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Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity

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Abstract

The interest in the consumption of pepper fruits (*Capsicum annuum* L.) is, to a large extent, due to its content of bioactive nutrients and their importance as dietary antioxidants. A greenhouse experiment was carried out to determine the effects of salinity and different ripening states of pepper fruits on several compounds with antioxidant properties. Fruits from plants grown under three saline treatments (0, 15, and 30 mM NaCl) were collected at three maturity states (green, turning, and red). Antioxidant activity in the hydrophilic (HAA) and lipophilic (LAA) fractions, lycopene, β-carotene, ascorbic acid, total phenolic compounds and reducing sugars were determined. From the nutritional point of view, the red state was the most appropriate state of maturation, since red peppers had the highest levels of lycopene, β-carotene, and sugars and the highest antioxidant activity for both hydrophilic and lipophilic fractions. The effect of salinity depended on the maturity state of the peppers: it had no effect on HAA, β-carotene or sugars, but decreased ascorbic acid and total phenolic compounds, and increased LAA and lycopene. The use of a moderately-saline water was beneficial when peppers were harvested in the red state, by increasing HAA and LAA in fruits, with no significant effects on other parameters.

Keywords: *Capsicum annuum* L.; Lycopene; β-carotene; Antioxidant activity; Ascorbic acid; Phenolic content; Ripening state; Salinity

1. Introduction

Pepper is an important agricultural crop, not only because of its economic importance, but also for the nutritional value of its fruits, mainly due to the fact that they are an excellent source of natural colours and antioxidant compounds (Howard, Talcott, Brenes, & Villalon, 2000; Lee, Howard, & Villalon, 1995). The intake of these compounds in food is an important health-protecting factor. They have been recognized as being beneficial for prevention of widespread human diseases, including cancer and cardiovascular diseases, when taken daily in adequate amounts (Bramley, 2000; Sies, 1991).

A wide spectrum of antioxidant compounds is present in pepper fruits. Phenolic compounds retard or inhibit lipid autoxidation by acting as radical scavengers (Namiki, 1990) and, consequently, are essential antioxidants that protect against propagation of the oxidative chain. It is also known that vitamin C, an important compound of pepper fruits, chelates heavy metal ions (Namiki, 1990), reacts with singlet oxygen and other free radicals, and suppresses peroxidation (Bielski, Richter, & Chan, 1975), reducing the risk of arteriosclerosis, cardiovascular diseases, and some forms of cancer (Harris, 1996). Carotenoids play an important role in fruit colouring and act as antioxidants, reacting with free radicals, mainly peroxide radicals and singlet molecular oxygen (Namiki, 1990). Lycopene is a powerful natural

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antioxidant that acts as the most efficient singlet oxygen quencher in vitro among common carotenoids (Di Mascio, Kaiser, & Sies, 1989) and as a determinant factor in reducing the mortality from several cancers (Gerster, 1997; Tsugane, Tsuda, Gey, & Watanabe, 1992; Zhang et al., 1997). For another major carotenoid in pepper, β-carotene, there is much in vitro evidence of its interaction with free radicals, acting as a chain-breaking antioxidant and as a scavenger and quencher of singlet oxygen (Conn, Lambert, Land, Schalch, & Truscott, 1992; Palozza & Krinsky, 1992).

From the agronomic point of view, for optimum production, pepper plants require environmental (temperature and light) conditions typical of arid and semi-arid regions, where irrigation water is usually limited, and saline. Salinity decreases pepper yield (Chartzoulakis & Klapaki, 2000; Navarro, Garrido, Carvajal, & Martinez, 2002) and imposes stress conditions on crop plants. Plants subject to harmful stress conditions produce cytotoxic activated oxygen that can seriously disrupt normal metabolism, through oxidative damage of lipids, proteins, and nucleic acids. In order to defend themselves against oxidants, plants have evolved specific protective mechanisms, involving antioxidant molecules and enzymes that protect against the potentially-cytotoxic species of activated oxygen. Plants with high levels of antioxidants, either constitutive or induced, have been reported as having greater resistance to this oxidative damage (Dhindsa & Matowe, 1981; Foyer, 1993).

