TOTAL POLYPHENOL, PROANTHOCYANIDIN AND FLAVONOID CONTENT, CARBOHYDRATE COMPOSITION AND ANTIOXIDANT ACTIVITY OF PERSIMMON (*DIOSPYROS KAKI* L.) FRUIT IN RELATION TO CULTIVAR AND MATURITY STAGE

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Abstract

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The current study investigates the total polyphenol, proanthocyanidin and flavonoid content, the carbohydrate composition, and antioxidant activity of persimmon fruit (*Diospyros kaki* L) from five cultivars (Hyakume, Moro, Hiratanenashi, Jiro C 24276 and Mandarino). Fruits were analyzed at two maturity stages - commercial harvest stage and physiological ripening stage. The HPLC analysis showed that glucose and fructose were the predominant sugars in the fruit. At commercial harvest stage total sugar content varied in the range 9.6 – 14.4 g/100g FW and increased slightly to 11.5-16.5 g/100g FW after maturation, being the highest in Hiratanenashi cultivar. Generally, the polyphenol content and particularly the proanthocyanidin content were strongly affected by the cultivar and maturity stage. The highest polyphenol and proanthocyanidin content at commercial harvest stage showed Hiratanenashi persimmon (916.8 mg GAE/100g FW and 540.2 CE/100g FW, respectively). The content of both polyphenols and proanthocyanins decreased proportionally during the maturation. At physiological ripening stage, again Hiratanenashi was the richest source of polyphenols and proanthocyanidins - 400.2 mg GAE/100g FW and 90.2 CE/100g FW, respectively. Persimmons showed different antioxidant activity depending on cultivars measured by Oxygen Radical Absorbance Capacity (ORAC) and Hydroxyl Radical Averting Capacity (HORAC) methods. At commercial harvest stage the pollination variant astringent cultivar Hiratanenashi has the highest ORAC and HORAC values – 49.2 μmolTE/g FW and 30.2 μmolGAE/g FW, respectively. After the maturation of the fruit of all cultivars, a more or less decrease in both ORAC and HORAC values was observed, proportionally to the decrease of the total polyphenol content.

Key words: Persimmon (*Diospyros kaki* L.), polyphenols, proanthocyanidins, antioxidant activity, ORAC, HORAC

Abbreviations: CE – Catechin equivalents; CV – Cultivar; FW – Fresh weight; GAE – Gallic acid equivalents; HORAC - Hydroxyl Radical Averting Capacity; HPLC – High performance liquid chromatography; ORAC – Oxygen Radical Absorbance Capacity; PCNA – Pollination constant non astringent; PVA – Pollination variant astringent; PVNA – Pollination variant non astringent; QE – Quercetin equivalents ; TE – Trolox equivalents

Introduction

Persimmon (*Diospyros kaki* L) belongs to the Ebenaceae family and originates from China (Giordani 2002). Persimmon culture is grown mostly because of the valuable taste qualities of the fruit, distinctive with low acidity and sweet flavour. Fruit are suitable for fresh consumption as well as dried or factory processed. Persimmon accumulates a large amount of condensed tannins (proanthocyanidins) in the vacuoles of specific cells called 'tannin cells' during fruit development. Proanthocyanidins cause astringency, which is a dry

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or puckering sensation due to the coagulation of oral proteins (Akagi et al., 2011). Generally, persimmon cultivars are divided into three main groups based on a combination of pollination types and astringency types. Pollination constant astringent (PCA) cultivars cannot be eaten firm at the commercial harvest stage because of the high levels of soluble tannins, which can be removed naturally or artificially (Bubba et al., 2009). Pollination constant non-astringent (PCNA) persimmon cultivars contain less soluble tannins than these belonging to the astringent group and may be consumed either when firm or when very soft at physiological ripening stage. The fruits of pollination variant non-astringent (PVNA) cultivars are not edible at harvest if parthenocarpic, whereas fruits are non-astringent when fertilized. The number of seeds can be relevant for fruit astringency at harvest. Normally few seeds guarantee edibility of the fruits.

