml of 7% Na_2CO_3 solution was added to each mixture and incubated at 40^{o}C for 60 min. Absorbance was measured at 750 nm using a microplate reader. Results are expressed as mg gallic acid equivalent (GAE) per 100 g fresh weight based on four replications per sample or standard.

Determination of total flavonoids (TF)

TF content of the samples was measured by the colorimetric method of Zishen et al. (1999). Properly diluted methanolic fruit extracts or catechin standard solutions (0.5 ml) were added to a test tube containing 2 ml Milli-Q water. After addition of

0.15 ml 5% $NaNO_2$ at zero min, followed by the 0.15 ml 10% $AlCl_3$, the reaction was initiated by adding 1 ml of 1M NaOH a minute later. Generation of a standard curve was achieved by constructing four different concentrations of catechin (10, 30, 60, and 100 mg/L). A blank was prepared using Milli-Q water instead of sample. The solution was vortexed for 30 sec and absorbance was determined at 510 nm using a microplate reader. Results are expressed as mg catechin equivalent (CE) per 100 g fresh weight based on four replications per sample or standard.

Extraction and determination of ascorbate (AsA)

Vitamin C was extracted and determined according to a procedure reported by Gossett et al. (1994). 20 g of fruit tissue was homogenized in 30 ml of pre chilled extraction buffer (30 g m-Phosphoric acid, 0.5 g EDTA and 80 ml glacial acetic acid diluted to 1L with Milli–Q water. The homogenate was centrifuged at 13,000 RPM for 15 min (4^{o}C) and then filtered through four layers of Miracloth. The supernatant was used for assay of AsA and total ascorbate. Total ascorbate was determined in a reaction mixture consisting 200 µl of supernatant, 500 µl of 150 mM KH_2PO_4 buffer (pH 7.4) containing 5mM EDTA and 100 µl of 10 mM dithiothreitol (DTT) to reduce DHAsA (dehydroascorbic acid) to AsA (ascorbic acid). After 10 min at room temperature, 100 µl of 0.5% N-ethylmaleimide is added to remove excess DTT. AsA was determined similarly except that 200 µl of Milli–Q water was substituted for DTT and N-ethylmaleimide. Color developed in both series of reaction mixtures with the addition of 400 µl of 10% TCA, 400 µl of 44% O-phosphoric acid, 400 µl of α,α'-dipyridyl in 70% ethanol and 200 µl of 3% ferric chloride. The final mixture was incubated at 37^{o}C for 60 min and absorbance of the colored solution was recorded at

525 nm. DHAA concentration was estimated from the difference of "total ascorbate" and "ascorbate" concentrations. A standard curve in the range 0-100 μl of ascorbate was used.

Extraction and determination of total carotenoids in fruit segment

Total carotenoids in citrus segments were measured using the method of Talcott and Howard (1999) was used. Total carotenoids were extracted (20 g/25 mL) with a solution of acetone/ethanol (1:1) containing 200 mgL^{-1} butylated hydroxytolulene (BHT). Samples were extracted using a Virtis Shear homogenizer at a speed of 70 rpm for 1 min. The homogenate was centrifuged at 13,500 rpm for 15 min at 4°C and filtered through four layers of Miracloth. The supernatant was collected and the remaining residue was re-extracted until the residue was colorless. Finally, all extracts were combined and brought to final volume 40ml. The absorbance was measured at 470 nm using a microplate reader. Total carotenoids were calculated using the equation

$$\frac{A \cdot V \cdot 10^6}{A^{1\%} \cdot 100 \cdot G}$$

where A is the absorbance at 470 nm, V is the total volume of extract, $A^{1\%}$ is the extinction coefficient for a mixture of solvents arbitrarily set at 2500, and G is the sample weight in grams (Talcott and Howard, 1999).

Extraction and determination of total carotenoid from peel

Extraction and determination of fruit pigments were performed according to Barry and van Wyk (2006). 10 g of rind samples (flavedo tissue) taken from each treatment were immersed in liquid nitrogen and finely ground in a pestle and mortar.

A 0.5 g sub sample of finely powdered flavedo tissue was added to 10mL of 80% acetone containing BHT (100 mg $L^{-1)}$ and Diethyl Dithio-Carbamate (DDC) (200 mg L^{-1}) antioxidants to prevent carotenoid degradation. Samples were vortexed for two 1 min bursts and stored at 4 °C to extract pigments for 1.5 h. The homogenate was centrifuged at 13,500 rpm for 15 min at 4°C and filtered through four layers of Miracloth. The absorbance was measured at 470, 649 and 664 nm using a microplate reader. Absorbance values were used to calculate total carotenoid concentrations (as $mg.g^{-1}$) according to Lichtenthaler (Lichtenthaler, 1987)**.**

$$C_a = (12.5 * A_{663}) - (2.79 * A_{646})$$

$$C_b = (21.5 * A_{646}) - (5.1 * A_{663})$$

$$C_{x+c} = \left(\frac{1000 \cdot A_{470} - 3.27C_a - 104 \cdot C_b}{229} \right)$$

Statistical analysis

Statistical analyses were conducted using The SAS System for Windows V9.1 (SAS Institute, Inc., Cary, N.C., 2004-2005) and Stata 10 (Stata Corp., College Station, Texas). Analysis of variance and protected least significance difference (LSD) test were performed via One-way Analysis of Variance (ANOVA) to identify differences among means, while Pearson Correlation test was conducted to determine correlations among the means. Statistical significance was determined at $P \leq 0.05$.

Results and Discussion

Total Phenolics (TPH)

Total phenolic content was expressed as mg of gallic acid equiv/100 g of fresh weight of the edible part of the fruit. The mean total phenolic content of Satsuma fruit

per 100 g was 41.388 mg GAE. The changes in TPH values of Satsuma samples over a 12 week period are shown in Fig 5.A. Among all fruit analyzed, those treated with resveratrol $1.6x10^{-5}$M had a significant effect on TPH followed by $1.6x10^{-3}$M and $1.6x10^{-4}$M ($p< 0.002$) with mean values of 42.51, 41.92 and 41.37 mg GAE/100g FW, respectively (Table 6). The TPH content in resveratrol treated fruit remained the same as initial values without much variation until week 8. However, after week 8, there was a significant decline ($p<0.001$) in TPH concentration in the fruit segments.

Results reported in the present study were lower than that of Sun et al. (2002) (56.8 mg/100 g), less than half the content reported by Xu et al. (2008) (1109.23 mg/L GAE) and Abeysinghe et al. (2007) (184 CAE/100 g). This discrepancy in reported TPH content could be due to several factors such as environmental conditions, maturity at harvest, genetic differences, different extraction and laboratory methods employed and tissue type (whole fruit vs. puree or juice extract). The over estimated values of TPH content in crude sample preparations may be due to non-phenolic reducing compounds such as sugars, organic acids, proteins and pigments reacting with FCR in fruit extract (Cho et al., 2005, Kim et al., 2002). In such cases, TPH values may be masked by interfering reductive substances, which act in either inhibitory, additive or augmenting manner (Singleton et al., 1999). Elevated relative concentrations of sugars greater than 2% in combination with high temperatures may produce unreliable results (Waterhouse, 2005). In addition, under acidic conditions (pH 3), ascorbic acid is reactive with polyphosphotungstan in FCR resulting in the appearance of blue color generated prior to the addition of alkali reagent in the assay (Kao, 2006). In such instances, solid phase extraction (SPE) technique is commonly used to fractionate and remove undesired components from bioactive compounds of interest (Robbin, 2003). For example, a four fold over estimated value of TPH content

in crude non-fractionated extracts of citrus fruit peels has previously been reported (Smith and Hossain, 2006). In the lemon cultivar 'Meyer', a TPH value of 156.37 GAE/ 100g FW of crude non-fractionated lemon peel extract and 37.47 GAE/100 g FW of fractionated lemon peel extracts were reported, respectively. Hence, further purification or fractionation may be required to obtain a more accurate approximation of TPH content in fruit extracts.

Total Flavonoids (TF)

Total flavonoid contents of Satsuma fruit after postharvest treatments is presented in Fig.5 B. Flavonoid contents are expressed as mg of catechin equiv/100 g of fresh weight of the edible part of the fruit. The content of total flavonoids in 100 g fresh fruit was at the level of 5.05 mg CE. The flavonoid content among the five postharvest treatments was not significantly different (p>0.1780).

Limited information is available with regards to Satsuma mandarin flavonoid composition. However, flavanone is the major flavonoid in oranges. Among flavanones, hesperidin was the most abundant followed by naringin and neohesperedin (Wang et al., 2006). Results reported in the present study were lower than that reported by Abeysinghe et al. (2007) (47.0 mg RE/100 g FW) and Wang et al. (2006) (11.2 mg/g db). It should be noted that this variation could be due to some factors such as different citrus variety, maturity, material preparation and analyzing methods (Xu et al., 2007). It could also be due to expression of flavonoids on a dry weight basis as by Wang et al. (2006). In addition, the selection of standards utilized may contribute to varied results of others and those reported within the present study.

