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Simultaneous HPLC determination of flavonoids and phenolic acids profile in Pêra-Rio orange juice

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ABSTRACT

The aim of this study was to develop and validate an HPLC-DAD method to evaluate the phenolic compounds profile of organic and conventional Pêra-Rio orange juice. The proposed method was validated for 10 flavonoids and 6 phenolic acids. A wide linear range (0.01–223.4 $\mu g \cdot g^{-1}$), good accuracy (79.5–129.2%) and precision (CV ≤ 3.8%), low limits of detection (1–22 $ng \cdot g^{-1}$) and quantification (0.7–7.4 μg), and overall ruggedness were attained. Good recovery was achieved for all phenolic compounds after extraction and cleanup. The method was applied to organic and conventional Pêra-Rio orange juices from beginning, middle and end of the 2016 harvest. Flavones rutin, nobiletin and tangeretin, and flavanones hesperidin, narirutin and eriocitrin were identified and quantified in all organic and conventional juices. Identity was confirmed by mass spectrometry. Nineteen non-identified phenolic compounds were quantified based on DAD spectra characteristic of the chemical class: 7 cinnamic acid derivatives, 6 flavanones and 6 flavones. The phenolic compounds profile of Pêra-Rio orange juices changed during the harvest; levels increased in organic orange juices, and decreased or were about the same in conventional orange juices. Phenolic compounds levels were higher in organic (0.5–1143.7 $mg \cdot 100\ g^{-1}$) than in conventional orange juices (0.5–689.7 $mg \cdot 100\ g^{-1}$). PCA differentiated organic from conventional FS and NFC juices, and conventional FCOJ from conventional FS and NFC juices, thus differentiating cultivation and processing.

1. Introduction

Orange is the main fruit produced in Brazil, with ca 450 million boxes in 2017. The majority of oranges are delivered for NFC (Not From Concentrate) and FCOJ (Frozen Concentrated Orange Juice) production and exportation. Of the total 1.01 million ton juice produced in 2016, 921,000 ton were from São Paulo, of which 699,000 ton FCOJ and 222,000 ton NFC (FCOJ equivalent). The prediction is 848,000 ton in 2017, 771,000 from São Paulo; 531,000 ton FCOJ and 240,000 ton NFC (FCOJ equivalent). More than 80% of the juice is exported to Europe and the USA (Fundecitrus, 2017; Neves et al., 2010; USDA, 2016).

Organic citrus fruit represents 0.9% of the total worldly production; amongst it, oranges are the most cultivated fruit and orange juice is the main organic product. Latin America holds the biggest area of organic cultivation in the world (ca 15,000 ha), where Brazil has a strong position as a productor and a potential consumption market (FAO, 2003; IFOAM, 2015).

There is still very little knowledge about the influence of the cultivation system on food quality, despite the increasing consumption of organic foods especially by environment-friendly and health-conscious consumers (Janzantti, Santos, & Monteiro, 2012; Macoris, De Marchi, Janzantti, & Monteiro, 2011; Macoris, Janzantti, Garruti, & Monteiro, 2011; Santos & Monteiro, 2004). It is necessary to assess organic foods from the chemical, nutritional and sensory points of view, to evaluate the characteristics and verify the potential health benefits associated with consumption.

Orange juice consumption has been associated with health benefits, mainly related to modulation of the human metabolism, and antioxidant and anti-inflammatory activities, which prevent chronic-degenerative diseases such as cardiovascular diseases, diabetes and cancer. Orange juice is a dietary source of ascorbic acid, and also contains flavonoids and phenolic acids, which highly prevent oxidative stress (Barreca et al., 2017; Khan, Huma, & Dangles, 2014; Medina-Remon, Estruch, Tresserra-Rimbau, Vallverdú-Queralt, & Lamuela-Raventos, 2013; Peterson et al., 2006; Tripoli, La Guardia, Giammanco, Di Majo, & Giammanco, 2007).