Pepper fruits can be consumed at different ripening stages (green, red or not fully-ripe). Free sugars play an important role in the flavour characteristics of fruits and ripening physiology has a considerable implication for the pattern of sugar accumulation of the fruits (Bognar, Bohling, & Forty, 1990; Schaffer, Rylski, & Fogelman, 1989). Apart from changes in carbohydrates, other events of nutritional importance for pepper take place during ripening. The scarcity of good-quality water in areas where peppers are grown makes necessary the use of saline waters for irrigation. Since hyperosmotic stress activates a physiological antioxidative response (Smirnoff, 1995), in the present work we have studied this response in pepper grown at different salinity levels, as well as the changes that take place during different maturity stages, in order to improve the management and harvesting of this crop and obtain fruits of a higher nutritional value.

2. Material and methods

2.1. Plant material and chemicals

The experiment was carried out in a greenhouse equipped with an automatic regulated computer system for drip irrigation. Pepper plants (*Capsicum annuum* L. cv. Orlando, a "California"-type pepper) obtained from a commercial nursery, were transplanted (13th December) into 1.2 m length perlite sacks. The base nutrient solutions used for irrigation (pH 5.6) had the following macronutrient composition (mM): NO_3^-, 14; $H_2PO_4^-$, 1.5; SO_4^{2-}, 1; Ca^{2+}, 4; K^+, 7.5; Mg^{2+}, 1. Micronutrient concentrations were (mg l^{-1}): Fe, 1.0; Mn, 0.5; B, 0.25; Cu, 0.02; Mo, 0.01. The plants were irrigated according to the demand detected in the appropriate trays.

Salinity treatments consisted of three NaCl levels (0, 15, and 30 mM NaCl), that constituted S1, S2, and S3 treatments, respectively. Salt was added on three consecutive days to the base nutrient solution to avoid osmotic shock. Each treatment was replicated three times and consisted of two sacks of perlite, each containing three plants and three 2-l h^{-1} emitters, about 40 cm apart. The pH and the conductivity of the nutrient solution were controlled during each irrigation period, while the amount of nutrient solution applied depended on the demand detected in the appropriate trays.

2.2. Samples

Three ripening stages were considered: green (fully developed fruit just before the onset of maturation), turning (approximately one-half green skin and the other half red) and red (completely red skin). Overmature and damaged fruits were discarded. During the middle of the harvest period, six uniform fruits in the green, turning, and red states were selected from each replicate. The six fruits of each replicate were divided into two subgroups, there being a total of six replicates per treatment and three fruits per replicate. Each fruit was weighed fresh, after being washed with deionized water, rinsed free of seeds and cut into two halves. One of them was liquefied, centrifuged and frozen at −20 °C. The three fruit extracts from the same replicate were combined; this constituted the water-soluble fraction for the sugars, ascorbic acid and phenolic acid contents and the antioxidant activity in hydrophilic fraction (HAA) determinations. The other half was cut into small pieces. A sample of these pieces was weighed fresh and then oven-dried for water content determination. The rest of the fruit pieces (frozen at −20 °C) from the same replicate were combined for determination of carotenoids, chlorophyll, and antioxidant activity of the lipophilic fraction (LAA).

2.3. Chemicals

2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) was obtained from Fluka Chemical Co. and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) from Sigma–Aldrich Chemical Co. Other reagents were of analytical grade.

2.4. Sugar determination

Reducing sugars (glucose, fructose) and sucrose concentrations were determined in the water-soluble fraction, by HPLC (Merck Hitachi). Samples were passed through a Sep-Pak C_{18} cartridge, preconditioned with methanol (4 ml) and water (10 ml), to remove interfering compounds (Navarro, Martinez, & Carvajal, 2000). Before use, the residual water in the cartridge was expelled with air. The first 2 ml of sample were discarded and the next 1 ml was used for analysis, after filtration through a 0.45-μm Millipore filter. HPLC analysis was performed using a LiCrospher 100 NH_2 5 μm column, coupled with a differential refractrometer detector. The mobile phase was acetonitrile:water (85:15), with a flow rate of 0.9 ml min^{-1}.