Ascorbic Acid (AsA)

The effect of *trans*-Resveratrol application on ascorbic acid content of mandarin fruit segments was also investigated and shown in Fig. 6 A-C. The mean L-ascorbic acid content content of Satsuma fruit per 100 g was 18.70 mg/100g FW. There were significant differences (p<0.033) among the treatments. Fruit treated with Wax+IMZ had the highest mean value of ascorbic acid (20.18 mg/100g FW) whereas, resveratrol $1.6x10^{-4}$M and $1.6x10^{-5}$M applications played an important role in maintaining uniform levels of vitamin C when compared to the initial value (Fig. 6 A). The mean of DHAA content for the five postharvest treatments was 12.95 mg/100g FW (Fig. 6B). The treatments had no effect on DHAA (p<0.5218). Total vitamin C content is the sum of ascorbic acid (AsA) and dehydroascorbic acid (DHAA). The mean of vitamin C in mandarins is 33.13 mg/100g FW.

Results reported in the present study were in range with those reported by Lee et al., (2000) (24-38 mg/100g FW). According to Lee and Kader (2000) light intensity during the growing season is one of the most important factors affecting vitamin C content. Satsumas grown under cool weather conditions (20-22^{o}C day, 11-13^{o}C night) had a higher vitamin C content than Satsuma fruit grown under warmer conditions (30-35^{o}C day, 20-25^{o}C night) (Lee et al., 2000). The loss of vitamin C can also be minimized by careful postharvest handling as losses are enhanced by extended storage, higher temperatures, low relative humidity, physical damage, and chilling injury (Lee and Kader, 2000).

Total Carotenoid Content in fruit segments (TCS)

The effect of *trans*-Resveratrol application on total carotenoid content of mandarin fruit segments is shown in Fig 5 D. The content of total carotenoids in fruit

segments was 4.36 mg per 100 g fresh Satsuma fruit. In all treatments, total carotenoid content increased by the end of 4 weeks of storage and thereafter it decreased. There were significant differences among the treatments (p<0.0001), with resveratrol 1.6x10^{-5}M retaining the highest amount of carotenoids (4.927 mg/100 g FW) with only a 6 % decline compared to the initial amount followed by Wax+IMZ which had approximately an 18% decline.

Total Carotenoid Content in fruit peel (TCP)

The effect of *trans*-Resveratrol application on total carotenoid content of mandarin fruit peels is shown in Fig 5 C. The total carotenoid content in fruit peels was 0.842 mg per g fresh weight (β-carotene equivalents). There was a significant effect (p<0.0001) between the treatments with resveratrol 1.6x10^{-4}M and 1.6x10^{-5}M retaining the highest amount of total carotenoid content in peels.

Conclusion

This study describes a possible application of the natural molecule resveratrol to maintain nutritional quality during postharvest storage. Results from this study indicated that resveratrol treatment at 1.6x10^{-5}M not only improved shelf life of fruit but also enhanced nutritional quality. Therefore, use of resveratrol as a natural fungicide offers a new simple and safe method to improve shelf life and postharvest quality of fruit.

V. POSTHARVEST TRANS-RESVERATROL APPLICATION ON THE ANTIOXIDANT ACTIVITY OF SATSUMA MANDARIN

Abstract

Satsuma mandarins (*Citrus unshiu* Marc., cv. Owari) were treated with *trans*-resveratrol ($1.6x10^{-3}$M, $1.6x10^{-4}$M, $1.6x10^{-5}$M), mixture of wax and Imazalil or tap water and were subsequently stored at 10^{o}C and 95% RH for up to 12 weeks. They were analyzed to determine the effect of *trans*-resveratrol on free radical scavenging activity as determined by ABTS, DPPH, FRAP and ORAC. The antioxidant capacity determined by the ABTS and DPPH methods ranged from 257.02 to 314.80 µmol TE/100g fw and 478.66 to 551.11 µmol TE/100g fw, FRAP from 519.73 to 607.62 µmol TE/100g fw and ORAC from 20.75 to 20.85 µmol TE/100g fw at the end of storage. Results from this study also indicated that vitamin C contributed significantly to the overall antioxidant capacities of Alabama grown Satsuma mandarins.

Keywords: *trans*- Resveratrol, Satsuma, mandarin, ABTS, DPPH, FRAP, ORAC.

Introduction

An antioxidant is defined as 'any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate' (Halliwell, Aeschbach et al. 1995).

Antioxidants are of significance, because they maintain cellular homeostasis, structure and function and protect against damaging effects of reactive oxygen species. Natural antioxidants, particularly in fruits and vegetables have gained interest among consumers and the scientific community because various epidemiological studies have indicated consumption of fresh fruits and vegetables is associated with a lower risk of cardiovascular disease and cancer (Temple 2000). The defensive role of natural antioxidants in fruits and vegetables is associated with three major groups: vitamins, phenolics, and carotenoids. Ascorbic acid, oxidized glutathione (GSSG) and reduced glutathione (GSH) are known as hydrophilic antioxidants, while carotenoids, phenolics and vitamin E are known as lipophilic antioxidants (Halliwell 1996).

Satsuma mandarin (*Citrus unshiu* Marc.) is a nutritious citrus fruit rich in vitamin C, phenolic acids, flavonoids and carotenoids (Xu, et al. 2007). Some studies have investigated the antioxidant capacity of citrus fruit, and it was assumed that total antioxidant capacity of citrus fruit were mainly attributed to AA and phenolic compounds, though there were some divergences as to which compound was the major contributor (Xu, et al. 2007). It is known that much of the nutritional value as well as the sensory quality of mandarin fruit can be lost as a result of current postharvest handling practices, which were developed primarily to maintain acceptable visual appearance with less regard to other quality aspects such as flavor and nutritional value. Despite numerous advances achieved in recent years in postharvest biotechnology in ameliorating commodity losses associated with postharvest decay, this aspect perhaps is the most important feature which defines commodity quality, value and consumer demand. Increased public awareness regarding the use of toxic fungicides has stimulated the need

for developing alternative non-chemical technologies reported to be effective in suppression of postharvest decay (Jimenez, Orea et al. 2005). Prior investigations have revealed that exogenous application of *trans*-resveratrol ($1.6x10^{-4}$M) to apples, grapes and tomatoes maintained their postharvest quality, sensory and nutritional value when compared to non treated control fruit (Jimenez, Orea et al. 2005).

To date, there has been little research investigating the effect of post-harvest resveratrol treatment on the antioxidant properties of citrus fruit. In the present study, Satsuma fruit treated with different concentrations of resveratrol, tap water or a mixture of wax and Imazalil were evaluated for their free radical scavenging activities. 2,2'-azino-bis(3-ethylbenzothiazoline-6 sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and the oxygen radical absorption capacity (ORAC) assays were employed to estimate antioxidant activities and were correlated with ascorbic acid, total phenolics, total flavonoids, and total carotenoids contents in fruit extracts.

Materials and Methods

Fruit source

Satsuma mandarin (*Citrus unshiu* Marc. Cv. "Owari") fruit were harvested from fully mature Satsuma mandarin trees budded onto *Poncirus trifoliate* rootstock and grown at the Gulf Coast Research Extension Centre in Fairhope, AL. After fruit reach 10:1 soluble solids to titratable acidity ratio, fruit are randomly picked. Fruit samples are washed and graded.

Sample preparation

Damaged and irregular fruit were discarded, and selected fruit were randomly divided into five treatment lots of 10 boxes each (each containing 50 fruit). The fruit were treated with varying concentrations of *trans*-Resveratrol ($1.6x10^{-3}$M, $1.6x10^{-4}$M and $1.6x10^{-5}$M), tap water or mixture of wax (StaFresh 4201, FMC technologies, Riverside, CA) and 1% Imazalil (IMZ) (Freshgard 700, FMC technologies, Riverside, CA) by submerging for 5 seconds and stored for a duration of 12 weeks at 10^{o}C and 95% RH. 100 fruit from each treatment were randomly sampled, peeled and separated at the end of 0, 4, 8, and 12 weeks of storage. The segments and peels were stored separately at -80^{o}C for further analysis.

Chemicals

2, 2'- azino-bis (3-ethylbenzothiazoline-6 sulfonic acid; ABTS) as diammonium salt, 2, 2'-azobis (2–amidinopropane) dihydrochloride (AAPH) and 1,1–diphenyl-2-picrylhydrazyl (DPPH) were obtained from Wako Chemicals USA Inc. (Richmond, VA, USA). All other chemical reagents, solvents or standards were either purchased from Sigma/ Aldrich Chemical Co. (St. Louis, MO), or Fischer Scientific (Fischer Scientific, Raleigh, NC) and were either high–performance liquid chromatography (HPLC) or analytical grade quality. Ultrapure Milli–Q water was used throughout this study and had electrical conductivity of 18.2 MΩ cm^2 obtained through a Millipore Direct -$Q^{TM.}$ 5 filter system (Millipore Corp., Bedford, MA).