Persimmon fruit possess high nutraceutical value and contain many biologically active substances including antioxidants, dietary fiber, triterpenoids and minerals (Gorinstein et al., 2001; Chen et al., 2008; Park et al., 2008; Akter and Eun, 2009; Veberic et al., 2010; Zhou et al., 2010; Dembitsky et al., 2011). Persimmons revealed considerable health and medicinal benefits, which are considered to be related to the various hydrophilic and lipophilic antioxidants including phenolic compounds, vitamin C and carotenoids, contained in the fruit (George and Redpath, 2008). Traditionally, persimmon fruit have been used for their medicinal properties, such as their blood pressure-lowering and diuretic effects. A persimmon-supplemented diet had a lipid-lowering effect (Matsumoto et al., 2006, 2008, 2010; Quan et al., 2012) and positively influenced organ functions in streptozotocin induced diabetic rats (Lee et al., 2006). Persimmon fruit improved also lipid metabolism in rats fed diets containing cholesterol (Gorinstein et al., 1998a, 1998b, 2000) and showed antigenotoxic effect (Jang et al., 2010). Furthermore, persimmon has been shown to have antitumor properties on several tumor cell lines *in vitro* (Kawase et al., 2003) and has been associated with an inhibitory effect on human lymphoid leukemia cells (Achiva et al., 1997). Phenols in fruit peel reduced the oxidative stress induced by hyperglycemia, indicating the potential benefits as a valuable source of antioxidants in diabetic conditions (Yokozawa et al., 2007). Persimmon tannin inhibited the incidence of stroke and prolonged the life span of spontaneously hypertensive rats (Uchida et al., 1995), revealed bile acid-binding ability (Matsumoto et al., 2011) and inhibited the catalytic activity and lethality of Chinese cobra PLA_2 (Xu et al., 2012).

Nowadays, persimmons are cultivated world widely with 90% of production being obtained in China, Japan and Korea. In Bulgaria the persimmon is one of the most recently introduced fruit-trees and probably because of this, there is only a scarce data about the chemical composition of Bulgarian persimmons (Mangarova, 2005; Yordanov, 2011). Several environmental and genetic factors such as cultivar, harvest time, habitat, fertilization, and climate affect the accumulation of biologically active substances in the fruit and precise informations for physiological activities of persimmons depend mainly on cultivars. Maturity stage is another factor determining the organoleptic and functional properties of the fruits. In the literature, several papers investigating the phytochemical and nutrient composition of persimmon fruit are available, but there are only few articles dealing with the changes of these parameters during the ripening process (Senter et al., 1991; Kondo et al., 2004; Salvador et al., 2007, Bubba et al., 2009). To our knowledge, such information for the cultivars Hyakume, Moro, Hiratanenashi, Jiro C 24276 and Mandarino, subjects of the current study, is not available. Therefore, the objective of the current study was to determine the total polyphenol, proanthocyanidin and flavonoid content, carbohydrate composition and antioxidant activity of five persimmon cultivars, grown in Bulgaria at two stages of maturity - commercial harvest stage and physiological ripening stage.

Materials and Methods

Trees growing conditions

Persimmon cultivars Hyakume, Moro, Hiratanenashi, Jiro C 24276 and Mandarino were planted in the spring of 2008 in Sliven (South Bulgaria) region. Date plum (*Diospyros lotus*) was used as a seedling rootstock. One-year-old nursery trees were planted at spacing 5x3 m. The experiment was set up in a randomized block design, with four replications and two plants per plot. Trees were trained as free growing crown. The experiment was conducted under drip irrigation on soil type: Fine-Silty, Mixed Mesic Mollic Xerofluvents, pH 6.5 (at 0-30 cm dept) and pH 6.4 (at 30-60 cm dept). 4050° is the average year temperature sum for the period with temperatures higher than 10° and the average year rain is 566 mm.

