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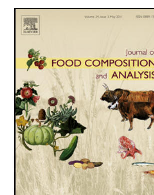

Original Research Article

Hydrophilic antioxidant compounds in orange juice from different fruit cultivars: Composition and antioxidant activity evaluated by chemical and cellular based (*Saccharomyces cerevisiae*) assays

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ABSTRACT

Antioxidant capacity was evaluated by a cellular model (*Saccharomyces cerevisiae*) and chemical methods (FRAP, TEAC and total phenols by Folin-Ciocalteu assay) in the hydrophilic fraction (phenolic compounds and ascorbic acid) of orange juices (OJs) from six varieties (Midknight, Delta Seedless, Rohde Red, Seedless, Early and clone Sambiasi), harvested in two seasons. The contents of phenolic compounds and ascorbic acid analyzed, respectively, by UPLC and HPLC were 370.04 ± 76.97 mg/L and 52.05 ± 6.69 mg/100 mL. Variety and season significantly influenced ($p < 0.05$) composition and antioxidant capacity. TEAC and FRAP values correlated well with individual hydrophilic compounds ($R^2 > 0.991$) but no correlation with cellular assay was observed. An increase in survival rates between 23% and 38% was obtained, excepting for two varieties that showed no activity (Rohde Red and Seedless). Narirutin, naringin-*d*, ferulic acid-d_2, didymin, neoeriocitrin and sinapic acid hexose and caffeic acid-d_1 were the phenolic compounds which contributed to survival rates ($R^2 = 0.979$, $p < 0.01$).

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1. Introduction

Orange juice (OJ) is the most consumed fruit juice worldwide and in addition to having a pleasant colour, flavour and aroma, OJ is an important source of compounds of nutritional relevance (carotenoids, phenolic compounds and vitamin C). The hydrophilic fraction is composed of vitamin C and phenolic compounds, and has been associated with the antioxidant capacity of citrus juices (Gardner et al., 2000). The principal phenolic compounds are hydroxycinnamic acids (ferulic, *p*-coumaric, sinapic and caffeic acids) and flavonoids, among which flavanones, mainly as glycosides (hesperidin and narirutin), are predominant (Gattuso et al., 2007).

Recently, these compounds have attracted increasing attention, not only for their antioxidant properties, but also as anti-inflammatory and anti-carcinogenic agents. These compounds are free radical scavengers, since they inhibit oxidative stress (Halliwell, 1996; Rice-Evans et al., 1997). They act synergistically with Vitamin C in order to maintain and regenerate antioxidant species. Besides phenolic compounds, Vitamin C is considered the most important water-soluble antioxidant that contributes to the antioxidant cellular defence against oxidative stress. The profile of the antioxidant compounds in OJ shows quantitative and qualitative differences related to the genotype (variety), environmental conditions (sunlight, rain, temperature), agronomic practices (type of crop and harvesting conditions), fruit maturity and technological processes (thermal, mechanical) and storage (Klimczak et al., 2007), all of which affect compound content and consequently antioxidant capacity (Dhuique-Mayer et al., 2005; Gil-Izquierdo et al., 2002; Mouly et al., 1997).

Several methods have been developed to determine the antioxidant capacity; the most frequently used are *in vitro* methods based on capturing or scavenging free radicals generated in the reaction or in the reduction of metal ions. Recent studies

Abbreviations: FRAP, ferric reducing ability of plasma; TEAC, Trolox equivalent antioxidant capacity; TP, total phenolic compound; SC, *Saccharomyces cerevisiae*; *EV*, Early Valencia; *cSV*, clone Sambiasi Valencia; *DSV*, Delta seedless Valencia; *SV*, Seedless Valencia; *MV*, Midknight Valencia; *RV*, Rohde red Valencia; *d*, derivate.

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suggest that the assessment of antioxidant capacity requires the parallel use of several methods, because different methods can produce divergent results (Niki, 2011; Prior et al., 2005; Tabart et al., 2009).

Although *in vitro* methods are widely used and accepted for determining the antioxidant capacity of a broad variety of compounds, these methods do not reflect cellular and physiological conditions such as bioavailability or metabolism. On the other hand, cellular models are considered a useful tool to provide valuable information on possible mechanisms of action and the protective effect of antioxidants. Models such as *Saccharomyces cerevisiae* (SC) or *Caenorhabditis elegans* allow a closer approximation to physiological conditions (Baroni et al., 2012; Jara-Palacios et al., 2013). In this sense, SC detects oxidative stress and generates a response at molecular level by inducing antioxidant defence systems (Amari et al., 2008; Costa and Moradas-Ferreira, 2008; Herrero et al., 2008; Niki, 2012).

Soares et al. (2003) found that BHT and vitamin C were able to protect the yeast cells against damage caused by the stressing agents (apomorphine, paraquat and hydrogen peroxide). Other studies on phenolic compounds (quercetin, resveratrol, catechin and hesperidin) reported an increased oxidative stress resistance in yeast cells by scavenging free radicals (Belinha et al., 2007; Dani et al., 2008; Wilmsen et al., 2005). However, to the best of the authors' knowledge, there are no data on the ability of hydrophilic compounds of OJ to reduce the oxidative stress caused by H_2O_2 in SC.

It is important to improve knowledge on the relationships between composition and the *in vitro* methods to evaluate the antioxidant capacity and the biological effects in cell models, since currently it is not so clear which characteristics are different between them. Thus, the aim of this work was to evaluate the content of hydrophilic compounds (phenolic compounds and AscA) of OJ serum from different orange varieties. Moreover, antioxidant capacity by three *in vitro* methods (FRAP, TEAC and Total phenols by Folin-Ciocalteu) were compared to estimate resistance of SC to oxidative stress.

2. Materials and methods

2.1. Chemicals

The analytic solvents HPLC-grade acetonitrile were procured from Merck (Darmstadt, Germany). Purified water was obtained from NANOpure® Diamond™ (Barnsted Inc. Dubuque, IO). L-ascorbic acid was purchased from Panreac, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, narginin, naringenin, hesperidin and apigenin from Sigma-Aldrich (Steinheim, Germany), and neoeriocitrin and didymin from Extrasynthese (Lyon-Nord, France).

Folin–Ciocalteu reagent, ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and potassium persulfate (di-potassium peroxdisulfate) were purchased from Sigma-Aldrich (Steinheim, Germany). TPTZ (2,4,6-tripyridyl-s-triazine) was purchased from Fluka (Buchs, Switzerland).

2.2. Samples

Six varieties of oranges (Citrus sinensis (L.) Osbeck): Midknight Valencia (*MV*), Delta Seedless Valencia (*DSV*), Rohde Red Valencia (*RV*), Seedless Valencia (*SV*), Early Valencia (*EV*) and clone Sambiasi Valencia late (*cSV*) were harvested from trees in the Agricultural Experiment Station (INTA) Concordia, Argentina in September 2010 and 2011.

Each sample consisted of 2 kg of fresh oranges with an appropriate stage of maturity, corresponding to 11–13 °Brix of soluble solid content. Thus OJ corresponding to 24 kg of oranges was extracted (6 samples/year × 2 years × 2 kg/sample).