Antioxidant activity determinations

Radical cation $ABTS^{\bullet+}$ scavenging activity

The method developed by Kim et al., (2004) was employed to evaluate the antioxidant capacity for the various polyphenolics. Briefly, 2.5 mM of ABTS [2, 2'-azino-bis (3- ethylbenzothiazoline-6 sulfonic acid) diammonium salt] was mixed with 1.0 mM of AAPH [2, 2'–azobis (2–amidinopropane) dihydrochloride] in 100 ml phosphate buffered saline (PBS) solution [100mM potassium phosphate buffer containing 150 mM NaCl adjusted to pH 7.4]. The ABTS radicals were generated by heating the mixture in a water bath at 68^{o}C for one hour with frequent agitation and filtered at reduced pressure through ZAPCAP®-CR filter unit (Whatman Inc., Florham Park, NJ). The concentration of the resulting blue-green ABTS radical solution was adjusted with fresh PBS to an absorbance of 0.650 ± 0.020 at 734 nm to 37^{o}C. 5 µl of sample solution or blank (80% HPLC grade methanol) was mixed with 225 µl of ABTS solution. The samples or standards were read every minute for 30 min in a microplate reader at 734 nm at 37^{o}C. The ability of antioxidants to decolorize (scavenge) the $ABTS^{\bullet-}$ radical was then determined. The standard curve was linear between 50-750 µM Trolox. Results are expressed in µM Trolox equivalents (TE)/100g fresh mass. Additional dilution was needed if the ABTS value measured was over the linear range of the standard curve.

Radical $DPPH^{\bullet}$ scavenging activity

Antioxidant radical scavenging activity was measured according to the method outlined by Kim et al., (2002) with slight modifications. Briefly, 100µM 2,2-Diphenyl-1-

picrylhydrazyl ($DPPH^{\bullet}$) was prepared in 200 ml of 80% HPLC grade methanol. The radical solution was stirred at room temperature for 20 min and the adjusted to 0.650 ± 0.020 absorbance value at 517 nm. The standard curve was linear between 50-750 μM Trolox. To determine the antioxidant capacity, 6.67 μl of clarified methanolic extract, blank, or L-ascorbic acid standard were mixed with 193.33 μl of DPPH radical solution. The decrease in absorbance of the resulting solution was measured at 517 nm for 30 min in a microplate reader. Results are expressed in μM Trolox equivalents (TE)/100g fresh mass. Additional dilution was needed if the DPPH value measured was over the linear range of the standard curve.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed according to Benzie and Strain (1996) with slight modifications. This assay is based on the reducing power of a compound (antioxidant). A potential antioxidant will reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}); the latter forms a blue complex (Fe^{2+}/TPTZ), which increases the absorption at 593nm (Benzie and Strain 1996). The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 ml glacial acetic acid), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 50 ml acetate buffer, 5 ml TPTZ solution and 5 ml $FeCl_3 \cdot 6H_2O$ solution and then warmed at 37°C before use. Fruit extracts (15 μl) were allowed to react with 285 μl of the FRAP solution for 30 min in the dark. Readings of the colored product (ferrous tripridyltriazine complex) were then taken at 593 nm. The standard curve was linear

between 50-750 μM Trolox. Results are expressed in μM Trolox equivalents (TE)/100g fresh mass.

Oxygen Radical Absorbance Capacity (ORAC) assay

The oxygen radical absorbance capacity assay (ORAC) was performed as described by Huang (Huang et al. 2002). It determines the peroxyl radical scavenging capacity of samples. In the reaction, antioxidants compete with the molecular probe fluorescein (FL) for peroxyl radical and are degraded by peroxyl radicals generated by the thermal decomposition of an azo compound (AAPH). This antioxidant scavenging of peroxyl radicals spares the FL from degradation which decreases its fluorescence. This scavenging capacity is calculated using the area under the curve (AUC). Results are expressed as trolox equivalents with the units of μmoles trolox per 100g FW. Briefly, AAPH (0.414g) was dissolved in 10ml of 75mN phosphate buffer (pH 7.4) to a final concentration of 153 mM and made fresh daily. A fluorescein stock solution (4×10^{-3} mM) was made in 75 mM phosphate buffer (ph 7.4) and stored wrapped in foil at 5°C. Immediately prior to use, the stock solution was diluted 1:1000 with 75 mM phosphate buffer (ph 7.4). The diluted sodium fluorescein solution was made fresh daily. In regards to the plate usage, the exterior wells were not used for experimental determinations. These wells were filled with 300 μl of water, while the interior wells were used for experimental determinations. To all experimental wells, 150 μl of working sodium fluorescein solution was added. In addition blank wells received 25 μl of 75 mM phosphate buffer (pH 7.4), while standards received 25 μl of Trolox dilution and samples received 25 μl of sample. The plate was then allowed to equilibrate by incubating for a

minimum of 30 minutes in the Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT) at 37°C. Reactions were initiated by the addition of 25 µl of AAPH solution using the microplate reader's injector for a final reaction volume of 200 µl. The fluorescence was then monitored kinetically with data taken every minute. Reactions were initiated by the addition of 25 µl of AAPH reagent (153 mM) followed by shaking at maximum intensity for 10 seconds. The fluorescence of each well was then measured every 60 seconds at a sensitivity setting of 35. (Cao and Prior 1999). The standard curve was linear between 0 and 50 µM Trolox. Results are expressed as µM TE/100g fresh mass. ORAC values were calculated as described by Cao and Prior (1999).

$$AUC=0.5+(R2/R1)+(R3/R1)+(R4/R1)+\ldots\ldots+0.5(Rn/R1) \qquad \text{(Eq.1)}$$

Where R1 is the fluorescence reading at the initiation of the reaction and Rn is the last measurement.

$$\text{Net AUC} = AUC_{sample} - AUC_{blank} \qquad \text{(Eq. 2.)}$$

The standard curve was obtained by plotting the Net AUC of different trolox concentrations against their concentration.

Statistical analysis

Statistical analyses were conducted using The SAS System for Windows V9.1 (SAS Institute, Inc., Cary, N.C., 2004-2005) and Stata 10 (Stata Corp., College Station, Texas). Analysis of variance and protected least significance difference (LSD) test were performed via One-way Analysis of Variance (ANOVA) to identify differences among

means, while Pearson Correlation test was conducted to determine correlations among the means. Statistical significance was determined at $P \leq 0.05$.

Results and discussion

Radical cation $ABTS^{\cdot+}$ scavenging activity

The effect of *trans*-resveratrol application on Satsuma mandarin antioxidative properties is shown in figure 7. In general, significant variation in antioxidant activities (Pr>F=0.0001) as quantified by ABTS assay were noted during 12 weeks of storage (figure 7 A). These changes occurred rapidly initially in fruit treated with Wax+IMZ which maintained the highest antioxidant activity 310.13 µmol TE/ 100g fw, with intermediate values for control treated (278.36 µmol TE/100g fw), $1.6x10^{-3}$M, (272.36 µmol TE/100g fw), $1.6x10^{-5}$M (259.24 µmol TE/100g fw), and $1.6x10^{-4}$M *trans*-resveratrol the least effective (242.80 µmol TE/100g fw). With continued storage, all treatments either increased or decreased with time. Initially the $1.6x10^{-4}$M treatment was the least effective in maintaining antioxidant activity however, at the end of the 12 week storage this treatment was the most significant effective treatment in sustaining overall fruit cellular antioxidant activity when compared to all other treatments.

Radical $DPPH^{\bullet}$ scavenging activity

During 12 weeks of storage significant changes (Pr>F=0.0008) in antioxidant radical scavenging capacities were observed among treatments (Fig. 7B). The antioxidant radical scavenging capacity was similar for all treatments at the beginning of storage and during the first four weeks. Thereafter, all treatments declined with respect to their initial

DPPH values within the initial four weeks. By the eighth week of storage, both control and fruit receiving postharvest applications of $1.6x10^{-3}$M *trans*-resveratrol retained significantly higher scavenging abilities when compared to other treatments. Applications of $1.6x10^{-5}$M and $1.6x10^{-4}$M *trans*-resveratrol at the end of storage, had the highest radical scavenging capacities (551.11 and 540.44 µmol TE/100g fw) respectively followed by control (512.44 µmol TE/100g fw), Wax+IMZ (479.1144 µmol TE/100g fw) and $1.6x10^{-3}$M (478.66 µmol TE/100g fw) *trans*-resveratrol, respectively.