Cultivars description

Hyakume: A pollination variant non-astringent (PVNA) cultivar, which originates from Japan. This cultivar produces large sized fruits and has very good grafting compatibility on *Diospyros lotus* rootstock. It bears female flowers only.

Moro: A pollination variant non-astringent (PVNA) cultivar of Italian origin. Moro bears female and male flowers and produces medium sized fruits. It has very good grafting compatibility on *Diospyros lotus* rootstock.

Hiratanenashi: A pollination variant astringent (PVA) cultivar, which originates from Japan. It has very good graft-

ing compatibility on *Diospyros lotus* rootstock, bears female flowers only and produces medium sized fruits.

Jiro C. 24276: A pollination constant non-astringent (PCNA) cultivar, which originates from the USA. The tree bears female flowers only and produces medium sized fruits. It has medium grafting compatibility on *Diospyros lotus* rootstock.

Mandarino: A pollination variant non-astringent (PVNA) cultivar with unknown origin. It bears female and male flowers and produces medium sized fruits. It has good grafting compatibility on *Diospyros lotus* rootstock.

Sampling and post harvest treatments

Samples of 40 fruits of each cultivar were harvested at commercial harvest period (beginning of November). Half of each sample was used immediately for analyzes. The other half of the sample was shelf stored and analyzed at physiological ripening stage. At both stages of maturity, samples were frozen and freeze-dried in laboratory freeze-drier. The freeze-dried samples were grinded to fine powder with a coffee mill and stored in plastic bags until analyzed.

Extraction and HPLC analysis of carbohydrates

One-gram freeze-dried powder was subjected to extraction with 30 ml water at 60°C and shaking on thermostatic water bath (NUVE, Turkey). After that, the samples were centrifuged (6 000 x g) and supernatants were used for HPLC analysis of sugars. HPLC determination was performed on Waters 484 system, connected to a refractometric Waters R401 detector and Aminex HPX – 87H column (300 x 7.8 mm, BioRad), eluent 0.004 mol/l H_2SO_4, flow 0.5 ml/min, temperature 23°C. The standard compounds were purchased from Sigma-Aldrich (Steinheim, Germany).

Extraction of polyphenols, proanthocyanidins and flavonoids

Approximately 0.5 g of freeze dried persimmon powders were weighted accurately and extracted with 20 ml of the extragent (80% acetone solution in 0.2% formic acid) (v:v). Extraction was conducted on an orbital shaker at room temperature for one hour. After that, the samples were centrifuged (6 000 x g) and supernatants were removed. The solid residue was subjected to the second extraction under the same conditions. Both supernatants were combined, acetone was evaporated and extract volume was adjusted to 40 ml with deionized water. Water extracts were analyzed for total polyphenol, proanthocyanidin and flavonoid contents, and antioxidant activity.

Total polyphenol content analysis

Total polyphenols were determined according to the method of Singleton and Rossi, (1965) with Folin-Ciocalteu's re-

agent. Gallic acid was employed as calibration standard and results were expressed as gallic acid equivalents (GAE) per 100 g fresh weight (FW).

Total proanthocyanidin content analysis

Total proanthocyanidins content was determined by the method of Sarneckis et al. (2006). To properly diluted persimmon extract (0.5 ml) was added 0.04 g/kg methylcellulose solution (1 mL) and the mixture inverted several times. To this solution was added saturated $(NH_4)_2SO_4$ solution (1 mL) and the total volume was made up to 5 mL with deionised water. The solution was allowed to stand for 10 minutes at room temperature and then centrifuged for 5 minutes at 4 000 x g. The absorbance of the solution was recorded at 280 nm and total proanthocyanidin content was calculated from a calibration curve with catechin solutions and as expressed as catechin equivalent (CE) per 100g FW.

Total flavonoid content analysis

Total flavonoids were determined using the method of Ordonez et al. (2006). Briefly, to 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoid content was calculated as quercetin equivalent from a calibration curve and expressed as quercetin equivalents (QE) per 100g FW.

