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INFLUENCE OF BIOTIC AND ABIOTIC FACTORS ON QUALITY AND SECONDARY METABOLITES OF 'VALENCIA' ORANGE FRUITS

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1. Introduction

In the last 30 years, consumers have shown interest in the benefits of eating fruit, especially in terms of prevention of degenerative human diseases. Awareness of fruit beneficial effects on health has substantially increased in recent times both by consumers and nutritionists, today also advising fruit consumption as a valuable tool for disease prevention and treatment.

The use of healthy foods in the human diet, for the role that they play in disease prevention, is linked to the possibility of accessing correct and updated information on the matter by both operators and consumers.

Fruit and vegetables are important sources of vitamins, carotenoids and antioxidant phenolic compounds, which are associated to a lower risk of developing cardiovascular, neurological and carcinogenic diseases (Scalbert and Williamson, 2000). This compounds have a wide range of biological effects, such as anti-inflammatory, antioxidant and antimicrobial activities (Harborne and Williams, 2000). Present in greater amounts in foods of plant origin (cereals, fruits and vegetables), but also in meat, eggs, milk and cheese, these compounds (mainly mineral salts, vitamins C, E, carotenoids, flavonoids, phenols, alkaloids, derivatives of chlorophyll, amino acids) are able to act even if present in small amounts and many of them also have the considerable advantage of resisting to cooking or other technological treatments (Kaur and Kapoor, 2001). For these reasons, nutritionists and dietitians call fruit and vegetable products 'functional food' or even 'pharmafood', and the compounds contained in fruits and vegetables 'nutraceuticals'.

Juices have high concentration of polyphenols and vitamins and for this reason they can be included among functional foods. They are prepared by mechanically squeezing or macerating fruit or vegetable flesh without the application of heat or solvents.

The main component of orange juice is water (85-90%), while more important soluble constituents are carbohydrates, organic acids and mineral substances. Mainly pectin and traces of protein can be found in suspension. Nutrients that provide energy are generally carbohydrates, proteins and fats. Presence of small amounts of protein (~ 0.6%) and fat (~ 0.2%) in the orange juice causes the greater contribution of the caloric value comes from carbohydrates present (10-12%). A substantial portion of carbohydrates in orange juice is composed of three simple sugars, sucrose, glucose and fructose, present in the ratio 2:1:1, respectively. They are an excellent source of readily available energy.

Citric acid and potassium, which are present in orange juice, function as a great buffer system for regulating pH in the stomach. High content of mineral elements, particularly potassium,

calcium, magnesium, phosphorus, as well as traces of iron, copper, zinc, manganese, cobalt, sulfur, bromine and iodine, make citrus juices essential in the human daily diet. Moreover, low sodium content gives particular dietary interest to citrus juices, especially for those people that suffer from hypertension and require a low-salt diet.

In an acid medium like the orange juice, sucrose can be easily hydrolyzed into glucose and fructose, and this may explain the low sucrose levels which are sometimes found in juices exposed to long-term storage.

Citric acid is the predominant organic acid; it contributes with 80% of the total acidity in an orange juice. Other organic acids are malic and isocitric acids. There are also traces of succinic, oxalic, formic and acetic acid, in free form or bound. The organic portion of the conjugated salts of these acids is metabolized, so cations are free to combine with other anions. For this reason the orange juice is classified as an alkaline food.

Many fruit juices have a higher sugar content than sweetened soft drinks; while soft drinks cause oxidative stress when ingested and may even lead to insulin resistance in the long term, the same thing cannot be attributed to fruit juices. On the contrary, fruit juices are actually known for their ability to raise antioxidant capacity and even offset the oxidative stress and inflammation normally caused by high-fat and high-sugar meals.

The perception of commercial fruit juice as equal in health benefit to fresh fruit has been questioned, mainly because it lacks fiber and has often been highly processed (Bibbins-Domingo et al., 2007); but fruit juice in moderate amounts can help children and adults meet daily recommendations for fruit consumption, nutrient intake and calories (O'Neil et al., 2010).

Orange juice is a drink in use all around the world, above all in USA where it is adopted as the official beverage. It is one of the most popular drinks to go with breakfast in the morning. Brazil and Florida are the major producers in the world and their production of orange juice makes up roughly 85% of the world market. Brazil exports 99% of its production, while 90% of Florida's production is consumed in the USA (Neves et al., 2012).

Orange juice is one of the most consumed fruit juices, thanks to the pleasantness of the taste, attractive color and for its perceived health benefits. It is a complex 'chemical mixture' of compounds. Aroma of fresh juice is unstable, due to a combination of chemicals, enzymes and microbial reactions that require stabilization treatments.

Fresh juice, for its perishability, is much less commercialized than treated one. Although alternative processes have been developed, almost all of the orange juice that is sold on the

market is heated, because this treatment is still the most cost effective means to reduce microbial populations and enzyme activity.

The concept of quality of orange juice has changed during the years. In the past, the presentation of the product and its safety were the main priority, while nowadays the expectations are more diversified, implying a strong focus on other aspects such as high quality taste and biological function to improve wellbeing and/or health. Therefore, the industry is currently facing the challenge of producing juices that meet new consumer preferences in order to ensure repeat purchase.

Over 50% of the production of orange juices is made with 'Valencia' oranges, which is the world's most important orange variety. It is a small sized, sweet and blond orange with a few seeds, thin skinned and the pulp is tender and very juicy. In the northern hemisphere, it is usually picked in April, but can stay on the tree until May or even June, depending on location and climate; this prolongs the season and somewhat marketing opportunities/processing period.

Valencia is one of the oranges that also thrives in the tropics. The fruit are of high quality but in the heat and without cool nights the color break does not occur and the fruit remain greenish in the tropics even when fully ripe.

Valencia orange juice, as other kinds of orange juices, is rich in many antioxidant categories of compounds like carotenoids, vitamin C and polyphenols, and these components are retained during processing of fruits into juice.

2. Antioxidants

The term 'antioxidant' is used to define those substances that can counteract oxidation. The most widely used definition is that of Halliwell and Whiteman (2004), according to which antioxidants are "substances which, in low concentrations compared to oxidizable substrate, and under specific conditions, are able to delay or prevent the oxidation of the substrate itself."

A substance with antioxidant activity, whether naturally present or assumed with the diet, generally acts on the activities of radical species in a direct way, i.e. giving electrons or hydrogen atoms and turning radicals into non-reactive forms, or indirectly, i.e. by binding metal ions such as copper or iron involved in the catalytic oxidation of lipids (Kaur and Kapoor, 2001).

The importance of antioxidants in foods is associated with the ability to both preserve the shelf-life of food by delaying the oxidation of polyunsatured fatty acids, and exert in vivo beneficial effects against chronic diseases induced by oxidative stress and age.

Chemically, antioxidants can be divided into:

- Natural: components of food where they play a regular protective action (phenolic compounds, tocopherols, flavonoids, phenolic acids, nitrogen compounds, alkaloids, derivatives of chlorophyll, amino acids, amines, carotenoids and ascorbic acid).
- Synthetic: molecules produced in the laboratory and widely used in food, cosmetic and pharmaceutical industries.

Based on their mechanisms of action, antioxidants can be classified in those that interrupt the chain of radical formation (or chain-breaking) and preventive antioxidants (Somogyi et al., 2007). The chain-breaking antioxidants inactivate free radicals by donating hydrogen or transferring a single electron to free radical species. These compounds, thanks to the negative potential, are able to supply electrons to the free radicals, thus restoring the chemical balance of the system in which they act. Their effectiveness depends on the stability of the radicals in which they are transformed, therefore, the more the delocalization of the unpaired electrons produced in the reaction with free radicals is efficient, the greater is their antioxidant power.

Preventive antioxidants have multiple mechanisms of action, and all are involved in slowing oxidation rate (initiation phase in particular), even if they do not convert the radicals in more stable compounds (Somogyi et al., 2007).

Antioxidants are further classified into hydrophilic, if they are soluble in water, or hydrophobic (or lipophilic), if soluble in lipids.

In general, water-soluble antioxidants react with oxidants in the cell cytoplasm and in the plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation. In foods of plant origin (cereals and vegetables), both hydrophilic and lipophilic antioxidants including vitamins, polyphenols, carotenoids, and minerals can be found (Nicita-Mauro and Basile, 2005).

3. Carotenoids

Carotenoids are organic pigments that are found in the chloroplasts and chromoplasts of plants. They are organized in pigment-protein complexes localized in chloroplasts (where their color is masked by the green chlorophyll); in other vegetables and fruit, the molecules are localized in chromoplasts, often fused to lipid droplets or bound to proteins (Takyi, 2001).

The basic structure of these molecules consists of a central portion, with 22 carbon atoms and two terminals, nine carbon atoms each. Carotenoids are generally tetraterpenoids (C40); they include eight isoprenic units (C5), connected head-to-tail, except in the center where the tie tail-to-tail reverses the order, giving a symmetric molecule (Rodriguez-Amaya and Kimura, 2004). Terminal units can be both acyclic, as in lycopene, or both cyclic, as in α and β-carotene, or one cyclic and the other acyclic, as in γ-carotene. The terminal cyclic units are rings with five or six atoms (Stahl and Sies, 1999) and they can also contain a wide variety of groups, for example, alcohol, ketone, epoxy and benzene. The combination of these end groups with functional groups containing oxygen and the changes in the level of hydrogenation generate the majority of the structures found in carotenoids.

They are extremely hydrophobic molecules with little or no solubility in water; for this reason, they are expected to be restricted to hydrophobic areas of the cell, such as the inner core of membranes, except when association with proteins allows them access to an aqueous environment.

Carotenoids play two key roles in plants: they absorb light energy for use in photosynthesis or protect chlorophyll from photodamage. The synthesis of various carotenoids, therefore, is a crucial metabolic process underlying these functions.

Among higher plants, these pigments are observed in flowers and fruits with yellow (zeaxanthine), orange (β-carotene) and red colors (lycopene). They are also present in the leaves, in the stems and in the grass, where their color is masked by that of chlorophyll. In fact, the typical change in leaf color of many deciduous trees in fall is due to chlorophyll degradation and appearance of carotenoid color. In higher plants, in fact, these pigments are normally present in smaller quantities than the chlorophyll and under normal conditions the predominant leaf color is green. However, when in the cold months the trees stop growing and prepare to lose their leaves, chlorophyll is rapidly degraded, carotenoids become the predominant pigments, and leaves show a typical reddish color.

In addition to their obvious role as visually attractive natural pigments, carotenoids perform a variety of essential biological functions. Universally, colored carotenoids provide photoxidative protection against the effects of singlet oxygen and radicals generated in the presence of light and endogenous photo-sensitizers such as chlorophylls and heme (El-Agamey et al., 2004).

During photosynthesis, carotenoids can transfer absorbed radiant energy to chlorophyll molecules with a light-harvesting function or dissipate excess energy via the xanthophylls

cycle in higher plants. Without carotenoids, photosynthesis and all life in an oxygen atmosphere would be impossible.

In humans, they are powerful antioxidants that are implicated in the prevention of or protection against serious health disorders such as cancer and heart diseases. These disorders, at some stage involve oxidation processes mediated by free radicals. To be an effective antioxidant, carotenoids would have to remove these radicals from the system either by reacting with them to yield harmless products or by disrupting free-radicals chain reactions. Many studies have identified that there is a close correlation between a low level of β-carotene in the blood and the possibility of developing cardiovascular diseases or cancer (Kontush et al, 1999).

It has been shown that carotenoids can be effective antioxidants in organic solution under defined conditions, especially at comparatively low oxygen concentrations (Burton and Ingold, 1984). Several competing reactions are possible, however, especially in complex systems. The concentrations of carotenoids in humans tissues generally are much lower than those used to demonstrate antioxidant behavior in model systems. To act as an antioxidant in vivo, carotenoids would need to be incorporated into the tissues in the correct location and at a suitable concentration relative to the oxidizing agent and the molecule that is to be protected.

There are many types of carotenoids, including carotenes and xanthophylls; some of these, like alpha-carotene, beta-carotene and zeaxanthin, are very important due to their ability to be converted into vitamin A, retinal and retinoic acid, thereby playing essential roles in nutrition, vision, and cellular differentiation, respectively (Britton, 1995).

4. Vitamin C

Vitamin C is an organic compound present in nature with antioxidant properties. It is an essential nutrient for humans, but mammals are not able to synthesize it due to the lack of L-gulonolactone oxidase enzyme that catalyzes the last reaction of ascorbic acid synthesis. It should be taken with the diet through consumption of raw fruits and vegetables because it is deteriorated during cooking processes.

Ascorbic acid is one of the components of vitamin C with rutin, tyrosinase, bioflavonoids and other compounds.

Vitamin C is well known for its antioxidant activity, acting as reducing agent to reverse oxidation in liquids. When there are more free radicals than antioxidants in the human body,

the condition is called oxidative stress, and has an impact on cardiovascular diseases, hypertension, chronic inflammatory diseases, diabetes as well as on critically ill patients and individuals with several burns.

Vitamin C is synthesized from glucose and it plays a role in many biological functions of the human body, such as absorption of iron (López and Martos, 2004), stimulation of immune defenses, bile acid synthesis (Iqbal et al., 2004), collagen production (Diegelmann and Evans, 2004), synthesis of steroid hormones (Murugesan et al, 2005), protection of the skin from UV rays (Placzek et al, 2005).

5. Polyphenols

Polyphenols represent more than 90% of the total antioxidant capacity in fruits and vegetables (Hervert-Hernandez et al., 2010) and they are products of the secondary metabolism of plants. There is a clear difference between primary and secondary metabolism. Primary metabolism includes all the indispensable ways to cell survival, and proteins, carbohydrates, lipids and nucleic acids are originated from it. The products of secondary metabolism are often present just in some kind of specialized and differentiated cells. They are not essential for the growth, development and reproduction of the organism, but they play a key role in ecological interactions that plants have with the biotic and abiotic environment in which they live. The two processes are closely linked, since plants draw from primary metabolism all the components needed for production of secondary metabolites.

Plants produce secondary metabolites in significant quantities. These compounds are synthesized in a well-defined part, frequently the roots, and they accumulate in an organ that can be different from the one where they were synthesized.

They are essential in the physiology of plants, contributing to the resistance against microorganisms and insects and to pigmentation and organoleptic characteristics. In particular, they are responsible for the pigmentation of flowers, fruits and seeds, thus attracting pollinators and seed dispersers; polyphenols promote the fertility of the plant and pollen germination; they act as signal molecule in the interaction between plant and microorganism; they protect against ultraviolet light and other defensive functions, including defense against pathogenic microorganisms. Polyphenols, interact with membrane proteins, enzymes and lipids of microorganisms, through different ways, by altering the permeability of their cells and determining the loss of protons, ions and macromolecules (Petti and Scully, 2009). It is also known that fruit and vegetables require a number of compounds to preserve

their integrity due to the continuous exposure to environmental stress, including UV radiation and high temperatures.

Chemically all phenolic compounds contain at least one aromatic ring with one or more hydroxyl groups (Petti and Scully, 2009; Naczk and Shahidi, 2006). They generally affect quality, acceptability and stability of the food by acting as flavoring, colorants and antioxidants. It is estimated that the average daily intake of polyphenols is about 1 g (Scalbert and Williamson, 2000).

Polyphenols are generally poorly absorbed, easily metabolized and rapidly eliminated. Isoflavones and gallic acid are the most absorbed polyphenols, followed by flavanones, catechins and quercetin (Petti and Scully, 2009).

Dietary intake of flavonols, flavones and flavanols is relatively low and plasma concentrations rarely exceed one mmol/l due to limited absorption and rapid elimination. Instead flavanones and isoflavones, even if they are contained only in citrus and soy, show the best bioavailability profile among polyphenols: in fact, their plasma concentration can reach five mmol/l. The low levels of polyphenols found in plasma, compared to 10-100 mg of each compound in the diet, bring out the complex mechanisms that regulate the bioavailability and it is estimated that the half-life of polyphenols in the circulation is between 2 and 6 hours (Carratù and Sanzini, 2005).

The study of the structure-function relationship of polyphenols shows that the structural characteristics affect their biological properties, bioavailability, antioxidant activity and interaction with specific cellular receptors.

The protective role exerted by polyphenols against the onset of chronic degenerative diseases is attributed mainly to their role as antioxidants. In fact, in vitro studies have shown that polyphenols act as antioxidants and are reducing agents, and together with other compounds introduced with the diet (such as vitamin C, vitamin E and carotenoids) contribute to the antioxidant potential of foods.

It is not shown an accumulation of polyphenols, therefore only the daily and regular consumption of foods rich in these compounds can help to prevent many human diseases linked to oxidative damage (Petti and Scully, 2009).

