

***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  $EC_{50} = 22.87 \mu\text{g/mL}$  and RP  $EC_{50} = 28.79 \mu\text{g/mL}$ ) 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.

**Keywords:** Stevia; storage conditions; tocopherols; sugars; phenolic compounds; 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, hydroxycinnamoyl 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, 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 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 loss 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; 750m 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 [ $\text{pH}_{(\text{H}_2\text{O})}$  6.3], and low in organic carbon (Walkley-Black)  $5.6 \text{ g kg}^{-1}$ . The soil was covered with anti-weed screen punched with holes spaced at  $50 \times 40 \text{ cm}$  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 13<sup>th</sup>, 2014. Plants were watered twice a week throughout the growing season. The harvest took place in August 5<sup>th</sup>, 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  $30^\circ\text{C}$  (Memmert oven, Edelstahl Rostfrei, Germany) for six days, being designated as oven-dried samples, while the other half was immediately frozen ( $-20^\circ\text{C}$ ) 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 ( $50 \text{ mg/mL}$ ) 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 NH<sub>2</sub> column (4.6 × 250 mm, 5 µm, Knauer), operating at 35 °C (7971 R 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<sub>18</sub> (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 a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

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 analyser that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 °C, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupoles were set at unit resolution. The ion spray voltage was set at -4500 V in the negative mode and spectra were recorded between  $m/z$  100 and 1500. The MS detector was programmed for recording in two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE)-10 V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -50 V, EP -6 V, CE -25 V, and collision energy spread (CES) 0 V.

The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standards, when available. Otherwise, peaks were tentatively identified from their absorption and mass spectra and comparison with data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the area of the peak at the detection wavelength used in the HPLC-DAD analysis. For the identified phenolic compounds for which a commercial standard was not available, the quantification was



performed through the calibration curve of a compound from the same phenolic group. The results were expressed in mg/g of extract.

### *2.7. Evaluation of in vitro antioxidant properties*

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA). Various concentrations of the extracts (30  $\mu$ L) were mixed with a methanol solution containing DPPH radicals ( $6 \times 10^{-5}$  mM, 270  $\mu$ L) in a 96-well plate and then were left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm and calculated as a percentage of DPPH discoloration using the formula:  $[(A_{\text{DPPH}} - A_{\text{S}})/A_{\text{DPPH}}] \times 100$ , where  $A_{\text{S}}$  is the absorbance of the solution containing the sample at 515 nm, and  $A_{\text{DPPH}}$  is the absorbance of the DPPH solution.

Reducing power was evaluated by the capacity to convert  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , measuring the absorbance at 690 nm in the microplate reader mentioned above. Various concentrations of the extracts (500  $\mu$ L) were mixed with sodium phosphate buffer (pH 6.6, 200 mM, 500  $\mu$ L) and potassium ferricyanide (1% w/v, 500  $\mu$ L) in an Eppendorf (2 mL) and incubated at 50 °C for 20 min. Trichloroacetic acid (10%, 500  $\mu$ L) was added and then the upper layer (800  $\mu$ L) was mixed with deionised water (800  $\mu$ L) and ferric chloride (0.1%, 160  $\mu$ L) in a 48-well plate; the absorbance was further measured at 690 nm. The results were expressed in  $\text{EC}_{50}$  values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay). Trolox was used as standard.

### *2.8. Statistical analysis*

Three samples were used for each preparation and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using a Student's *t*-test to determine the significant difference among two different samples, with  $\alpha = 0.05$ . This treatment was carried out using SPSS v. 22.0 program (IBM, Armonk, NY, USA).

### 3. Results and discussion

#### 3.1. Tocopherols and sugars composition

The tocopherol contents determined in the oven-dried and frozen fresh samples of *S. rebaudiana* are given in **Table 1**, with the presence of three isoforms ( $\alpha$ -,  $\gamma$ - and  $\delta$ -tocopherol), being  $\alpha$ -tocopherol the majority compound in both samples. The highest value of total tocopherols was obtained in the frozen fresh samples, which is in concordance with [Pinela et al. \(2011\)](#), who reported a decrease in tocopherols content in *Cytisus* and *Genista* plant species after shade drying. The higher concentration of  $\alpha$ -tocopherol in frozen fresh samples in comparison with the oven dried ones is also supported by the work of the previously mentioned authors.