The orange fruits were immediately hand-squeezed with a domestic squeezer (Clatronic Model ZP3066, International GMBH, Germany). Juices were strained to remove seeds. Then, freshly squeezed juices were centrifuged at 12,500 × *g* in a centrifuge RC5C model (Sorvall Instruments, DuPont Co., Wilmington, DE, USA) for 10 min at 4 °C to remove pulp, and supernatants were used for analyses.

2.3. Ascorbic acid analysis

The ascorbic acid (AscA) was determined by HPLC with isocratic elution (Oruña-Concha et al., 1998). First 500-μL aliquots of the OJ were gently mixed with 500 μL of 10% metaphosphoric acid and centrifuged at 18,000 × *g* for 5 min. Eventually, the supernatant was filtered through a 0.45 μm pore size membrane filter before injection. An HPLC-DAD analysis was carried out on an Agilent 1200 system (Agilent, Palo Alto, CA) using a C18 column (2.5 μm, 10 cm × 4.6 mm) (Análisis Vínicos, Ciudad Real, Spain) kept at 20 °C. The mobile phase was 0.02 M orthophosphoric acid and the isocratic flow was set at a rate of 1 mL/min. The chromatograms were monitored at 254 nm and the injection volume was 20 μL. AscA peaks were identified by comparison of their retention times and spectra with those of the standard, and the concentrations were worked out by external calibration. The results were expressed as milligrams of AscA per 100 mL of juice. All samples were analyzed in triplicate.

2.4. Phenolic compounds analysis

2.4.1. Chromatography

All individual phenolics were analyzed by ultra-high performance liquid chromatography (UPLC) with direct injection of the sample. Samples were centrifuged at 18,000 × *g* for 15 min at 4 °C and subsequently filtered through a 0.45-μm pore size membrane filter before injection. The UPLC analyses were carried out on an Agilent 1260 system equipped with a diode-array detector, which was set to scan from 200 to 770 nm. Open lab ChemStation software was used and the chromatograms were monitored at 280, 320 and 370 nm. A C18 Poroshell 120 column (2.7 μm, 5 cm × 4.6 mm) (Agilent, Palo Alto, CA) kept at 25 °C was used as stationary phase, and the injection volume was set at 20 μL. The mobile phase was pumped at 1.5 mL/min and consisted of two solvents: solvent A, water/formic acid (99:1; v/v) and solvent B, acetonitrile. The linear gradient elution was 0 min, 100% A; 5 min, 95% A + 5% B; 20 min, 50% A + 50% B; 22 min, 100% A; 25 min, 100% A.

The identification of phenolic compounds were carried out according the method described by Rodríguez-Pulido et al. (2012). MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. The quantification was carried out by external calibration considering the following wavelengths: 320 nm for hydroxycinnamic acids and flavones and 280 nm for flavanones. The results were expressed in mg/L of OJ, as mean ± standard deviation. All samples were analyzed in triplicate.

2.4.2. Method validation

The proposed chromatographic method was validated to determine the linearity, limits of detection (LOD), limits of quantification (LOQ), and precision (repeatability and reproducibility) of each compound.

The linearity was examined through the calibration curves that were obtained by plotting concentration against peak area. LOD and LOQ were calculated as three and ten times the relative standard deviation of the analytical blank values calculated from the calibration curve, respectively. These were calculated using the

Microcal Origin ver. 3.5 software (OriginLab Corporation, Northampton, MA, USA) and were expressed in mg/L.

Intra-day precision (repeatability) of peak areas was calculated from the successive injection ($n = 6$) of a mix of pure standards performed on the same day, and was repeated in three different days ($n = 18$) for assessment of inter-day precision (reproducibility).

2.5. *In vitro* antioxidant capacity of hydrophilic compounds

Three methods were employed to measure the *in vitro* antioxidant capacity of the OJs: Ferric reducing/antioxidant power (FRAP) assay, Trolox equivalent antioxidant capacity (TEAC) assay and Total phenols by Folin-Ciocalteu method. All samples were analyzed in triplicate.

2.5.1. FRAP assay

The FRAP assay was carried out according to the procedure described by Benzie and Strain (1996). This assay is based on the ability of substances (antioxidant) to reduce Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ. Briefly, juices were diluted 20-fold, and 100 μL of dilution was added to 3 mL of FRAP reagent measuring the absorbance at 593 nm on a spectrophotometer Shimadzu Model MultiSpec-1501 (Tokyo, Japan) equipped with temperature controller model PSC-240-A after incubation at room temperature for 6 min, using the FRAP reagent as a control.

The FRAP reagent contained 100 mL of 0.3 mol/L acetate buffer, pH 3.6, 10 mL of a 10 mmol/L TPTZ solution in 40 mmol/L HCl plus, and 10 mL of 20 mmol/L $FeCl_3{\cdot}6H_2O$ was freshly prepared. A calibration curve of Trolox was used and results are expressed as mM of Trolox equivalent per L of OJ. All samples were analyzed in triplicate.

2.5.2. TEAC (Trolox Equivalent Antioxidant Capacity Assay) method

The method used is based on the scavenging of the radical cation ABTS?$^+$ generated in the reaction medium, compared to a standard antioxidant (Re et al., 1999). The 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS?$^+$) was produced by reacting an ABTS aqueous solution (7 mM) with potassium persulfate (2.45 mM final concentration). The mixture stood in the dark at room temperature for 12–16 h before use. The ABTS+ solution was diluted with phosphate-buffered saline (PBS) pH = 7.4 to an absorbance of 0.7 at 734 nm (30 °C). Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) was used as a standard for comparison of the scavenging capacity. One mL of the ABTS radical solution was added to the cuvette and the absorbance was measured at time 0. Subsequently, 10 μL and 25 μL of the supernatant (obtained by centrifugation at 3280 × *g* for 15 min at 4 °C and subsequently diluted 10-fold) were added. The mixtures were stirred and incubated at 30 °C. After 6 min, the absorbance was measured at 734 nm on a spectrophotometer Shimadzu Model MultiSpec-1501 (Tokyo, Japan) equipped with temperature controller model PSC-240-A. The dose-response curve for Trolox consisted of plotting the absorbance at 734 nm as a percentage of the absorbance of the uninhibited radical cation (blank) and the analysis was made in triplicate. The Trolox equivalent antioxidant capacity (TEAC) was calculated by dividing the gradient of the curve of the sample and the gradient of the standard Trolox curve, taking into account the dilution used. The antioxidant capacity was expressed in mM of Trolox equivalent per L of OJ. All samples were analyzed in triplicate.

2.5.3. Total phenols

Total phenol content was determined using the Folin-Ciocalteu method by Singleton and Rossi (1965), adapted microscale by Arnous et al. (2001). First, 790 μL of distilled water and 10 μL of each sample (dilution 1:2) were combined in an Eppendorf tube. After that, 50 μL of Folin-Ciocalteu reactive was added. Subsequently after stirring for one minute, 150 μL sodium carbonate solution 20% was added, stirred (vortexed) and allowed to stand at room temperature (24 °C) in the dark, for 120 min.

The absorbance at 750 nm in a spectrophotometer Shimadzu Model MultiSpec-1501 (Tokyo, Japan) and in quartz cuvettes from 10 mm light path was measured. TP concentration from a calibration curve constructed using gallic acid as standard and the results were expressed as mg of gallic acid equivalents per 1 L of juice. All analyses were carried out in triplicate.