5.1 Antioxidant activity

Polyphenols protect cells from damage caused by free radicals, which are developed with the normal cellular metabolism and because of several factors such as radiation, smoke, pollutants, UV rays, emotional or physical stress, chemical additives, viral and bacterial

attacks.

Some polyphenols are highly reactive to deactivate singlet oxygen (Kahkonen et al., 1999), to neutralize free radicals by giving a hydrogen atom or an electron, and to chelate metal ions in aqueous solutions (Petti and Scully, 2009). Their antioxidant activity in vitro is considered superior to the one of vitamins (Wang et al., 1996). The effectiveness of their antioxidant activity is due to the presence of hydroxyl groups linked to the aromatic structures and to molecule geometry.

The formation of stable phenolic radicals through electron delocalization on the aromatic and aliphatic structures is the good condition to explicate the antioxidant activity of polyphenols (Halliwell and Gutteridge, 1990). Antioxidant activity of flavonoids and their in vitro metabolism also depend on the position of the functional groups in each compound (Heim et al., 2002). Gallic acid, resveratrol and catechins are the compounds with the highest antioxidant activity among polyphenols.

In addition to combat free radicals, phenolic compounds carry out many biological activities such as protection of the blood capillaries, anti-inflammatory, antibacterial, immunostimulant, antiallergenic, antiviral, anti-estrogenic and anticancer action. It is also known their inhibitory action against some enzymes, such as phospholipase, cyclooxygenase, lipoxygenase, glutathione reductase and xanthine oxidase (Waladakhani and Clemens, 2001).

5.2 Anti-carcinogenic activity

Anti-carcinogenic activity of polyphenols is due to the ability of these molecules to inhibit the enzymes involved in carcinogenesis and tumor development (Petti and Scully, 2009). In general, they influence the step of initiation of cancer development, protecting cells against the direct attack by carcinogens or altering their activation mechanism (in vitro).

5.3 Anti-atherogenic activity

It is widely reported as the oxidation of lipids and in particular of LDL is the cause of the development of atherosclerosis and its related diseases (stroke, thrombosis and cardiovascular diseases in general).

Epidemiological studies suggest that a high intake of polyphenols decreases the mortality due to cardiovascular disease and also decreases the risk of stroke, as well as lung and rectum cancers (Petti and Scully, 2009). The first mechanisms of action are the reduction of platelets and coagulation of LDL and the inhibition of the lipoproteins oxidation.

5.4 Anti-inflammatory activity

The antioxidant activity of flavonoids might be at the basis of an anti-inflammatory and anti-platelet role (Robak and Grygliwski, 1996), both thanks to the structure of the polyphenols and to their ability to penetrate the lipid membrane of the cell (Saija et al., 1995).

5.5 Antibacterial and antiviral activity

Polyphenols have anti-viral activity against HIV, Herpes Simplex, various influenza viruses and rhinovirus (Havsteen, 2002). Their supposed anti-HIV activity could be due to the inhibition of enzymes, such as reverse transcriptase, integrase and proteinase (Petti and Scully, 2009). The action against cytomegalovirus is related to the inhibition of the epidermal growth factor receptor (Petti and Scully, 2009).

The beneficial effects of polyphenols in animals or in vitro are generally not confirmed in human studies. This discrepancy could be explained because the mechanism of action of the polyphenols in vivo may be different from the mechanism in vitro. In fact, the classic antioxidant activity of polyphenols is most likely not the main explanation for the beneficial effects on man, but rather mechanisms of modulation and inhibition of proteins/enzymes, receptors and transcription molecules are involved in their activity (Petti and Scully, 2009).

6. Flavonoids

Flavonoids belong to polyphenol group, like other classes of compounds. They are synthesized from glucose that is the principal product of photosynthesis. Their synthesis follows two different pathways; the first starts with glucose, then acetic acid and polimalonic acid; the second one, after glucose continues with shikimic acid and ends with flavonoids (Ververidis et al., 2007).

In nature, flavonoids are in form of glycosides, so a simple part, called aglycone, is linked to one or more sugar molecules, called glycone. Glycosides are hydrolyzed by specific enzymes of the intestinal bacterial flora that separate them from sugars; this allows the absorption of the aglycone that is the biologically active portion. Then this last molecule can be modified by different enzymes to produce the different classes of flavonoids.

Many flavonoids absorb visible light; in nature these compounds give flowers and ripe fruits a yellow-orange, red, violet and blue color (Miller and Owens, 2011). They act as chemical signals for some insects and microorganisms (Simmonds, 2003); some are intended to inhibit the germination of fungal spores that are on the surface of leaves (Norton, 1999). Flavonoids

also play a protective function against UV rays (Agati and Tattini, 2012) and are involved in many biological activities such as transport of energy (Zhao and Dixon, 2010), growth regulation (Stafford, 1991), respiratory processes, photosynthesis (Ghasemzadeh et al, 2010) and regulation of hormonal activity (Mathesius et al, 1998).

In plants they have a defensive function, acting as an antioxidant (Hernandez et al, 2009); in humans they exert several beneficial properties. Flavonoids have, both in vitro e in vivo, antitumor and anti-inflammatory activity and provide cardiovascular protection. Moreover, they can protect against high blood pressure and cholesterol increase (Mennen et al, 2004).

Epidemiological studies have shown that a regular intake of flavonoids is associated with a reduced risk of cardiovascular diseases because the protective effects of flavonoids include antithrombotic, anti-ischemic and antioxidant capacity. Reduction of the risk of coronary heart disease is due to three main actions: improving coronary vasodilatation, decreased platelet aggregation and preventing oxidation of low-density lipoproteins (LDL).

Anti-inflammatory properties are due to the inhibition of the synthesis of some pro-inflammatory mediators such as prostaglandins and arachidonic acid. First is a group of lipid compounds that are derived enzymatically from fatty acid and have important functions, such as constriction or dilatation in vascular smooth muscle cells, aggregation or disaggregation of platelets, sensitization of spinal neurons to pain, reduction of intraocular pressure, regulation of the movement of calcium and hormonal regulation control. Arachinodic acid is a polyunsatured fatty acid and it is necessary for the repair and growth of skeletal muscle tissue.

Among all compounds of citrus fruit, flavonoids have, in vitro, the greatest capacity of inhibiting the enzyme glycogen synthase kinase-3-β (Johnson et al., 2011), which means a reduction of cell growth of pancreatic cancers. Different types of flavonoids are identified in citrus: flavanones, flavones, flavonols and other minor ones. Flavanones, such as hesperedin, narirutin and didymin, are in higher quantity than the other flavonoids. They are mostly in the epicarp, less in the mesocarp and endocarp. However flavones, such as nobiletin and tangeretin have a higher biological activity, even if they are in much lower concentration.

6.1 Hesperidin

Hesperidin is in greater amounts than others flavonoids in citrus fruits. It is a flavanone constituted by a sugar molecule, rutinose, linked to hesperetin, which is the corresponding aglycone. This flavonoid has significant antioxidant, hypotensive and hypoglycemic properties. Hesperidin can modulate the plasma levels of lipids, increasing high density lipoproteins (HDL) and decreasing low density lipoproteins (LDL) and triglycerides. It is

used to reduce fragility of capillary walls and it has a conservative role against the symptoms of varicose veins. To satisfy the average daily consumption, each person should consume, at least, 850 mg of hesperidin.

Various preliminary studies reveal new pharmaceutical properties, none of which has been confirmed as applicable to humans. Hesperidin reduces cholesterol (Monforte et al., 1995) and blood pressure (Ohtsuki, et al., 2003) and it decreases bone density loss (Chiba et al., 2003) in rats. Another animal study shows protective effects against sepsis (Kawaguchi et al., 2004). Hesperidin is also a potential sedative, probably acting through adenosine receptors (Guzmán-Gutiérrez and Navarrete, 2009).

6.2 Narirutin

Narirutin is a flavanone constituted by a sugar molecule, rutinose, linked to naringenin, which is the corresponding aglycone. It is considered to have a bioactive effect on human health as antioxidant, free radical scavenger, anti-inflammatory, carbohydrate metabolism promoter, and immune system modulator (Felgines et al., 2000). It is the predominant flavanone in grapefruit, but it is also present in orange and tomato.

Narirutin can reduce hepatitis C virus production by infected liver cells in cell culture. This seems to be secondary to narirutin's ability to inhibit the secretion of very-low-density lipoprotein by the cells (Nahmias et al., 2008).

Naringenin lowers the plasma and hepatic cholesterol concentrations by suppressing HMG-CoA reductase and ACAT in rats fed a high-cholesterol diet (Lee et al., 1999).

6.3 Didymin

Didymin is a flavanone constituted by a sugar molecule, rutinose, linked to neoponcirin, which is the corresponding aglycone. Didymin is richly expressed in citrus fruits such as orange, lemon as well as mandarin and bergamot. In rats, it plays an important role against cancer diseases, like neuroblastomas, causing significant reductions in tumors size (Singhal et al., 2012).

6.4 Nobiletin

Nobiletin is a flavone with many methoxy groups. It is soluble in organic solvents and not in water. Several studies indicate that it is a molecule with chemopreventive properties for very interesting tumors. It interferes with certain pathways of signal transduction in cancer cells.

Nobiletin, in detail, seems to be an inhibitor of the pathway components of the Mitogen-Activated Protein Kinases (MAPK), such as c-Raf and MEK1 (Miyata et al., 2008). In addition it would block the use of phosphoinositides by phospholipase C and kinases activated by phosphoinositide (PI-3K) (Nakajima et al., 2007). Both ways are actively used by cancer cells in their uncontrolled growth. There are studies, finally, proving that nobiletin is neuroprotective in animal models. If administered before an induced ischemic brain, it allows a faster recovery of metabolic infarcted tissue and a reduced production of oxygen free radicals (Yamamoto et al., 2009).

6.5 Tangeretin

Tangeretin is a flavone with many methoxy groups and it is mostly presented in tangerines and other citrus. In plants, it strengthens the cell wall and it acts as a defensive mechanism against disease-causing pathogens. It is also used as a marker compound to detect contamination in citrus juices (Uckoo et al., 2011).

Animal research shows the potential of tangeretin as a cholesterol lowering agent (Kurowska and Manthey, 2004). A study on rats demonstrated potential protective effects against Parkinson's disease (Datla et al., 2001).

Tangeretin also shows enormous potential as an anticancer agent. In vitro, tangeretin appears to contrast some adaptations of cancer cells. Tangeretin induces apoptosis in leukemia cells while sparing healthy cells (Hirano et al., 1995). In a study on two human breast cancer cell lines and one colon cancer cell line, tangeretin blocked cell cycle progression at the G1 (growth) phase in all three cell lines, without inducing apoptosis in the tumor cell lines. Once tangeretin was removed from the tumor cells, their cell cycle progression returned to normal (Morley et al., 2007).

7. Factors affecting carotenoids and polyphenol content

The quantity and quality of carotenoids and polyphenols present in fruit and vegetable can vary significantly due to intrinsic and extrinsic factors such as the genus, species and cultivar, soil composition and growing conditions, different management practices, diseases, ripeness and post-harvest conditions (Faller and Fialho, 2010).

The distribution of polyphenols in tissues and cells of plants is not homogeneous; the outer layers of the plants have a higher polyphenol content than the inner layers; some fruits have a higher polyphenol content in the peel than in the pulp. Also, the insoluble phenols are

localized in cell walls, while the soluble ones in cell vacuoles. The insoluble phenols, linked to the different components of the cell, contribute to the mechanical strength of the cell as well as to the regulation of plant growth and morphogenesis and to cell defense from biotic and abiotic stress.

According to Naczk and Shahidi (2006) summer crops have a higher polyphenol content than those grown in autumn. In tomato, flavonols are located in the peel; cherry tomatoes also contain a higher amount of flavonoids than other larger varieties.

The stage of maturation is one of the factors that significantly affect the composition of carotenoids (Rodriguez-Amaya and Kimura, 2004). During maturation, there are changes in color in some fruits due to changes of some components such as carotenoids (Calvo, 2005). In fact, fruit and vegetable ripening is generally accompanied by carotenoid accumulation.

Generally high temperatures and large exposure to sunlight increase the biosynthesis of carotenoids in fruits. Similarly, some leafy vegetables produced in greenhouses or in covered ground with plastic roofs show high concentrations of carotenoids during the summer. On the contrary, the levels of carotenoids in leafy vegetables grown in outdoor are significantly higher in winter than in summer, suggesting that carotenoid photodegradation prevails on synthesis (Rodriguez-Amaya and Kimura, 2004).

Contrasting results have been reported in response to pesticides and fertilizers. Some studies show no significant changes in the carotenoid content, while other studies report a decrease in their concentration (Rodriguez-Amaya and Kimura, 2004; Calvo, 2005).

The major cause of the destruction of carotenoids during processing and storage is non-enzymatic and enzymatic oxidation. Carotenoids are natural protectors of plant tissues (Rodriguez-Amaya and Kimura, 2004) and as a result of their antioxidant activity, they are subject to oxidation, a phenomenon which is accelerated by organic and inorganic catalysts and physical factors such as temperature and light radiation. Therefore, a significant loss of these compounds can take place during technological transformations, where heat treatment and favorable conditions for oxidative processes can be present.

Vegetables and fruits are subjected to preliminary processing, such as sorting and removal of non-edible parts, washing, cutting or chopping, stabilization and preservation treatments, such as freezing and cooking (Cappelli and Vannucchi, 2005). Also cut, chopped, squeezed and pureed fruits and vegetables experience substantial carothenoid loss, often higher than that observed after heat treatments. This is primarily due to the concomitant exposure of carotenoids to oxygen and the enzymes that catalyze the oxidation, released after cell rupture.

8. Greening

Citrus greening disease, or Huanglongbing, or HLB, is probably the worst disease of citrus caused by a vectored pathogen. The responsible agents are motile bacteria, *Candidatus Liberibacter* spp. Transmission is by insects: the Asian citrus psyllid *Diaphorina citri* or, in Africa, the African citrus psyllid *Trioza erytreae*, a tiny insect that feeds on the leaves and stems of citrus trees.

Candidatus Liberibacter spp. are phloem-limited plant pathogenic bacteria. The phloem system of the plant transports sugars (food) bidirectionally through the plant, although products of photosynthesis (sugars) are primarily transported from sources of photosynthetic activity (leaves) to sinks (flowers, fruits, roots, seeds).

The disease was first described in 1929 and first reported in China in 1943 (Lin, 1956). The African variation was first reported in 1947 in South Africa (Moll and Van Vuuren, 1977), where it is still widespread. Symptoms of HLB were first reported in São Paulo State in 2004 (Coletta-Filho et al., 2004). In August 2005, the disease was found in the south of Florida. HLB has not been reported in Australia or in the Mediterranean basin. Wherever the disease has appeared, citrus production has been compromised with the loss of millions trees.

Detection of HLB can be difficult, as symptoms may not show up for more than a year (up to five) after the tree has become infected. The early symptoms on leaves are vein yellowing and an asymmetrical chlorosis referred to as "blotchy mottle." This symptom is the most diagnostic symptom of the disease. Leaves may be small with a variety of chlorotic patterns that often resemble mineral deficiencies such as those of zinc, iron, and manganese. Some leaves may be totally devoid of green or with only green islands. The blotchy mottle symptom also may be confused with other diseases or damage such as severe forms of citrus Tristeza virus. Root systems of infected trees are often poorly developed and new root growth may be suppressed. Early symptoms of yellowing may appear on a single branch. The yellowing usually spreads throughout the tree over a year, especially on young trees, and affected trees may show die-back of twigs, causing a decrease of the production within a few years. Fruit are often few in number, small, with a curved central core, and fail to color properly. Many fruit drop prematurely from infected trees. A yellow stain may be present just beneath the peduncle on a cut fruit. Infected fruit often contain aborted seeds and have a salty bitter taste There is general consensus throughout the world literature that three general practices must be adopted in order to have a successful citrus greening management program. These include the

planting of certified, clean nursery stock, effective control of psyllid populations and removal of infected trees that serve as an inoculum source for psyllid acquisition.

There is no cure for HLB and efforts to control the disease have been slow because infected citrus plants are difficult to maintain, regenerate, and study. There is little or no evidence of genetic resistance to HLB in citrus. Apparently, citrus has had only a recent association with *Liberibacter* species, an association too short to have built up resistance to the bacterium.

It is not easy to study the cure for HLB because in each growing region where psyllid is, there are differences in climate, cultural practices, and even strains of the pathogen or vector species which makes direct comparison of results difficult. In China, for example, greening disease has severely limited citrus production in the lowland areas where *Diaphorina citri* is relatively abundant. In contrast, in the highland areas of China greening disease is not a problem (Hung et al., 2004). In these areas of higher altitude, *Diaphorina citri* survival is low and thus spread of *Candidatus liberibacter asiaticus* is also low. The situation in Florida is much different as the climate and current production practices are ideal for buildup of large psyllid populations.