Regarding free sugars composition (**Table 1**), oven-dried and frozen fresh samples revealed the presence of eight different sugars: rhamnose, xylose, arabinose+fructose, glucose, sucrose, trehalose, and raffinose. In general, the highest value of total sugars was registered in frozen fresh samples, mostly due to its higher content of xylose, the major sugar in this sample. It also showed the highest concentration of glucose. No significant differences were obtained for rhamnose and trehalose concentrations. Otherwise and despite the lower value of total sugars, oven-dried samples gave higher concentration of arabinose+fructose, sucrose and raffinose than frozen fresh samples.

Pinela et al. (2011) also reported a decrease in free sugars content of plant species from *Cytisus* genus and *Genista* genus, after shade drying. Not only free sugars and tocopherols are affected by the drying process but also other important compounds in *S. rebaudiana* such as steviol glycosides (Periche et al., 2015).

Sucrose was the major free sugar in the oven-dried samples (4.26 g/100 g), which is in agreement with the results reported by Pereira et al. (2015), who studied a dried and commercial sample of *S. rebaudiana* (3.93 g/100 g). However, the profile obtained was dissimilar between both samples, with the commercial one showing only sucrose, fructose, glucose and trehalose. Karimi et al. (2015) also studied soluble sugars in *S. rebaudiana*, describing an increasing content (mainly glucose) with soil moisture depletion. Fructose and sucrose were also identified by those authors, but in low levels. Indeed, differences in the chemical composition might be related with photoperiod (Ceumen and Geuns, 2013), planting times, plant stands and harvest regime (Serfaty et al., 2013; Karimi et al., 2015). It should be highlighted that the majority of studies in *S. rebaudiana* that are available in literature address to the evaluation of steviol (diterpene compound) glycosides (Brandle and Telmer 2007; Gardana et al. 2010; Geuns et al. 2003; Periche 2015), the responsible for the sweet taste of this plant, and not the herein studied free sugars.

### 3.2. Phenolic composition

The phenolic compounds profile of the hydroalcoholic extract of *S. rebaudiana* leaves is shown in **Figure 1**. Data (retention time,  $\lambda_{\text{max}}$  in the visible region, pseudomolecular ion and main fragment ions observed in MS<sup>2</sup>) obtained by HPLC-DAD-ESI/MS analysis regarding phenolic compounds identification and individual quantification are presented in **Table 2**. Compounds 3, 6, 7 and 10 were positively identified as 5-*O*-caffeoylquinic

acid, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside respectively, by comparison with authentic standards and analysis of their MS fragmentation pattern, retention time and UV-vis characteristics. The presence of these compounds has already been reported in *S. rebaudiana* by different authors (Cacciola et al., 2011; Muanda et al., 2011; Karaköse et al., 2011, 2015; Shivanna et al., 2013; Barba et al., 2015; Gawel-Bęben et al., 2015).

Compounds 1 and 2 ( $[M-H]^-$  at  $m/z$  353) were identified as 3-*O*-caffeoylquinic acid and 4-*O*-caffeoylquinic acid based on the fragmentation pattern and characteristics reported by Clifford et al. (2003, 2005). Peaks 8, 11 and 15 ( $[M-H]^-$  at  $m/z$  515) could be assigned as 3,4-*O*-, 3,5-*O*- and 4,5-*O*-dicaffeoylquinic acids, respectively, based on their elution order, fragmentation pattern and relative abundances (Clifford et al., 2003, 2005). All those compounds have also been described in *S. rebaudiana* (Muanda et al., 2011; Karaköse et al., 2011, 2015; Shivanna et al., 2013), also confirming their identification in this sample. Peaks 4 ( $[M-H]^-$  at  $m/z$  381) and 5 ( $[M-H]^-$  at  $m/z$  391) presented a fragmentation pattern and UV-vis spectra similar to caffeic acid. Compounds showing a pseudomolecular ion at  $m/z$  381 were detected in other Asteraceae like *Erigeron breviscapus* and identified as caffeoyl-2,7-anhydro-3-deoxy-2-octulopyranosonic acids (Zhang et al., 2007; Liao et al., 2011), identity that was tentatively assumed for the compound 4 detected herein. No structure could, however, be matched for peak 5 that remained unidentified as a caffeic acid derivative.