2.5. L-Ascorbic acid determination

For L-ascorbic acid determination, samples obtained for sugar determination were used. L-Ascorbic acid concentration was then determined by HPLC (Merck-Hitachi), using a Chromsil C18 (10 μm) 25 × 0.4 cm column, coupled with a UV–Vis wavelength detector set at 245 nm (Carvajal, Martínez, Martínez-Sánchez, & Alcaraz, 1997). The mobile phase was di-ammonium hydrogen phosphate (20 g l^{-1}), adjusted to pH 2.8 with *ortho*-phosphoric acid. The flow rate was 0.9 ml min^{-1}.

2.6. Total phenolics content

Total phenolics content was determined in pepper juice samples using the Singleton and Rossi (1965) colourimetric procedure with phosphotungstic–phosphomolybdic acid reagent, and measurement of the optical density of samples at 660 nm. The total phenolics were determined by a comparison of the values obtained with a standard curve of *p*-coumaric acid. Results were expressed as grammes of *p*-coumaric acid per kg of dry weight.

2.7. Carotenoids determination

Fresh samples of pepper fruit were homogenized using a pestle and mortar in the presence of liquid N_2. Sixteen millilitres of acetone–hexane (4:6) solvent were added to 1.0 g of pepper homogenate and mixed in a test-tube. Automatically, two phases separated, and an aliquot was taken from the upper solution for measurement of optical density at 663, 645, 505, and 453 nm in a spectrophotometer. Lycopene and β-carotene contents were calculated according to the Nagata and Yamashita (Nagata & Yamashita, 1992) equations: Lycopene (mg 100 ml^{-1} of extract) = $-0.0458 * A_{663} + 0.204 * A_{645} + 0.372 * A_{505} - 0.0806 * A_{453}$. β-Carotene (mg 100 ml^{-1} of extract) = $0.216 * A_{663} - 1.22 * A_{645} - 0.304 * A_{505} + 0.452 * A_{453}$. Lycopene and β-Carotene were finally expressed as mg kg^{-1} DW, using the fruit water content.

2.8. Antioxidant activity determination

The test used to determine the antioxidative capacities of the HAA and LAA of pepper fruit was the $ABTS^{\bullet+}$ radical cation assay (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993). $ABTS^{\bullet+}$ radical cation was prepared by adding an excess of manganese dioxide (Sigma Chemical Co.) to a 5 mM aqueous stock solution of ABTS. This solution was diluted in 5 mM phosphate-buffered saline (PBS), pH 7.4, and pre-incubated at 30 °C prior to use.

The system was standardized by means of trolox (Miller et al., 1993; Miller, Sampson, Candeias, Bramley, & Rice-Evans, 1996). After addition of 1.0 ml of $ABTS^{\bullet+}$ solution to aliquots of trolox or the pepper extract (extract used for carotenoids determination for LAA or pepper juice for HAA determination), the solutions were vortex-mixed for exactly 30 s and the absorbance at 734 nm was taken, exactly 30 min after initiation of mixing, in a spectrophotometer. A dose–response curve was derived for trolox by plotting the absorbance at 734 nm as a percentage of the absorbance of the uninhibited radical cation solution (blank) according to the equation:

$$\text{Inhibition of } A_{734}\ (\%) = (1 - A_f/A_0) \times 100,$$

where A_0 is the absorbance of uninhibited radical cation and A_f is the absorbance measured 30 min after the addition of antioxidant samples.

Each sample was analyzed in triplicate and referenced to the trolox dose–response curve. The trolox equivalent antioxidant capacity (TEAC index) was calculated as the concentration (mM) of trolox in phosphate buffer, and shown as the antioxidative potential equivalent to the ml of juice used in the 30th reaction minute.

2.9. Statistical analysis

All data were analyzed statistically by ANOVA and by the Waller–Duncan multiple range test, to determine differences among means, using the SPSS software package (SPSS 7.5.1 for Windows, standard version, 1996).

3. Results and discussion

3.1. Antioxidant activity of the pulp and pepper juice

The antioxidant activity of fruits and vegetables is important for assessing their nutritional value (Rice-Evans, Miller, & Paganga, 1996) and its measurement allows the evaluation of this nutritional variable without analysis of each antioxidant compound (Pellegrini, Re,

Yang, & Rice-Evans, 1999; Scalfi et al., 2000). A significant interaction between salinity and the maturity state of the pepper fruits was found for HAA and LAA (Fig. 1 and Table 1). Salinity increased both HAA and LAA in the red state and decreased these parameters in green fruits. However, at the turning maturity state, salinity decreased HAA but increased LAA. In general, regardless of salinity level, HAA and LAA significantly increased with maturation, as found by Howard et al. (2000). Since it is well-recognized that the role of antioxidant molecules is critical in the detoxification of free radicals (Shen, Jensen, & Bohnert, 1997; Smirnoff, 1995), these results indicate that a moderate salt treatment may significantly improve the beneficial nutritional properties of peppers, regarding the prevention of free radical-related diseases, when they are harvested in the red state, but not the green or turning states.

