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Original Research Article

Cold storage of blueberry (*Vaccinium* spp.) fruits and juice: Anthocyanin stability and antioxidant activity

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ABSTRACT

Blueberries (*Vaccinium* spp.) may be considered one of the best potential sources of antioxidants in the diet. This characteristic results from the presence of flavonoids (especially anthocyanins), tannins and phenolic acids in the fruits. The objective of this work was to analyze the anthocyanin stability and antioxidant activity of refrigerated whole blueberry juice stored at 4 °C for 10 d, and of fruits stored frozen (−18 °C) for 6 months, in order to determine the changes occurring during storage. The frozen fruits showed a significant increase ($p < 0.05$) in antioxidant activity during the 3rd month of frozen storage, followed by a decrease up to the end of the 6-month period. The juice was analyzed every other day, and the antioxidant activity changed on the 8th d of refrigerated storage, remaining stable up to the 10th d. There were significant losses of anthocyanins both in the frozen fruits (59%) and refrigerated juice (83%). The antioxidant capacity was shown to be stable during cold storage both in the case of the fruits and whole juice, whereas the anthocyanins were degraded, possibly due to oxidations and/or condensation reactions with other phenolic compounds. More studies are required to optimize the storage time and temperature of these products with respect to nutrient stability.

1. Introduction

The blueberry (*Vaccinium* spp.) is a fruit native to North America and Europe, where it is widely cultivated and commercialized. It is known for its elevated antioxidant capacity against free radicals and reactive species, being considered as one of the best sources of antioxidants in the diet (Prior et al., 1998; Vrhovsek et al., 2012). This activity is related to the presence of bioactive compounds such as flavonoids (especially anthocyanins), tannins and phenolic acids, as well as to various beneficial health properties attributed to blueberry (Heinonen et al., 1998; Smith et al., 2000; Seeram, 2008). Anthocyanins are natural pigments present in fruits and vegetables and widely distributed in nature. They are water-soluble, belong to the flavonoids group, and contain the flavylium cation or 2-phenylbenzopirilium as part of their basic structure (Bridle and Timberlake, 1997). They can be found in the form of glucosides, hydrolyzed in sugars, and as aglycones, known as anthocyanidins (Schwartz et al., 2008).

The anthocyanins are differentiated from one another by the number and position of hydroxyl and/or methyl groups and by the nature, number, position and acylation of the sugars present in their structure. Due to this enormous variety, there are reports of more than 500 anthocyanins and 23 anthocyanidins, of which only 6 are frequently found distributed in nature and in foods: cyanidin (50%), delphinidin, pelargonidin and peonidin (12%) and petunidin and malvidin (7%) (Castañeda-Ovando et al., 2009).

However, the anthocyanins are extremely unstable and easily degraded in the isolated form (Giusti and Wrolstad, 2003), thus restricting their use as food dyes. An increase in hydroxylation increases their instability, whereas an increase in glucosylation confers greater stability (Schwartz et al., 2008). The anthocyanins are normally stable at pH values between 1 and 4. At pH 1, the predominant structure corresponds to the flavylium cation, conferring red and purple colors, whereas at values between pH 2 and 4, blue quinoid bases predominate. They are generally

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degraded at pH values above 7 (Castañeda-Ovando et al., 2009). In addition to pH and type of chemical structure, the following factors also influence their stability: temperature, light, oxygen, ascorbic acid, sulfur dioxide, the presence of enzymes (peroxidase, polyphenoloxidase and glucosidase), metal ions, proteins and other flavonoids (Bridle and Timberlake, 1997), as also food processing and storage (Schwartz et al., 2008).

Freezing is characterized by a reduction in temperature of the food to below its freezing point, with the formation of ice crystals, thus increasing the preservation time by reducing the water activity. Cooling corresponds to a reduction in temperature of a food to between −1 °C and 8 °C, so as to decrease enzymatic and microbial activities, conferring a longer shelf life on the product (Fellows, 2000).

Many studies have been carried out on the behavior of the anthocyanins and of the antioxidant capacity of blueberries during cold storage (Kalt et al., 2000; Connor et al., 2002; Srivastava et al., 2007; Piljac-Žegarac et al., 2009; Piljac-Žegarac and Šamec, 2011), but there are still some doubts about a suitable storage time and temperature for such products in relation to their bioactive compounds.

Thus the objective of the present study was to analyze the stability of the antioxidant activity and anthocyanins in whole blueberry juice stored under refrigeration at 4 °C for 10 d, and in fruits stored frozen at −18 °C for a total period of 6 months, so as to verify possible changes occurring during domestic storage and thus avoid significant losses of the bioactive compounds and properties of these products.

2. Materials and methods

2.1. Samples

Samples of fresh blueberries (*Vaccinium* spp.) when fully mature were pre-selected for color and size uniformity, and berries with visible injuries and infections were discarded. The fruits were acquired in March 2010 on the retail market in the city of Porto Alegre in the state of Rio Grande do Sul (RS), Brazil, commercialized by the Berrygood Company (São Paulo, SP, Brazil). The samples were washed with water and 1% sodium hypochlorite, then divided into portions of 250 g, stored frozen at −18 °C for 6 months in a domestic freezer and analyzed after 0, 1, 3 and 6 months.