The rest of phenolic compounds were assigned to flavonol derivatives based on their characteristic UV spectra showing maximum wavelength of absorption ( $\lambda_{max}$ ) around 345-360 nm and the release of MS<sup>2</sup> fragment ions at  $m/z$  301 (quercetin) or 285 (kaempferol). Peaks 9, 13 and 14 ( $[M-H]^-$  at  $m/z$  579, 433 and 447, respectively) were assigned to quercetin glycosides, and peaks 12 ( $[M-H]^-$  at  $m/z$  447), 16 and 17 ( $[M-H]^-$

at  $m/z$  417), and 18 ( $[M-H]^-$  at  $m/z$  431) to kaempferol derivatives. They presented  $MS^2$  fragments corresponding to distinct losses of hexosyl (-162 u), deoxyhexosyl (-146 u) or pentosyl (-132 u) residues, although mass spectral characteristics did not allow characterising the position and nature of the sugar moieties. Similar compounds have already been reported in leaves of *S. rebaudiana* (Cacciola et al., 2011; Muanda et al., 2011; Karaköse et al., 2015; Shivanna et al., 2013). Among them, peaks 13 and 14 might be tentatively associated to quercetin-3-*O*-xyloside and quercetin-3-*O*-rhamnoside, previously identified in leaves of *S. rebaudiana* by Shivanna et al. (2013) and Cacciola et al. (2011), respectively.

The most abundant phenolic compounds in the studied samples were 5-*O*-caffeoylquinic acid (chlorogenic acid) and 3,5-*O*-dicaffeoylquinic acid. Oven-dried samples presented the highest total phenolic compounds concentration, due to the presence of higher concentration of phenolic acids, whereas the concentration of total flavonoids in both samples is similar. As for the qualitative composition, oven-dried samples did not reveal the presence of quercetin-3-*O*-rutinoside (rutin) and quercetin-*O*-pentosyl-deoxyhexoside, while the frozen fresh sample did not present peak 12 (kaempferol-*O*-hexoside).

### 3.3. Antioxidant activity

**Figures 2 and 3** show the results obtained in the antioxidant activity evaluation of *S. rebaudiana* extracts, measured by the DPPH radical scavenging activity (RSA) and reducing power (RP) assays, respectively. The extracts from oven-dried samples gave higher RSA and RP (i.e., lower  $EC_{50}$  values of 22.87  $\mu\text{g/mL}$  and 28.79  $\mu\text{g/mL}$ , respectively) than those of frozen fresh samples (50.66  $\mu\text{g/mL}$  and 39.73  $\mu\text{g/mL}$ ), which is in disagreement with other authors (Pinela et al., 2011), that reported a higher

antioxidant activity in frozen fresh samples of four shrubby flowering plants widely consumed in folk medicine. However, our results are in accordance with the contents of phenolic compounds observed in both samples, once the extract of the oven-dried samples possessed higher concentration of total phenolic compounds (**Table 2**). Other authors have reported the high potential of *S. rebaudiana* leaves as a natural antioxidant, with associated health benefits (Xi et al 1998; Tadhani et al., 2007; Jahan et al 2010; Kim et al 2011).

*S. rebaudiana* is widely known for its natural sweeteners content, although this plant could also be considered as a good source of antioxidants, such as phenolic compounds. However, appropriate methodologies have to be applied to preserve the chemical constituents of *S. rebaudiana*. This study proved that oven-dried samples showed higher antioxidant activity and phenolic compounds contents than frozen fresh samples, whereas these latter possessed higher levels of tocopherols and free sugars. Overall, it is very important to establish suitable conditions to cultivate *S. rebaudiana* in order to standardize its chemical composition, but not least important is the preservation process selected for this plant.

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### **References**

- Abou-Arab, E.A., Abu-Salem, F.M., 2010. Evaluation of bioactive compounds of *Stevia rebaudiana* leaves and callus. *Afr. J. Food Sci.* 4, 627-634.
- Barba, F.J., Grimi, N., Vorobiev E., 2015. Evaluating the potential of cell disruption technologies for green selective extraction of antioxidant compounds from *Stevia rebaudiana* Bertoni leaves. *J. Food Eng.* 149, 222-228.
- Barros, L., Pereira, E., Calhelha, R.C., Dueñas, M., Carvalho, A.M., Santos-Buelga, C., Ferreira, I.C.F.R., 2013. Bioactivity and chemical characterization in hydrophilic and lipophilic compounds of *Chenopodium ambrosioides* L. *J. Funct. Food.* 5, 1732-1740.
- Brandle, J.E., Telmer, P.G., 2007. Steviol glycoside biosynthesis. *Phytochem.* 68, 1855-1863.
- Cacciola, F., Delmonte, P., Jaworska, K., Dugo, P., Mondello, L., Rader, J.I., 2011. Employing ultra high pressure liquid chromatography as the second dimension in a comprehensive two-dimensional system for analysis of *Stevia rebaudiana* extracts. *J. Chromatogr. A* 1218, 2012–2018
- Carocho, M., Morales, P., Ferreira, I.C.F.R., 2015. *Natural food additives: Quo vadis?* *Trends Food Sci. Technol.* 45, 284-295.
- Ceunen, S., Geuns, J.M.C., 2013. Influence of photoperiodism on the spatio-temporal accumulation of steviol glycosides in *Stevia rebaudiana* (Bertoni). *Plant Sci.* 198, 72-82.
- Clifford, M.N., Johnston, K.L., Knight, S., Kuhnert, N.A., 2003. A hierarchical scheme for LC-MS<sup>n</sup> identification of chlorogenic acids. *J Agric. Food Chem.* 51, 2900-2911.
- Clifford, M.N., Knight, S., Kuhnert, N.A., 2005. Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS<sup>n</sup>. *J. Agric. Food Chem.* 53, 3821-3832.