Antioxidant capacity as measured by the DPPH radical scavenging assay was higher than that of ABTS radical scavenge assay by approximately 45%. The ABTS radical method is an excellent tool for determining the ability of antioxidants to quench free radicals both in aqueous and organic phase (Kim et al., 2002; Leong et al., 2002). High free radical quenching activities associated with DPPH radicals indicates the antioxidants were more lipid soluble and had preference toward the lipid peroxyl radicals. As a result, the antioxidant scavenging capacities as determined via the DPPH radical scavenging method was higher than that of the ABTS radical scavenging method.

Treatments that had high antioxidant capacities in the DPPH radical scavenging assay also had high antioxidant capacities in the ABTS radical scavenge assay (Table 8). This correlation might result partially from a similar mechanism of antioxidants (hydrogen donating ability to free radicals) and also a similar solubility in aqueous/ethanol systems (Leong et al., 2002).

Ferric Reducing Antioxidant Power (FRAP) Assay

The antioxidant activity as assessed by FRAP was significantly (Pr>F=0.0001) affected by treatment during 12 weeks of storage (Fig. 7C). Initial FRAP values were highest in control (618.06 µmol TE/gfw) and Wax+IMZ treatments (616.06 µmol TE/100g fw) and lowest in $1.6x10^{-3}$ M (518.95 µmol TE/100g fw), $1.6x10^{-4}$ M (513.84 µmol TE/100g fw) and $1.6x10^{-5}$M (540.28 µmol TE/100g fw) *trans*-resveratrol respectively. Following four weeks of storage the trend in FRAP values varied significantly with respect to postharvest treatments. Although FRAP values declined in control fruit, this treatment remained the highest (570.17 µmol TE/100g fw) during the fourth week of storage along with an observed increase in FRAP values in fruit treated with $1.6x10^{-3}$M *trans*-resveratrol (575.28 µmol TE/100g fw) followed by Wax+IMZ (562.28 µmol TE/100g fw), $1.6x10^{-5}$M (545.17 µmol TE/100g fw) and $1.6x10^{-4}$M (468.17 µmol TE/100g fw) *trans*-resveratrol respectively. During week 8, Wax+IMZ treated fruit increased significantly in FRAP value (616.73 µmol TE/100g fw) and was the highest when compared to all other treatments. However, by the end of 12 weeks of storage fruit treated with $1.6x10^{-4}$ M *trans*-resveratrol maintained significantly higher FRAP values (607.62 µmol TE/100g fw).

Oxygen Radical Absorbance Capacity (ORAC) assay

In general, the antioxidant capacity of all Satsuma mandarin fruit extract values expressed as ORAC increased significantly (Pr>=0.0001) during storage in response to treatments (Fig. 7 D). However, treatment by week interaction was not significant. Initial ORAC values were equally highest in fruit receiving applications of $1.6x10^{-3}$M (20.71

μmol TE/100g fw) and $1.6x10^{-5}$M (20.70 μmol TE/100g fw) followed by control (20.63 μmol TE/100g fw) and Wax+IMZ (20.64 μmol TE/100g fw) with the lowest ORAC value for $1.6x10^{-4}$M (20.56 μmol TE/100g fw) *trans*-resveratrol, respectively. During the fourth week of storage the antioxidant capacity was not influenced by postharvest treatments. However, following four weeks of storage all treatments increased. During week 8 of storage, discernable differences in ORAC values were noted in response to treatments. Satsuma fruit treated with $1.6x10^{-5}$M (20.88 μmol TE/100g fw) *trans*-resveratrol were highest, followed by control (20.86 μmol TE/100g fw) intermediate for $1.6x10^{-3}$ M (20.83 μmol TE/100g fw) and Wax+IMZ (20.81 μmol TE/100g fw) and lowest for $1.6x10^{-4}$M (20.79 μmol TE/100g fw) *trans*-resveratrol respectively. By the end of storage control (20.85 μmol TE/100g fw) and Wax+IMZ (20.84 μmol TE/100g fw) treated fruit had the highest ORAC values with intermediate values for $1.6x10^{-3}$M (20.80 μmol TE/100g fw) and $1.6x10^{-4}$M (20.83 μmol TE/100g fw) and lowest for $1.6x10^{-5}$M *trans*-resveratrol, respectively.

Conclusion

This study describes a possible application of the natural molecule resveratrol to maintain nutritional quality during postharvest storage. In general *trans*-resveratrol application significantly maintained antioxidant capacities (ABTS, DPPH and FRAP) during a 12 week storage period with the exception noted for ORAC assay. Therefore, use of resveratrol as a natural fungicide offers a new simple and safe method to improve shelf life and postharvest quality of fruit.

CONCLUSIONS

Results from our study demonstrate that *trans*-resveratrol application could potentially serve as a postharvest conservation method in extending shelf-life and maintaining the nutritional quality of Satsuma mandarin during 12 weeks of storage. In general *trans*-resveratrol application significantly maintained antioxidant content (Total Phenolic, Total Flavonoid, Vitamin C, and Total Carotenoid content) and antioxidant capacities (ABTS, DPPH and FRAP) during a 12 week storage period with the exception noted for ORAC assay. The overall effect of *trans*-resveratrol application was concentration dependent. In most assays the higher *trans*-resveratrol concentration ($1.6x10^{-3}$M) was the least effective in maintaining nutritional quality. Therefore, when considering cost, this concentration is not advisable as a postharvest antimicrobial treatment. As noted by Jimenez (2005), application of *trans*-resveratrol assists in maintaining turgidity and fruit firmness by creating a modified atmosphere surrounding individual fruit tissues. Whether this effect directly alters cellular oxidative metabolism has not been established. Our preliminary findings are the first to report the beneficial impact on antioxidant properties of citrus.

Our ongoing research focuses on the physicochemical and antioxidant properties of *trans*-resveratrol application to Satsuma mandarin. Further research is needed to establish whether appropriate concentrations of *trans*-resveratrol in combination with

conventional wax application could provide a synergistic effect during storage. These studies would compliment other desired research efforts involving antiproliferative and cryoprotective toxicological properties as well as microbial effects of juice extracts obtained from treated citrus fruit and their potential beneficial health effects. The use of cell culture model system would further enhance our understanding of cellular protection afforded by juice extracts when cellular oxidative stress is induced *in vivo*.

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APPENDICES

APPENDIX A: TABLES

Table 1. Nomenclature of all possible reactive oxygen species (ROS). Adapted from Wilcox et al., 2004.

Free Radicals	**Non Radicals**
Superoxide, $O_2^{\cdot-}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\cdot}$	Hypobromous acid, HOBr
Hydrogen peroxyl, $HO_2^{\cdot}$	Hypochlorous acid, HOCl
Peroxyl, $RO_2^{\cdot}$	Ozone, O_3
Alkoxyl, $RO^{\cdot}$	Singlet oxygen, O_2^1
Carbonate, $CO_3^{\cdot-}$	Organic peroxide, ROOH
Carbon dioxide, $CO_2^{\cdot-}$	Peroxy nitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH

Table 2. Fresh citrus- energy and major nutrients (content per 100 g of edible portion)

	Amount in 100 g of edible portion*						
Citrus fruit	Calories (Kcals)	Water (g)	Protein (g)	Total fiber (g)	Total fat (g)	Carbohydrates (g)	Sugars (g)
Orange	47	86.8	0.94	2.4	0.12	11.8	9.4
Grapefruit (white)	33	90.5	0.69	1.1	0.10	8.4	7.3
Grapefruit (pink and red)	42	88.1	0.77	1.6	0.14	10.7	6.9
Tangerine	88.1	85.2	0.80	1.8	0.31	13.3	10.6

* Data source: The USDA Database for Standard Reference Release 17, 2004

Table 3. Fresh citrus fruit – Mineral contents

	Amount in 100 g of edible portion*									
Citrus fruit	Ca (mg)	Fe (mg)	Mg (mg)	P (mg)	K (mg)	Na (mg)	Zn (mg)	Cu (mg)	Mn (mg)	Se (mg)
Orange	40	0.10	10	14	181	0	0.07	0.05	0.03	0.50
Grapefruit (white)	12	0.06	9	8	148	0	0.07	0.05	0.01	1.40
Grapefruit (pink and red)	22	0.08	9	18	135	0	0.07	0.05	0.02	0.10
Tangerine	37	0.15	12	20	166	2	0.07	0.05	0.04	0.10

* Data source: The USDA Database for Standard Reference Release 17, 2004

Table 4. Changes in external color attributes and carotenoid content of satsuma mandarin fruit treated with *trans*-resveratrol ($1.6x10^{-4}$M, $1.6x10^{-5}$M), wax+IMZ and water during storage.