In Vietnam, it has been found that the presence of guava trees surrounding citrus trees (inter-planting) prevents or at least retards HLB. Volatiles from guava repel the Asian citrus psyllid, thus explaining the guava effect. However, recent experiments in Vietnam have shown that efficacy of guava inter-planting on HLB is limited, as the effectiveness of guava against disease invasion breaks down after one year.

The management of infected orchard involves three classic measures:

- insecticide sprays of all trees, several times a year to decrease the insect-vector population;
- identification and immediate removal of symptomatic trees, to rid orchards of sources of inoculum;
- replacement of removed trees with new certificated ones.

This kind of management, based on these three measures, can be influenced by different crucial factors, like the region where the farm is located, the percentage of disease incidence, the proximity to other infected orchards, the size and the age of the orchard (control is easier with larger and old orchards than smaller and young ones).

There are other factors that can be modified and through which management can change the HLB incidence in the farm, like the number of inspections for early detection and removal of symptomatic trees; number of psyllid control operations, nature of insecticides, application methods, prevention of insecticide resistance.

9. Maturation promoters

Fruit ripening is characterized by a complex series of physiological and biochemical processes that ultimately determine organoleptic and commercial quality. The main processes that take place during ripening are color development, due to degradation of chlorophyll and accumulation of anthocyanins (red, orange and purple) and carotenoids (yellow and orange); accumulation of sugars (glucose, fructose and sucrose); decrease of organic acids, mainly transformed into sugars; softening of the pulp, due to the activation of enzymes that degrade the main components of cell walls (Brady, 1987)

9.1 Phenilalanine

Anthocyanins are polyphenols and their synthesis starts from phenylalanine (Sakuta et al., 1994). Precisely, conversion of L-phenylalanine to trans-cinnamic acid is the initial step of the phenylpropanoid pathway and flavonoids, including anthocyanins and condensed tannins, are derived from coumaric acid of the phenylpropanoid pathway (Ishikura et al., 1984). Some phases of this pathway are catalyzed by L-phenylalanine ammonia-lyase (PAL), a key regulatory enzyme in the biosynthesis of phenolics (Jones, 1984). High PAL activity is associated with the accumulation of anthocyanins and other phenolic compounds in fruit tissues of several species (Billet et al., 1978; Blankenship and Unrath, 1988; Kataoka et al., 1983). Studies of Given et al. (1988) show that accumulation of anthocyanins in ripening strawberry fruit requires high PAL activity. Anthocyanins synthesis begins when the color change of the fruit occurs, often described as veraison phase.

9.2 Methionine

Ethylene is synthesized from methionine by the action of synthetase (ACS) and oxidase (ACO) enzymes (Bleecker and Kende, 2000). During ethylene synthesis, plants are stimulated by auxin. Ethylene is essential for the maturation because it regulates and coordinates most of the metabolic pathways activated during this process. The biosynthesis of ethylene in fact promotes the uniformity of maturation, the accumulation of sugars and the synthesis of carotenoids (Alexander and Grierson, 2002; Alba et al., 2005). This effect is reinforced by the oxylipins, which stimulate the activity of ACS and ACO enzymes (Kondo et al., 2007).

Ethylene, is the simplest olefin, exists in the gaseous state under normal physiological conditions, it is biologically active in trace amounts, and its effects are commercially important.

9.3 Oxylipins

During fruit maturation, chlorophyll is degraded by chlorophyllase, and only after that carotenoids and anthocyanins may become visible. The oxylipins stimulate the expression of the genes from which depends the formation of chlorophyllase (Tsuchiya, 1999) accelerating the degradation of chlorophyll and the consequent appearance of color. Both phenylalanine and oxylipins are involved in determining cell wall structure and resistance (Galliano et al., 1993) with consequences on fruit flesh firmness.

Oxylipins, and in particular the pool of precursors and derivatives of jasminic acid, operate as activators of the enzymes of the phenylpropanoid pathway. Oxylipins, in fact, acting on DNA, increase the expression of genes from which the formation of these enzymes, PAL in particular, depends (Petroni and Tonelli, 2011; De Geyter et al., 2012). Post-harvest treatments with methyl derivatives of the jasminic acid, for example, have improved peel color in 'Golden Delicious' apples (Fan et al., 1998).

9.4 Mono-saccharides

In addition to all these specific ripening processes, externally supplied simple sugars may favor an increase of the primary metabolism (Roitsch and Gonzalez, 2004). The increase in the primary metabolism can, in turn, lead to an increase in the capacity of the fruit to attract and accumulate (sink) sugars produced in the leaves (source) during photosynthesis, as well as an increase in the synthesis of organic acids that, during maturation, are converted into sugars (Roitsch, 1999). These phenomena may lead to greater fruit growth and final size, but also to an improvement of fruit organoleptic and nutritional qualities since the end products of primary metabolism (aminoacids, sugars, organic acids) are in turn a substrate for synthesis of secondary metabolites such as pigments and vitamins

10. Water management

Irrigation is the application of water to living plants. Uphoff (1986) defines irrigation as a practice of applying water to the soil to supplement the natural rainfall and provide moisture for plant growth. According to FAO (1994), irrigation is defined as the artificial application of water to the crop for the purpose of food and fiber production overcoming deficiencies in rainfall and help in creating stabilized agriculture.

10.1 Irrigation scheduling

The irrigation scheduling indicates how much irrigation water has to be given to the crop, and how often or when water is given. This ensures that water is applied to the crop when needed with the required amount (Brouwer et al., 1989; Evans et al., 1991). There are many factors to consider when determining a successful irrigation schedule (Doorenbos and Kassam 1979; Vedula and Nagesh Kumar 1996; Fereres and Evans, 2006) such as soil water holding capacity, crop water use and crop sensitivity to moisture stress at different growth stages, effective rainfall and availability of irrigation water and prevailing.

Water availability strongly influences flowering and fruit set and can affect fruit drop, fruit size, yield, internal fruit quality characteristics and canopy development (Falivene et al., 2006). Therefore, the planning of seasonal supply of the available amount of water must be directed towards meeting the water requirements of the plant during the most sensitive growth stages instead of spreading the available water to the plant equally over the all growing periods. For the non-sensitive growth stages, the amount of water allocated must be sufficient enough to prevent any effect on the yield and quality.

Reducing water supply during the initial and final stages of fruit development will delay size increases in *Citrus limon*, but the final yield will be unaffected (Torrecillas et al., 1993).

Koo (1969), Tucker (1986), and Parsons (1989) report that optimal citrus production requires the maintenance of available soil water during the growing stages as follows:

- Flowering, fruit setting and new flush development should have good soil moisture by applying water if the absence of rain does at this time;
- Fruit maturation: a high moisture content may have a harmful effect on fruit quality and flower initiation so that, it is recommended to reduce the soil water content at this stage and the soil should be kept relatively dry;
- Post-harvest: after the crop has been harvested, trees require less amounts of water to restore growth.

On the other hand, irrigation scheduling saves water, energy, labor and maximizes yield response to other management practices. A careful use of irrigation water may also help reduce pollution and environmental risks (Ali, 2010). Irrigation scheduling can also help prevent several problems, such as water loss by deep percolation, soil salinity, water availability for irrigation and low crop yield (Trimmer and Hansen, 1994).

Wample and Smithyman (2002) define the irrigation management as a tool for improving the production that can have many goals such as, control of tree vigor, prevention of occasional

periods of water stress, improvement of fruit quality through its influence on the content of soluble solids and pH.

10.2 Water budgeting for irrigation scheduling

Irrigation scheduling involves determining both the timing of irrigation and the quantity of water to apply. It is an essential daily management practice for a farm manager growing irrigated crops. Proper timing of irrigation can be done by monitoring the soil water content or monitoring the crop in the field. Plant stress responses provide the most direct measure of identifying the plant demand for water. However, it should be noted that while plant stress indicators provide a direct measure of when water is required, they do not provide a direct volumetric measure of the volume of water required to be applied.

The crop water requirement is defined as the amount of water required to compensate the water loss from the cropped field (Allen et al., 1998). Many researchers describe it as the total water needed for evapotranspiration (ET). Therefore, the water requirement can be decided by determining the actual ET.

The crop water requirement can be related to the amount of water used by a reference crop. The reference crop typically is grass or alfalfa that is well irrigated and covers 100 % of the ground. The reference evapotranspiration (ET_0) includes the water evaporated from the soil surface and the water transpired by the plants.

The daily ET_0 can also be calculated from daily climate data like temperature, wind speed, sunshine and relative humidity. There are several methods used to calculate or measure ET_0. The most common methods are Penman method, Pan Evaporation and Blaney-Criddle method. The climate data can be obtained from a weather station.

The successful irrigation scheduling requires good understanding to the knowledge of soil water holding capacity, crop water use, and crop sensitivity to moisture stress at different growth stages. This requires consideration about the effective rainfall and availability of irrigation water (Waskom, 1994).

A good irrigation schedule requires an accurate quantification of ET. The most common approach to calculate ET is given by multiplying ET_0 by the crop coefficient (k_c) which depends on ground cover and crop characteristics (Doorenbos and Pruitt, 1977; Allen et al., 1998; Villalobos et al., 2000).

10.2.1 Crop coefficient (Kc) for Citrus

According to Allen et al. (1998), in the arid and semi-arid regions of the Mediterranean, the recommended K_c values for citrus range from 0.70 in winter to 0.65 in summer with no ground cover. With growing ground cover or weed, the values range from 0.75 to 0.70, respectively for winter and summer.

The average yearly K_c value of 0.69 is in good agreement with reports from Arizona (Hoffman et al., 1982), Valencia (Castel and Buj, 1993), Cyprus (Eliades et al, 1994), Iran (Sepaskhah and Kashefipour, 1995), Crete (Chartzoulakis et al., 1999), California (Grismer, 2000) and Uruguay (Garcia Petillo and Castel, 2007).

Garcia Petillo and Castel, (2007) indicate that K_c has a clear seasonal trend. The minimum K_c is 0.60 in summer, intermediate values in autumn and spring (0.77 and 0.80, respectively) and a maximum of (0.87) in winter.

10.3 Basics of water relations in plants

Water relations are important to the functioning of trees, as water is the greatest component of the active tree (by mass), and all biological processes and growth may be limited by an inappropriate water status (Lakso, 2003). Literature shows that changes in plant water relation parameters, like leaf water status and stomatal response, can be explained in terms of changes in the hydraulic architecture of trees (Tyree and Cochard, 1996; Salleo et al., 2000).

Plant water status is usually described by two basic parameters: the content of water in the plant or the energy status of the water in the plant, expressed as the (total) water potential, ψ (Kirkham, 2005). It can be regarded as a very sensitive indicator for the degree of water stress experienced by the plant (McCutchan and Shackel, 1992).

Different irrigation regimes and their effect on water relations have been studied in many tree crops and other perennial and annual plants. The emphasis of most studies has mainly been on yield, vegetative growth and water relations in response to reducing water supply. Citrus fruits are frequently grown in areas where water supply limits optimum growth and production. Many experiments considering water relation of citrus trees have been carried out under different conditions (Levy, 1980; Habermann et al., 2003; Pérez-Pérez et al., 2007). Although these results may be of scientific value, they are of limited applicability to orchard conditions due to different environmental conditions within orchards.

10.3.1 The soil-plant-atmosphere continuum (SPAC)

The Soil-Plant-Atmosphere Continuum (SPAC) is the pathway for water moving from soil through the plant to the atmosphere. The transport of water along this pathway occurs in components only as separate defined and differently between a scientific discipline in the environment (John, 1966):

- Soil physics that characterizes water in soil in terms of tension;
- Physiology of plants and animals that characterizes water in organisms in terms of diffusion pressure deficit;
- Meteorology uses vapor pressure or relative humidity to characterize atmospheric water.

The water absorbed by the root hairs is translocated upwards through the xylem. The ascent of sap or movement of water from root to leaf is explained by the cohesion-tension theory. A number of theories have been put forward at various times to explain the mechanism of ascent of sap. These are vital theories, root pressure theory and transpiration pull (Rajan, 2003).

10.3.2 Water potential concepts

Water potential denotes the state of water in the plant or soil as compared to that of pure water (0 MPa). It depends on three factors: concentration, pressure and gravity. The following equation expresses the components of water potential (Tromp et al., 2005):

$$\Psi_t = \Psi_p + \Psi_s + \Psi_g + \Psi_m$$

Where Ψp is the pressure or hydrostatic potential, Ψs the osmotic potential, Ψg the gravitational potential and Ψm the matric potential.

Leaf water potential (Ψ_l) and stomatal conductance (g_s) are responding to the interacting environmental factors such as radiation, temperature, availability of moisture and vapor pressure deficit (VPD) (Elfving et al., 1972; Hall et al., 1975; Pereira and Kozlowski, 1978). Such stomatal responses to VPD generally act to minimize the effect of changing environment on ET, and hence Ψ_l. The response of leaf g_s to these environmental and edaphic parameters has been reviewed (Camacho, 1977). Low Ψ soil has an overriding influence on leaf (Fereres et al., 1979) g_s. This relationship has been observed to various degrees by others (Kriedemann and Barrs, 1981). A humidity response is probably also involved in the gradual closure of stomata during the day as this has been observed in apple (Landsberg et al., 1975; Lakso, 1986) and in citrus (Sinclair and Allen, 1982; Cohen and Cohen, 1983).

However, water relations of citrus are also influenced to a large extent by high resistances to water transport within the plant. As the soil dries, and water uptake rate from the soil falls below the potential transpiration rate, so the actual transpiration rate falls. This situation arises when the soil-plant conducting system cannot sustain the rate of water loss driven by atmospheric demand, and so g_s must fall. Therefore, the effects of falling gravimetric water content θ_g in the root zone are reflected in changing leaf g_s values of canopy/stomata conductance values.

In citrus, changes in plant water potential and atmospheric demand (humidity and temperature) influence primarily stomatal behavior (Hall et al., 1975; Levy, 1980). Transpiration of citrus trees is much more sensitive to changes in total leaf g_s than in many other agricultural crops due to the generally high boundary layer conductance of orchard canopies (Jarvis et al., 1981). This high boundary layer conductance implies a close coupling with the environment.

Measurement of plant water status by compensation, sap flow, porometry and pressure chamber methods are useful in establishing the degree of water stress in plants (Slavik, 1974; Kramer, 1983).

10.4 Deficit irrigation

Deficit irrigation practices differ from traditional irrigation practices. The manager needs to know the level of transpiration deficiency allowable without significant reduction in crop yield. Deficit irrigation is a promising water management strategy to achieve increased crop water productivity (English, 1990). It is an optimization strategy in which irrigation water is applied during sensitive growth stages of a crop and whereby, outside these periods, irrigation is limited or can even be absent if rainfall guarantees a minimum supply of water.

10.4.1 Regulated deficit irrigation (RDI)

Behboudian and Mills (1997) define Regulated Deficit Irrigation (RDI) as a system of managing soil water supply to impose periods of predetermined plant or soil water deficit that can result in some economic benefit. Over the last two decades, extensive research work conducted on fruit trees has demonstrated a positive yield response to mild water deficits (Marsal et al., 2002; Chalmers et al., 1981).

Originally, RDI was developed primarily to control vegetative growth in high density orchards, and secondarily to maximize fruit size, fruitfulness and fruit quality (Goodwin and Jerie, 1992). Thus, it consists on deliberately applying an amount of water at a level below

water requirement during the irrigation season in order to save water and control vigor (Goodwin and Boland, 2000). A certain reduction in yield is observed, but fruit quality (e.g. sugar content) tends to be equal to or even greater than that of fully irrigated trees (Marouelli and Silva, 2007; Spreer et al., 2007; Cui et al., 2008; Hueso and Cuevas, 2008).

A certain level of drought stress is maintained in the root zone and the primary objective of its use in tree crops is to control the canopy size and save water. Usually a fraction of ET_c is replaced during each irrigation rather than applying the full ET_c rate. Successful implementation of the RDI strategy depends on a precise knowledge of the vegetative and reproductive development of the fruit tree under investigation. For example, periods when vegetative growth is normally fast and fruit growth is slow are ideal for reducing irrigation rates (RDI) since a significant portion of resources can be allocated in favor of fruit development rather than toward vegetative growth. On the other hand, application of RDI during the sensitive periods, e.g. flowering and fruit set, is not advisable. For those reasons, much research has focused on the appropriate timing of the application of this strategy, also in citrus (Goldhamer and Salinas, 2000).