3.2. Lycopene and β-carotene

Lycopene and β-carotene in the pulp of fruits were correlated with the hydrophilic ($P < 0.001$) ($r^2 = 0.896$ and 0.918, respectively) and lipophilic ($P < 0.01$) ($r^2 = 0.499$ and 0.423, respectively) antioxidant activities. The concentrations of these carotenoids significantly increased with ripening (Fig. 2 and Table 1). The β-carotene concentrations were higher than for lycopene but the latter increased more than β-carotene as a result of the ripening process. Pepper ripeness has been associated with carotenoids accumulation (Howard, Smith, Wagner, Villalon, & Burns, 1994; Márkus, Daood, Kapitany, & Biacs, 1999) and the highest levels of β-carotene in pepper have been found in fully-ripe fruits (Gnayfeed, Daood, Biacs, & Alcaraz, 2001).

Our results show that salinity significantly modified the lycopene contents in fruits (Fig. 2 and Table 1). Moderate salinity enhances fruit carotenoid content in tomato (De Pascale, Maggio, Fogliano, Abrosino, & Ritieni, 2001), possibly via activation of the biosynthetic pathway, inducing upregulation of the genes encoding the enzymes related to lycopene level (Giuliano, Bartley, & Scolnik, 1993). The increase of carotenoids observed in tomato

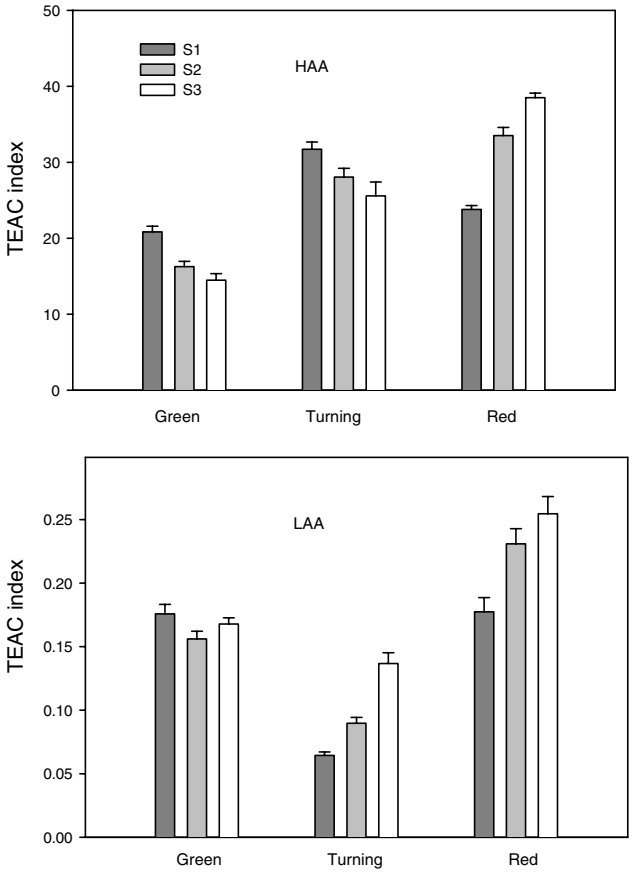

Fig. 1. HAA and LAA (TEAC index) in green, turning, and red peppers as affected by 0, 15, and 30 mM NaCl (S1, S2, and S3, respectively). Data are means ± SE ($n = 6$).