The phenolic composition of orange juice is influenced by the variety and maturity of the fruit, edaphoclimatic conditions, cultivation system, post-harvest and processing conditions (Baldwin, Scott, Shewmaker, & Schuch, 2000; Haard, 1984; Macoris, De Marchi, et al.,

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2011; Zou, Xi, Hu, Nie, & Zhou, 2016). The main *Citrus* phenolic compounds are flavonoids and cinnamic acid derivatives (Gattuso, Barreca, Gargiulli, Leuzzi, & Caristi, 2007; Khan et al., 2014; Peterson et al., 2006). Most of the methods for phenolic compounds analysis are based on HPLC technique in reversed phase and gradient mode, with DAD and/or MS detection. Several methods have been applied to honey (Escriche, Kadar, Juan-Borrás, & Domenech, 2011), grapes (Burin, Ferreira-Lima, Panceri, & Bordignon-Luiz, 2014), bayberry (Fang, Wang, Hao, & Guo, 2009), dates (Abu-Reidah, Ali-Shtayeh, Jamous, Arráez-Román, & Segura-Carretero, 2015), guava (Rojas-Garbanzo, Zimmermann, Schulze-Kaysers, & Schieber, 2016), mango (López-Cobo, Gómez-Caravaca, Svarc-Gajíc, Segura-Carretero, & Fernández-Gutiérrez, 2015), cherry (Martini, Conte, & Tagliazucchi, 2017), strawberries (Pinto, Lajolo, & Genovese, 2008) and tomato (Vallverdú-Queralt, Arranz, Medina-Remón, Casals-Ribes, & Lamuela-Raventos, 2011; Vallverdú-Queralt, Jáuregui, Medina-Remon, & Lamuela-Raventos, 2012). Furthermore, flavonoids and phenolic acids have been reported in Kozan, Navel, Moro, Tarocco, Sanguinello, Salustiana, Cara-Cara, Baia, Natal and Lima orange juices. Hesperidin is by far the major flavonoid, followed by narirutin. Amongst the acids, ferulic, caffeic and coumaric acids have been reported in Kozan and Navel orange juices (Gattuso et al., 2007; Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán, 2001; Kelebek, Selli, Canbas, & Cabaroglu, 2009; Leuzzi, Caristi, Panzera, & Licandro, 2000; Rapisarda et al., 1999).

Despite many studies on flavonoid composition in orange juice from several varieties, the Pêra-Rio variety was not included and there is still little knowledge about the compounds other than hesperidin and narirutin. Pêra-Rio is the most important variety cultivated in Brazil and is responsible for the uniqueness of the Brazilian juice. To the best of our knowledge, there is a lack of studies regarding the phenolic profile of orange juice, as well as the Pêra-Rio variety.

The aim of this work was to develop and validate an HPLC-DAD method for simultaneous flavonoid and phenolic acids profile analysis in Pêra-Rio orange juice from organic and conventional cultivation systems during the 2016 harvest.

2. Material and methods

2.1. Chemicals

Pure standards (95–99%, Sigma Aldrich) of six phenolic acids (gallic, protocatechuic, caffeic, syringic, coumaric and ferulic acids) and ten flavonoids (rutin, eriocitrin, quercitrin, narirutin, naringin, hesperidin, naringenin, hesperitin, nobiletin, and tangeretin) were used, and benzoic acid was used as the internal standard.

Acetonitrile, methanol and ethyl acetate were of HPLC grade, formic acid was of analytical grade, and ultrapure water was obtained from a Direct Q-3 UV system (Millipore, USA).

2.2. Orange juice samples

Organic freshly-squeezed (FS) and NFC Pêra-Rio orange juices from the 2016 harvest were provided by a certified producer (no CA5897/15, IBD Certification) from Itirapina, SP, Brazil (22° 15′ 10″ S, 47° 49′ 22″ W). Conventional FS, NFC and FCOJ Pêra-Rio orange juices were provided by a citrus industry from Araraquara region, SP, Brazil (21° 47′ 40″ S, 48° 10' 32″ W).