10.4.2 Citrus and water deficit

Water deficit is the physiological condition to which a tree is subjected whenever the rate of water loss from the leaves by transpiration exceeds the rate of water absorbed by the root system; this reduces plant growth and biomass production. McCarthy et al. (2002) reported that vegetative growth (shoot growth) is more sensitive to water deficit than fruit growth.

A readily available soil water supply appears essential for the development of optimum fruit size and yield in citrus (Levy et al., 1978; Moreshet et al., 1983). Plant water deficit affects a wide range of physiological and developmental processes involved in fruit production, including reduced growth due to inhibited cell division and cell expansion, and reduced photosynthetic capacity due to stomatal closure (Slatyer, 1967; Kozlowski, 1974; Hsaio, 1973).

Pérez-Pérez et al. (2007) shows that the effect of water deficit on growth and yield depends on its severity and time of occurrence. It also varies with crop species and variety. The yield response to water shortage can vary depending on the particular growth period. The initial period of fruit growth is the most sensitive phase to drought stress. Total soluble sugars and titratable acidity increase when a severe drought stress occurs in the final period of fruit growth, near ripening and harvest. On the contrary, it only increases the peel/pulp ratio if it occurs in the initial period of fruit growth.

Citrus trees have the tendency to conserve water through relatively low transpiration rates and relatively high leaf and root resistances (Kriedemann and Barrs, 1981). But to a large extent, drought resistance in citrus also appears to be due to its ability to adapt to water deficits under a range of environmental conditions. There is, however, little unequivocal information available concerning the effect of decreasing soil water potential on total root hydraulic resistance within citrus trees. These characteristics can potentially be manipulated to improve WUE, but to achieve this goal there is a continuing need for more knowledge about the physiological processes that control water use.

Moss and Muirhead (1971a, b) in their study on 'Valencia' orange show that temperature and evaporation rates during November were the most consistent predictors of yield in New South Wales. Similar responses from one season to the next are reported by Jones and Cree (1964) who show that yield was negatively correlated with the previous year's crop and with daily maximum temperature following bloom.

On the other hand, Hilgeman (1977) showed that fruit quality (total soluble solids and total acidity) improves when late summer water stress is induced. Also other studies indicate that reduced water supply, particularly late in fruit development, is associated with higher total soluble solids and titratable acidity (Castel and Buj, 1990; Peng and Rabe, 1998; Hutton et al., 2007). Mantell et al. (1976) report similar results in 'Shamouti' orange where fewer summer irrigations resulted in improved juice quality (higher levels of vitamin C, sugar, citric acid, and higher total soluble solids to acid ratio), despite increasing the number of small fruit per tree.

Manner et al., (2006) indicate that citrus growth in dry Mediterranean climates, with average rainfall less than 250 mm/yr and dry summers, is only possible with irrigation. Citrus can generally tolerate 3-4 months of minimal rainfall. Drought tolerance depends on temperatures, soils, wind, and the desired level of fruit production. Citrus loses productivity in drought and requires irrigation during the summer months to sustain production also in Florida and Central and Southern California.

Syvertsen (1982) reported that citrus leaf water potential is related to age, and minimum leaf water potential for stomatal closure of young leaves were as low as -1.6 MPa which coincides with previously measured leaf potentials at zero turgidity. As for mature leaves, water potential is been as low as -3.5 MPa in three- to six-month-old leaves. The rate at which new leaves are produced and become mature may be an important mechanism by which the tree can adjust to water deficit.

González-Altozano and Castel (1999) report that Clementine yields are reduced when water stress was applied at an early stage of fruit ontogeny through increased fruit drop. However, stress applied at a later stage can affect fruit yield by decreasing fruit weight. Syvertsen (1985) showed that vegetative growth in citrus is very sensitive to water deficit. Also, long water deficit periods reduce yield of orange about 25% (Pires et al., 2008).

Shalhevet and Levy (1990) reported that size, rind appearance and internal maturity are the major quality parameters for citrus fruit, and these variables are strongly influenced by irrigation management. Water application may enhance performance of some variables while minimizing effects on others.

Research on citrus irrigation has been reviewed by several authors (Doorenbos and Kassam, 1979; Kriedemann and Barrs, 1981; Shalhevet et al., 1981). Functional relationships between yield and water deficits in citrus have been given by Shalhevet and Bielorai (1978) and Doorenbos and Kassam (1979). In some of these irrigation studies, the sensitivity of yield to water stress has been found to depend on the phenological growth stage at which the deficit occurred.

10.4.3 Partial rootzone drying

PRD is a deficit irrigation strategy designed to maintain half of the root system in a dry or drying state, while the other half is irrigated. The theory behind PRD is based on root-to-shoot chemical signaling in drying soil, which is supposed to reduce stomatal conductance (Gowing et al., 1990; Düring et al., 1996; Loveys et al., 2000). This strategy involves exposure of roots to alternate drying and wetting cycles and enables plants to grow with reduced stomatal conductance but without signs of water stress, (Zhang et al., 1987; Davies et al., 1994; Davies et al., 2002; Santos et al., 2003; Kang and Zhang 2004; Talluto et al., 2008).

A practical inconvenience of PRD is that it requires the use of twice the amount of tubing than RDI or conventional irrigation, thus increasing installation costs. Nevertheless, the underlying mechanisms of PRD functioning are still a matter of discussion. Bravdo (2005) states that it is not possible to have absolute control of root drying under field conditions and that hydraulic redistribution from deeper to shallower roots may prevent the clear results that can be obtained in potted plants.

The PRD seems to give better results in soils that ensure high rates of infiltration and deep roots. However, the depth of the root-zone is not necessarily directly correlated with the infiltration rate. Low infiltration rates may be associated with both superficial and deep root systems.

The partial root-zone drying irrigation technique reduces the need for pruning (Dry et al., 1996) due to a reduced vegetative vigor. In addition, opening up of the canopy increases light penetration to the fruit which increases the color e.g. of grape fruit and increases the content of compounds associated with flavor and aroma. Most important also, half the amount of water applied to control plants can be added to apple trees under PRD without significant yield reductions (Talluto et al., 2008). Interesting results of the application of PRD on cotton showed that the crops were ready for harvest three weeks earlier than the control treatment (Mingo and Davies, 2001).

PRD improves WUE in various tree crop species (Kang et al., 2002; Grant et al., 2004; Romero et al., 2004; Van Hooijdonk et al., 2004; Cifre et al., 2005; Tognetti et al., 2005). PRD clearly improves yield per unit of applied water with respect to conventional irrigation when high irrigation volumes are applied (Davies et al., 2002; Kirda et al., 2007; Morison et al., 2008).

11. Relationship between water deficit and polyphenol content

There are some interesting and conflicting experiments that regard the relationship between water deficit in different species and their polyphenol content. According to Walia et al. (2005), the increase in the expression of F3'H, which leads to the biosynthesis of ortho-dihydroxylated B-ring antioxidant flavonoids, is superior in the salt sensitive genotype than in the salt tolerant rice. Tattini et al. (2006) state that the increase in carbon allocated to myricetin and quercetin glycosides, two well known antioxidant flavonols, is significantly greater in the salt sensitive *Myrtus communis* than in salt tolerant *Pistacia lentiscus*. Conversely, antioxidant activity and polyphenols content is higher in a less salt sensitive cv of *Carthamus tinctorius* than in another salt sensitive (Karray-Bouraoui et al., 2011).

In grape berries, a steep induction of the whole set of genes involved in the biosynthesis and transport of flavonoids was found in respnse to water deficit (Castellarin et al., 2007 a, b). Polyphenols oxidase and non-enzymatic antioxidants increase under drought stress also in *Vigna unguiculata* (Manivannan et al., 2007). Bettaieb et al. (2011) show that water deficiency cause a reduction of biomass, plant eight and total chlorophyll contents in *Salvia officinalis*, but also an increase of the level of total and individual polyphenols that is more pronounced under moderate water deficit. In tomatoes, plants grown with no irrigations, show fruits with an higher antioxidant activity, due to a decline in enzyme activity, and a rise in vitamin C and total phenolic contents, than in full irrigated plants (Barbagallo et al., 2012). Olive trees that receive a seasonal water equivalent to 100% of estimated crop

evapotranspiration show a decrease of total polyphenols than trees treated with a continuous deficit irrigation (30% ET_c) and trees with no irrigation (Fernandes-Silva et al., 2013).

In *Ligustrum vulgare*, the roozone salinity stress and UV-radiation enhance in a similar way the flavonoids metabolism (Agati et al., 2011). In *Andrographis paniculata*, the content of two anticancer phytochemicals, including andrographolide and neoandrographolide, increases with salinity (Talei et al., 2013).

On the contrary, declining soil water content reduces growth and content of polyphenols in different cultivars of *Camellia sinensis* (Cheruiyot et al., 2007). In cherry tomatoes, polyphenols oxidase activity seems to be related to the watering regime employed, showing a maximum under normal watering conditions and pectin methylesterase enzymatic activity decreases greatly with increasing water stress (Barbagallo et al., 2008). Water stress causes a decrease in shikimic acid, and so in polyphenols content, in salt sensitive cultivars of cherry tomato (Sánchez-Rodríguez et al., 2011).

Despite some apparently contrasting responses, it is generally observed that, under prolonged stress, the activities of key components of the antioxidant machinery of stress-sensitive tend do decrease (Hatier and Gould 2008).

12. The aim of the research

The aim of the research was to determine, in 'Valencia' oranges, the level of secondary metabolites like flavonoids and carotenoids in relation to different abiotic factors, such as irrigation and use of metabolic promoters, and biotic factors, such as greening disease. It would be interesting to regulate the content of flavonoids and carotenoids, which are useful for the human body, using different agricultural techniques. Also, it could assume a different use for orange fruits infected by greening disease; not only for juice extraction, but also how pharmafood with with an importance against degenerative human diseases. The effect of the assumption of orange juices obtained from trees under different irrigation treatments on human dermal carotenoids was also studied.

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EXPERIMENTAL PART

Effect of Sunred metabolic promoter and deficit irrigation on fruit quality of 'Valencia' oranges

Quality at harvest is a key factor for fruit production in general and citrus orchards in particular. The unevenness of the fruit maturation level, particularly evident in Valencia orange, may determine significant cost increases, especially in the case of manual harvesting, and a substantial decline of product quality.

Fruit ripening is characterized by a complex series of physiological and biochemical processes that ultimately determine organoleptic and commercial quality. The main processes that take place during ripening are color development, due to degradation of chlorophyll and accumulation of anthocyanins (red, orange and purple) and carotenoids (yellow and orange); accumulation of sugars (glucose, fructose and sucrose); decrease of organic acids, mainly transformed into sugars; softening of the pulp, due to the activation of enzymes that degrade the main components of cell walls (Brady, 1987).

Anthocyanins are polyphenols and their synthesis starts from phenylalanine (Sakuta et al., 1994). Biosynthesis of carotenoids involves several steps catalyzed by ethylene-promoted enzymes (Alba et al., 2005). Ethylene synthesis, in turn, depends on methionine (Bleecker and Kende, 2000) and is known to regulate maturation, also by acting on sugar accumulation and carotenoid synthesis (Alexander and Grierson, 2002).

During maturation, chlorophyll is degraded by chlorophyllase, which is regulated by oxylipins (Tsuchiya, 1999), thus indirectly responsible for accelerating fruit color development. Both phenylalanine and oxylipins are involved in determining cell wall structure and resistance (Galliano et al., 1993) with consequences on fruit flesh firmness.

Preliminary field trials with foliar applications of phenylalanine, methionine, oxylipins and sugars have shown improvements in external color, sugar content and uniformity of maturation in grapes (González-Herranz et al, 2009), pome fruits (Fan et al, 1998; Kondo et al, 2007), tomato (Alba et al, 2005) and melon (Nafie et al, 2011). It was hypothesized that similar improvements could be obtained in 'Valencia' oranges with applications of those metabolic promoters.

Materials and Methods

The trial was carried out in one of the experimental fields of the Department of Agricultural and Forest Sciences, University of Palermo (30°06' N, 13°21' E, and 31 m a.s.l.). Forty-five

adult orange trees (*Citrus sinensis*, 'Valencia') grown on sour orange (*Citrus aurantium*) were selected and divided according to a randomized block design.

Three irrigation treatments were imposed in spring 2011: irrigation with volumes corresponding to 100% of crop evapotranspiration applied to entire root-zone (CI), partial root-zone drying (PRD) with 50% of CI water applied to one alternated side of the root-zone, and continuous deficit irrigation (DI) with 50% of CI water applied to both sides of the root-zone.

In March 2012, one half of the trees was selected from each block and divided into two treatments (Control and Sunred). About 30 and 20 days before harvest (7 May 2012), Sunred trees were sprayed with a 4 ml/l solution of the commercial product Sunred (Biolchim, Bologna, Italy) using a shoulder sprayer. Trees of the two treatments were separated by an appropriate number of buffer trees to avoid drifts and contamination of control. Trees were sprayed at sundown of dry days and in absence of wind.

At harvest, all fruits were collected, counted and weighed to obtain an average production per tree. A sub-sample of fruits from each tree (at least 10%) was randomly taken and brought to the laboratory for qualitative determinations, such as weight, peel color, juice yield, juice color, total soluble solids (TSS) and titratable acidity (TA). Intensity of peel and juice color of each fruit was determined by digital image analysis.

Yield and fruit quality data were compared by two-way analysis of variance with Sunred and irrigation as main factors, blocks as replicate factors, and Sunred ´ irrigation as the sole interaction. All tests were performed using Systat procedures (Systat Software Inc., Richmond, Ca., USA).

Results and Discussion

No significant interaction between irrigation and foliar spray was detected. Also, yield and fruit quality was mostly unaffected by irrigation treatments (data not shown). On the other hand, Sunred spray somewhat influenced fruit quality, whereas yield parameters were not affected (Table 1). Specifically, Sunred significantly increased TSS/TA in the juice of 'Valencia' oranges with respect to untreated trees. The average intensity of peel or juice color was not improved by Sunred sprays.

Yet, a test for differences between variances (Levene) as well as the coefficient of variation indicated greater peel color uniformity in Sunred treated fruits as compared to control fruits (Table 2). Such higher degree of peel color uniformity may be related to a more even allocation of carbon to fruits and more favorable conditions for those metabolic processes

involved in color development. We also detected less variability in number of fruits of Sunred trees, but this should not be related to the treatment.

The experiment demonstrated that there is no synergy or interaction between irrigation level and Sunred foliar sprays. The latter influenced fruit quality and specifically it increased juice TSS/TA ratio. This improvement was primarily due a decrease in TA rather than an increase in TSS, and it may be assumed as an indicator of maturity advancement. Also, no significant peel or juice color improvement was observed in our 'Valencia' oranges, as hypothesized by the metabolic function of applied promoters. The Sunred effect, in this case, was expressed in terms of enhanced color uniformity, which may be considered an improvement if we relate it to a more uniform maturation stage.

Our results suggest that, thanks to the action of oxyilipins and phenylalanine (activating biosynthesis of anthocyanins and flavonols) and methionine (stimulating ethylene biosynthesis and in turn fruit ripening), Sunred foliar metabolic promoter in pre-harvest may improve internal fruit quality of 'Valencia' oranges, especially by advancing and uniforming fruit maturation, and regardless of irrigation management.

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Table 1. Production and fruit quality of adult 'Valencia' orange trees sprayed (Sunred) or non-sprayed (Control) with Sunred metabolic promoter in pre-harvest. TSS: total soluble solids; TA: titratable acidity.

	Control	Sunred	P value
Production per tree (kg)	43.6	40.6	0.614
Number of fruits per tree	383	331	0.329
Fruit weight (g)	116	121	0.299
Juice yield (%)	57.5	58.5	0.570
Specific weight (g/g)	0.344	0.345	0.630
TSS (°Brix)	12.8	12.8	0.959
TA (g/L)	12.2	11.2	0.049
TSS/TA	1.06	1.16	0.046
Peel color index	0.963	0.960	0.376
Juice color index	0.799	0.798	0.660

Table 2. Coefficients of variation (c.v.) and significance (P value) from Levene's test for production and fruit quality parameters measured in adult 'Valencia' orange trees sprayed (Sunred) or non-sprayed (Control) with Sunred metabolic promoter in pre-harvest. TSS: total soluble solids; TA: titratable acidity.