ORAC (Oxygen Radical Absorbance Capacity) assay

The method measures the antioxidant scavenging activity against peroxyl radical induced by *2,2'*-azobis (*2*-amidinopropane) dihydrochloride at 37°C. Fluorescein was used as the fluorescent probe. The loss of fluorescein fluorescence was an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of an antioxidant was measured by assessing the area under the fluorescence decay curve as compared to that of blank in which no antioxidant is present. ORAC was measured according to the method of Ou et al. (2002) with some modifications described by Denev et al., (2010). Activity of the sample is expressed as ìmol trolox equivalents (TE) per gram of fresh fruit

HORAC (Hydroxyl Radical Averting Capacity) assay

HORAC measures the metal-chelating activity of antioxidants under the conditions of Fenton-like reactions employing a Co(II) complex and hence the protecting ability against formation of hydroxyl radical (Ou et al., 2002). One HORAC unit was assigned to the net protection area provided by 1μmol/l gallic acid and the activity of the sample is expressed as ìmol gallic acid equivalents (GAE) per gram of fresh fruit. ORAC and HORAC analyses were carried out using a FLU-

Ostar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany), excitation wavelength of 485 nm and emission wavelength of 520 nm were used.

Results and Discussion

Sugar content and composition

The content of carbohydrate determines the sweetness of persimmons, which is very important for the overall organoleptic appearance of the fruit. The carbohydrate composition of the investigated five persimmon cultivars are shown on Table 1. Among the investigated sugars, glucose was the predominant representative at both commercial harvest and physiological ripening stage in all investigated cultivars. At commercial harvest stage, glucose concentrations varied in the range 5.8 g/100g FW (cv Jiro C 24276) – 8.9 g/100g FW (cv Hiratanenashi). Fructose was the second most abundant sugar found in concentrations 3.8 – 5.6 g/100g FW, whereas sucrose and one unidentified disaccharide were found in very low concentrations only in cv Hyakume. The total amount of sugars in the commercial harvest stage samples was in the range 9.6 – 14.4 g/100g FW being the highest in Hiratanenashi persimmon. After the deastringency at physiological ripening stage the content of individual representatives as well as the total amount of sugars increased to values in the range 11.5-16.5 which is characteristic for fully ripe persimmons. Some authors found also low amounts of arabinose in selected persimmon cultivars (Bubba et al., 2009), but in the five cultivars investigated in our study, this sugar was not detected neither at commercial harvest nor at physiological ripening stages. The prevalent abundance of glucose and fructose was a result commonly found in almost all studies in the literature, even though a strong variability of the sucrose content was observed, depending on the variety investigated and the extraction method used (Ittah, 1993; Senter et al., 1991, Veberic et al., 2010; Bubba et al., 2009).

Polyphenol, proanthocyanidin and flavonoid content

In spite of the incomplete knowledge of the chemical structure of persimmon polyphenols, it has been demonstrated that simple polyphenols (benzoic acid derivatives, monomeric flavanols, flavonols and flavanones) together with highly polymerized flavanols are present in persimmons. Among the phenolic compounds in fully ripe persimmon fruit, catechin and gallic acid were predominant (Park et al., 2006). Epicatechin, caffeic acid, quercetin-3-rutinoside, quercetin-3-galactoside and quercetin-3-glucoside were also detected in small amounts (Gorinstein et al., 2001; Park et al., 2006). The contents of total polyphenols, proanthocyanidins and flavonoids in five persimmon cultivars from our study at commercial harvest stage and physiological ripening stage are presented on Figure 1. Generally, the polyphenol content and particularly the proanthocyanidin content were strongly affected by the cultivar and maturity stage. The highest polyphenol content at commercial harvest stage showed Hiratanenashi persimmon (916.8 mg GAE/100 g FW), followed by Moro (805.3 mg GAE/100 g FW). These two cultivars were characteristic also with the highest content of total proanthocyanidins - 540.2 CE/100 g FW and 520.4 CE/100 g FW respectively. The lowest concentration of proanthocyani-