- Dacome, A.S., Silva, C.C., Costa, C.E.M., Fontana, J.D., Adelmann, J., Costa, S.C., 2005. Sweet diterpenic glycosides balance of a new cultivar of *Stevia rebaudiana* (Bert) Bertoni: isolation and quantitative distribution by chromatographic, spectroscopic and electrophoretic methods. *Process Biochem.* 44, 3587-3594.
- Fernandes, Â., Barreira, J.C.M., António, A.L., Santos, P.M.P., Martins, A., Oliveira, M.B.P.P., Ferreira, I.C.F.R., 2013. Study of chemical changes and antioxidant activity variation induced by gamma-irradiation on wild mushrooms: Comparative study through principal component analysis. *Food Res. Int.* 54, 18-25.
- Gardana, C., Scaglianti, M., Simonetti, P., 2010. Evaluation of steviol and its glycosides in *Stevia rebaudiana* leaves and commercial sweetener by ultra-high-performance liquid chromatography-mass spectrometry. *J Chromatogr. A* 1217, 1463-1470.
- Ghanta, S., Banerjee, A., Poddar, A., Chattopadhyay, S., 2007. Oxidative DNA damage preventive activity and antioxidant potential of *Stevia rebaudiana* (Bertoni) Bertoni, a natural sweetener. *J. Agric. Food Chem.* 55, 10962-10967.
- Gawel-Bęben, K., Bujak, T., Nizioł-Łukaszewska, Z., Antosiewicz, B., Jakubczyk, A., Karaś M., Rybczyńska, K., 2015. *Stevia rebaudiana* Bert. leaf extracts as a multifunctional source of natural antioxidants. *Molecules* 20, 5468-5486.
- Geuns, J.M.C., Augustijns, P., Mols, R., 2003. Metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. *Food Chem. Toxicol.* 41, 1599-1607.
- Jahan, I.A., Mostafa, M., Hossain, H., Nimmi, I., Sattar, A., Alim, A., Moeiz, S.M.I. (2010). Antioxidant activity of *Stevia rebaudiana* Bert. leaves from Bangladesh. *Bangladesh Pharm. J.* 13, 67-75.



- Karakose, H., Jaiswal, R., Kuhnert, N., 2011. Characterization and quantification of hydroxycinnamate derivatives in *Stevia rebaudiana* leaves by LC-MS<sup>nt</sup>. J. Agric. Food Chem. 59, 10143-10150.
- Karakose, H., Muller, A., Kuhnert, N., 2015. Profiling and quantification of phenolics in *Stevia rebaudiana* leaves. J. Agric. Food Chem. 63, 9188-9198.
- Karimi, M., Ahmadi, A., Hashemi, J., Abbasi, A., Tavarini, S., Guglielminetti, L., Angelini L.G., 2015. The effect of soil moisture depletion on *Stevia (Stevia rebaudiana* Bertoni) grown in greenhouse conditions: Growth, steviol glycosides content, soluble sugars and total antioxidant capacity. Sci. Hort. 183, 93-99.
- Kim, I., Yang, M., Lee, O., Kang, S., 2011. The antioxidant activity and the bioactive compound content of *Stevia rebaudiana* water extracts. Food Sci. Technol. 44, 1328-1332.
- Komissarenko, N.F., Derkach, A.I., Kovalyov, I.P., Bublik, N.P., 1994. Diterpene glycosides and phenylpropanoids of *Stevia rebaudiana* Bertoni. Rastitel'Nye Resursy 1, 53-64.
- Koyama, E., Kitazawa K., Ohori, Y., Izawa, O., Kakegawa, K., Fujino, A., Ui, M., 2003. *In vitro* metabolism of the glycosidic sweeteners, Stevia mixture and enzymatically modified Stevia in human intestinal microflora. Food Chem. Toxicol. 41, 359-374.
- Liao, S.-G., Zhang, L.-J., Li, C.-B., Lan, Y.-Y., Wang, A.-M., Huang, Y., Zhen, L., Fu, X.-Z., Zhou, W., Qi, X.-L., Guan Z.-Z., Wang Y.-L., 2010. Rapid screening and identification of caffeic acid and its esters in *Erigeron breviscapus* by ultra-performance liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 24, 2533-2541.