2.6. OJ serum effect on the survival of yeast submitted to oxidative stress

The survival assay was performed using cells of SC ATCC36900 (American Type Culture Collection). Cells were grown in liquid YPD medium using an orbital shaker at 28 °C and 160 rpm, with the ratio for flask volume/culture medium of 5/1. Yeast cells at the exponential phase (Abs_{600nm}: 0.5–0.7) were transferred to fresh medium (Abs_{600nm}: 0.2), either with serum OJ or without it, and incubated for 1 h at 28 °C/160 rpm. Both cultures were subjected to oxidative stress with H_2O_2 (0.75 mM) and incubated 1 h at 28 °C/160 rpm. Optimal dose was determined in adaptive treatments, exposing cells to increased concentrations of serum. The concentration chosen was the lowest that improves cell growth in comparison to cohorts exposed to H_2O_2 (0.75 mM) without the addition of serum. Two control groups were used: a control plate (untreated cells), and serum control plate (yeast exposed to serum alone, without H_2O_2).

Cell viability was analyzed by plating, in triplicate, on solid YPD medium, after proper dilution. Plates were incubated at 28 °C for 72 h and the colonies counted. 100% survival was considered the number of colonies observed in the control plate (untreated cells). The number of colonies in each plate was between 150 and 200 (Silva et al., 2005).

2.7. Statistical analysis

Results were expressed as the mean and standard deviation of three independent determinations. Appropriate statistical models were applied to evaluate the existence of significant differences among OJ variety and harvest season. Specifically, a two-factor ANOVA (season and variety) was carried out. Correlations between *in vitro* antioxidant capacity or the resistance to oxidative stress of SC and hydrophilic compounds were evaluated by both, simple and multiple regressions. All statistical analyses were performed with Statistica v.8.0 software (StatSoft, 2007).

3. Results and discussion

3.1. Phenolic compounds and ascorbic acid

The UPLC method used to determine phenolic compounds was validated by defining the linearity, limits of quantification and detection, and precision (repeatability and reproducibility). The validating parameters of each calibration curve (regression equation, correlation coefficient (R^2), lineal range, LOD and LOQ) are described in Table 1. Excellent linearity was observed for all these compounds between peak areas and concentrations ($R^2 \geq 0.999$) over the range tested. LOD for all the compounds was in the range 0.01–0.88 mg/L. LOQ for all the compounds ranged from 0.03 at 2.93 mg/L. The repeatability and reproducibility were evaluated by the relative standard deviation (RSD) and the good precision of the UPL method is also shown in Table 1.

The identification of phenolic compounds was carried out by comparison of their mass characteristics and also chromatographic

Table 1
Validation data for determination of phenolic compounds in orange juices.

Phenolic compounds	Compound	Wavelength (nm)	Regression equation	R^{2}[(a)]	Linear range (mg/L)	LOD[(b)] (mg/L)	LOQ[(c)] (mg/L)	Intra-day ($n=6$)	Inter-day ($n=18$)
HCA	*p*-Coumaric acid	320	$y=86.605x-13.445$	0.9998	0.4–23.0	0.14	0.46	0.03	0.11
	Caffeic acid	320	$y=67.011x-3.609$	0.9998	0.2–25.0	0.15	0.50	0.04	0.06
	Ferulic acid	320	$y=69.321x-5.692$	0.9997	0.2–22.0	0.13	0.43	0.05	0.09
	Sinapic acid	320	$y=59.353x+1.346$	1.0000	0.8–9.4	0.01	0.03	0.08	0.10
Flavone	Apigenin	320	$y=19.494x+2.359$	0.9986	24.0–122.0	0.43	1.43	0.45	0.61
Flavanones	Naringin	280	$y=19.741x+1.023$	0.9991	0.41–48.6	0.35	1.16	0.13	0.14
	Neoeriocitrin	280	$y=21.238x-7.079$	0.9992	0.8–47.0	0.65	2.18	0.15	0.22
	Hesperidin	280	$y=2.179x+2.267$	0.9998	4.5–290.0	0.88	2.93	0.32	0.43
	Naringenin	280	$y=37.914x-2.057$	0.9998	2.7–27.0	0.17	0.58	0.08	0.13
	Dydimin	280	$y=19.889x-7.795$	0.9988	1.2–36.0	0.60	2.00	0.14	0.16

(a) Coefficient of determination
(b) Limits of detection
(c) Limits of quantification

behaviour and absorption spectra, in comparison with available standards and our library data (Supplementary material, Table S1). Table 2 summarizes the mean levels of phenolic compounds determined by UPLC. A total of 24 phenolics were identified, which can be classified into three major categories: hydroxycinnamic acids (HCA) (caffeic, ferulic, *p*-coumaric, sinapic acids and derivates), flavones (vicenin-2 and apigenin derivate) and flavanones (hesperidin, narirutin, naringin, neoeriocitrin and didymin). A typical chromatogram at different wavelengths (320 nm for the HCA and flavones, and 280 nm for flavanones) is depicted in Fig. 1. Total phenolic compounds determined as the sum of individual compounds ranged from 308.06 ± 30.62 to 476.10 ± 26.10 mg/L, with the highest amounts being recorded in *SV* variety. These results are in agreement with those reported in OJs from different varieties (Dhuique-Mayer et al., 2005; Kebelek et al., 2009; Rapisarda et al., 2008).

The AscA contents of Valencia varieties ranged from 42.52 to 63.30 mg/100 mL, in accordance with other studies which reported 45–64 mg/100 mL (Dhuique-Mayer et al., 2005; Rapisarda et al., 2008).

A two factor ANOVA (season and variety) was performed. Significant effect ($p < 0.001$) was obtained for both season ($F = 66{,}387$) and variety ($F = 238{,}318$). Also the interaction between factors ($F = 29{,}588$) was significant ($p < 0.001$).

Table 2
Summary of the mean phenolic compounds and ascorbic acid levels on the different orange juices varieties analyzed (mean values corresponding to 2010–2011 harvest).