Storage time (weeks)	Treatment	[v]Color attributes						[w]Carotenoid content	
		L*	a*	b*	C*	h*	CI[x]	CP[y]	CS[z]
0	Control	63.342a	25.445a	62.909a	67.90a	68.006a	6.3898b	1.0392a	3.097a
	Wax+IMZ	64.084a	25.652b	63.141a	68.222c	67.920a	6.3535b	0.9238b	2.154c
	$1.6x10^{-4}$M	63.472a	25.987b	62.908a	68.19ab	67.48ab	6.527ab	0.8932c	1.958d
	$1.6x10^{-5}$M	64.177a	28.373b	64.387a	70.38bc	66.348b	6.8988a	0.8515d	2.405b
4	Control	63.643a	30.46ab	61.891a	69.037b	63.78ab	7.7641a	0.9914a	2.9788d
	Wax+IMZ	63.242a	29.45bc	60.132b	67.013b	63.91ab	7.7687a	0.6746d	3.6175b
	$1.6x10^{-4}$M	63.461a	28.893c	60.72ab	67.305a	64.594a	7.5071a	0.8930b	3.2966c
	$1.6x10^{-5}$M	63.611a	31.034a	61.631a	69.029b	63.265b	7.9292a	0.6980c	3.7472a
8	Control	62.338a	32.307a	60.126a	68.275a	61.744a	8.6398a	0.8603a	2.7129b
	Wax+IMZ	62.964a	32.165a	60.500a	68.540c	61.995a	8.4622a	0.7181c	2.8555a
	$1.6x10^{-4}$M	62.695a	32.852a	60.663a	69.00bc	61.570a	8.6453a	0.5556d	2.3673c
	$1.6x10^{-5}$M	62.791a	32.619a	60.949a	69.14ab	61.839a	8.5306a	0.7682b	2.8579a
12	Control	61.265a	34.933a	66.35bc	75.010a	62.217a	8.6211a	0.7018b	1.46 d
	Wax+IMZ	61.752a	34.586a	63.207c	72.087a	61.277b	8.9139a	0.7524a	2.418b
	$1.6x10^{-4}$M	61.837a	34.837a	65.35ab	74.079a	61.92ab	8.6454a	0.6872b	1.668c
	$1.6x10^{-5}$M	61.788a	35.01a	63.865a	72.857a	61.260b	8.8996a	0.7687a	2.635a

L*, lightness; a*, redness and blueness; b*, greenness and yellowness; C*, chroma; h*, hue angle.

[x] Color Index, CI = (1000a*)/L*b*;

[y] Total Carotenoid content in peels, $CP = (1000A_{470}-3.27C_a-104C_b)/229$ expressed in mg/100 g fresh weight.

[z] Total carotenoid content in segments, $CS = (A.V.10^6)/(A^{1\%}.100.G)$ expressed in mg/100 g fresh weight.

Means followed by same letter within columns are not significantly different.

[v,w] Values are means of 25 replicates and 4 replicates, respectively.

Table 5. Pearson's correlation coefficients of external color attributes and carotenoid content in satsuma mandarin fruit treated with *trans*-resveratrol ($1.6x10^{-4}$M, $1.6x10^{-5}$M), wax+IMZ and water during storage.

Variable	L*	a*	b*	C*	h*	CI[x]	CP[y]	CS[z]
L*		-0.66*	-0.05[ns]	-0.43*	0.63*	-0.72*	0.45*	0.30[ns]
a*			0.19[ns]	0.67*	-0.94*	0.95*	-0.52*	-0.16[ns]
b*				0.79*	0.09[ns]	-0.09	0.08[ns]	-0.50*
C*					-0.45*	0.46*	-0.22[ns]	-0.46*
H*						-0.98*	0.55*	-0.05[ns]
CI							-0.56*	-0.05[ns]
CP								0.01[ns]
CS								

L*, lightness, a*, redness and blueness; b*, greenness and yellowness; C*, chroma; h*, hue angle.
[x] Color Index, CI = (1000a*)/L*b*;
[y] Total Carotenoid content in peels, CP = $(1000A_{470}-3.27C_a-104C_b)/229$ expressed in mg/100 g FW.
[z] Total carotenoid content in segments, CS = $(A.V.10^6)/(A^{1\%}.100.G)$ expressed in mg/100 g FW.
* Significant at $p \leq 0.05$.
[ns] non significant
- negative correlation.

Table 6. Changes in total phenolic, total flavonoid, ascorbic acid and total carotenoid contents of satsuma mandarin fruit treated with *trans*-resveratrol ($1.6x10^{-3}$M, $1.6x10^{-4}$M and $1.6x10^{-5}$M), wax+IMZ and water during storage.

Storage time (weeks)	Treatment[a]	TP[b]	TF[c]	AA[d]	TCP[e]	TCS[f]
0	Control	38.904a	5.5704a	20.82ab	0.9781a	5.158a
	Wax+IMZ	39.742a	5.2788a	19.42bc	0.9419a	3.4873c
	$1.6x10^{-3}$	41.997b	5.2911a	25.134a	0.9231a	3.184d
	$1.6x10^{-4}$	43.157b	5.0226a	19.58bc	0.8435b	3.0006e
	$1.6x10^{-5}$	42.126b	4.9383a	15.600c	0.9716a	3.804b
4	Control	40.902c	5.092ab	18.81bc	0.9644a	4.956d
	Wax+IMZ	42.89ab	4.7182b	25.777a	0.7488c	6.496ab
	$1.6x10^{-3}$	41.93bc	5.5534a	14.514d	0.760bc	6.3866b
	$1.6x10^{-4}$	41.224c	4.633b	16.40cd	0.8289b	5.928c
	$1.6x10^{-5}$	43.479a	4.7112b	19.663b	0.8301b	6.592a
8	Control	39.935c	4.9179a	18.013a	0.8213b	4.6046c
	Wax+IMZ	43.543a	5.249a	19.301a	0.6451c	5.0546a
	$1.6x10^{-3}$	43.350a	5.1748a	19.019a	0.9653a	3.828e
	$1.6x10^{-4}$	41.546b	4.8961a	19.1a	0.6486c	4.0193d
	$1.6x10^{-5}$	43.286a	4.9919a	14.996b	0.8680b	4.788b
12	Control	39.8abc	4.875ab	13.870d	0.766bc	2.188b
	Wax+IMZ	38.714c	4.6440b	16.24bc	0.791bc	4.1553a
	$1.6x10^{-3}$	40.40ab	5.106ab	14.19cd	0.640c	2.4526b
	$1.6x10^{-4}$	39.558c	5.2836a	20.507b	1.0000a	2.594b
	$1.6x10^{-5}$	41.181a	5.089ab	23.122a	0.910ab	4.526a

[a] Water treated control, mixture of wax and Imazalil, three concentrations of resveratrol; $1.6x10^{-3}$M, $1.6x10^{-4}$M and $1.6x10^{-5}$M.
[b] Total phenolic (TP) content expressed in mg gallic acid equivalents/ 100 g fresh weight.
[c] Total flavonoid (TF) content expressed in mg catechin equivalents/ 100 g fresh weight.
[d] Ascorbic acid (AA) expressed in mg/100 g fresh weight.
[e], [f] Total carotenoids content expressed in mg/100 g fresh weight in peels (TCP) and segments (TCS), respectively.
Means followed by same letter within columns are not significantly different.

Table 7. Pearson's correlation coefficients of total phenolics, total flavonoids, ascorbic acid, and total carotenoids in satsuma mandarin fruit treated *trans*-resveratrol ($1.6x10^{-3}$M,$1.6x10^{-4}$M, $1.6x10^{-5}$M), wax+IMZ and water during storage.

Variable	TP	TF	AA	TCP	TCS
TP		0.4063*	0.3483*	0.3706*	0.0739 ns
TF			0.0659 ns	0.1231 ns	-0.2872 ns
AA				-0.2256 ns	-0.2772 ns
TCP					0.0179 ns
TCS					

TP= total phenolics, TF = total flavonoids, AA= ascorbic acid, TCP= total carotenoid content measured in peels, TCS= total carotenoid content measured in segments.
* =significant at $p \leq 0.05$
ns =non significant
-negative correlation.

Table 8. Antioxidant activities of satsuma mandarin fruit extracts as determined by the ABTS, DPPH, FRAP and ORAC assays after treatment with *trans*-resveratrol ($1.6x10^{-3}$M $1.6x10^{-4}$M, $1.6x10^{-5}$M), wax+IMZ and water during storage.