	Control c.v.	Sunred c.v.	P value
Production per tree	0.378	0.293	0.396
Number of fruits per tree	0.399	0.259	0.039
Fruit weight	0.095	0.119	0.307
Juice yield	0.039	0.083	0.133
Specific weight	0.021	0.019	0.701
TSS	0.046	0.060	0.603
TA	0.107	0.073	0.201
TSS/TA	0.102	0.071	0.813
Peel color index	0.008	0.002	0.021
Juice color index	0.009	0.007	0.851

Effect of HLB on flavonoid content in peel, pulp and juice of 'Valencia' oranges

Citrus Greening disease, or Huanglongbing, or HLB, is considered to be the most harmful of all Citrus diseases. Pratically, all commercial citrus species and cultivars are sensitive, regardless of rootstocks, and it represents a dangerous threat for regions still free of the disease, such as the Mediterranean basin, Western Asia, Australia and Pacific Ocean islands. The causal agent of HLB is the endogenous bacterium *Candidatus Liberibacter* spp., vectored by two psyllid species: *Diaphorina citri* in Asia and in America and *Trioza erytreae*, in Africa (Jagoueix et al., 1994; Jagoueix et al., 1996). Both psyllid species can transmit the bacterium (Bovè, 2006), carrying it inside the salivary glands from which it is injected into the phloem (Bonani et al., 2010).

A tree, which begins to show symptoms in an orchard in early stages of infections, can be identified, among many symptomless neighboring tress, by the presence of one or several yellow shoots that give rise to the Chinese name ‘yellow dragon disease’ or Huanglongbing. These yellow shoots are very typical and unique to HLB.

In leaves, the most typical symptom of HLB is the so called ‘blotchy mottle’. In addition to these symptoms, the leaves may become thicker and leathery.

HLB induces also very characteristic symptoms to fruits which remain small, asymmetric, misshapen and poorly colored. Also, in a cross-sectioned fruit, it is easy to see the small, brownish/black aborted seeds, highly typical of HLB (Bovè, 2006).

After transmission of the bacterium, infected Citrus trees respond with a wide array of physiological and chemical reactions resulting in a sequence of symptoms, from mild chlorosis to distinct mottling associated with an increase in bacterial concentrations (Coletta-Filho et al., 2010).

Content of carbohydrates, whose concentration is directly proportional to that of secondary metabolites, changes significantly in healthy, HLB-symptomatic and HLB-asymptomatic sweet orange leaves, although those are not symptoms unique to HLB (Cimò et al., 2013). Specifically starch, sucrose and fructose concentrations increase in HLB-asymptomatic and HLB-symptomatic leaves when compared to healthy ones (Fan et al., 2010). Disease symptom development in HLB-affected plants is associated with starch accumulation in the leaf tissue (Albrecht and Bowman, 2008).

Juice from symptomatic fruit has higher acidity, lower sugars and lower Brix/acid ratio, resembling juice from less mature fruits (Dagulo et al., 2010). Analysis of secondary

metabolites, flavonoids above all, in HLB infected fruits compared to healthy ones reveals increases in terpenes, hesperidin, naringenin, quercetin, limonin and nomilin aglycones (Dagulo et al., 2010).

Flavonoids are secondary metabolites synthesized from glucose via the acetic acid or shikimic acid pathways (Ververidis et al., 2007). In plants they have a defensive function, acting as antioxidants (Hernandez et al, 2009). In humans, they exert several beneficial properties such as antitumor and anti-inflammatory activity and cardiovascular protection. Moreover, they can protect against high blood pressure and cholesterol (Mennen et al, 2004).

The aim of this study was to determine the effect of HLB disease on flavonoid content in peel, pulp and juice of 'Valencia' oranges. Studying these responses could on one side, improve our understanding of plant defense mechanisms against HLB infection, and on the other side, suggest alternative uses for unmarketable, infected oranges, above all the fruit peel, where flavonoid content should concentrate.

Materials and Methods

'Valencia' orange fruits used in this study were obtained from groves at the Citrus Research and Education Center (CREC), Lake Lafred, FL, USA. Fruits were harvested on 29 March and 27 May, and divided into three groups: oranges from trees showing clear symptoms of HLB infection, oranges from HLB-infected trees with no visual symptom, and oranges from healthy trees (control).

A sub-sample of fruits from each group was randomly taken and divided into 5 sets of 4 fruits. Each set of fruit was weighed with an electronic scale, washed, photographed, split into two halves and squeezed with an electric juicer to obtain juice.

Pictures were taken under controlled light conditions. Digital images were edited, cropped to one fruit per image, labeled and used to determine intensity of peel color in each fruit. Digital images were analyzed using an algorithm that converts images from RGB to CIE 1976 L*a*b format, extracts the fruit from the image (removing the image background) and quantifies color characteristics as the weighted distance of each pixel in the image from a reference sample (best colored area interactively chosen from a well-colored fruit). The output is an index ranging from 0 (no orange) to 1 (full orange).

Juice of each set of fruits was weighed to determine juice yield as a percentage of total fruit weight. Total soluble solids (TSS) were measured in the juice of each set using a manual refractometer. Juice pH and titratable acidity were determined using a pH meter and a calibrated column. Then juices were stored in a refrigerator at 4 °C for subsequent analysis.

Pulp was removed by hand from each fruit, weighed and stored at -29 °C. Similarly, peel of each set of fruits was cut in small pieces, weighed and stored at -29 °C. Pulp and peel were freeze-dried at -50 °C, then they were weighed again and ground into powder with an electric coffee grinder. Pulp and peel powder was stored at -29 °C. Subsequently, 1 g of peel powder for each set of fruits was mixed with 20 ml of pure methanol and the suspension was sonicated for 60 minutes in an ultrasonic bath at room temperature. The sonicated suspension was filtered and methanol extracts were stored at -29 °C. The same protocol was adopted for 1 g of pulp.

A solution containing 5 ml of unfiltered orange juice and 20 ml of 70% methanol (v:v) was centrifuged at 4000 rpm for 15 minutes at 20 °C. The supernatant was filtered and stored at -29 °C.

The same procedure was repeated with fruit harvested at the end of May, for a total of 90 methanolic extracts.

Hesperidin, nobiletin, tangeretin, narirutin and didymin were quantified using HPLC equipment (Agilent Technologies 1200 Series). The analysis was performed on an Agilent Zorbax SB-C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size) at room temperature using a gradient elution of acetonitrile (solvent A) and millipore water (solvent B) at a flow rate of 1 ml min^{-1}. Re-equilibration time between two individual runs was 2 min and a gradient program was used as follow:

time (min)	solvent A (%)	solvent B (%)
0.00	22.00	78.00
10.00	22.00	78.00
35.00	61.00	39.00
40.00	100.00	0.00
45.00	22.00	78.00

Retention times were investigated and sample chromatograms with their compounds were compared with standard chromatograms to get flavonoid concentrations contained in orange peel, pulp and juice.

Hesperidin, nobiletin and tangeretin standards (HPLC grade) were purchased from Thermo Fisher Scientific (Waltham, MA, USA), narirutin and didymin standards from Indofine Chemical Company (Hillsborough, NJ, USA). Standards were diluted to obtain 5 different solutions: 6 mg of hesperedin in 1 ml of dimethyl sulfoxide; 3.8 mg of narirutin, 1 mg of

tangeretin, 1 mg of nobiletin and 1 mg of didymin each in 1 ml of methanol. A mixed standard stock solution was prepared by mixing 1 ml of each previous solution with 1 ml of pure methanol. Then, the mixed solution was diluted step by step with methanol to give six different concentrations of working standard solutions.

Data were compared by three-way analysis of variance with group (control, asymptomatic, and infected), tissue (peel, pulp, and juice) and harvest period as main factors, and all possible interactions; statistical tests were performed using SYSTAT procedures (Systat Software Inc., Richmond, Ca., USA).

Results and Discussion

Peel color and TSS were higher in healthy and asymptomatic fruits than in infected ones, whereas acidity was higher in infected fruits than in control and asymptomatic ones (Tab. 1). The TSS/AC ratio was highest in control fruits, followed by asymptomatic and lowest in infected fruits. Also, peel color, juice TSS and acidity increased with maturation (Tab. 1). There was no significant change in TSS/AC over the considered period.

Citric acid was linearly related to peel color but with different slopes depending on fruit type (Fig. 1); one steeper for infected fruits and the other less steep for asymptomatic and control fruits.

There was also a direct linear relationship between peel color and soluble solids common to all fruit types, suggesting that sweeter fruit are also better colored (Fig. 2). As expected, values of infected fruit lay at the low left end of the line.

HPLC chromatograms show retention times of various flavonoid compounds. They also show that flavonoids were most abundant in peel, juices exhibited only traces of hesperidin, narirutin and didymin, and hesperidin was the most abundant flavonoid in all tissues and fruit types (Figs. 3, 4, 5).

In the fruit peel, narirutin and dydimin contents were higher in infected fruits than asymptomatic and control ones (Tab. 2). Hesperidin content was higher in infected fruits than in control ones; asymptomatic fruits had an intermediate content. Nobiletin content was different in all groups, with infected fruits showing the highest level and control fruits showing the lowest. The level of all four compounds decreased with fruit maturation (Tab. 2). No interaction between fruit type and harvest was detected.

In March, tangeretin content was higher in infected and asymptomatic fruits than in control ones (Tab. 3). In May, tangeretin content was highest in infected fruits and lowest in control

fruits, with asymptomatic fruits at an intermediate level. There is, also, a general decrease in tangeretin with fruit maturation (Tab. 3).

In the pulp, narirutin content tended to decrease with fruit maturation, especially in infected fruits, while there was no change over time in control fruits (Tab. 4). Narirutin level was higher in infected fruits than in control and asymptomatic fruits, only in less ripe fruits (March). Hesperidin content was higher in infected fruits than in control or asymptomatic fruits harvested in March (Tab. 4). In addition, hesperidin content decreased with fruit maturation in infected and control fruits, while there was no significant change with maturation in asymptomatic fruits. In March, dydimin level was higher in infected fruits than in control or asymptomatic fruits. Dydimin content decreased with maturation only in infected fruits (Tab. 4).

In the juice, narirutin content increased with fruit maturation only in control fruits. It also tended to be highest in infected fruits at both harvest times (Tab. 5). Hesperidin content was highest in infected fruits and lowest in control fruits, only in May. Dydimin content was higher in infected fruits than in asymptomatic ones, only in May. Also, there is a general increase in hesperidin and dydimin with fruit maturation (Tab. 5).

When flavonoid contents in the three tissues were averaged considering the incidence of each tissue on the whole fruit (weighed average, mg g^{-1} fruit), asymptomatic fruits harvested in March had a higher level of narirutin than control and infected fruits (Tab. 6). Narirutin content increased with maturation in infected fruits, while it decreased in asymptomatic ones. In March, hesperidin content was higher in control and asymptomatic fruits than in infected ones (Tab. 6). Hesperidin content increased with maturation in asymptomatic and infected fruits, while it decreased in control fruits. In March, control and infected fruits had a lower content of didymin than asymptomatic fruits (Tab. 6). Dydimin content increased with maturation in infected fruits only. Asymptomatic fruits exhibited a higher level of tangeretin than control and infected ones (Tab. 6). Also, tangeretin content decreased with maturation in asymptomatic fruits, while it increased in infected ones. Nobiletin content was higher in asymptomatic fruits than in control and infected ones (Tab. 6). Nobiletin content increased with maturation only in infected fruits. In May, flavonoid levels were similar in all fruit types, except for hesperidin content that was lower in control fruits than asymptomatic and infected ones (Tab. 6).

HLB appears to arrest or slow down fruit maturity as evidenced by lower sugars and elevated acid levels in fruits. HLB interferes with starch and sugar metabolism in the leaves and fruits, this determines the chaos in the networks of plant hormones, such as ethylene, causing the

sugar accumulation in leaves and deprivation in fruits where they act as precursors of flavonoids. Also HLB stops the transport of nutrients in the phloem, reducing photosynthesis (Fan et al., 2010). Asymptomatic fruits seem to be closer to healthy fruits than to infected fruits, both in terms of sugars and acidity. Also the content of nariritun, hesperidin, nobiletin and dydimin is similar in healthy and asymptomatic fruits (regardless of tissue), especially when early-harvested in March.

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Table 1. Fruit quality of healthy, asymptomatic and HLB-infected 'Valencia' oranges harvested at the end of March and end of May. P-values are from analysis of variance. Different letters indicate significant differences among levels of a given factor for each compound (Tukey's test, $P \leq 0.05$).

Treatment	Peel Color	TSS (°Brix)	Citric acid ($g\ L^{-1}$)	TSS/AC
Healthy	0.785 a	12.4 a	7.19 b	1.10 a
Asymptomatic	0.785 a	12.2 a	7.48 b	1.04 b
Infected	0.677 b	8.5 b	12.2 a	0.45 c
P	<0.001	<0.001	<0.001	<0.001
March	0.729	9.35	7.52	0.858
May	0.769	12.7	10.4	0.871
P	<0.001	<0.001	<0.001	0.407

Table 2. Narirutin, hesperidin, didymin and nobiletin content (mg g^{-1}) in the peel of healthy, asymptomatic and HLB-infected 'Valencia' oranges harvested at the end of March and end of May. P-values are from analysis of variance. Different letters indicate significant differences among levels of a given factor for each compound (Tukey's test, $P \leq 0.05$).

Factor level	Narirutin	Hesperedin	Didymin	Nobiletin
Healthy	2.53 b	25.9 b	1.66 b	0.799 c
Asymptomatic	3.38 b	27.8 ab	1.89 b	1.036 b
Infected	4.85 a	33.6 a	2.42 a	1.267 a
P	< 0.001	0.009	< 0.001	< 0.001
March	4.47	31.3	2.28	1.20
May	2.69	26.9	1.70	0.863
P	< 0.001	0.009	< 0.001	< 0.001

Table 3. Tangeretin content (mg g^{-1}) in the peel of healthy, asymptomatic and infected 'Valencia' oranges harvested at the end of March and end of May. P-value for harvest X fruit type is from analysis of variance. Different letters indicate significant differences among types of fruit within each harvest (Tukey's test, $P \leq 0.05$).

Harvest	Fruit type	Tangeretin
March	Healthy	0.118 b
	Asymptomatic	0.173 a
	Infected	0.204 a
May	Healthy	0.099 b
	Asymptomatic	0.106 ab
	Infected	0.141 a
	P	0.032

Table 4. Narirutin, hesperidin and didymin content (mg g^{-1}) in the pulp of healthy, asymptomatic and HLB-infected 'Valencia' oranges harvested at the end of March and end of May. P-value for harvest X fruit type is from analysis of variance. Different letters indicate significant differences among types of fruit within each harvest (Tukey's test, $P \leq 0.05$).

Harvest	Fruit type	Narirutin	Hesperedin	Didymin
March	Healthy	3.42 b	14.5 b	1.62 b
	Asymptomatic	3.86 b	14.2 b	1.71 b
	Infected	8.48 a	26.9 a	3.12 a
May	Healthy	3.43 a	9.89 c	1.69 a
	Asymptomatic	2.83 a	14.2 b	1.46 a
	Infected	4.01 a	19.2 a	2.03 a
	P	< 0.001	< 0.001	< 0.001

Table 5. Narirutin, hesperidin and didymin content (mg g^{-1}) in the juice of healthy, asymptomatic and HLB-infected 'Valencia' oranges harvested at the end of March and end of May. P-value for harvest X fruit type is from analysis of variance. Different letters indicate significant differences among types of fruit within each harvest (Tukey's test, $P \leq 0.05$).

Harvest	Fruit type	Narirutin	Hesperedin	Didymin
March	Healthy	0.033 b	0.135 a	0.012 a
	Asymptomatic	0.042 ab	0.128 a	0.014 a
	Infected	0.051 a	0.125 a	0.014 a
May	Healthy	0.053 a	0.157 a	0.019 ab
	Asymptomatic	0.049 a	0.182 ab	0.015 b
	Infected	0.060 a	0.192 a	0.020 a
	P	0.016	0.005	0.009

Table 6. Flavonoid content (mg g^{-1} of fruit, weighed average of peel, pulp and juice) in healthy, asymptomatic and HLB-infected 'Valencia' oranges harvested at the end of March and end of May. P-value for harvest X fruit type is from analysis of variance. Different letters indicate significant differences among types of fruit within each harvest (Tukey's test, $P \leq 0.05$).