Peak	Phenolic compounds*	*EV* ($n=2$)	*cSV* ($n=2$)	*DSV* ($n=2$)	*SV* ($n=2$)	*MV* ($n=2$	*RV* ($n=2$)
1	*p*-Coumaric acid-d_1	1.59 ± 0.39^{a}	1.05 ± 0.20^{b}	0.94 ± 0.06^{c}	0.87 ± 0.21^{d}	0.93 ± 0.14^{c}	0.79 ± 0.05^{e}
2	Caffeic acid dimer	0.40 ± 0.10^{a}	0.25 ± 0.08^{b}	0.23 ± 0.06^{bce}	0.21 ± 0.03^{ce}	0.25 ± 0.03^{b}	0.21 ± 0.02^{e}
3	*p*-Coumaric acid-d_2	0.92 ± 0.25^{a}	0.60 ± 0.05^{b}	0.67 ± 0.09^{c}	0.73 ± 0.03^{d}	0.64 ± 0.07^{e}	0.61 ± 0.03^{b}
4	Ferulic acid-d_1	0.63 ± 0.02^{a}	0.38 ± 0.08^{bc}	0.40 ± 0.10^{b}	0.36 ± 0.11^{c}	0.29 ± 0.02^{d}	0.32 ± 0.03^{e}
5	Ferulic acid dimer	1.32 ± 0.03^{a}	1.48 ± 0.20^{b}	1.69 ± 0.13^{c}	1.67 ± 0.50^{d}	1.81 ± 0.15^{e}	1.80 ± 0.22^{f}
6	*p*-Coumaric acid-d_3	1.82 ± 0.58^{a}	1.05 ± 0.12^{b}	0.93 ± 0.09^{c}	0.90 ± 0.24^{d}	1.02 ± 0.11^{e}	0.82 ± 0.01^{f}
7	Ferulic acid-d_2	3.49 ± 0.55^{a}	3.49 ± 0.19^{a}	3.63 ± 0.20^{b}	4.88 ± 0.60^{c}	3.75 ± 0.27^{d}	3.87 ± 0.24^{e}
8	Caffeic acid-d_1	0.85 ± 0.22^{a}	0.94 ± 0.01^{b}	1.03 ± 0.02^{c}	1.22 ± 0.14^{d}	0.91 ± 0.05^{e}	1.10 ± 0.01^{f}
9	*p*-Coumaric acid dimer	2.44 ± 1.13^{a}	1.02 ± 0.01^{b}	0.93 ± 0.18^{c}	0.82 ± 0.16^{d}	1.08 ± 0.03^{e}	0.74 ± 0.10^{f}
10	Sinapic acid-*d*	2.20 ± 0.20^{a}	2.11 ± 0.06^{b}	2.40 ± 0.10^{c}	2.42 ± 0.67^{d}	2.90 ± 0.13^{e}	2.67 ± 0.14^{f}
11	Ferulic acid-d_3	3.43 ± 0.08^{a}	2.98 ± 0.10^{b}	3.30 ± 0.10^{c}	3.10 ± 0.63^{d}	4.04 ± 0.10^{e}	3.55 ± 0.18^{f}
12	Caffeic acid	4.82 ± 1.92^{a}	6.14 ± 0.01^{b}	5.97 ± 0.21^{c}	7.76 ± 0.34^{d}	4.72 ± 0.32^{a}	5.05 ± 0.09^{e}
14	Ferulic acid-d_4	1.53 ± 0.07^{a}	0.72 ± 0.05^{b}	0.93 ± 0.01^{c}	0.85 ± 0.28^{d}	1.46 ± 0.03^{e}	0.96 ± 0.09^{f}
15	Ferulic acid hexose	6.13 ± 0.75^{a}	14.81 ± 0.14^{b}	14.04 ± 0.04^{c}	15.26 ± 1.35^{d}	12.04 ± 2.04^{e}	16.48 ± 0.12^{f}
16	Sinapic acid hexose	3.33 ± 0.24^{a}	2.69 ± 0.52^{b}	3.37 ± 0.02^{a}	3.56 ± 0.54^{c}	3.39 ± 0.119^{a}	2.97 ± 0.47^{d}
	∑Hydroxycinnamic acid	34.92 ± 6.40^{a}	39.72 ± 0.89^{b}	40.47 ± 0.72^{c}	44.63 ± 4.47^{d}	39.23 ± 1.98^{e}	41.96 ± 0.53^{f}
13	Apigenin-*d*	9.98 ± 0.14^{a}	9.62 ± 0.13^{b}	9.73 ± 0.19^{c}	10.05 ± 0.41^{d}	9.98 ± 0.21^{a}	9.79 ± 0.26^{e}
17	Vicenin-2	23.40 ± 3.60^{a}	19.60 ± 0.80^{b}	23.42 ± 0.20^{a}	24.70 ± 2.53^{c}	23.49 ± 1.51^{d}	27.29 ± 2.74^{e}
	∑Flavones	33.38 ± 3.74^{a}	29.22 ± 0.74^{b}	33.15 ± 0.40^{c}	34.75 ± 2.12^{d}	33.47 ± 1.30^{e}	37.08 ± 3.00^{f}
18	Narirutin hexose	9.37 ± 0.24^{a}	8.25 ± 0.04^{b}	11.66 ± 0.23^{c}	12.73 ± 5.14^{d}	11.16 ± 3.68^{e}	12.14 ± 1.40^{f}
19	Neoeriocitrin	2.67 ± 1.58^{a}	1.34 ± 0.29^{b}	0.84 ± 0.32^{c}	0.82 ± 0.28^{c}	4.10 ± 0.82^{d}	3.07 ± 0.10^{e}
20	Hesperidin-*d*	12.99 ± 4.64^{a}	5.56 ± 0.24^{b}	7.37 ± 0.25^{c}	6.35 ± 0.30^{d}	8.84 ± 2.44^{e}	5.31 ± 0.46^{b}
21	Naringin-d	0.90 ± 0.21^{a}	0.46 ± 0.10^{b}	0.89 ± 0.18^{a}	0.74 ± 0.43^{c}	0.66 ± 0.41^{d}	0.53 ± 0.16^{e}
22	Narirutin	8.64 ± 0.84^{a}	9.43 ± 0.04^{b}	12.92 ± 1.90^{c}	16.22 ± 5.00^{d}	12.93 ± 2.09^{c}	18.11 ± 4.57^{e}
23	Hesperidin	241.68 ± 128.07^{a}	211.10 ± 32.49^{b}	253.65 ± 20.09^{c}	355.93 ± 16.90^{d}	232.29 ± 7.78^{e}	256.37 ± 1.73^{f}
24	Didymin	1.99 ± 0.31^{a}	2.97 ± 0.20^{b}	3.58 ± 0.86^{c}	3.92 ± 0.39^{d}	3.37 ± 0.25^{e}	4.41 ± 1.17^{f}
	∑Flavanones	278.24 ± 135.49^{a}	239.11 ± 32.45^{b}	290.90 ± 22.37^{c}	396.72 ± 28.45^{d}	273.33 ± 16.96^{e}	299.93 ± 4.73^{f}
	∑Flavanoids	311.62 ± 139.23^{a}	268.34 ± 31.51^{b}	324.05 ± 22.76^{c}	431.47 ± 30.57^{d}	306.80 ± 18.27^{e}	337.01 ± 7.73^{f}
	∑Total Phenols	346.54 ± 145.63^{a}	308.06 ± 30.62^{b}	364.52 ± 22.05^{c}	476.10 ± 26.10^{d}	346.03 ± 16.28^{a}	378.97 ± 8.24^{e}
	Total Phenols by Folin-Ciocalteu	690.38 ± 63.82^{a}	715.50 ± 70.98^{a}	756.75 ± 11.57^{b}	774.67 ± 96.06^{bd}	609.25 ± 18.03^{c}	795.50 ± 14.22^{d}
	Ascorbic acid[ψ]	54.04 ± 9.82^{a}	46.39 ± 4.47^{b}	56.69 ± 0.20^{c}	53.02 ± 1.37^{d}	45.91 ± 2.53^{e}	56.22 ± 8.18^{f}

a,b,c,d,f,e Different superscripts within the same row indicate statistically significant differences ($p < 0.05$).
Key: *EV*, Early V.; *cSV*, Clon sambiasi V.; *DSV*, Delta seedless V.; *SV*, Seedless V.; *MV*, Midknight V.; *RV*, Rohde red V.
* mg of phenolic compound/L,
ψ mg Ascorbic acid/100 mL.