For the juice analyses, organic blueberries of the cultivar Rabbiteye were used, supplied by the Fazenda Viva o Verde in the city of Camaquã, RS, Brazil (latitude: 30°46′25.26″ S and longitude: 51°42′38.78″ O), picked between December 2009 and January 2010. The fruits were pre-selected and then stored at −18 °C until used. The whole juice was prepared using a domestic centrifugal-type extractor (Walita-Philips®), then individually stored in amber jars under refrigeration at 4 °C for 10 d, and analyzed every other day.

2.2. Antioxidant activity

The DPPH method (Brand-Williams et al., 1995), based on free radical scavenging by antioxidants, and the ABTS method (Re et al., 1999), where the free radical is generated by a chemical reaction with potassium persulfate, were used to determine the antioxidant activity (AA), with modifications.

The extract was obtained from about 10 g of sample in 40 mL 50% aqueous methanol (Vetec, Duque de Caxias, RJ, Brazil, P.A.) and 40 mL 70% aqueous acetone (Vetec, P.A.), centrifuged twice at 15,000 × *g* for 15 min (Hitachi, model Himac CR21E centrifuge, Tokyo, Japan), and three dilutions prepared using the supernatant (1:5, 1:10 and 1:15). This procedure was adapted from Larrauri et al. (1997).

For the DPPH method, a 100 μL aliquot of each dilution was added to 3.9 mL of DPPH radical (Sigma–Aldrich, St. Louis, MO, USA), and the reading made at 515 nm in an Ultrospec 3100 pro spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK) after 30 min, using methanol as the blank. For the ABTS method, an aliquot of 30 μL of each dilution was added to 3.0 mL of ABTS radical (Sigma–Aldrich), and the reading made in the spectrophotometer at 734 nm after 6 min of reaction, using ethanol as the blank and a standard curve prepared using Trolox (Sigma–Aldrich). The analyses of the extract were carried out in triplicate and the results presented in g or mL of sample/g of DPPH or in μM of Trolox equivalents (TE)/g or mL of sample.

2.3. Anthocyanin extraction, identification and quantification

The anthocyanins were exhaustively extracted from a sample (fruit) of about 2 g using a 1% solution of HCl (Vetec, 32%) in methanol (Vetec, P.A.). The solution was then filtered and vacuum concentrated ($T < 38$ °C) in a rotary evaporator (Fisatom, model 801/802, São Paulo, SP, Brazil) (Francis, 1982). The concentrated crude extract was transferred to a 25 mL flask, and completed to volume with the acidified methanol solution. A 1 mL aliquot of this solution was removed, dried in nitrogen gas (N_2) and stored frozen ($T < -18$ °C) until analyzed. This extract, dried in N_2 (or 1 mL of previously filtered juice), was diluted in chromatographic grade methanol (J.T. Baker-Mallinckrodt, Center Valley, PA, USA), homogenized in an ultrasound bath (Unique, model USC 1400, Indaiatuba, SP, Brazil) and filtered into a vial through a polyethylene membrane (Millex PTFE, Millipore, Barueri, SP, Brazil) with a pore size of 0.45 μm and diameter of 13 mm.

The anthocyanins were identified and quantified by high performance liquid chromatography (HPLC), by comparing with the respective standards. All the solvents used in the HPLC separation were of chromatographic grade, and previously filtered through the Millipore vacuum filtration system using a 0.45 μm membrane for organic solvents.

An Agilent series 1100 chromatograph (Santa Clara, CA, USA) equipped with a quaternary solvent pumping system and UV/Vis detector was used for the HPLC analyses. The pigments were separated on a Shim-pak 5 μm C_{18} CLC-ODS reverse phase column, 250 mm × 4.6 mm (Shimadzu, Kyoto, Japan), using linear gradient elution with a mobile phase of 4% aqueous phosphoric acid (Vetec, 85%)/acetonitrile (J.T. Baker-Mallinckrodt), from 85:15 (v/v) to 20:80 (v/v) in 25 min, in a chromatographic run of 15 min, according to the conditions established experimentally by Zanatta et al. (2005). The flow rate of the mobile phase was 1.0 mL/min and the injection volume 10 μL. The column temperature was maintained at 29 °C and the chromatograms processed at 520 nm. The anthocyanins were extracted and injected into the chromatograph in duplicate, and identified by comparison of the retention times (tR) with those of commercial standards obtained from Sigma–Aldrich® and Santa Cruz Biotechnology® (Santa Cruz, CA, USA). The anthocyanins were quantified by constructing standard curves with glycosylated anthocyanins, such as cyanidin-3-glucoside (Sigma–Aldrich, ≥95% purity), cyanidin-3,5-diglucoside (Sigma–Aldrich, ≥90% purity), delphinidin-3-glucoside (Santa Cruz Biotechnology, ≥95% purity), pelargonidin-3-glucoside (Sigma–Aldrich, ≥97% purity) and malvidin-3-glucoside (Sigma–Aldrich, ≥90% purity), and the aglycone delphinidin (Sigma–Aldrich, ≥95% purity). The regression coefficients (R^2) for the standard curves were >0.99. The results were presented in mg/100 g or mL of fresh weight (FW).

The limits of detection (LOD) and quantification (LOQ) were calculated according to Long and Winefordner (1983), and were,