Table 1
Effect of salinity and maturity stage on HAA and LAA (TEAC index), lycopene and β-carotene (mg kg^{-1} DW), total phenolics (g kg^{-1} DW), ascorbic acid (mM), and fruit water content in green, turning, and red peppers as affected by 0, 15, and 30 mM NaCl (S1, S2, and S3, respectively)

Main effect	HAA	LAA	β-Carotene	Lycopene	Total phenolics	Ascorbic acid	% H_2O
Salinity							
S1	25.4	0.142 a	391	133 a	5.45	24.5 b	86.7 c
S2	25.9	0.160 b	402	156 b	5.32	22.7 b	86.2 b
S3	26.2	0.188 c	407	158 b	5.37	19.9 a	85.1 a
Maturity stage							
Green	17.4 a	0.166 b	139 a	–	5.47	1.8 a	87.8 c
Turning	28.4 b	0.096 a	395 b	138	5.41	35.2 c	85.6 b
Red	32.2 c	0.228 c	666 c	322	5.25	30.1 b	84.6 a
Analysis of variance (F values)							
Salinity	ns	17***	ns	5*	ns	9***	31***
Maturity stage	189***	144***	457***	629***	ns	552***	127***
Salinity × maturity stage	39***	7***	ns	ns	4*	11***	ns

*, ** and *** Significant differences between means at the 0.5%, 0.1% and 0.01% level of probability, respectively. ns = not significant. Values are the means of 6 replicates. Values followed by the same letter within a column are not significantly different at the 0.05 level of probability according to the Waller–Duncan test.

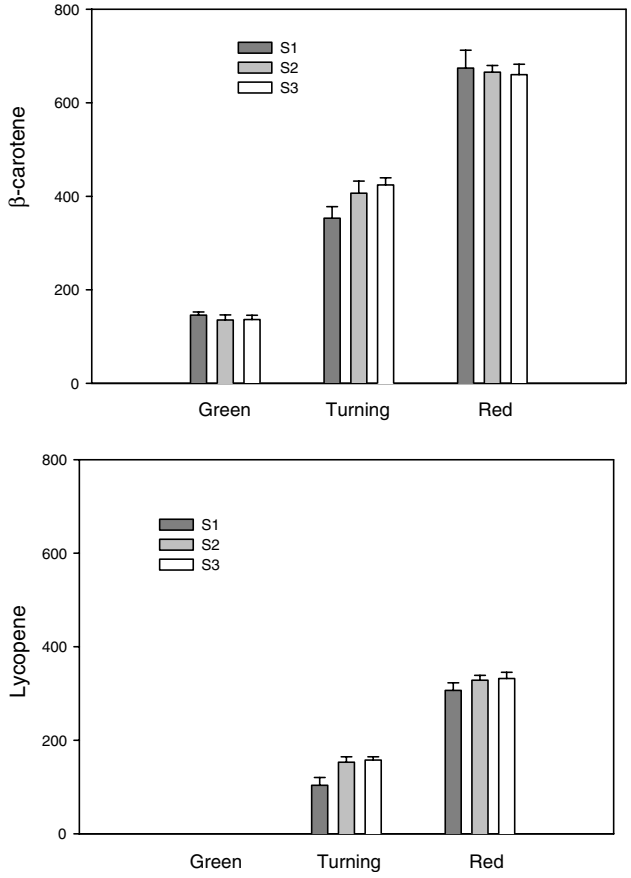

Fig. 2. Lycopene and β-carotene concentrations (mg kg^{-1} DW) in green, turning, and red peppers as affected by 0, 15, and 30 mM NaCl (S1, S2, and S3, respectively). Data are means ± SE ($n = 6$).