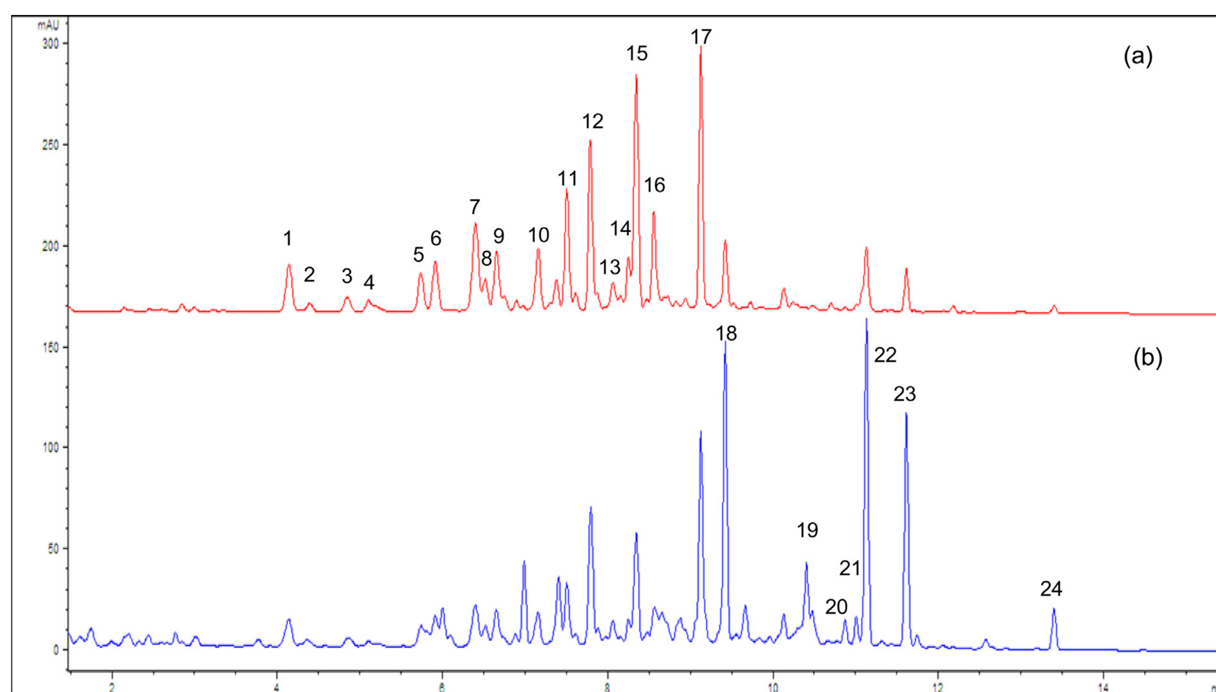

Fig. 1. Chromatograms of the phenolic compounds at 320 nm (a) and at 280 nm (b) from orange juice sample. Peak identification in Table 2.

3.1.1. Varietal effect on the hydrophilic fraction of OJ

An ANOVA analysis was carried out, considering the phenolic compounds as grouping factor. It can be observed (Table 2) that there were significant differences in HCA, flavones, flavanones and total phenolic compounds among varieties.

Total HCA was higher ($p < 0.05$) in *SV* and *RV* and lower in *EV* varieties, although slightly lower values than reported previously in several studies were found (Rapisarda et al., 1999, 2008). Ferulic acid was the most abundant HCA, followed by caffeic, sinapic and *p*-coumaric acid which represented approximately 58%, 17%, 14% and 10%, respectively, of total HCA in accordance with data reported by other authors (Kebelek et al., 2009; Rapisarda et al., 2008; Stinco et al., 2013). When considered as a sum of the same group of acids, ferulic acid showed the highest levels and *p*-coumaric acid the lowest levels for *SV* and *RV* varieties, while *EV* variety showed the opposite tendency (the highest value for *p*-coumaric and the lowest level for ferulic acid).

Five flavanones (narirutin, naringin, hesperidin, neohesperidin and didymin) were identified in this study. In accordance with published data (Dhuique-Mayer et al., 2005; Tomás-Barberán and Clifford, 2000) for sweet oranges, hesperidin (hesperetin-7-O-rutinoside) and narirutin (naringenin-7-O-rutinoside) were the main ones. Total flavanones ranged from 239.11 mg/L (*cSV*) to 396.72 mg/L (*SV*) in agreement with literature data that reported values ranging from 202.3 ± 21.3 mg/L (Washington navel) to 244.1 ± 19.6 mg/L (Valencia late) (Rapisarda et al., 1999). Significant differences ($p < 0.05$) were observed in the flavanone content among varieties. Hesperidin was the most abundant, while neoeriocitrin and didymin were the least abundant in all the varieties. The concentration of hesperidin was 1.7-fold higher in *SV* than *cSV* (Table 2). Narirutin, the second most abundant flavanone, was significantly different in all varieties, except between *DSV* and *MV*. Narirutin levels were lower than those previously reported for Valencia variety (Dhuique-Mayer et al., 2005; Gattuso et al., 2007).

In the group of flavones, vicenin-2 and an apigenin derivate were identified. The total flavones ranged from 29.22 to 37.08 mg/L. These results were found to be slightly lower than those reported in the literature (Gattuso et al., 2007). The higher contents were found in *RV* and *SV* varieties. Vicenin-2 was the main flavones in *RV*, while *cSV* showed the highest content of apigenin derivate.

Significant differences ($p < 0.05$) were observed among varieties regarding AscA, the lowest content corresponded to the *MV* variety, while the highest level to *DSV*.

3.1.2. Harvest season effect

When evaluating season effect, significant differences ($p < 0.05$) were detected in the hydrophilic compounds. Total flavones and total flavanone contents were higher in season 2010 than in season 2011, with differences about 2.5% and 20% respectively between them. Conversely, the total HCA content was higher in season 2011 (Table 3). Total phenolic compounds, determined as the sum of individual compounds, were higher in samples harvested in 2010. This was a foreseeable result, since flavanones are the predominant compounds. The variation in phenolic content may be due to climatic conditions such as temperature, rainfall and relative humidity, *etc.*, during fruit development. Other factors such as the species, geographical location and physiological maturity play a decisive role in the final characteristics of the fruit (Dhuique-Mayer et al., 2005; Tang and Tigerstedt, 2001).

Taking into account the meteorological data (monthly temperature and precipitation provided by the local meteorological Station (INTA, Concordia, Argentina) for both seasons (Supplementary

Table 3
Analysis of variance performed on test set samples composition data: harvest season effect.