Table 1
Carbohydrate composition of five persimmon cultivars at commercial harvest and physiological ripening stages

Cultivar	Total dry solids, g/100g	Fructose, g/100g	Glucose, g/100g	Sucrose, g/100g	Unidentified disaccharides, g/100g	Total sugars, g/100g
Commercial harvest stage						
Hyakume	18.25	5.2	7.0	0.2	0.2	12.6
Moro	18.46	4.9	7.2	nd*	nd	12.1
Hiratanenashi	21.6	5.6	8.9	nd	nd	14.4
Jiro C 24276	17.8	3.8	5.8	nd	nd	9.6
Mandarino	18.7	5.2	6.1	nd	nd	11.4
Physiological ripening stage						
Hyakume	19.56	5.7	8.1	0.2	0.3	14.2
Moro	19.62	5.1	7.2	0.2	0.3	12.9
Hiratanenashi	25.76	6.6	9.7	0.2	nd	16.5
Jiro C 24276	21.1	5.8	8.3	0.2	0.2	14.5
Mandarino	20.8	4.4	7.1	nd	nd	11.5

*nd - not detected

dins was detected in the pollination constant non astringent cultivar Jiro C 24276, which also had the lowest polyphenol content at the commercial harvest stage. The content of both polyphenols and proanthocyanins decreased proportionally during the deastingency and maturation, which is in line with the observations of Bubba et al. (2009). In our study Hyakume cultivar lost about 33% of its polyphenols and 69% of the total tannins. The most significant drop in the polyphenols was observed for Moro and Hiratanenashi persimmons – 72% and 56% respectively. The chabge of proanthocyanidin content was also the most significant in these two cultivars. Moro proanthocyanidins decreased from 520.4 to 87.1 CE/100 g FW, whereas Hiratanenashi proanthocyanidins dropped from 540.2 to 90.2 CE/100 g FW at physiological ripening stage. The smallest changes either in polyphenol or pranthocyanidin contents during the maturation were observed in the non-astringent cultivar Jiro C 24276. At physiological ripening stage Hiratanenashi was the richest source of polyphenols and proanthocyanidins - 400.2 mg GAE/100g FW and 90.2 CE/100g FW, respectively while Mandarino was the cultivar with the lowest polyphenol content (132.9 GAE/100g FW). Jiro C 24276 had the lowest amount of proanthocyanidins - 6.2 CE/100g FW. Generally, all investigated cultivars had less proanthocyanidins at the physiological ripening stage than the threshold of 0.1 g/100 g FW, which is usually considered as sensorially astringent (Taira, 1996).

In the literature, the data about total polyphenol content of persimmons is quite different. Soluble polyphenol concentrations are reported to vary in the range from 1.3 mg to 1550 mg/100g FW of total polyphenols even for the same astringent cultivar Triumph (Gorinstein et al., 2001; Park et al., 2006). The wide range of variability observed can be explained based on environment effects, even though the different applied extraction methods and the analytical protocols could also significantly influence the results (Giordani et al., 2011). Our results support the findings of Bubba et al., 2009 that non-astringent cultivars appear to have less polyphenols and tannins than the astringent ones. Total condensed tannin levels of both astringent and non-astringent cultivars declined after maturation. Katsube et al. (2004) who reported that non-astringent persimmon fruit showed less polyphenols than astringent observed similar findings. Suzuki et al. (2005) also reported that polyphenols of astringent persimmons were 4-6 times higher than those of nonastringent persimmons. As it could be seen from Figure 1, flavonoids represent just a very small part of the total polyphenols in persimmons. Their content vary in the range 1.3 – 4.2 mg QE/100 g FW at commercial harvest stage and decrease slightly to 0.9 – 3.7 QE/100 g FW after maturation. At both stages, fruits of Hyakume and Mandarino cultivars are the richest source of flavonoids.