- Madan, S., Ahmad, S., Singh, G.N., Kohli, K., Kumar, Y., Singh, R., Garg, M., 2010. *Stevia rebaudiana* (Bert.) Bertoni - A Review. Indian J. Natural Prod. Res. 1, 267-286.
- Muanda, F.N., Soulimani, R., Diop, B., Dicko, A., 2011. Study on chemical composition and biological activities of essential oil and extracts from *Stevia rebaudiana* Bertoni leaves. Food Sci. Technol. 44, 1865-1872.
- Pereira, C., Barros, L., Ferreira, I.C.F.R., 2015. A comparison of the nutritional contribution of thirty-nine aromatic plants used as condiments and/or herbal infusions. Plant Foods Hum. Nutr. 70, 176-183.
- Periche, A., Castelló, M.L., Escriche, I., 2015. Influence of drying method on steviol glycosides and antioxidants in *Stevia rebaudiana* leaves. Food Chemistry, 173, 1-6.
- Pinela, J., Barros, L., Carvalho, A.M., Ferreira, I.C.F.R., 2011. Influence of the drying method in the antioxidant potential of four shrubby flowering plants from the tribe Genisteae (Fabaceae). Food Chem. Toxicol. 49, 2983-2989.
- Savita, S.M., Sheela, K., Sunanda, S., 2004. *Stevia rebaudiana* - A functional component for food Industry. J Hum. Ecol. 15, 261-264.
- Serfaty, M., Ibdah, M., Fischer, R., Chaimovitsh D., Saranga, Y., Dudai N., 2013. Dynamics of yield components and stevioside production in *Stevia rebaudiana* grown under different planting times, plant stands and harvest regime. Ind. Crop Prod. 50, 731-736.
- Shivanna, N., Naika, M., Khanum, F., Kaul, V.K., 2013. Antioxidant, anti-diabetic and renal protective properties of *Stevia rebaudiana*. J. Diabetes Complications 27, 103-113.

- Shukla, S., Mehta, A., Bajpai, V.K., Shukla, S., 2009. *In vitro* antioxidant activity and total phenolic content of ethanolic leaf extract of *Stevia rebaudiana* Bert. Food Chem. Toxicol. 47, 2338-2343.
- Tadhani, M., Patel, V., Subhash, R., 2007. *In vitro* antioxidant activities of *Stevia rebaudiana* leaves and callus. J. Food Com. Anal. 20, 323-329.
- Xi, Y., Yamaguchi, T., Sato, M., Takeuchi, M., 1998. Antioxidant activity of *Stevia rebaudiana*. J. Japanese Soc. Food Sci. Technol. 45, 310-316.
- Zhang, Y., Shi, P., Qu, H., Cheng, Y., 2007. Characterization of phenolic compounds in *Erigeron breviscapus* by liquid chromatography coupled to electrospray ionization mass spectrometry. Rapid Commun. Mass Spectrom. 21, 2971-2984.