Hydrophilic compounds	Harvesting season	
	2010 ($n = 6$)	2011 ($n = 6$)
Ascorbic acid	49.34 ± 5.00^a	54.76 ± 7.24^b
∑Hydroxycinnamic acid	40.05 ± 1.45^a	40.26 ± 5.90^b
∑Flavones	33.92 ± 2.92^a	33.10 ± 3.36^b
∑Flavanones	323.26 ± 64.19^a	269.49 ± 79.25^b
∑Flavanoids	357.18 ± 66.44^a	302.59 ± 74.15^b
∑Total phenols	397.22 ± 66.90^a	342.85 ± 79.36^b

[a,b] Different superscripts within the same row indicate statistically significant differences ($p < 0.05$).

material, Fig. S1), significantly higher rates of precipitation values and relative humidity from January to March were observed in 2010 season. These higher levels match with the higher levels of phenolic content, mainly flavonoids. Wang and Zheng (2001) reported that high growing temperatures (25 and 30 °C) significantly enhanced the flavonoid content and antioxidant capacity in wild strawberries. In the case of the AscA, the content was 1.1-fold higher in oranges harvested in 2011 than in the fruit harvested in 2010, probably due to a dilution effect.

All varieties showed higher individual flavanones levels in the 2010 season, except for didymin, HCA and AscA showed an opposite trend, which suggests that differences in the hydrophilic fraction composition could be related to the others factors mentioned above.

3.2. In vitro *antioxidant activity*

Taking into account the widespread recommendation of considering at least two different methods of assessment (Prior et al., 2005) we evaluated the *in vitro* antioxidant capacity using three different chemical methods: FRAP, TEAC and total phenols (TP) by the Folin-Ciocalteu method.

Fig. 2 shows the values of the hydrophilic antioxidant capacity measured by FRAP (a), TEAC (b) and TP (c) methods in different varieties and seasons. It can be observed that the highest antioxidant capacity measured by the three assays was obtained for *RV* variety, while the lowest values were observed for *MV* and *cSV* varieties.

The antioxidant capacity measured by TEAC and TP assays were higher in OJs from the 2010 harvest (5.4% and 4.3% respectively) than for the 2011 OJs ($p < 0.05$). The opposite trend was observed for FRAP assay, with the highest values observed in 2011 season. According to composition data (Table 3), the antioxidant capacity determined by FRAP would be more related to the ascorbic acid content, while phenolic compounds would have more influence on the antioxidant capacity measured by TEAC and TP methods.

ANOVA analysis of the antioxidant capacity among varieties showed that FRAP values were significantly different ($p < 0.05$) except for in *EV* and *DSV*. Likewise, antioxidant capacity by TEAC was significantly different among *EV*, *MV* and *RV* varieties. This was a foreseeable result, since, as discussed earlier, significant differences were found in the phenolic compounds and ascorbic acid contents in these varieties.

When comparing the total phenol content determined by UPLC with the values obtained by Folin-Ciocalteu method, it can be observed that the last ones were considerably higher (Table 4). This could be due to the presence in OJ of non-phenolic nature substances like ascorbic acid, that interfere and can react with Folin reagent overestimating the phenolic compound content (Prior et al., 2005).

The relationship between the antioxidant capacity and the composition depends on several factors such as type of compound, chemical structure, synergistic effects, and specific conditions applied (Escobedo-Avellaneda et al., 2013; Huang et al., 2005). Multiple correlations were carried out between the antioxidant capacity (by the three methods assayed) and the composition. Table 4 shows the multiple regression coefficients, the equations and *p*-values obtained. In a fist stage, the composition was considered as groups of compounds. No correlation was found between the antioxidant capacity determined by Folin-Ciocalteu and the hydrophilic composition. It can also be observed that there were good correlations between FRAP method and the different fractions considered. Regarding TEAC method, correlation coefficients improved when considering all the groups of compound in the hydrophilic fraction.

In order to further characterize the relationship between the antioxidant capacity (FRAP and TEAC) and the composition, a stepwise multiple regression model was used. Individual phenolic compounds and AscA content were considered as predictors of both methods. The results obtained showed that there was a significant correlation between the FRAP values and AscA and some phenolic compounds (neoeriocitrin, ferulic acid-d_4, narirutin, vicenin-2, *p*-coumaric acid-d_2, didymin, *p*-coumaric acid-d_1, ferulic acid-d_1, caffeic acid, ferulic acid-d_3, di-caffeic acid) ($R^2 = 0.995$, $p < 0.001$). Similarly, a good correlation coefficient ($R^2 = 0.991$, $p < 0.001$) was obtained between the antioxidant capacity assessed by TEAC, and some phenolic compounds (vicenin-2, apigenin-*d*, ferulic acid-d_1, hesperidin-d_1, narirutin, didymin, di-caffeic acid, narirutin hexose, *p*-coumaric acid-d_3 and neoeriocitrin).

Thus, as it was suggested before, in these equations, it can be observed that when FRAP values are considered, the AscA was selected, while the phenolic compounds only contributed to the antioxidant capacity determined by TEAC. These observations are in agreement with those reported by Rice-Evans et al. (1995), who informed that the relative antioxidant capacity determined by TEAC method in flavonoids and antioxidant vitamins was higher for HCA, followed by flavones, flavanones and vitamin C.

3.3. *OJ serum effect on the survival of yeast submitted to oxidative stress*

As a previous step, the possible toxic effect of OJ serum was evaluated on SC cells in culture media supplemented with different volumes of OJ (data not shown). The noncytotoxic volume of serum was determined to be 35 μL (meaning 5.8% of total volume).

Subsequently, the concentration of hydrogen peroxide that was able to cause 50% of death in the yeasts was determined. With this aim, two concentrations (0.75 mM and 1 mM) were used. It was determined that the optimum concentration was 0.75 mM, since concentration 1 mM produced a higher cell death (data not shown).

Thus, in order to analyze the capacity of OJ to protect SC cells against damage, after having induced oxidative stress with H_2O_2, determination of cell viability with or without OJ pre-incubation was done. Results in the experiment without OJ showed that, as expected, yeast cells showed sensitivity to H_2O_2 and only 46% survival to the oxidative agent (Fig. 3). This result is in agreement with the 45% of survival rates of SC treated with H_2O_2 at 0.75 mM reported by Baroni et al. (2012).

Pretreatment with OJ serum reduced the damaged caused by the oxidant (H_2O_2). For the assays conducted with OJs from 2010 season, *EV* and *DSV* varieties increased 38% and 35% the survival rate compared to yeast cells exposed to H_2O_2 (without OJ serum), while in *cSV* and *MV* varieties these rates were lower (23% and 26%, respectively). Regarding other varieties (*SV* and *RV*), no significant differences were found as a consequence of the OJ presence (Fig. 3).

In season 2011, the varieties EV, *cSV*, *DSV* and *MV* had a significant increasing effect in the survival rates, which ranked from 23% to 27%. As in the previous harvest, varieties *SV* and *RV* showed no significant effect in the survival rate.

Previous studies have reported that different phenolic compounds (quercetin, resveratrol, catechin and hesperidin) and vitamin C are able to protect yeast cells against damage caused by different stressing agents (apomorphine, paraquat and hydrogen peroxide) (Belinha et al., 2007; Dani et al., 2008; Soares et al., 2003; Wilmsen et al., 2005). Also, phenolic compounds extracted from red wine, musts or skin extracts from dried grapes, showed the ability to rescue SC from the oxidative stress induced by H_2O_2, by either capturing free radicals or by activating endogenous defence system (Baroni et al., 2012; López de Lerma et al., 2013; Stefenon et al., 2010).