Antioxidant activity

There are several reports regarding the antioxidant activity of persimmons (Chen et al., 2008; Fukai et al., 2009; Gorinstein et al., 2011; Jang et al., 2010, 2011; Jung et al., 2005; Park et al., 2008). The majority of the studies employed DPPH and ABTS assays, which are very rapid and easy to perform but have some serious limitations. For example, DPPH· radical is resistant nitrogen radical in contrast to highly reactive physiologically relevant radicals (Figure 2). Often antioxidants, which react fast with peroxyl radicals, react very slowly with DPPH. There are also evidences that DPPH· reacts reversibly with some polyphenols resulting in altered radical-scavenging value (Huang et al., 2005). Depending upon the reactions involved, antioxidant assays can roughly be classified into two types: assays based on hydrogen atom transfer reactions and assays based on electron transfer. Since physiologically antioxidants scavenge reactive oxygen and nitrogen species by hydrogen atom transfer, hydrogen atom transfer methods are considered as more physiologically relevant. ORAC methods assess the radical scavenging activity of the sample against peroxyl radicals, which
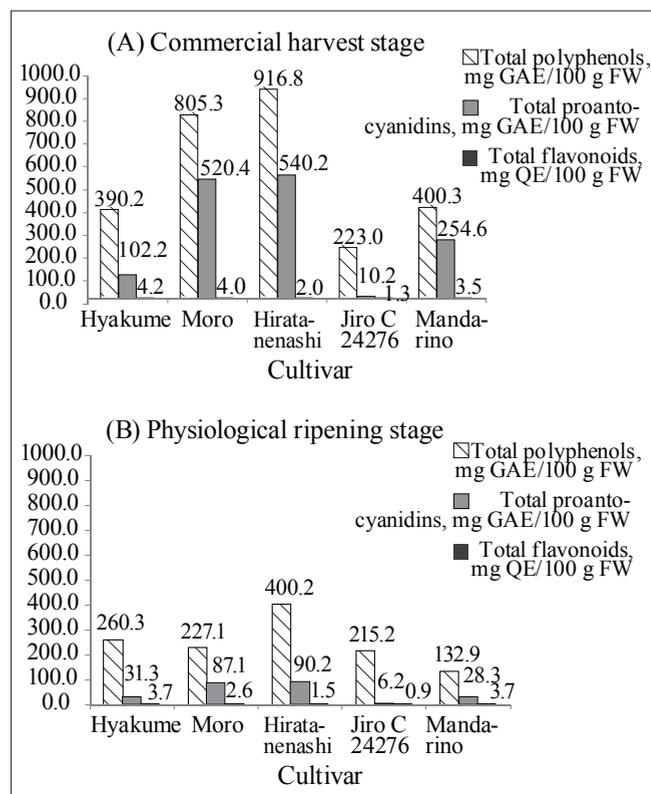

Fig. 1. Total polyphenol, proanthocyanidin and flavonoid content in five persimmon cultivars at (A) commercial ripening and (B) physiological ripening stages

physiologically are the most important ones. HORAC method measures the metal-chelating activity of antioxidants under the conditions of Fenton-like reactions and hence the protecting ability against formation of hydroxyl radical. The chosen methods embrace different aspects of the antioxidant action and give a comprehensive view on the antioxidant potential of the investigated extracts. This is important since it is recommended to use more than one antioxidant assay for the detailed understanding the principles of antioxidant properties of antioxidants (Ciz et al., 2010). The ORAC and HORAC antioxidant activities of the five investigated cultivars at both maturity stages are depicted on Figure 2. Similarly, to the polyphenol content our results suggest that persimmons have different antioxidant activity depending on cultivars. It is evident that at commercial harvest stage the pollination variant astringent cultivar Hiratanenashi has the highest ORAC and HORAC values – 49.2 µmolTE/g FW and 30.2 µmolGAE/g FW, respectively followed by pollination variant non astringent cultivar Moro with ORAC – 48.3 µmolTE/g FW and HORAC – 30.1 µmolGAE/g FW. The pollination constant non-astringent cultivar Jiro C 24276 had the lowest ORAC

