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# *Stevia rebaudiana* Bertoni cultivated in Portugal: A prospective study of its antioxidant potential in different conservation conditions

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ABSTRACT

Studies with well-established cultivation conditions are of utmost importance in order to standardize the plants production and, therefore, its chemical composition. However, the conservation conditions may influence some of the bioactive compounds present in those plants. *Stevia rebaudiana* Bertoni samples cultivated in the north-eastern of Portugal, were exposed to different conservation conditions (oven-dried at 30 °C, for six days, or kept fresh by freezing (−20 °C) in the same period), and then studied for their antioxidant potential (including antioxidant compounds such as reducing sugars, tocopherols and phenolic compounds, free radical scavenging activity (DPPH) and reducing power (RP)). Oven-dried samples gave the highest antioxidant activity (DPPH $EC_{50}$ = 22.87 μg/mL and RP $EC_{50}$ = 28.79 μg/mL) and the highest total phenolic compounds concentration. The most abundant phenolic compounds in both samples were two caffeic acid derivatives, 5-*O*-caffeoylquinic acid (chlorogenic acid) and 3,5-*O*-dicaffeoylquinic acid. Otherwise, the highest values of total tocopherols and total sugars were registered in frozen fresh samples.

This study gives clues to choose the most appropriate methodology to preserve primary or secondary metabolites of *S. rebaudiana*, involved in its antioxidant activity.



## 1. Introduction

The demand for natural sweeteners has been gaining more and more importance due to the great controversy associated with the use of some synthetic sweeteners as cyclamate, aspartame or acesulfame-K. The steviol glycosides (E-960) are a group of natural sweeteners of generalized availability. These compounds are obtained from *Stevia rebaudiana* Bertoni, a sweet plant native to South America (Carocho et al., 2015), belonging to the *Compositae* (*Asteraceae*) family. It is one of the 154 members of the *Stevia* genus and one of the only two species that produce sweet glycosides (Madan et al., 2010). This plant is also cultivated in China and Southeast Asia (Koyama et al., 2003).

The commercial exploitation of *S. rebaudiana* has become stronger since the 1970′s, after the development in Japan of processes for the extraction and refinement of its leaf sweetener (Dacome et al., 2005). The natural sweeteners of *S. rebaudiana* can be 300 times sweeter than sucrose (Gardana et al., 2010) and with the advantages of not being caloric and avoiding high levels of blood sugar (Periche et al., 2015). In addition, *S. rebaudiana* contains other metabolites with bioactive potential, such as alkaloids, water-soluble chlorophylls, xanthophylls, hydroxycynnamoyl derivatives (caffeoyl and chlorogenic acid derivatives), neutral water-soluble oligosaccharides, free sugars, amino acids, lipids, essential oils and trace elements (Komissarenko et al., 1994). It has also been shown to possess antimicrobial and antioxidant activities (Xi et al., 1998; Tadhani et al., 2007). *In vitro* antioxidant activity and preventive activity against DNA oxidative damage were reported for crude methanolic and ethyl acetate extracts from *S. rebaudiana* leaves (Ghanta et al., 2007).

Previous studies have shown that stevia leaves contain different antioxidant compounds, such as ascorbic acid (Kim et al., 2011), phenolic compounds (Shukla et al., 2009) including flavonoids (Jahan et al., 2010; Abou-Arab and Abu-Salem, 2010) and tannins (Savita et al., 2004). However, it is important to evaluate if the

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chemical composition of the plant is affected by the conservation process.

In fact, the plants with commercial value are often stored for some time before use and an efficient conservation is very important to avoid the loose of bioactive molecules during this process. One of the main conservation methodologies is drying, which prevents the growth of microorganisms and avoids certain biochemical reactions that may alter the plant organoleptic characteristics (Pinela et al., 2011).

Accordingly, the present work evaluated the effects of dehydration process on the *S. rebaudiana* antioxidant potential, by comparing the free radical scavenging activity, reducing power, and antioxidant compounds of fresh frozen and dried plants. It should be highlighted that the studied samples were obtained in an experimental field with defined cultivation conditions in order to standardize *S. rebaudiana* production and, therefore, chemical composition.