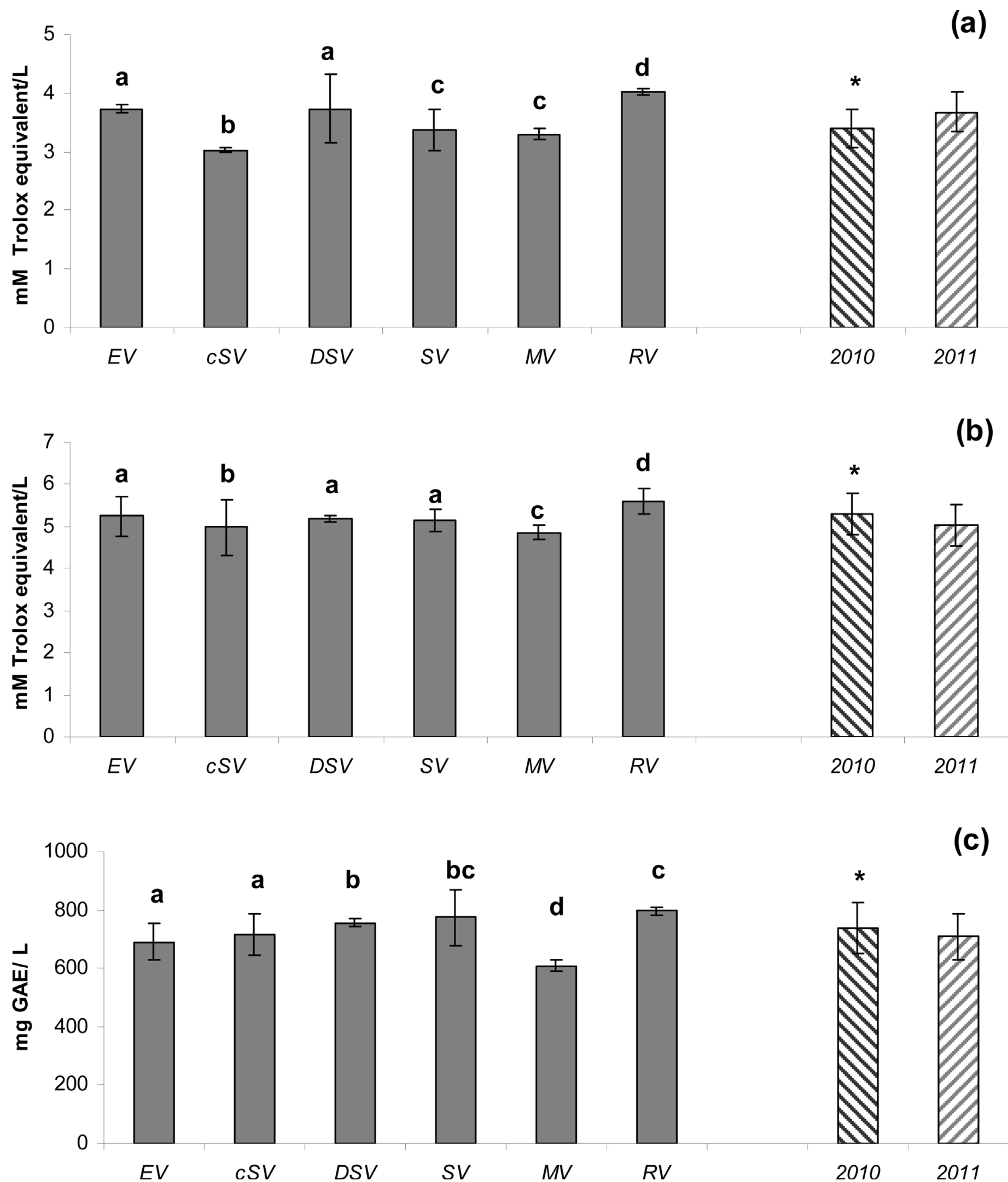

Fig. 2. Antioxidant capacities of orange juices assessed by (a) FRAP, (b) TEAC assay expressed as mM of Trolox equivalent per L of orange juice and TP (c) expressed as mg gallic acid equivalents (GAE) per L of orange juice. **Key**: *EV*, Early V.; *cSV*, Clon sambiasi V.; *DSV*, Delta seedless V.; *SV*, Seedless V.; *MV*, Midknight V.; *RV*, Rohde red V. * indicate significant differences ($p < 0.05$) between seasons for each variety. [a,b,c,d] different letters indicate significant differences ($p < 0.05$) among varieties.

According to the assay used in this study, the bioactive compounds from OJ could act as antioxidant by two ways: scavenging the H_2O_2 in the solution media and/or protecting the cells against the reactive species induced by H_2O_2. In the last case, it is involved the uptake of the phenolic compounds by the cell.

Considering the composition of the serum of the different varieties and the biological effect on SC, it can be highlighted that the varieties with the highest contents of HCA and flavanones (*SV* and *RV*) had no effect on the survival rates. This fact may suggest that these compounds could exert a toxic effect in the yeast depending on its concentration. As it is known, the antioxidant capacity of these compounds is related to their concentration, since they may act as pro-oxidants when exceed certain levels in the cell (Procházková et al., 2011). So, it could be deduced that the increase in survival rates observed in the varieties with lower phenolic content is more related to the second mechanism (protection of the cells against reactive species) than to the first one (scavenging H_2O_2 of the solution). Slatnar et al. (2012) have reported phenolic uptakes by yeast cells after incubation with different berry juices ranging from 62.9 and 100%. Also they have pointed out that increases in phenolic uptake do not trigger a higher *in vivo* antioxidant activity.

Table 4
Variables in models, determination coefficients (R^2) and fitted equations in the prediction of antioxidant activity (FRAP and TEAC assay) by the best subsets multiple linear regressions based on ascorbic acid (AAsc), hidroxycinnamic acid (∑HCA), Flavones (∑FV), Flavanones (∑FN), ferulic acid (∑FA), sinapic acid (∑SA), caffeic acid (∑CA), *p*-coumaric acid (∑pCA) and Flavonoids (∑FL) content.

Model	Variables	Equations	R^2	*p*-value
1	AAsc, ∑HCA, ∑FL	FRAP = 0.060 (AscA) – 0.009 (∑HCA) – 0.001 (∑FL) + 0.757	0.74	<0.001
2	AAsc, ∑HCA, ∑FL	TEAC = 0.019 (AscA) – 0.004 (∑HCA) + 0.001 (∑FL) + 3.972	0.16	<0.001
3	AAsc, ∑HCA, ∑FV, ∑FN	FRAP = 0.041 (AscA) – 0.007 (∑HCA) + 0.093 (∑FV) – 0.003 (∑FN) – 0.580	0.88	<0.001
4	AAsc, ∑HCA, ∑FV, ∑FN	TEAC = 0.008 (AscA) – 0.002 (∑HCA) + 0.057 (∑FV) – 0.001 (∑FN) + 3.163	0.26	<0.001
5	AAsc, ∑FA, ∑SA, ∑CA, ∑pCA, ∑FV, ∑FN	FRAP = 0.038 (AscA) – 0.022 (∑FA) – 0.0011(∑SA) + 0.079 (∑CA) – 0.013 (∑pCA) + 0.121 (∑FV) – 0.005 (∑FN) – 0.964	0.89	<0.001
6	AAsc, ∑FA, ∑SA, ∑CA, ∑pCA, ∑FV, ∑FN	TEAC = –0.005 (AscA) – 0.052 (∑FA) – 0.356 (∑SA) + 0.229 (∑CA) – 0.067 (∑pCA) + 0.185 (∑FV) – 0.006 (∑FN) + 2.835	0.64	<0.001