and HORAC antioxidant activities, which slightly decrease after the maturation of the fruits. In all other cultivars, the decrease in both ORAC and HORAC values were more significant and proportional to the drop of the total polyphenol content. Our results are in line with Katsube et al. (2004), who also reported that astringent persimmon fruit showed higher DPPH radical scavenging activity than non-astringent one. It could be concluded that at commercial harvest stage proanthocyanidins are the main contributors to the antioxidant activity of persimmons, whereas after maturation antioxidant properties are due to other polyphenol compounds, present in fruit. In the literature there is only one source reporting ORAC value for Singaporean persimmon – 7.42 µmol TE/g FW, but however the cultivar is not mentioned (Isabelle et al., 2010). To our knowledge, our study reports for the first time information about the HORAC antioxidant activity of persimmon fruit.

Conclusion

The current study is the most detailed study on the carbohydrate, polyphenol, pranthocyanidin and flavonoid content, and antioxidant activity of persimmon fruit from different cultivars grown in Bulgaria. Similar studies are available in the literature, but the information regarding environmental and postharvest factors, that can strongly affect ripening time and chemical composition of fruits, are often omitted. Our study provides information for the chemical composition and antioxidant activity of five persimmons cultivars at both commercial harvest stage and physiological ripening stage. It is evident that the maturity stage strongly affects the investigated parameters and fruit in commercial harvest stage are richer source of polyphenol compounds and in particular proanthocyanidins. Such kind of fruits could be used for extraction of tannins, which as already mentioned possess a broad range of biological activities. For nutritional purposes, fruits should be analysed at the "ready to eat" ripening stage, following the general consumer preference (Giordani et al., 2011). On the other hand, the differences in the concentrations of sugars and polyphenol compounds between the different cultivars determine their nutritional and functional importance and provide information on their marketing potential.

Fig. 2. ORAC and HORAC antioxidant activity of five persimmon cultivars at (A) commercial ripening and (B) physiological ripening stages

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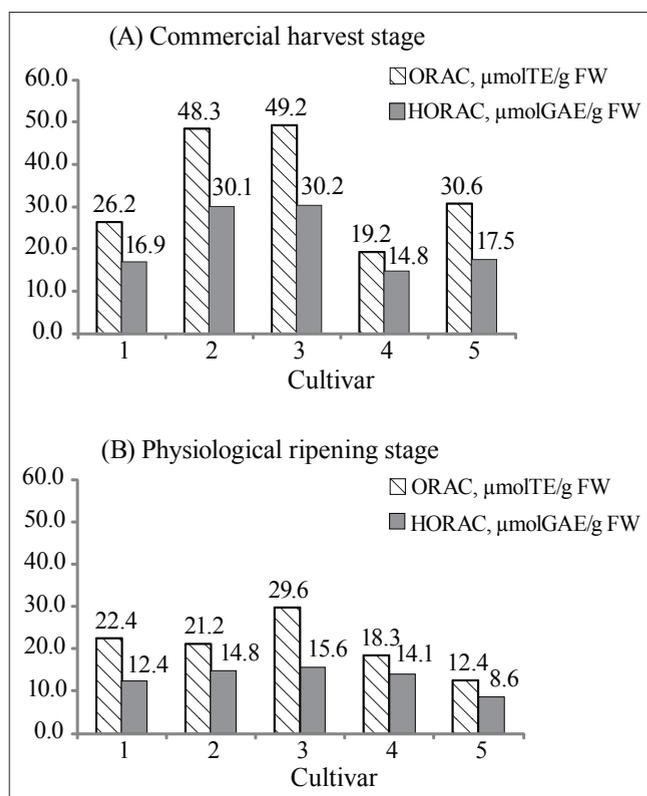
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