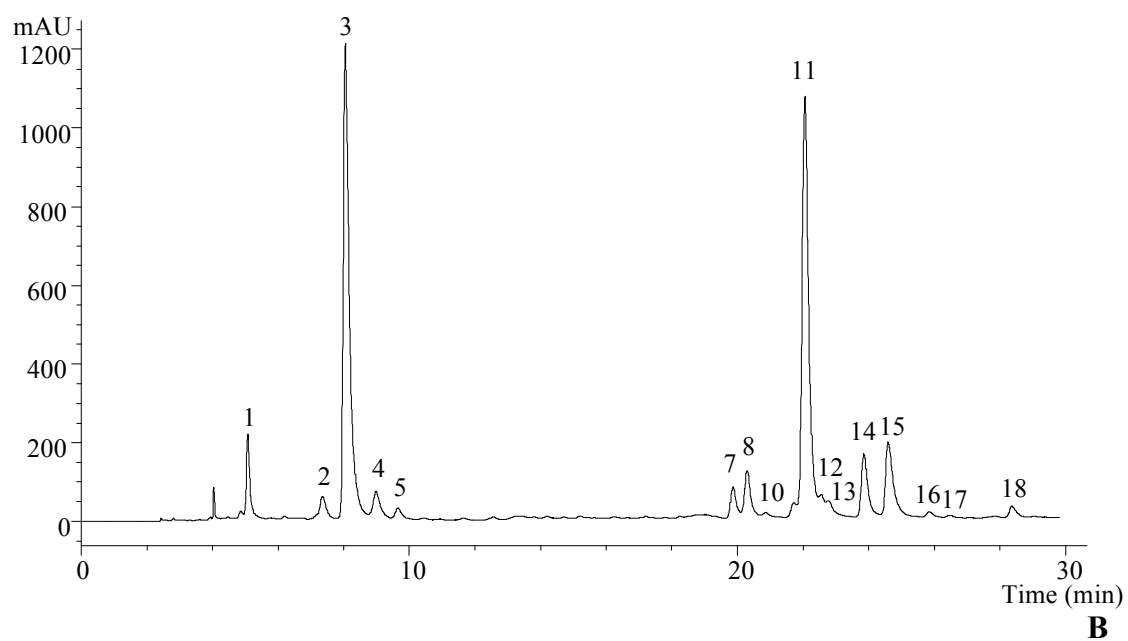
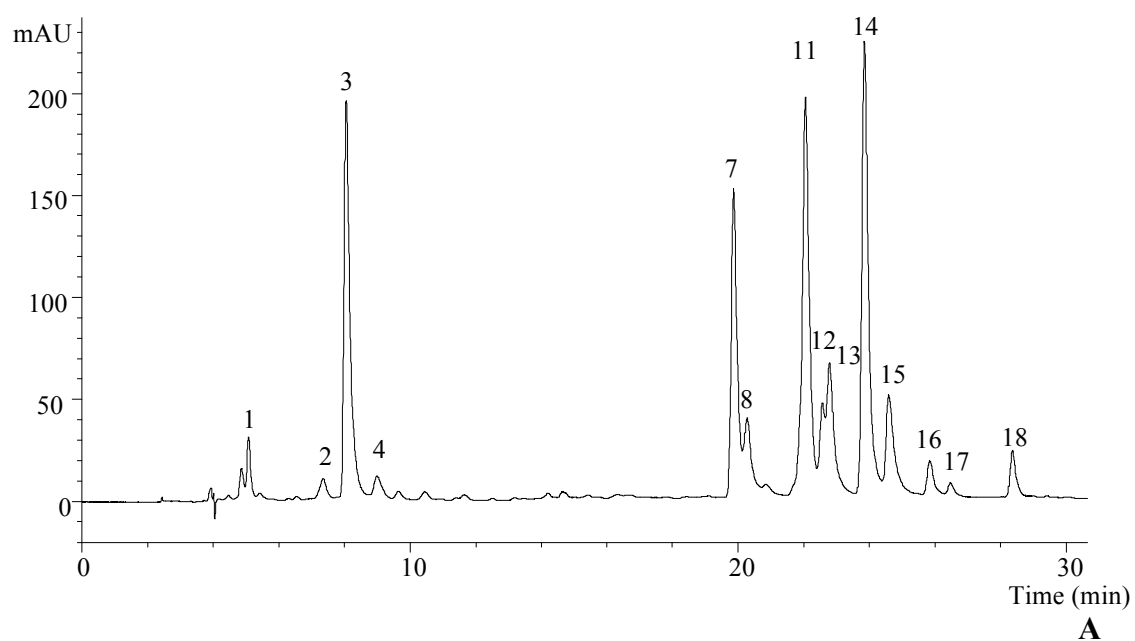
**Table 1.** Composition of *Stevia rebaudiana* leaves in tocopherols and free sugars (mean  $\pm$  SD).