FS juices (2 L) were collected from the finishing step, NFC juices (2 L) from the pasteurization step and FCOJ (500 mL) from the concentration step. The collection of the juices from each step was performed from the same load of oranges, so that all the juice collected was from the same processing batch. All juices were sampled from July to October 2016 at the beginning, middle and end of harvest. Juices were frozen and lyophilized. Phenolic compounds were extracted immediately after lyophilization.

2.3. Phenolic compounds extraction

Lyophilized orange juice was weighted (3–4.0000 g) and added of a methanol aqueous solution (90%, v/v), homogenized (1 min) and extracted in ultrasonic bath (20 min), then centrifuged at 9000 rpm at 20 °C (20 min). Supernatant was collected and the extraction was repeated. Supernatants were combined and submitted to a cleanup step using solid phase extraction (SPE).

SPE conditions were evaluated employing conventional NFC juice. A standard solution of the phenolic compounds (3.106–23.368 $\mu g \cdot g^{-1}$) was added to the juice prior to the extraction for recovery evaluation. C18 (Bond Elut, Agilent Technologies, USA) and polymeric (Strata X, Phenomenex, USA) cartridges, both of 500 mg phase and 6 mL volume, were conditioned with acetonitrile (18 mL) and aqueous formic acid (0.1% v/v) (18 mL) or ethyl acetate (18 mL) and aqueous formic acid (0.1%, v/v) (18 mL). Extracts were introduced in the cartridges and collected, and then cartridges were washed with acetonitrile (30 mL) or ethyl acetate (30 mL) for the elution of the retained compounds. Eluted extracts and solvents were combined, dried under nitrogen flow and reconstituted with aqueous formic acid (0.1%, v/v).

SPE conditions were selected based on peak area repeatability and recovery of phenolic compounds. C18 cartridges conditioned with ethyl acetate (18 mL) and aqueous formic acid (18 mL), and washed with ethyl acetate (30 mL) were selected. After drying and reconstitution as previously described, aqueous extracts were weighted, filtered through 0.22 μm regenerated cellulose disk filters and stored at − 20 °C until analysis. Extracts were obtained in duplicate for each juice.

2.4. HPLC-DAD and MS conditions

Liquid chromatography was carried out in an Acquity ARC system (Waters, USA) with a diode array detector, using a BEH X-Bridge C18 column (250 × 4.6 mm, 5 μm) and guard column (20 × 4.6 mm, 5 μm). For development purposes, mobile phase was water acidified with formic acid (0–5%, v/v) and acetonitrile or methanol. Column temperature (25–50 °C), volume of injection (5–20 μL) and flow rate (0.7–1.0 $mL \cdot min^{-1}$) conditions were tested and selected based on peak number, symmetry and resolution. Wavelengths were monitored from 210 to 400 nm and chromatograms were acquired at 255, 270 and 280 nm. Final working conditions were: mobile phase of aqueous formic acid solution (0.1%, v/v) and acetonitrile, column temperature of 50 °C, 20 μL of injection volume, flow rate of 1.0 $mL \cdot min^{-1}$ and gradient of 6–10% acetonitrile (0–16 min), 10–22% (16–36 min), 22–100% (36–38 min) and held for 5 min. Column was equilibrated for 10 min between injections.

An Acquity HPLC system (Waters, USA) with a single-quadrupole QDa mass detector using the same column and separation conditions as in HPLC-DAD was used. Electrospray ionization and MS analysis conditions were as follows: capillary voltage 1.5 kV (positive mode) and 0.8 kV (negative mode), probe temperature of 500 °C, N_2 as drying gas and MS Scan from 100 to 900 *m/z*. Mass spectra were obtained in both positive and negative ionization modes.

2.5. Validation

The method was validated using analytical figures of merit, based on international validation protocols (ICH, 2005; Magnusson & Örnemark, 2014; Thompson, Ellison, & Wood, 2002). Calibration, linearity, limit of detection, precision, accuracy, limit of quantification and ruggedness were evaluated.

2.6. Phenolic compounds profile of Pêra-Rio orange juice

Organic and conventional orange juice extracts were injected in HPLC-DAD system in triplicate. Phenolic compounds were identified based on retention time and DAD spectra. In order to confirm peak