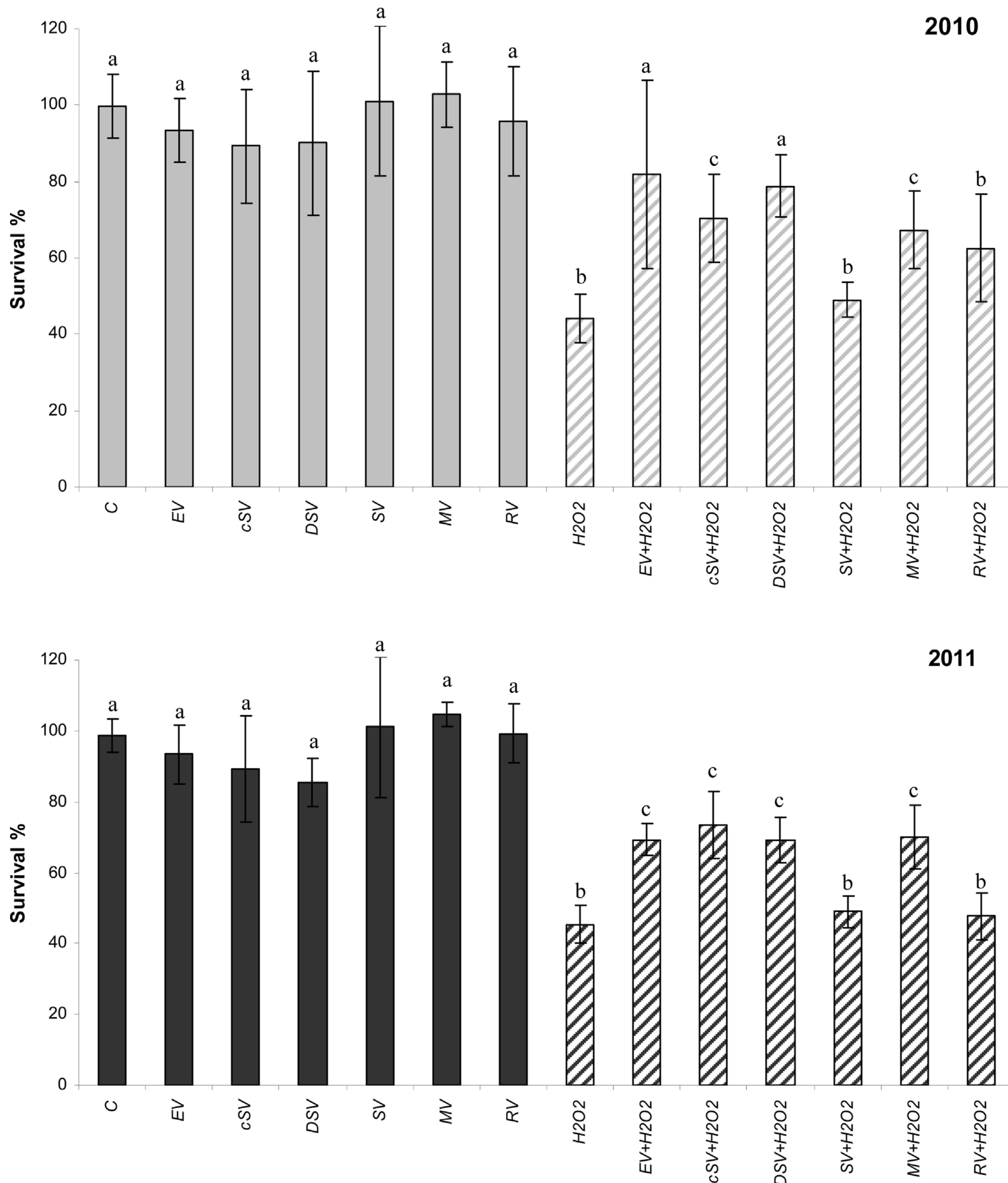

Fig. 3. Survival rates of *S. cerevisiae* treated and untreated with orange juice serum and/or H_2O_2 (mean ± SD). **Key**: *C*, untreated cells; *EV*, Early V.; *cSV*, Clon sambiasi V.; *DSV*, Delta seedless V.; *SV*, Seedless V.; *MV*, Midknight V.; *RV* Rohde red V. Black columns represent untreated cells and striped columns represent treated cells. [a,b,c,d,e] different letters indicate significant differences ($p < 0.05$).

In order to evaluate the possible relationship between the *in vitro* antioxidant capacity and stress resistance in SC cells, simple correlations were explored. Results showed no significant correlation between different methodologies. These results are in agreement with others reported in the literature, who did not find correlation between antioxidant capacity measured by both methodologies (Baroni et al., 2012; Silva et al., 2005; Stefenon et al., 2010).

The relationships between the survival increase and each antioxidant compound were analyzed by several simple and multiple correlations. As regards survival rates *vs* individual composition of the different fraction, only simple correlations with *p*-coumaric acid dimer (r = 0.592), narirutin (r = 0.728) and didymin (r = 0.703) were significant.

On the other hand, we applied multiple regression analysis to determine the relationship between resistance to oxidative stress through survival rate of SC and the composition (AscA and individual phenolic compound). Surprisingly, the mathematical model included seven phenolic compounds (narirutin, naringin-*d*, ferulic acid-d_2, didymin, neoeriocitrin and sinapic acid hexose and caffeic acid-d_1), which contributed to survival rates ($R^2 = 0.979$, $p < 0.01$). Narirutin, ferulic acid-d_2 and sinapic acid hexose had a negative contribution to the survival rate, while the didymin, naringin-*d* and caffeic acid-d_1 had a positive one.

4. Conclusion

Orange variety and harvest season influences the hydrophilic compounds composition and thus the antioxidant capacity of orange juice. Good correlations were found among *in vitro* antioxidant capacity by FRAP and TEAC and the composition. The best estimation of antioxidant capacity is achieved when the individual compounds are considered jointly. These observations indicate that although both techniques (FRAP and TEAC) are useful to assess the antioxidant capacity, there are some differences between them. FRAP assay seemed to be more related to the ascorbic acid content, while phenolic compounds would have more influence on TEAC values. Results showed that there were no significant correlations between *in vitro* methods and stress resistance in SC. This suggests that although *in vitro* methods are widely used due to their simplicity, they just provide a slight approximation to the antioxidant capacity, thus, the results can not be directly extrapolated to *in vivo* system. Cellular models, even with some limitations (O'Brien et al., 2000), allow a better approximation to the antioxidant protection in more complex organisms, whereby they can be used as a good prediction tool of antioxidant capacity of different compounds. More research is needed to assess the real antioxidant capacity of hydrophilic compounds in OJs, the relationship among them (potential synergistic effect) and the levels at which they could lose their health-promoting effect.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jfca.2014.09.006.

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