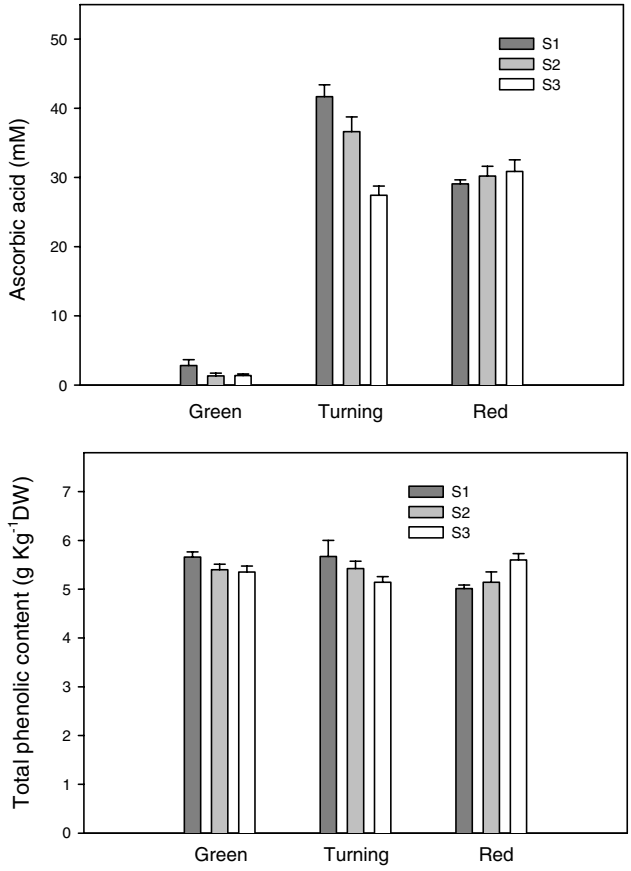

Fig. 3. Ascorbic acid (mM) and total phenolic contents (g kg^{-1} DW) in green, turning, and red peppers as affected by 0, 15, and 30 mM NaCl (S1, S2, and S3, respectively). Data are means ± SE ($n = 6$).

fruits due to salinity could be due to the water content reduction (Petersen, Willumsen, & Kaack, 1998). However, our results show that salinity produces new synthesis of lycopene since its concentration was calculated on a dry weight basis. Lutein is synthesized by the action of cyclase enzymes (Cunnigham et al., 1996), and, when synthesis of lutein was blocked by decreases in these enzyme activities in tomato, an accumulation of lycopene was observed (Ronen, Cohen, Zamir, & Hirschberg, 1999). Lutein is intimately linked with photosynthesis as part of the light-harvesting system (Hornero-Méndez, Gómez-Ladrón, & Mínguez-Mosquera, 2000), and it is well-known that salinity inhibits photosynthetic processes (Chartzoulakis & Klapaki, 2000; Bethke & Drew, 1992). Although the salinity treatments could have increased lycopene concentration by decreasing lutein levels, due to a lower photosynthetic activity, further studies are necessary to confirm this hypothesis.

3.3. Ascorbic acid

Chemical contributors to HAA in peppers are numerous and may include ascorbic acid. We found a significant ($P < 0.001$) correlation of these parameters ($r^2 = 0.6706$). Our results show that the ascorbic acid content was dependent on the maturity state (Fig. 3 and Table 1). Some authors have found differences in ascorbic acid content of 30% between red and green peppers (Howard et al., 1994). We found that turning or red peppers had more than one order of magnitude more than green peppers. On the other hand, other authors have found that ascorbic acid increased or remained constant as pepper fruits matured (Howard et al., 1994, 2000; Osuna-Garcia, Wall, & Waddell, 1998; Rahman, Cuckle, & Edwardsn, 1978), and declined with further ripening (Gnayfeed et al., 2001). Our results show a non-linear increase of ascorbic acid concentration with maturation. Control fruits had a large increase from the green to the turning state, and exhibited a loss much earlier than the full colour intensity stage, similar to results of Yahia, Contreras-Padilla, and Gonzalez-Aguilar (2001). This decreased concentration of ascorbic acid, from turning to red fruits, was not due to a dilution effect, since total volume or fruit juice did not increase, as found in other species (Nagy, 1980). Márkus et al. (1999) attributed this increase at the start of ripening, and the later decrease with advanced ripening, to the

antioxidant role of ascorbic acid, which increases with the increasing respiration rate in climacteric fruits.

The salinity effect on ascorbic acid was dependent on the maturity stage (Table 1), since salinity application decreased its concentration when peppers were collected in the green or turning state, but did not modify it for red peppers. The water content of fruits was dependent on salinity treatment and maturity state (Table 1), but ascorbic acid, expressed on a dry weight basis (data not shown), showed the same behaviour, indicating no concentration effect due to salinity.