## 2. Materials and methods

### 2.1. Cultivation procedure, collection and treatment of the samples

The field trial supporting this work was carried out in Bragança (41.796742; −6.761418; 750 m a.s.l.), Northeast Portugal. The region benefits from a Mediterranean type climate, somewhat influenced by the Atlantic regime. Average annual air temperature and annual precipitation for 1971–2000 period were, respectively, 12.3 °C and 758 mm. The soil is a Regosol of colluvial origin, loamy textured (23.9% clay, 21.8% silt, 54.4% sand), slightly acidic [$pH_{(H2O)}$ 6.3], and low in organic carbon (Walkley-Black) 5.6 g $kg^{-1}$. The soil was covered with anti-weed screen punched with holes spaced at 50 × 40 cm after the installation of the drip irrigation system. Cuttings of 10–15 cm height, previously placed on rooting in a greenhouse, were planted in the holes opened in the screen. The cuttings were obtained from a farmer's field grown with cv. Morita. Crop plantation occurred in June 13th, 2014. Plants were watered twice a week throughout the growing season. The harvest took place in August 5th, 2014 and subsamples consisting of three whole plants were separated in fresh in stems and leaves.

After the collection, the specimens (leaves) were separated in two approximately equal halves and placed in paper bags. Half of the samples were dried at 30 °C (Memmert oven, Edelstahl Rostfrei, Germany) for six days, being designated as oven-dried samples, while the other half was immediately frozen (−20 °C) for the same period, being only lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) before analysis, in order to express the results in dry weight basis to be comparable with the oven-dried samples. These last samples were designated as fresh frozen samples. All the samples were kept in a desiccator and protected from light for subsequent use.

### 2.2. Standards and reagents

HPLC-grade acetonitrile and formic acid were obtained from Lab-Scan (Lisbon, Portugal). Racemic tocol (50 mg/mL) and tocopherol standards were purchased from Matreya (Pleasant Gap, PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic compound standards were from Extrasynthèse (Genay, France). Trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid) and free sugar standards were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from common chemical suppliers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

### 2.3. Free sugars

Free sugars were determined by high-performance liquid chromatography coupled to a refraction index detector (HPLC-RI) after an extraction method described by Barros et al. (2013), using melezitose as internal standard (IS). The equipment consisted of a combined system with a pump (Knauer, Smartline system 1000; Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD) and an RI detector (Knauer Smartline 2300). The chromatographic separation was achieved with a Eurospher 100-5 NH2 column (4.6 × 250 mm, 5 μm, Knauer), operating at 35 °C (7971R Grace oven). The mobile phase used was a mixture of acetonitrile/deionized water, 70:30 (v/v), at a flow rate of 1 mL/min. Sugars were identified by chromatographic comparison with standards and quantitation was performed by the IS methodology using a Clarity 2.4 Software (DataApex, Prague, Czech Republic). Results were expressed in g per 100 g of dry weight (dw).

### 2.4. Tocopherols

Tocopherols were determined after an extraction procedure previously described by Fernandes et al. (2013), using tocol as IS. The analysis was carried out using the HPLC system described above connected to a fluorescence detector (FP-2020; Jasco, Tokyo, Japan) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II normal-phase column (250 × 4.6 mm, 5 μm; YMC, Kyoto, Japan), operating at 35 °C. The mobile phase used was a mixture of *n*-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min. Tocopherols were identified by chromatographic comparison with standards and quantitation was performed by the IS methodology using a Clarity 2.4 Software. The tocopherols content were expressed in g per 100 g of dry weight (dw).

### 2.5. Extracts preparation

Hydroalcoholic extractions were performed by stirring the plant material (leaves) (1 g) with 30 mL of methanol/water (80:20, v/v) at 25 °C, with a stirring agitation of 150 rpm for 1 h, and filtered through Whatman No. 4 paper. The residue was re-extracted with an additional 30 mL portion of the hydroalcoholic mixture. The combined extracts were evaporated at 35 °C under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and then further lyophilized.

The lyophilized extracts of both samples were re-dissolved in methanol/water (80:20, v/v, final concentration 5 mg/mL), for phenolic compounds determination and antioxidant activity evaluation. The final solutions were further diluted to different concentrations to be submitted to distinct *in vitro* assays.

### 2.6. Phenolic compounds

The extracts were analysed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2$C_{18}$ (4.6 mm × 150 mm, 3 μm; Lisbon, Portugal) column thermostatted at 35 °C was used for separation. The mobile phase consisted of two solvents: (A) 0.1% formic acid in water, and (B) acetonitrile, establishing the following elution gradient: 15% B for 5 min, 15% B to 20% B over 5 min, 20–25% B over 10 min, 25–35% B over 10 min, 35–50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in