Compounds	Frozen fresh Samples	Oven-dried Samples	<i>t</i> -Students test <i>p</i> -value
$\alpha$ -Tocopherol	12.25 $\pm$ 0.08	5.83 $\pm$ 0.01	<0.001
$\gamma$ -Tocopherol	0.20 $\pm$ 0.01	0.22 $\pm$ 0.01	0.013
$\delta$ -Tocopherol	0.25 $\pm$ 0.01	0.11 $\pm$ 0.01	0.001
Total (mg/100 g)	12.70 $\pm$ 0.10	6.16 $\pm$ 0.02	<0.001
Ramnose	0.55 $\pm$ 0.02	0.50 $\pm$ 0.01	0.001
Xylose	9.76 $\pm$ 0.15	3.81 $\pm$ 0.18	<0.001
Arabinose+Fructose	2.04 $\pm$ 0.13	3.30 $\pm$ 0.15	<0.001
Glucose	0.26 $\pm$ 0.01	0.040 $\pm$ 0.001	<0.001
Sucrose	1.79 $\pm$ 0.16	4.26 $\pm$ 0.17	<0.001
Trehalose	0.45 $\pm$ 0.05	0.38 $\pm$ 0.03	0.005
Raffinose	0.19 $\pm$ 0.04	0.88 $\pm$ 0.04	<0.001
Total (g/100 g)	15.04 $\pm$ 0.18	13.17 $\pm$ 0.42	<0.001

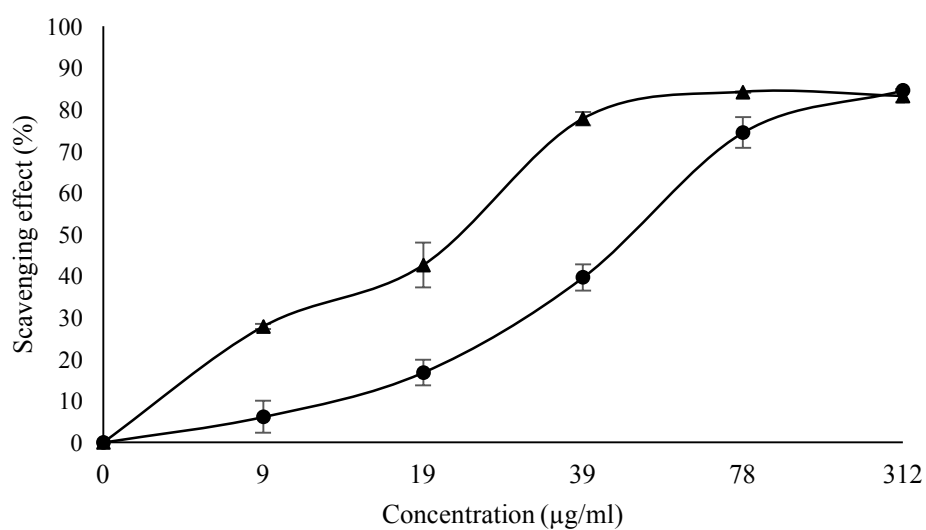
**Table 2.** Phenolic compounds identification and quantification in *Stevia rebaudiana* leaves.

Compounds	Rt	$\lambda_{\max}$	Pseudomolecular ion	MS <sup>2</sup>	Tentative identification	Quantification (mg/g extract)		<i>t</i> -Students test <i>p</i> -value
						Frozen fresh samples	Oven-dried samples	
	(min)	(nm)	[M-H] <sup>-</sup> ( <i>m/z</i> )	( <i>m/z</i> )				
1	5.1	328	353	191(100),179(45),161(6),135(66)	3- <i>O</i> -Caffeoylquinic acid	1.79±0.03	5.54±0.03	<0.001
2	7.2	328	353	191(72),179(80),173(100),161(9),135(77)	4- <i>O</i> -Caffeoylquinic acid	1.48±0.01	2.85±0.01	<0.001
3	7.9	328	353	191(100),179(3),161(6),135(4)	5- <i>O</i> -Caffeoylquinic acid	27.85±0.05	51.0±0.2	<0.001
4	8.7	328	381	179(35),161(100),135(33)	Caffeoyl-2,7-anhydro-3-deoxy-2-octulopyranosonic acid	0.22±0.01	2.82±0.02	<0.001
5	9.5	320	391	217(50),179(38),135(63)	Caffeic acid derivative	0.20±0.02	0.84±0.05	<0.001
6	19.0	354	609	301(100)	Quercetin-3- <i>O</i> -rutinoside	4.32±0.01	nd	-
7	20.2	354	463	301(100)	Quercetin-3- <i>O</i> -glucoside	0.51±0.01	5.38±0.04	<0.001
8	20.7	328	515	353(87),335(45),191(41),179(76),173(91),161(17),135(25)	3,4- <i>O</i> -Dicafeoylquinic acid	1.24±0.04	5.52±0.03	<0.001
9	21.0	358	579	301(100)	Quercetin- <i>O</i> -pentosyl-deoxyhexoside	1.08±0.02	nd	-
10	21.3	348	447	285(100)	Kaempferol-3- <i>O</i> -glucoside	2.10±0.02	0.39±0.03	<0.001
11	22.4	328	515	353(90),335(8),191(100),179(89),173(14),161(8),135(46)	3,5- <i>O</i> -Dicafeoylquinic acid	18.1±0.1	49.2±0.2	<0.001