3.4. Total phenolics

Peppers contain moderate to high levels of other phytochemicals that can contribute to antioxidant activity, such as phenolic acids; important components that may reduce the risk of degenerative diseases (Hasler, 1998; Larson, 1988). The total phenolics content increased with salinity level in red fruits but was unaltered or slightly decreased for green or turning peppers (Table 1 and Fig. 3). However, when ascorbic acid concentration is high, total phenolics could be overestimated due to the response of the ascorbic acid to the Folin–Ciocalteau assay. In this way, when the impact of ascorbic acid on the total phenolics concentration was corrected (Asami, Hong, Barret, & Mitchell, 2003), total phenolics increased with salinity and decreased during maturation (data not shown). Although an increase of phenolic compounds in pepper fruits with maturation has been described (Lee et al., 1995), other authors have found that it depends on the pepper cultivar (Howard et al., 2000). In this way, Gnayfeed et al. (2001) found differences between cultivars with respect to capsaicinoids evolution during maturation, and the loss during maturation of some pepper cultivars was related to the activity of peroxidase.

3.5. Soluble sugars

It is important to study the role of sugars, as natural ingredients of foods, in vitamin C stability (Birch & Pepper, 1983). In general, foods containing vitamin C usually are characterized by high carbohydrate content. Pepper has high vitamin C content (Lee & Kader, 2000; Vanderslice, Higgs, Hayers, & Block, 1990) and a high sugar concentration. Levels of fructose and glucose were similar and sucrose concentration was minimal, decreasing with maturation to non-detectable levels in red fruits (Table 2), as Lee, Kim, and Howard

Table 2
Effect of salinity and maturity stage on fructose, glucose, sucrose, and total sugar concentrations (g l^{-1}) in green, turning, and red pepper as affected by 0, 15, and 30 mM NaCl (S1, S2, and S3, respectively)

	Fructose	Glucose	Sucrose	Total sugars
Salinity				
S1	24.5	24.3	0.4	49.2
S2	24.0	24.2	0.3	48.5
S3	23.7	24.5	0.3	48.4
Maturity stage				
Green	11.7 a	14.7 a	0.7 c	27.1 a
Turning	24.2 b	24.7 b	0.3 b	49.2 b
Red	36.3 c	33.6 c	0.0 a	69.9 c
Salinity × maturity stage				
S1				
Green	12.2	14.9	1.0	28.1
Turning	25.0	24.0	0.3	49.3
Red	36.4	34.0	–	70.4
S2				
Green	10.6	13.5	0.5	24.6
Turning	24.1	24.8	0.3	49.2
Red	37.4	34.4	–	71.8
S3				
Green	12.5	15.7	0.6	28.8
Turning	23.5	25.3	0.3	49.1
Red	35.0	32.4	–	67.4
Analysis of variance (F values)				
Salinity	ns	ns	ns	ns
Maturity stage	597***	420***	24***	622***
Salinity × maturity stage	ns	ns	ns	ns

*** Significant differences between means at the 0.1% level of probability. ns = not significant. Values are the means of 6 replicates. Values followed by the same letter within a column are not significantly different at the 0.05 level of probability according to the Waller–Duncan test.

(1999) found in pepper. The total sugars concentration significantly increased with maturation, red pepper fruits having the highest levels. Although salinity increases reducing sugar accumulation in others species, such as cucumber, melon or tomato fruits (Adams & Ho, 1989; Navarro, Botella, Cerdá, & Martinez, 1999; Schaffer et al., 1989), in our experiment peppers exhibited slightly decreased concentrations with salt application. Similar results were found in previous studies (Navarro et al., 2002) and could be explained by the increase in fruit respiration observed when the ionic strength of the nutrient solution increases (Tadesse, Nichols, & Fisher, 1999).

4. Conclusions

In summary, antioxidant activity increased with fruit maturation. Biologically-active carotenoids, such as β-carotene, lycopene, fructose, and glucose, reached their highest levels in red fruits. Although the turning peppers had the greatest amount of vitamin C, the losses of ascorbic acid at the end of pepper fruit maturation were not great and the beneficial effect of maturation on the other antioxidant compounds showed the red state to be optimal from the nutritional point of view. Salinity decreased ascorbic acid, produced no effect on HAA, β-carotene, total phenolics or sugars, and increased LAA and lycopene. We have not found a general positive effect of irrigation with saline water on antioxidant response, since this effect was maturity state-dependent. Only when peppers were collected in the red state did the use of moderately-saline irrigation water produce increases of antioxidant activity (HAA and LAA), total phenolics, ascorbic acid, lycopene and β-carotene.

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