Harvest	Fruit type	Narirutin	Hesperedin	Didymin	Tangeretin	Nobiletin
March	Health	0.716 b	5.52 a	0.401 b	0.017 b	0.123 b
	Asymptomatic	1.180 a	6.18 a	0.559 a	0.026 a	0.186 a
	Infected	0.787 b	3.50 b	0.325 b	0.013 b	0.093 b
May	Health	0.714 a	5.05 b	0.441 a	0.019 a	0.137 a
	Asymptomatic	0.791 a	6.81 a	0.476 a	0.020 a	0.149 a
	Infected	0.834 a	5.81 a	0.446 a	0.017 a	0.130 a
	P	0.005	0.013	0.022	< 0.001	< 0.001

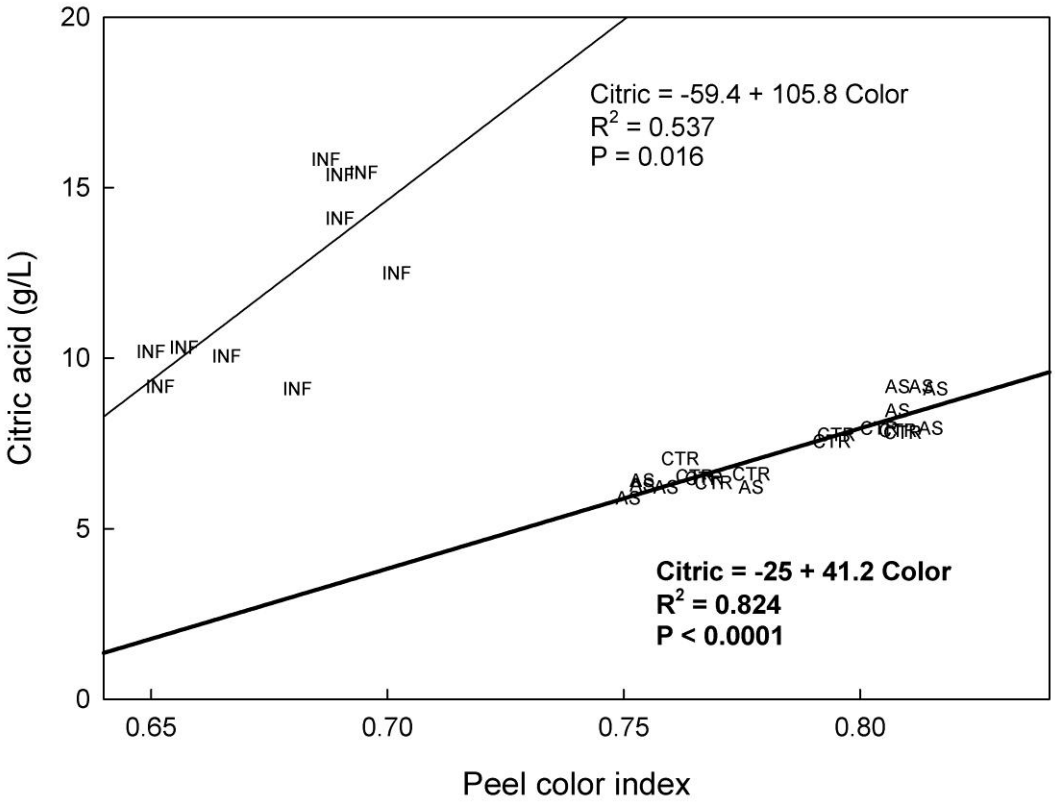

Figure 1. Relationships between peel color index and citric acid of healthy (CTR), asymptomatic (AS) and HLB-infected (INF) 'Valencia' orange fruits.

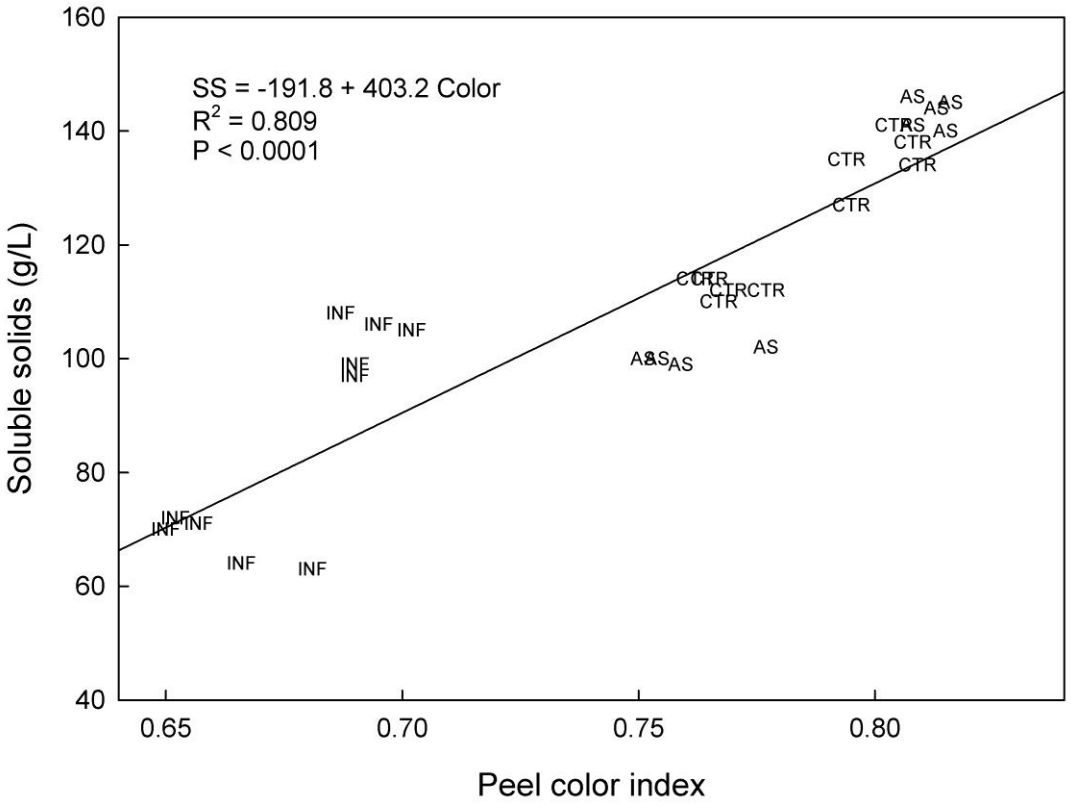

Figure 2. Relationship between peel color index and soluble solids of healthy (CTR), asymptomatic (AS) and HLB-infected (INF) 'Valencia' orange fruits.

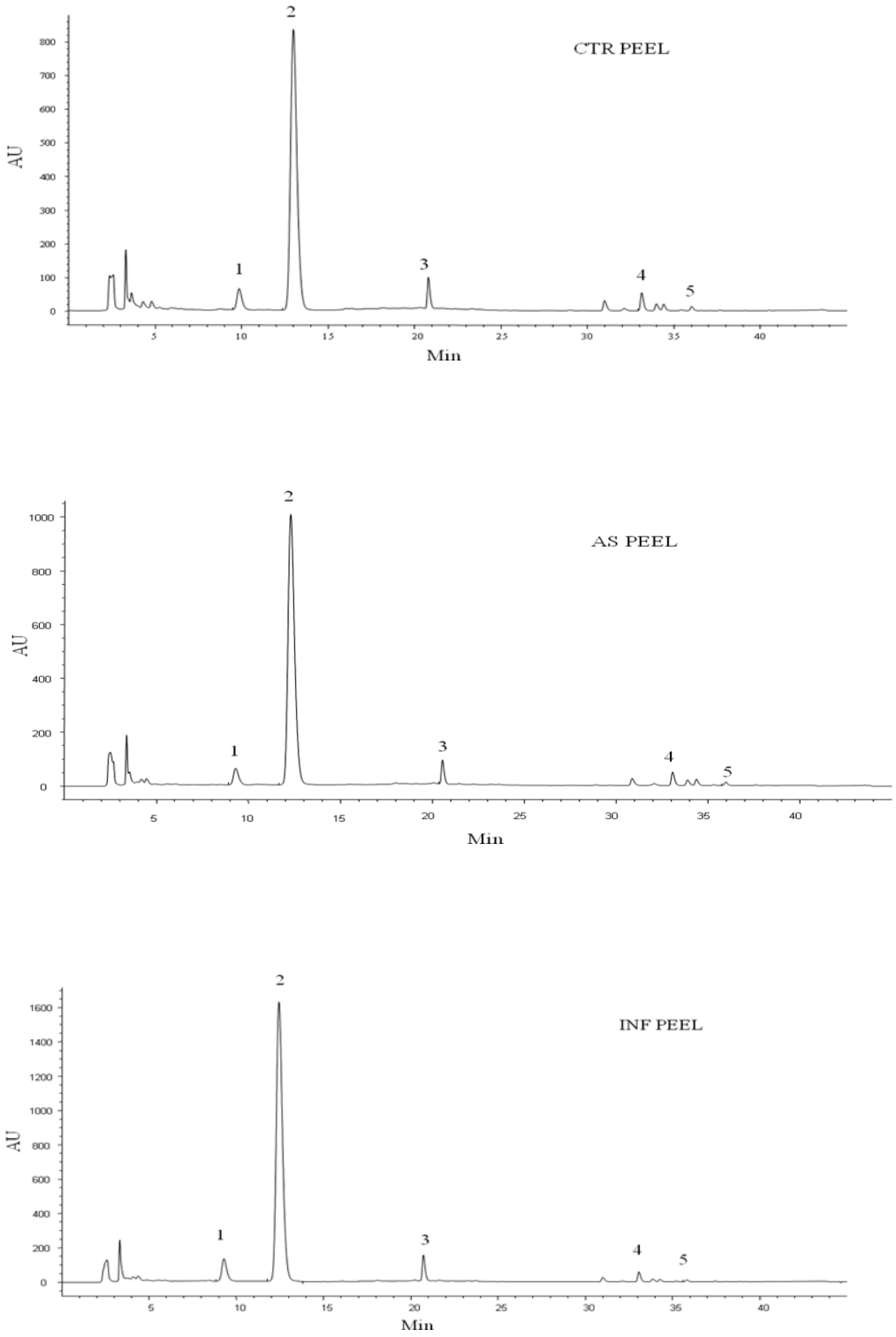

Figure 3. HPLC chromatograms for peels of healthy (CTR), asymptomatic (AS) and infected (INF) 'Valencia' oranges. Peaks 1, 2, 3, 4 and 5 correspond to narirutin, hesperidin, didymin, tangeretin and nobiletin, respectively.

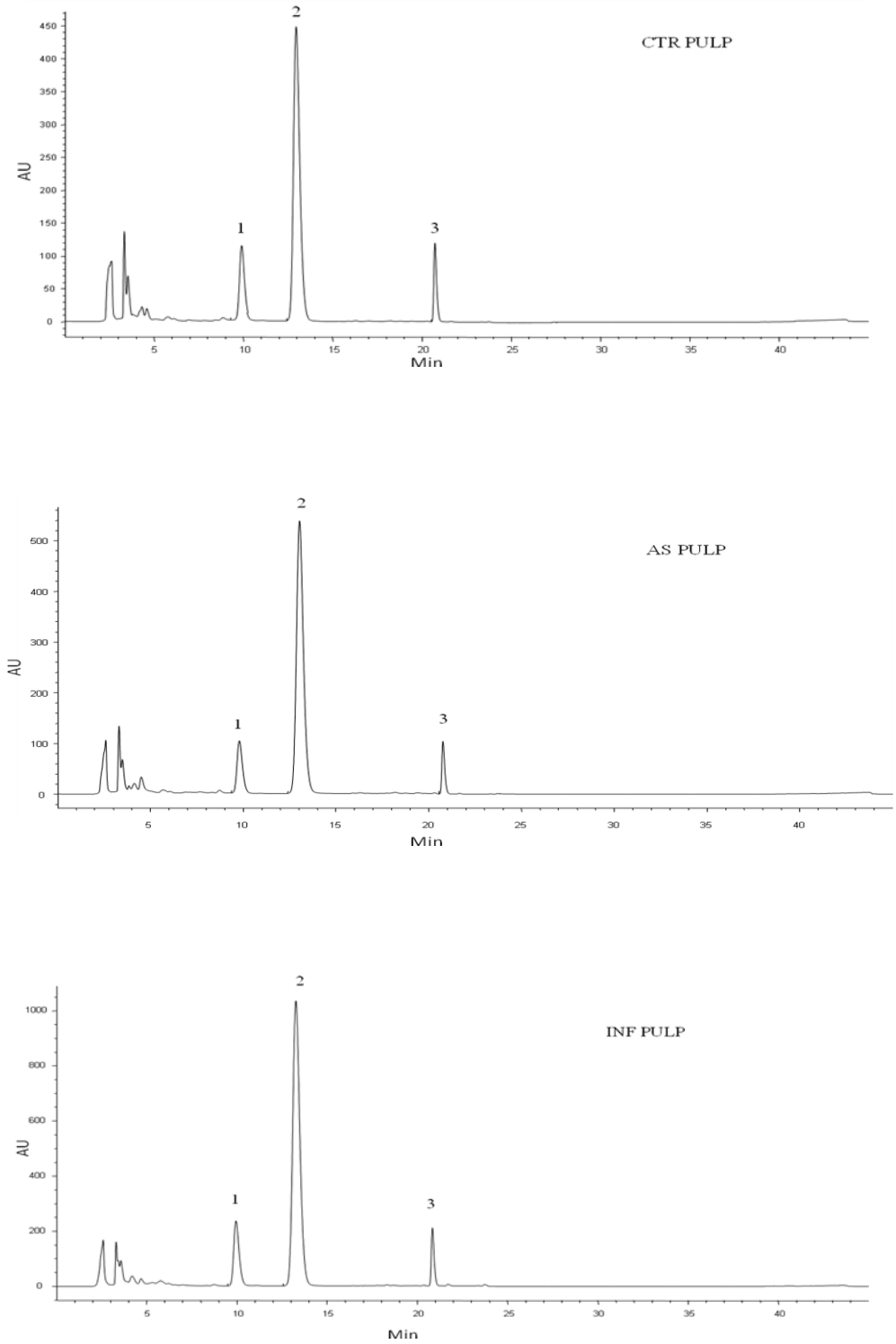

Figure 4. HPLC chromatograms for pulps of healthy (CTR), asymptomatic (AS) and infected (INF) 'Valencia' oranges. Peaks 1, 2 and 3 correspond to narirutin, hesperidin and didymin, respectively.

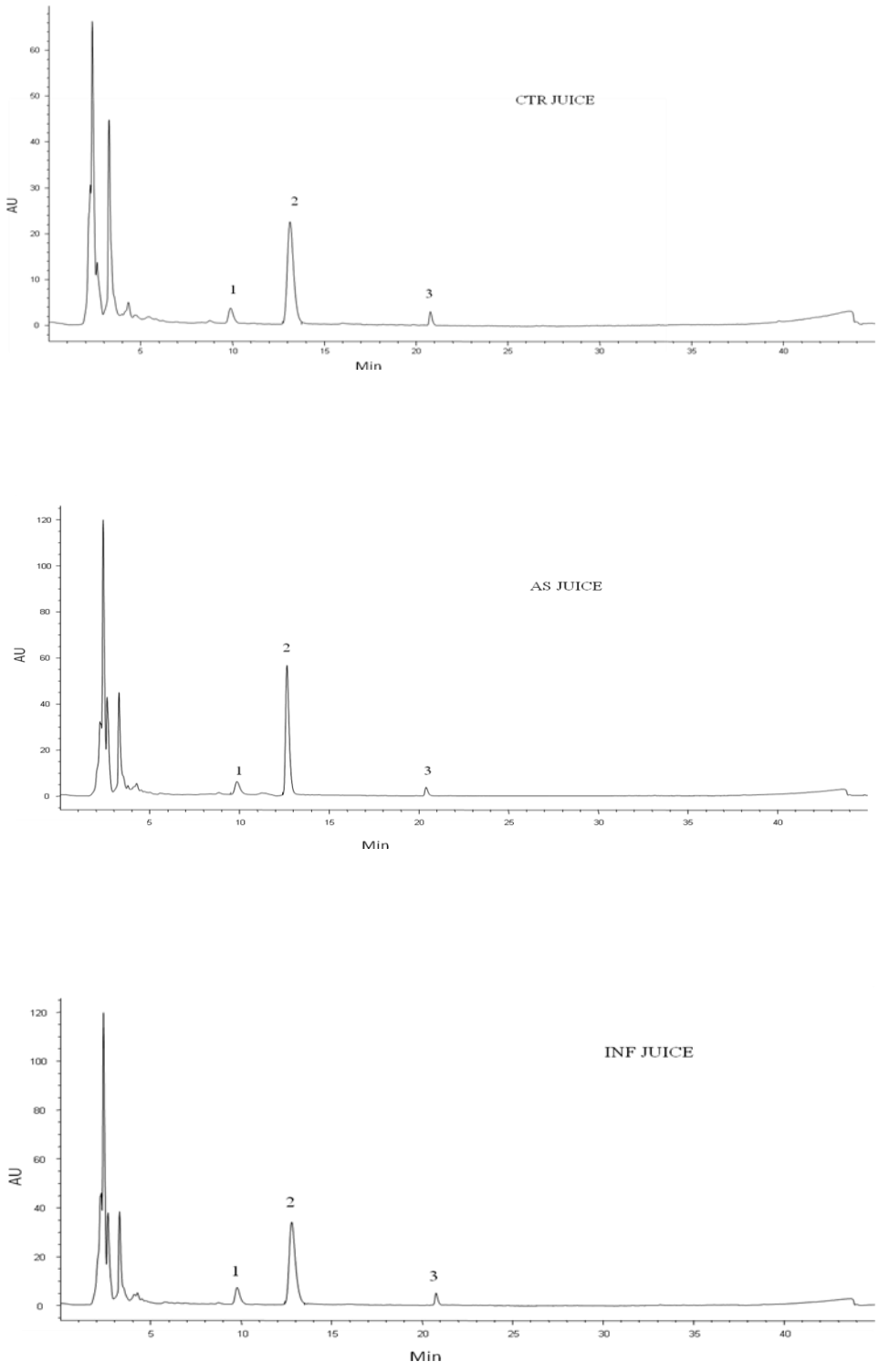

Figure 5. HPLC chromatograms for juices of healthy (CTR), asymptomatic (AS) and infected (INF) 'Valencia' oranges. Peaks 1, 2 and 3 correspond to narirutin, hesperidin and didymin, respectively.