12	23.1	350	447	285(100)	Kaempferol- <i>O</i> -hexoside	nd	2.1±0.1	-	
13	23.3	358	433	301(100)	Quercetin-3- <i>O</i> -xyloside	2.2±0.1	2.97±0.01	0.007	
14	24.3	350	447	301(100)	Quercetin-3- <i>O</i> -rhamnoside	9.84±0.03	9.29±0.03	0.001	
15	25.0	328	515	353(81),335(3),191(21),179(73),173(100),135(21)	4,5- <i>O</i> -Dicaffeoylquinic acid	2.95±0.04	10.83±0.05	<0.001	
16	26.4	350	417	285(100)	Kaempferol- <i>O</i> -pentoside	1.35±0.01	1.03±0.01	<0.001	
17	27.1	350	417	285(100)	Kaempferol- <i>O</i> -pentoside	0.29±0.01	0.35±0.01	0.015	
18	29.0	346	431	285(100)	Kaempferol- <i>O</i> -deoxyhexoside	1.58±0.02	1.20±0.02	0.001	
						Total phenolic acid derivatives	53.8±0.1	128.5±0.4	<0.001
						Total flavonoids	23.3±0.1	22.7±0.2	0.029
						Total phenolic compounds	77.1±0.2	151±1	<0.001

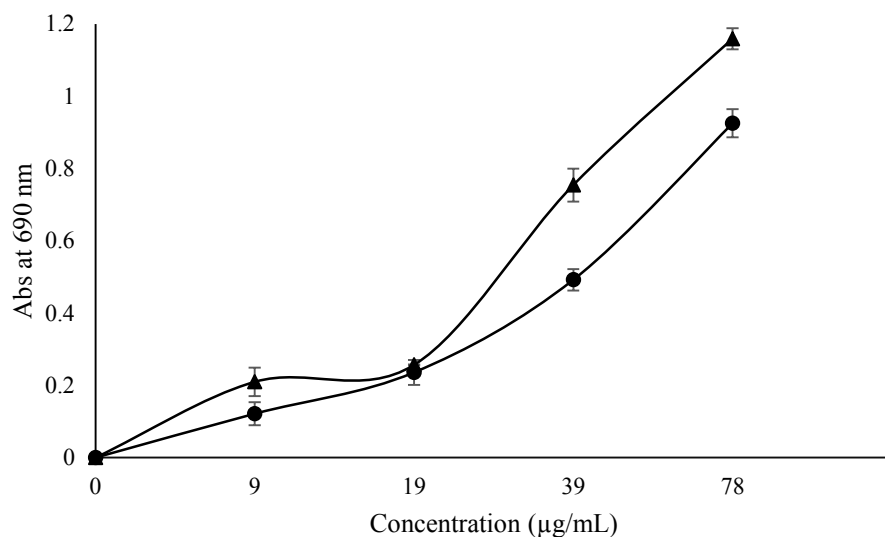


**Figure 1.** Phenolic compounds profile of oven-dried *Stevia rebaudiana* leaves recorded at 370 (A) and 280 (B) nm.



**Figure 2.** Percentage of scavenging activity on DPPH radicals in extracts of frozen fresh (●) and oven-dried samples (▲) of *Stevia rebaudiana*. EC<sub>50</sub>: extract concentration corresponding to 50% of DPPH scavenging activity. Frozen fresh samples: EC<sub>50</sub> = 50.66 ± 3.64 µg/mL; oven-dried samples: EC<sub>50</sub> = 22.87 ± 2.17 µg/mL.





**Figure 3.** Reducing power in extracts of frozen fresh (●) and oven-dried samples (▲) of *Stevia rebaudiana* (higher absorbance indicates higher reducing power).  $EC_{50}$ : extract concentration corresponding to 0.5 of absorbance. Frozen fresh samples:  $EC_{50} = 39.73 \pm 0.10 \mu\text{g/mL}$ ; oven-dried samples:  $EC_{50} = 28.79 \pm 0.09 \mu\text{g/mL}$ .