Storage time(weeks)	Treatment[a]	Antioxidant activity (µM TE/ 100g FM)			
		ABTS	DPPH	FRAP	ORAC
0	Control	278.36b	509.33b	618.06a	20.63ab
	Wax+IMZ	310.13a	512.44b	616.06a	20.64ab
	$1.6x10^{-3}$M	272.36b	536.44ab	518.95b	20.71a
	$1.6x10^{-4}$M	242.80c	528.00ab	513.84b	20.56b
	$1.6x10^{-5}$M	259.24bc	546.22a	540.28b	20.70a
4	Control	250.36bc	511.55a	570.17a	20.65a
	Wax+IMZ	259.24b	504.88a	562.28b	20.67a
	$1.6x10^{-3}$M	277.69a	502.22a	575.28a	20.68a
	$1.6x10^{-4}$M	239.47c	518.22a	468.17d	20.68a
	$1.6x10^{-5}$M	281.91a	508.88a	545.17c	20.61a
8	Control	243.02d	514.66a	525.51b	20.86ab
	Wax+IMZ	283.47b	497.77ab	616.73a	20.81bc
	$1.6x10^{-3}$M	268.59c	513.33a	487.84c	20.83abc
	$1.6x10^{-4}$M	259.47c	494.22b	491.62c	20.79c
	$1.6x10^{-5}$M	302.58a	478.22b	531.95b	20.88a
12	Control	275.69bc	512.44ab	584.62ab	20.85a
	Wax+IMZ	257.02b	479.11b	519.73c	20.84a
	$1.6x10^{-3}$M	267.69b	478.66b	520.73c	20.80ab
	$1.6x10^{-4}$M	314.80a	540.44a	607.62a	20.83ab
	$1.6x10^{-5}$M	292.36b	551.11a	563.84b	20.75b

[a] Water treated control, mixture of wax and Imazalil, three concentrations of resveratrol; $1.6x10^{-3}$M, $1.6x10^{-4}$M and $1.6x10^{-5}$M.

ABTS =antioxidant activity measured based on ABTS assay, DPPH =antioxidant activity measured based on DPPH assay, FRAP=antioxidant activity measured based on FRAP assay, ORAC=antioxidant activity measured based on ORAC assay.

Means followed by same letter within columns are not significantly different.

Table 9. Pearson's correlation coefficients of antioxidant activities measured in satsuma mandarin fruit treated with *trans*-resveratrol ($1.6x10^{-3}$M, $1.6x10^{-4}$M, $1.6x10^{-5}$M), wax+IMZ and water during storage

Variable	ABTS	DPPH	FRAP	ORAC
ABTS		0.70*	0.6496*	-0.27
DPPH			0.40	-0.50
FRAP				0.07
ORAC				

ABTS =antioxidant activity measured based on ABTS assay, DPPH =antioxidant activity measured based on DPPH assay, FRAP=antioxidant activity measured based on FRAP assay, ORAC=antioxidant activity measured based on ORAC assay.
* =significant at $p \leq 0.05$
^{ns} =non significant
-negative correlation.

Table 10. Pearson's correlation coefficients of ABTS, DPPH, FRAP, ORAC and other dependent variables measured in satsuma mandarin fruit treated with *trans*-resveratrol ($1.6x10^{-3}$M, $1.6x10^{-4}$M, $1.6x10^{-5}$M), wax+IMZ and water during storage

Variable	TP	TF	AA	TCS	TCP
ABTS	0.25	0.083	0.7089*	-0.35	0.44*
DPPH	0.22	-0.24	0.70*	-0.12	0.53*
FRAP	0.14	-0.23	0.47	-0.57*	0.63*
ORAC	-0.09	0.026	-0.07	0.54*	-0.79

ABTS =antioxidant activity measured based on ABTS assay, DPPH =antioxidant activity measured based on DPPH assay, FRAP=antioxidant activity measured based on FRAP assay, ORAC =antioxidant activity measured based on ORAC assay, TP= total phenolics, TF = total flavonoids, AA= ascorbic acid, TCP= total carotenoid content measured in peels, TCS= total carotenoid content measured in segments.
* =significant at $p \leq 0.05$
^{ns} =non significant
-negative correlation.

APPENDIX B: FIGURES

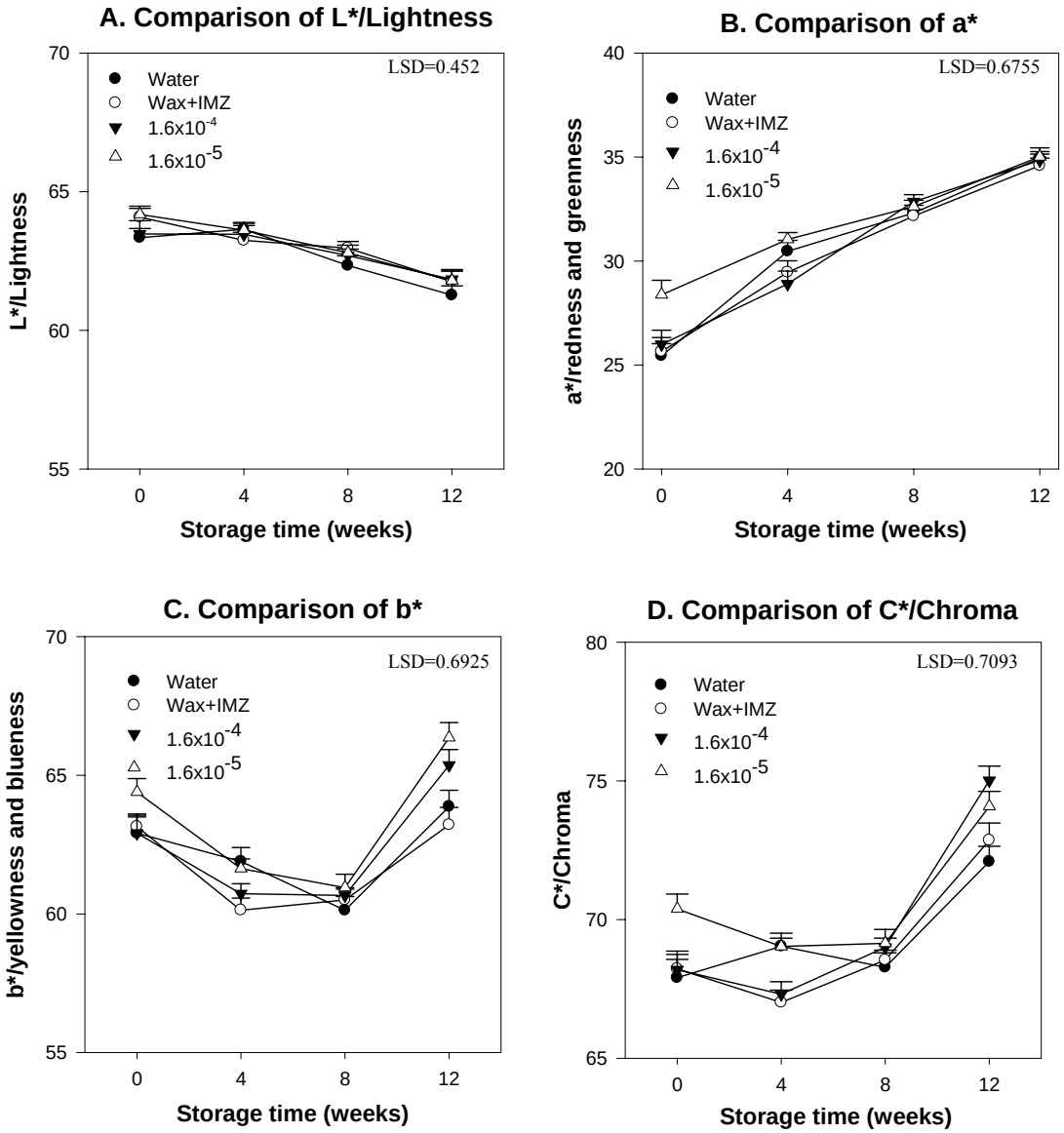

Fig.3. Comparison of external color attributes L*, a*, b* C* of satsuma mandarin fruit treated with *trans*-resveratrol ($1.6x10^{-4}$M, $1.6x10^{-5}$M), wax+IMZ and water during storage at 10^{o}C for 12 weeks. Each data point represents the average and standard deviation. Error bars show the standard deviation.