Influence of water deficit and fruit maturation on flavonoid content in peel, and juice of 'Valencia' oranges

The sustainable production of citrus depends on the availability of adequate water throughout the year and orange plant growth is closely linked to soil water availability. From another side, climatic changes and demographic pressure can influenced water availability for cultivation of fruit trees. For these reasons increasing crop water use efficiency through the rationalization of irrigation becomes critical also for fruit production and a readily available soil water supply appears essential for the development of optimum fruit size and yield (Levy et al., 1978; Moreshet et al., 1983).

Plant water deficits, resulting from a reduction in the flow of water through the soil-plant system, affect a wide range of physiological and developmental processes involved in fruit production, including reduced photosynthetic capacity due to stomatal closure (Slatyer, 1967; Kozlowski, 1974; Hsaio, 1973).

Behboudian and Mills (1997) define deficit irrigation (DI) as a system of managing soil water supply to impose periods of predetermined plant or soil water deficit that can result in some economic benefit. Over the last two decades, extensive research work conducted on fruit trees have demonstrated a positive yield response to mild water deficits (Marsal et al., 2002; Chalmers et al., 1981).

Originally, DI was developed primarily to control vegetative growth in high density orchards, and secondarily to maximize fruit size, fruitfulness and fruit quality (Goodwin and Jerie, 1992). Thus, it consists on deliberately applying an amount of water at a level below water requirement during the irrigation season in order to save water and control vigor (Goodwin and Boland, 2000). A certain reduction in yield is observed, but fruit quality (e.g. sugar content) tends to be equal to or even greater than that of fully irrigated trees (Marouelli and Silva, 2007; Spreer et al., 2007; Cui et al., 2008; Hueso and Cuevas, 2008).

Moss and Muirhead (1971a, b) in their study on ‘Valencia’ orange show that temperature and evaporation rates during November were the most consistent predictors of yield in New South Wales. Similar responses (ranging from 26 to 150 kg per tree) from one season to the next, are reported by Jones and Cree (1964) who show that yield appeared negatively correlated with the previous year’s crop and with daily maximum temperature following bloom.

On the other hand, (Hilgeman, 1977) shows that fruit quality (total soluble solids and total acidity) improves when late summer water stress is induced. Also other studies indicate that reduced water supply, particularly late in fruit development, is associated with higher total

soluble solids and titratable acidity (Castel and Buj, 1990; Peng and Rabe, 1998; Hutton et al., 2007).

In grape berries a steep induction of the whole set of genes involved in the biosynthesis and transport of flavonoids, because of water stress, is found by Castellarin et al. (2007a, b). Water stress causes a decrease in shikimic acid, and so in polyphenols content, in salt sensitive cultivars of cherry tomato (Sánchez-Rodríguez et al., 2011).

Bettaieb et al. (2011) show that water deficiency cause a reduction of biomass, plant eight and total chlorophyll contents in *Salvia officinalis*, but also an increase of the level of total and individual polyphenols that is more pronounced under moderate water deficit.

The aim of this study is to determine the influence of water deficit and fruit maturation on flavonoid content in peel, and juice of 'Valencia' oranges. Understanding these responses, it could be possible to regulate flavonoid content under different irrigation treatments.

Materials and Methods

The trial was carried out in one of the experimental fields of the Department of Agricultural and Forest Sciences, University of Palermo (30°06' N, 13°21' E, and 31 m.a.s.l.). Thirty-two adult orange trees (*Citrus sinensis*, 'Valencia') grown on sour orange (*Citrus aurantium*) were selected and divided according to a randomized block design with four blocks of four trees each.

Water deficit was imposed by applying two different irrigation levels in spring 2011: irrigation with volumes corresponding to 100% of crop evapotranspiration (CI) and irrigation (DI) with 50% of CI water volumes, by using sprinklers with half the delivery rate.

On 9 and 23 April and 9 May, five fruits per tree, from four CI trees and four DI trees (one in each block), were randomly harvested. Samples were brought to the laboratory, where they were washed and squeezed with an electric juicer. Juices and peels were stored at -18 °C. In May, a solution containing 5 ml of unfiltered orange juice and 20 ml a 70% methanol (v:v) was centrifuged at 4000 rpm for 15 minutes at 20 °C. The supernatant was filtered and stored at -18 °C.

Peel was cut in small pieces, weighed and freeze-dried at -50 °C, then it was weighed again and ground into powder with an electric coffee grinder. Subsequently, 1 g of peel powder was mixed with 20 ml of pure methanol and the suspension was sonicated for 60 minutes in an ultrasonic bath at room temperature. The sonicated suspension was filtered and methanol extracts were stored at -18 °C.

Hesperidin, narirutin and didymin were quantified LC/MS using Thermo TSQ Quantum Access equipment. The analysis was performed on a Luna C18(2) column (150 x 2.0 mm, 5µm) at ambient room temperature using a gradient elution of H_2O + 0.1% HAF (solvent A) and CH_3CN + 0.1% HAF (solvent B) at a flow rate of 0.2 ml/min. A gradient program was used as follow:

2 min.	90% (A)	10% (B)
5 min.	90% (A)	10% (B)
5 min.	80% (A)	20% (B)
26 min.	80% (A)	20% (B)
28 min.	50% (A)	50% (B)
31 min.	50% (A)	50% (B)
32 min.	60% (A)	40% (B)
38 min.	60% (A)	40% (B)
40 min.	90% (A)	10% (B)

Retention times were investigated and sample chromatograms with their compounds were compared with standard chromatograms to get flavonoid concentrations containing in orange peel, pulp and juice.

Hesperidin, narirutin and didymin standards were purchased from Indofine Chemical Company (Hillsborough, NJ, USA). Standards were diluted to obtain 3 different solutions: 6 mg of hesperedin with 1 ml of dimethyl sulfoxide; 3.8 mg of narirutin with 1 ml of methanol and 1 mg of didymin with 1 ml of methanol. A mixed standard stock solution was prepared by transferring 1 ml of each previous solution with 1 ml of pure methanol. Then, the mixed solution was diluted step by step with methanol to give five different concentrations of working standard solutions.

Data were compared by three-way analysis of variance with irrigation, tissue and harvest period as main factors, and all possible interactions were included in the model; statistical tests were performed using SYSTAT procedures (Systat Software Inc., Richmond, Ca., USA).

Results and Discussion

Water deficit influenced hesperidin, narirutin and didymin content in ‘Valencia’ oranges peel, infact oranges DI had in peel higher content of flavonoids than oranges CI (Fig. 1). In peel, narirutin content decreased with fruit matiruation and these results confirm what had been seen in US with healthy, asymptomatic and HLB infected oranges harvested in two different periods. Hesperidin and dydimin contents were not influenced by fruit maturation (Fig. 2). In juice, narirutin and dydimin content were higher in oranges DI, while hesperidin content was not influenced by water deficit (Fig. 3). Narirutin content decreased with fruit maturation,

while hesperidin and dydimin content were not influenced by fruit maturation. This work showed how water deficit can influenced in the same way both orange peel and juice and all flavonoids contained in them, on the contrary flavonoids are differently influenced by fruit maturation.

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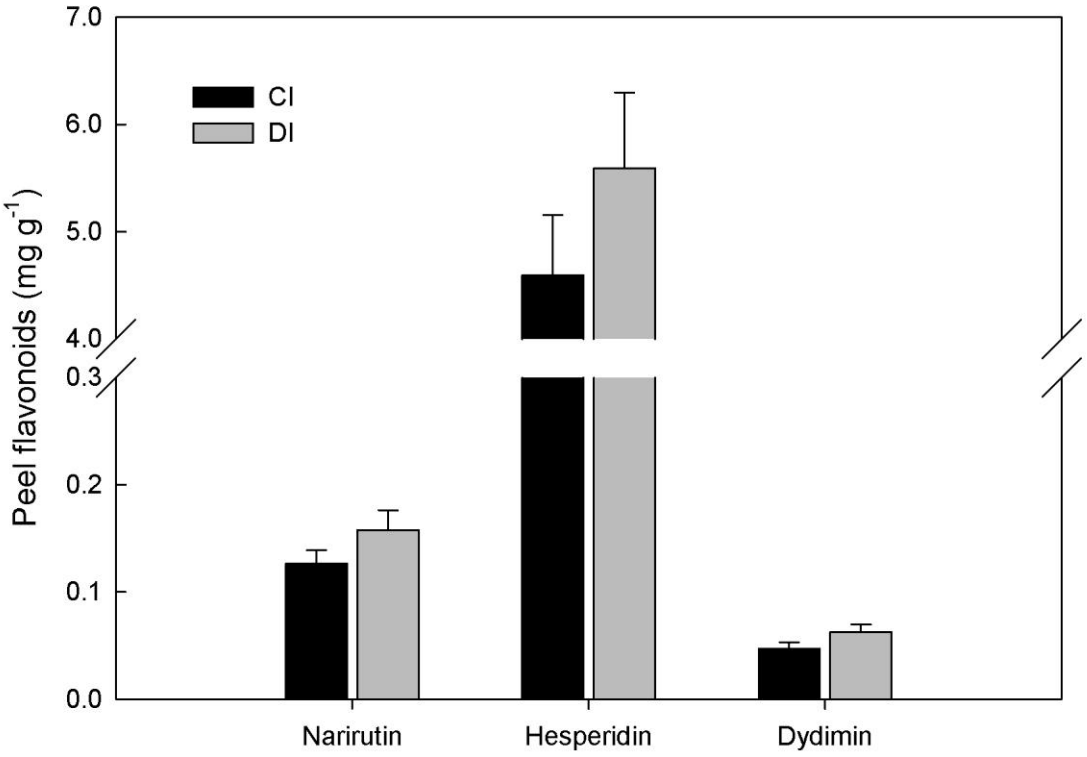

Figure 1. Narirutin, hesperidin and dydimin content in peel of 'Valencia' oranges in response to water deficit.

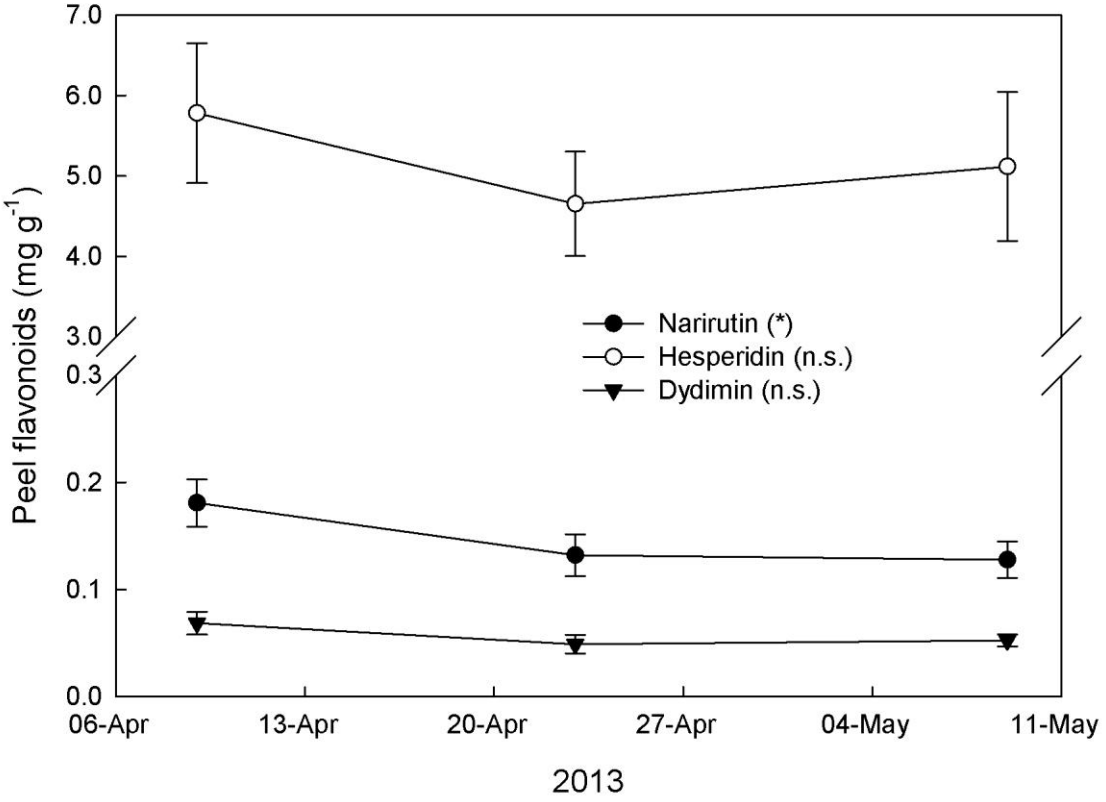

Figure 2. Narirutin, hesperidin and dydimin content in peel of 'Valencia' oranges in response of fruit maturation.

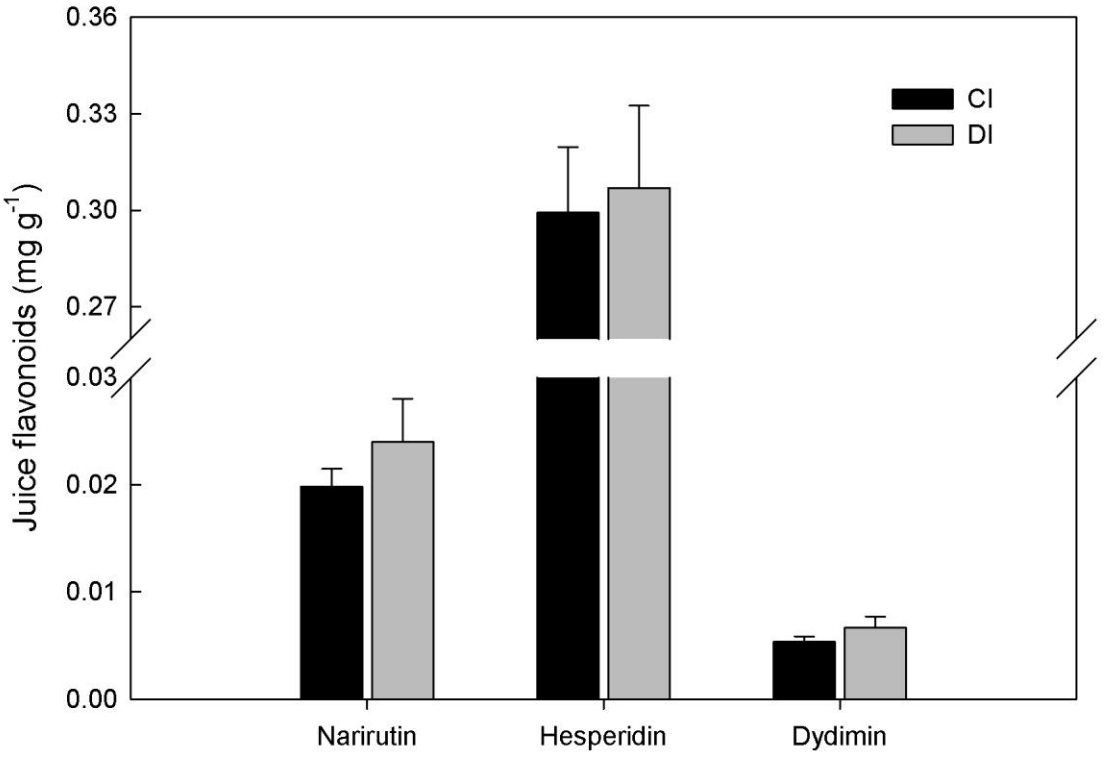

Figure 3. Narirutin, hesperidin and dydimin content in juice of 'Valencia' oranges in response to water deficit.

