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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 ($R^2 = 0.979$, $p < 0.01$).

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