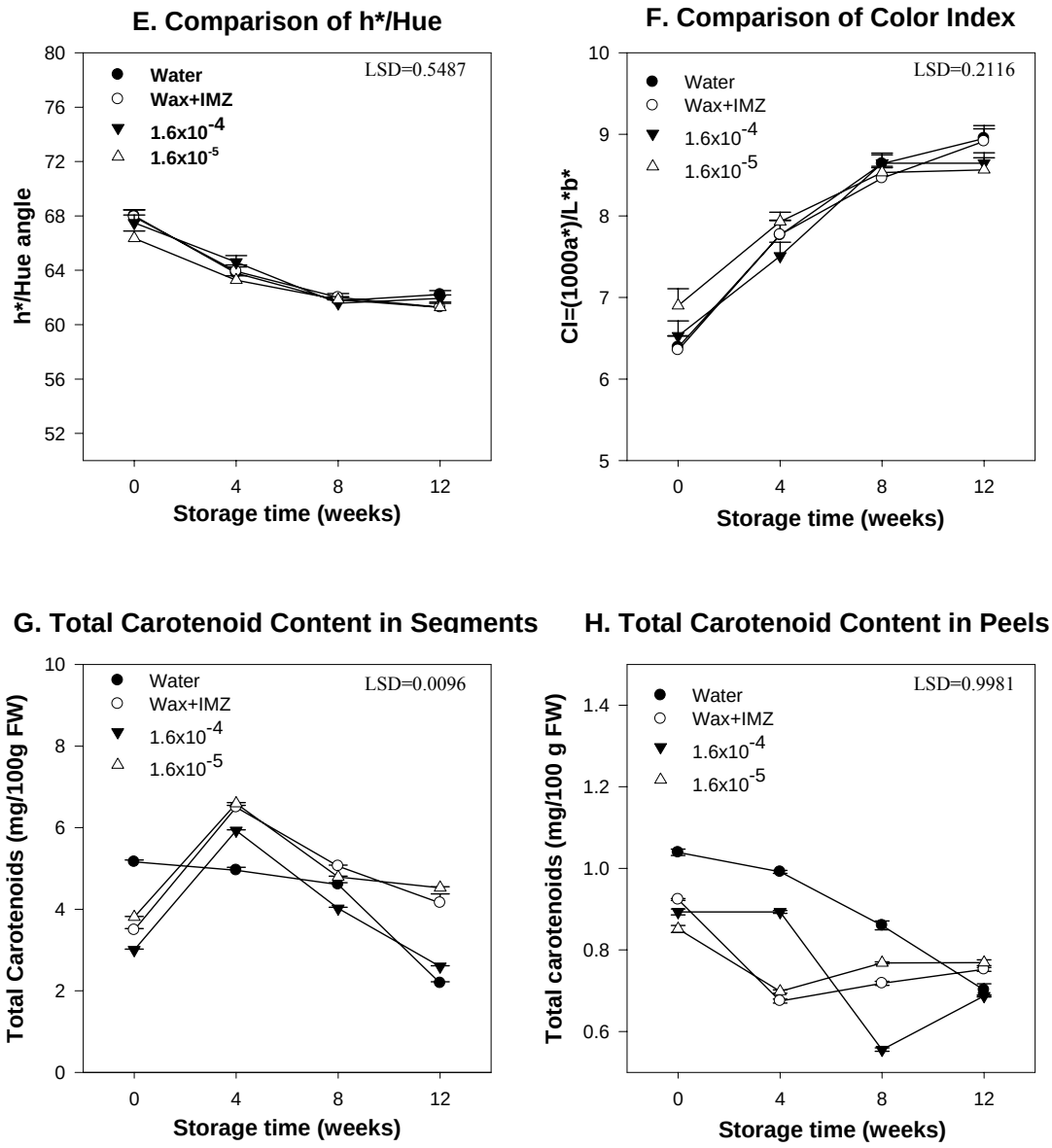

Fig 4. Comparison of external color attributes hue, color index and total carotenoid content in satsuma peels and segments treated with various concentrations of trans-resveratrol ($1.6x10^{-4}$M, $1.6x10^{-5}$M), wax and Imazalil and water during storage at 10^{o}C for 12 weeks. Each data point presents the average and standard deviation. Error bars show the standard deviation.

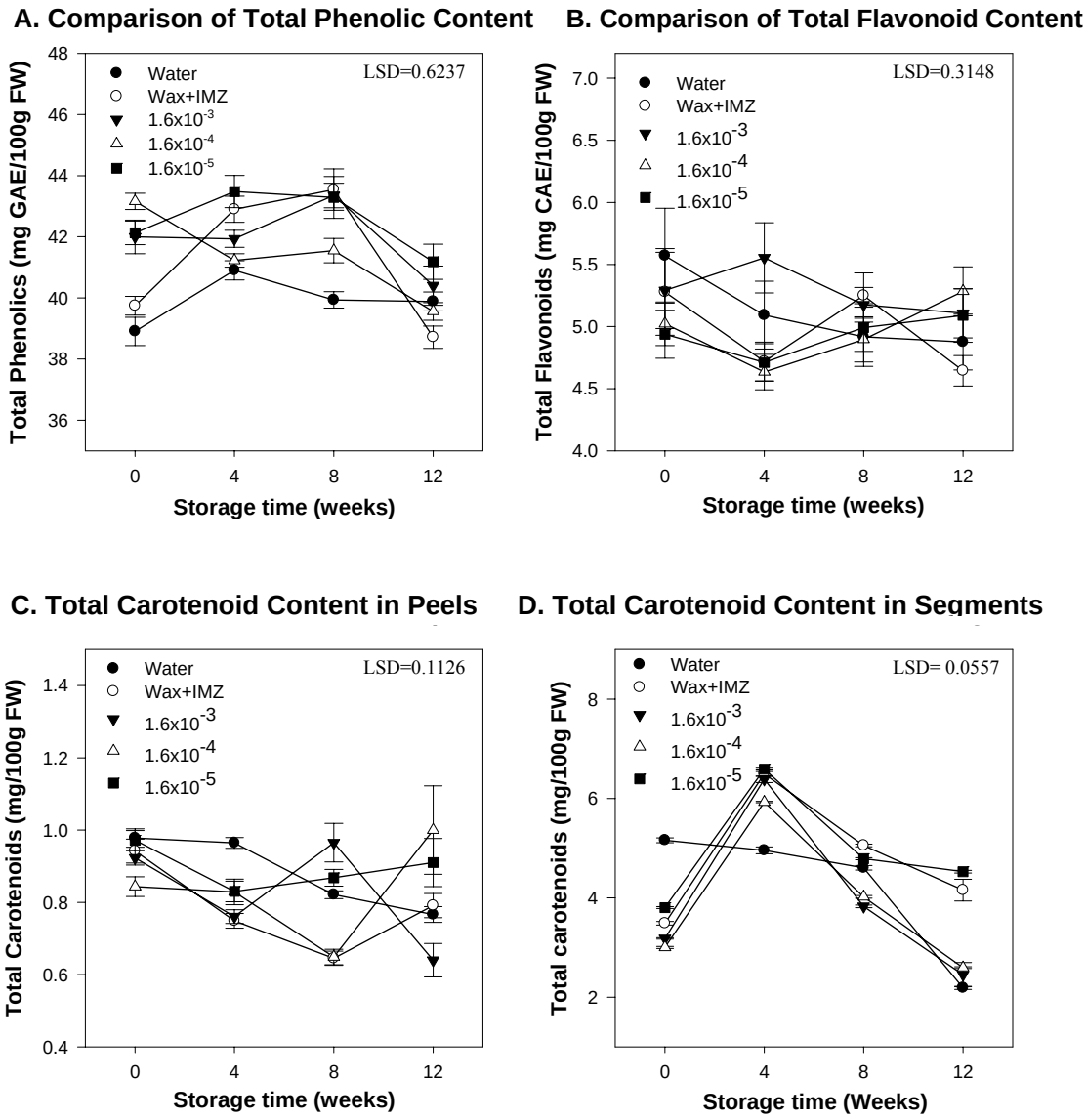

Fig 5. Comparison of means of total phenolic content, total flavonoid content, total carotenoid content in peels and segments in satsuma fruit treated with *trans*-resveratrol ($1.6x10^{-3}$M,$1.6x10^{-4}$M and $1.6x10^{-5}$M), wax+IMZ and water during storage at 10^{o}C for 12 weeks. Values are the mean ±SD (standard deviation) of three determinations