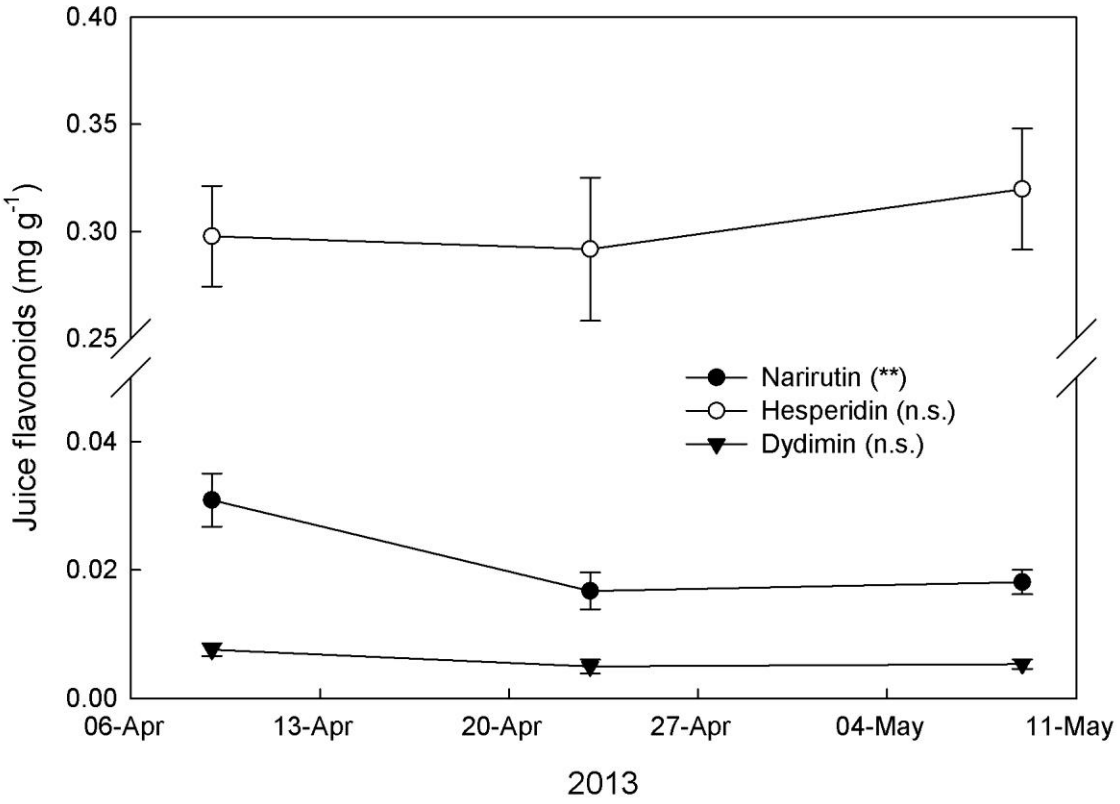

Figure 4. Narirutin, hesperidin and dydimin content in juice of 'Valencia' oranges in response of fruit maturation.

Influence of orange juice from differently irrigated trees on human dermal carotenoid content

In the last 30 years, consumers have shown increasing attention in the benefits of fruit consumption in terms of prevention of degenerative human diseases. Fruit and vegetables are important sources of vitamins, carotenoids and phenolic compounds, which are associated with a lower risk of developing cardiovascular, neurological and oncological diseases (Scalbert and Williamson, 2000). This compounds have a wide range of biological effects, such as anti-inflammatory, antioxidant and antimicrobial activities (Harborne and Williams, 2000).

Juices have high concentration of polyphenols and vitamins, for this reason they can be included among functional foods. Orange juice is one of the most consumed fruit juices, thanks to the pleasantness of its taste, attractive color and for its perceived health benefits. It is a complex 'chemical mixture' of compounds. Aroma of fresh juice is unstable, due to a combination of chemical, enzymes and microbial reactions that require stabilizations treatments.

The concept of quality of orange juice has changed during the years. In the past, the presentation of the product and its safety were the main priorities, while nowadays the expectations are more diversified, implying a strong focus on high quality taste and biological function to improve wellbeing and/or health.

Over 50% of the world's production of orange juice is made with 'Valencia' orange, that is the world's most important orange variety. It is a small sized, sweet and blond orange with a few seeds, thin skin, tender pulp and very juicy. It is usually picked in April, but can stay on the tree without any problem or quality loss until the end of May; this prolongs juice processing and marketing season.

Carotenoids serve two key roles in plants: they absorb light energy for use in photosynthesis and protect chlorophyll from photodamage (El-Agamey et al., 2004). The synthesis of various carotenoids, therefore, is a crucial metabolic process underlying these functions.

In humans, they are powerful antioxidants that are implicated in the prevention of or protection against serious health disorders such as cancer and heart diseases. Many studies have identified that there is a close correlation between a low level of β-carotene in the blood and the possibility of developing cardiovascular diseases or cancer (Kontush et al, 1999).

The human skin has a balanced and developed defense mechanism, which acts effectively against the harmful effects of free radicals and other reactive species produced in the human

body as a result of UV radiation, contacts with environmental hazards and by metabolic processes (Bickers and Athar, 2006). It is possible to determine in vivo carotenoid content in the human skin with a Raman spectrometer; the device uses a fast and non-invasive optical method of measuring the absolute concentrations of β-carotene in living human skin (Gardiner and Graves,1989).

The aim of this study was to determine whether irrigation level/strategy can influence carotenoid content in 'Valencia' orange juice and how orange juice consumption can influence human dermal carotenoid content.

Materials and Methods

The trial was carried out in one of the experimental citrus groves of the Department of Agricultural and Forest Sciences, University of Palermo, in the 2011-2012 cropping season. Forty-five adult orange trees (*Citrus sinensis*, 'Valencia') grown on sour orange (*Citrus aurantium*) were selected and divided according to a randomized block design. Three irrigation treatments were imposed in spring 2011: irrigation with volumes corresponding to 100% of crop evapotranspiration applied to entire root-zone (CI), partial root-zone drying (PRD) with 50% of CI water applied to one alternated side of the root-zone, and continuous deficit irrigation (DI) with 50% of CI water applied to both sides of the root-zone.

Before harvesting a sample of CI, PRD and DI fruit was collected and brought to the lab, where each fruit was washed, squeezed with an electric juicer and 10 ml of juice were placed in Petri dishes and photographed with a digital camera against a white background and under standard light conditions. Digital images were edited, cropped to one fruit per image, labeled and used to determine intensity of juice color in each fruit. Digital images were analyzed using an algorithm that converts images from RGB to CIE 1976 L*a*b format, extracts the Petri dish with juice from the image (removing the image background) and quantifies color characteristics as the weighted distance of each pixel in the image from a reference sample (best colored area interactively chosen from a well-colored juice). The output is an index ranging from 0 (no orange) to 1 (full orange).

The same juice was used to determine total carotenoid content by UV-visible spectrophotometry using a Beckman DU-640 spectrophotometer (Beckman Coulter, Inc., CA, USA) and according to Benk's method (Benk, 1961). Briefly, 0.5-1.0 g of orange juice was mixed with 50 ml of a 1:1 (v: v) methanol and petroleum ether solution in presence of sand. The mix was filtered on cotton wool and residues were repeatedly extracted with the methanol-petroleum ether solvent until the elute was colorless. Five ml of water were added,

the solution was mixed well and the aqueous layer was removed. The remaining petroleum-ether phase was washed with small quantities of methanol (90%) until colorless. After washing with 25 ml of water and filtering, the extract containing all the carotenoids was re-diluted with petroleum ether to 50 ml final volume. The optical density of the solution was read on the spectrophotometer at 450 nm. Potassium bichromate was used to construct standard curves and quantify total carotenoids.

Only CI and PRD fruit samples were used to test the level of skin carotenoids. A pre-trial involved two people that had been drinking 500 ml of orange juice daily for a month. Before and after each drinking, their hand was scanned with a Raman spectrometer to determine the dermal level of carotenoids.

In the final trial, 38 individuals were randomly selected and were divided into two groups. One group drank 500 ml of fresh orange juice from CI fruits every other day for 16 days. The second group drank the same amount of orange juice from PRD fruits. At the beginning of the experiment, all people volunteering to the test were interviewed on their eating habits, and their hands scanned with a Raman spectrometer to establish their initial level of dermal carotenoids. The measurement was repeated two and four days after the last consumption of orange juice.

Data were compared by two-way analysis of variance with treatment and day of measurement as main factors, and statistical tests were performed using SYSTAT procedures (Systat Software Inc., Richmond, Ca., USA).

Results and Discussion

There was a non-linear relationship between juice color index and carotenoids present in juice. In particular, juice carotenoids increased exponentially as color index increased (Fig. 1). Juice deriving from PRD fruits had the highest level of carotenoids, while CTR had the lowest and DI had an intermediate level (Tab. 1). For this reason juices from PRD and CTR were used for the Raman trial.

Dermal carotenoid score generally increased with consumption of orange juice (Fig. 2). Trends for the two people were different probably due to the unequal initial values of dermal carotenoids and personal living habits (diet and stress). In one case, high initial values of dermal carotenoids determined a linear increase of carotenoid score with juice consumption (Fig. 2A); in the other case, low initial values of dermal carotenoids determined a logaritmic increase of carotenoid score with juice consumption (Fig. 2B).

In the final trial, dermal carotenoid content was highest at the end of the experiment, regardless of juice type (CI or PRD). Four days after the end of juice consumption carotenoid score decreased, but its level remained higher than at the beginning of the experiment (Fig. 3). Dermal carotenoid level was generally increased by juice consumption and may be considered an effective method of estimating in vivo human carotenoid level. The study also showed that carotenoids are quickly processed by the human body, and their persistence most likely depends on living habits, diet and stress. Further trials with alternated periods of juice drinking and no juice drinking would give us a better understanding of carotenoid persistence in the human body.

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Table 1. Carotenoid content in orange juices from fruits of 'Valencia' trees under control irrigation (CI), deficit irrigation (DI) or partial rootzone drying (PRD). Different letters indicate significant differences among levels of a given factor for each compound (Tukey's test, $P \leq 0.05$).

Irrigation	Total carotenoids (mg L^{-1} juice)
CI	22.2 b
DI	22.4 ab
PRD	23.0 a

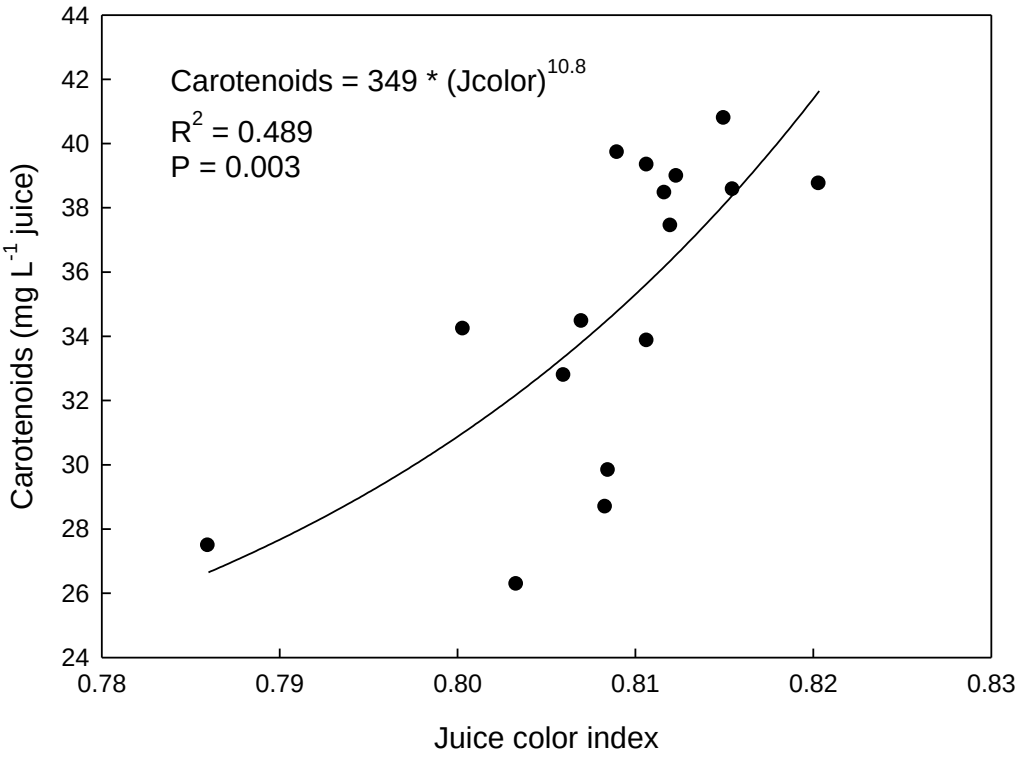

Figure 1. Relationship between juice color index and total carotenoids in 'Valencia' orange fruits.

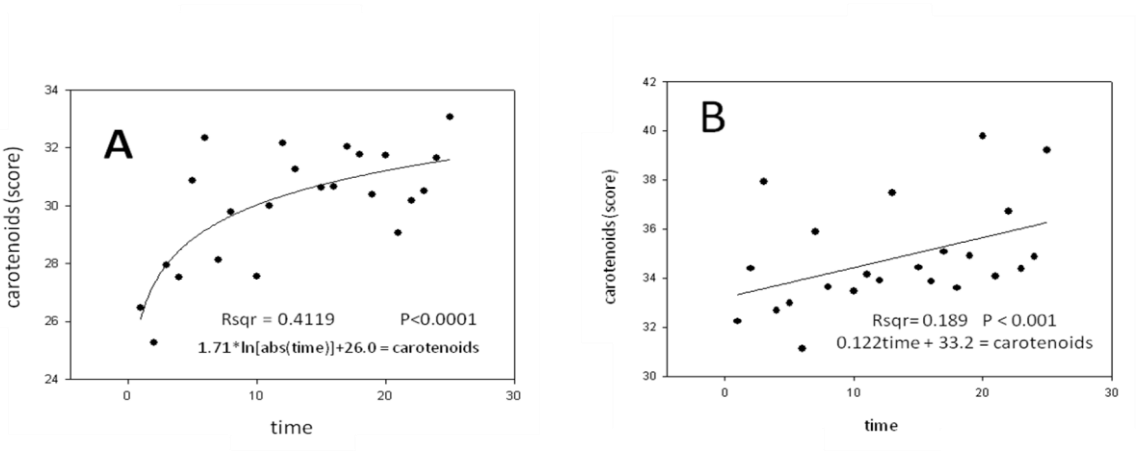

Figure 2. Relationship between consumption of orange juice and dermal carotenoid score in two different people.

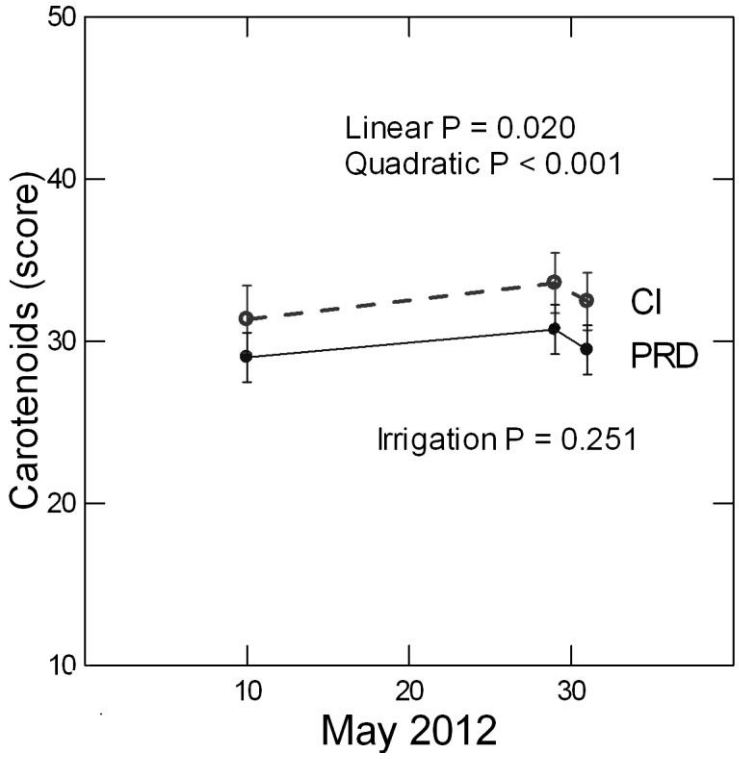

Figure 3. Changes in dermal carotenoid score during and after the period of orange juice consumption; orange juice obtained from fruits of 'Valencia' trees under control irrigation (CI) or partial rootzone drying (PRD). P values from ANOVA and orthogonal polynomial contrasts.