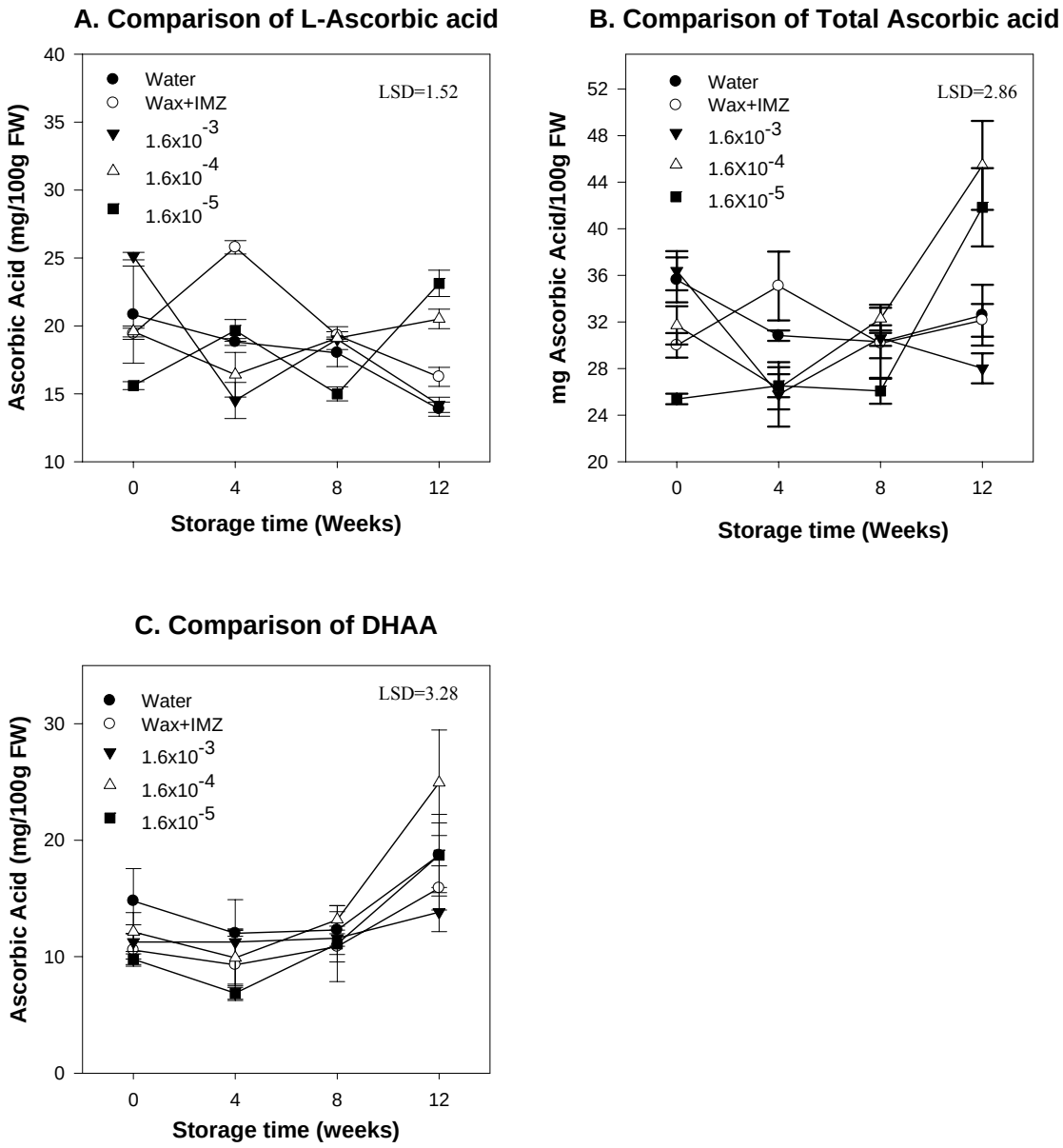

Fig 6. Comparison of means of L-Ascorbic acid (reduced form), total ascorbic acid content and DHAA (oxidized form) in satsuma fruit treated with various concentrations of *trans*-resveratrol ($1.6x10^{-3}$M, $1.6x10^{-4}$M and $1.6x10^{-5}$M), wax+IMZ and water during storage at 10^{o}C for 12 weeks. Values are the mean ±SD (standard deviation) of three determinations.

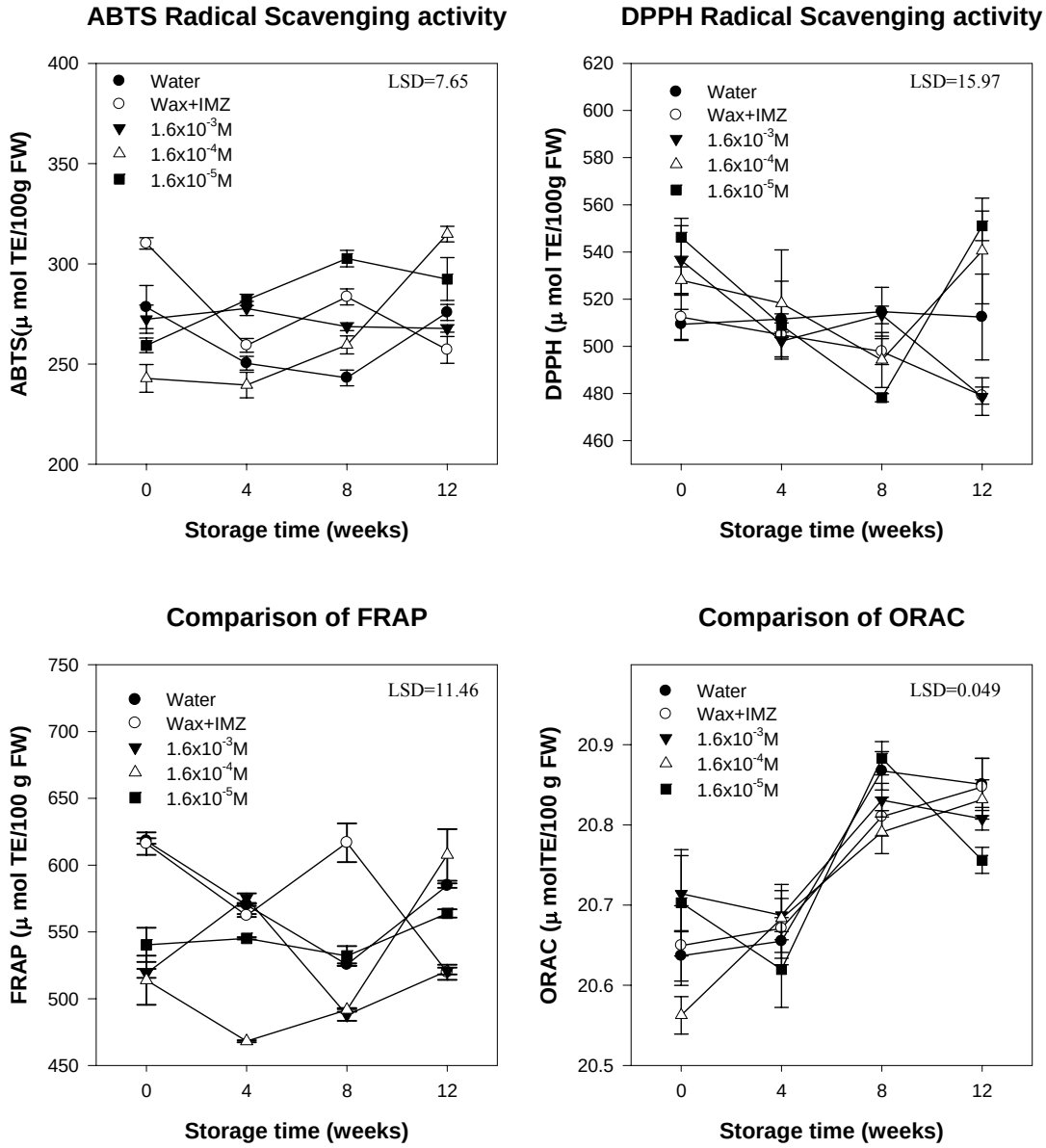

Fig 7. Comparison of means of ABTS, DPPH, FRAP and OREC assays in satsuma fruit treated with *trans*-resveratrol ($1.6x10^{-3}$M, $1.6x10^{-4}$M and $1.6x10^{-5}$M), wax+IMZ and water during storage at 10^{o}C for 12 weeks. Values are the mean ±SD (standard deviation) of three determinations.

APPENDIX C: STANDARDIZED SUMMARY COMPARISON OF METHODS FOR DETERMINATION OF BIOACTIVE COMPOUNDS IN SATSUMA MANDARIN FRUIT.

Antioxidant Method	BASIS FOR SELECTION Advantage	BASIS FOR SELECTION Disadvantage
ABTS Radical Anion Scavenging Activity	Inexpensive and easy to use, based on the scavenging ability of antioxidants, rapid, applicable over wide pH ranges, soluble in both aqueous and organic solvents; total antioxidant value can be estimated.	Many compounds have low redox potentials and can thus not be the same for slow reactions, and thus may take a long time to reach endpoint.
DPPH Radical Scavenging Activity	Simple, needs only a UV-vis spectrophotometer	DPPH radicals may interact with other radicals, i.e. alkyl, may require long reaction time. Carotenoids, Anthocyanins may interfere, thus pH dependent, not standardized
FRAP	Simple, inexpensive, robust, fast, measures only the reducing capability based on ferric ion and does not require specialized equipment.	Does not detect compounds that act by radical quenching, i.e. thiols, and proteins oxidizable substrates.
VCEAC	Simple, reliable, correlates with ORAC, Trolox, and phenolics, calculated on weight basis familiar with scientists and general, soluble in aqueous and organic phases.	

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