Periche Santamaría, A.; Koutsidis, G.; Escriche Roberto, MI. (2014). Composition of antioxidants and amino acids in stevia leaf infusions. Plant Foods for Human Nutrition. 69(1):1-7. doi:10.1007/s11130-013-0398-1.

Composition of Antioxidants and Amino Acids in Stevia Leaf Infusions

Angela Periche[1], Georgios Koutsidis[2], Isabel Escriche[1]*

[1]Universitat Politècnica de València. Institute of Food Engineering for Development. Food Technology Department. P.O.Box:46022 Valencia, Spain

[2]Northumbria University, School of Life Sciences (Newcastle upon Tyne, UK) NE1 8ST

*Corrresponding author: iescrich@tal.upv.es Tel: +34963873661. Fax: +34963877956

Abstract

Stevia, a non-caloric natural sweetener with beneficial properties and considerable antioxidants and amino acids, is increasingly consumed as an infusion. This work evaluates the influence of the conditions (temperature: 50, 70 or 90ºC and time: 1, 5, 20 or 40 min) applied to obtain *Stevia* infusions, on antioxidants (total phenols, flavonoids and antioxidant activity) and amino acids. The total concentration of the eleven amino acids found was 11.70 mg/g in dried leaves and from 6.84 to 9.11 mg/g per gram of Stevia in infusions. However, infusions showed higher levels of certain amino acids (alanine, asparagine, leucine and proline), and greater values of the three antioxidant parameters in comparison with dry leaves. Temperature had more influence (minimum values at 50 °C and maximum at 90 °C) than time in the case of antioxidants. At 90ºC there were no important increases in the extraction of antioxidant compounds after 5 min; each gram of *Stevia* having117 mg trolox (Total antioxidant activity), 90 mg gallic acid (total phenols) and 56 mg catechin equivalents (flavonoids). Varying the temperature and time conditions no notable differences were observed in the concentrations of the majority of amino acids. However, the infusion treatment 90ºC for 5 minutes was the best, as it gave the highest yield of 8 of the 11 amino acids. Therefore, with respect to the compounds analyzed in this study, the best way to obtain *Stevia* leaf infusions is the same as the domestic process, almost boiling water for a short time.

Keywords: antioxidant activity, total phenols, flavonoids, amino acids, *Stevia*.

Introduction

Stevia rebaudiana Bertoni, (Asteraceae family) is a perennial plant from Brazil and Paraguay [1]. The main characteristic of *Stevia* leaves is high sweetness (250-300 times sweeter than sucrose) due to the diterpene compounds, called steviol glycosides [2]. The most common use of *Stevia* leaves is the extraction and purification of steviosides to obtain a non-caloric natural sweetener, as a sugar substitute, or as an alternative to artificial sweeteners [3]. Other authors have demonstrated that *Stevia* leaves also have beneficial properties, showing them to be: anti-inflammatory, diuretic, antihypertensive, antihyperglycemic, antidiarrehic, antitumoral and antioxidant [4]. These antioxidant effects, as in other plants, are in part due to the presence of flavonoids and phenolic compounds [5, 6]. Although several authors have studied the antioxidant capacity of extracts from different plant leaves such as tea [7], mate [8] or mint [9], there are fewer works related specifically to infusions, except for the results given by Atuoi et al. [10] and Gorjanovic et al. [11] for tea and herbal teas [12,13], and by Samaniego et al. [14] for green tea.

In addition to the before mentioned properties, *Stevia* leaves, like other herbs such as Chinese tea [15] and black tea [16], have considerable amino acid content. In fact, Rafiq et al. [17] and Abou-Arab et al. [18] identified seventeen amino acids in *Stevia* leaves (glutamic acid, aspartic acid, lysine, serine, isoleucine, alanine, proline, tyrosine, arginine, histidine, methionine, phenylalanine, leucine, valine, threonine, glycine, cystine). *Stevia* leaves contain all the indispensable amino acids [19] with the exception of tryptophan.

Due to these therapeutic properties, *Stevia* leaves are consumed more and more as an aqueous extract of dried leaves. These extracts are drunk as a simple infusions or incorporated in different food formulations: juices, biscuits, jams, sweets, etc. This has become an option for the European industry as EFSA (European Food Safety Authority) recognized the safety of *Stevia* leaf extracts for alimentary use in November 2011 [20]. However, the use of *Stevia* was authorized in different Asian and American countries one decade ago.

As far as the authors know, there is no research related to the antioxidant properties and the free amino acid content of *Stevia* leaf infusions. For this reason, the aim of this study was to evaluate how the

conditions (time and temperature) used to obtain infusions (from dehydrated *Stevia* leaves), affect amino acids, antioxidant capacity, total phenolic content, and total flavonoid content.

Material and Methods

Plant material and infusion preparation

Organically produced (based on minimizing the use of external inputs, avoiding the use of synthetic fertilizers and pesticides) dried leaves of *Stevia rebaudiana* (Raab, Vitalfood, Rohrbach, Germany) were used in this study. Aqueous extracts of dried *Stevia* leaves were obtained at atmospheric pressure and different temperatures using a thermostatic bath (JPSelecta Precisdig, Spain). 1g of dried *Stevia* leaf powder (ground in a grinding mill, A11 Basic, IKA, Germany) was dispersed in 100 mL of water. Different temperatures (50, 70 and 90 ˚C) and times (1, 5, 20 and 40 minutes) were applied to obtain the infusions. It was decided to use these combinations of time and temperature in order to cover a wide range of possible treatment conditions, from less aggressive (low temperatures and short time) to more aggressive (high temperatures and long time). Subsequently, the aqueous extracts were filtered through filter paper and cooled before the analytical determinations. Although treatment at 50ºC and 70ºC is not really infusion, in this work in order to facilitate the terminology, all the thermal treatments are called "infusions". All the analyses were performed in triplicate.

Standard compounds and Reagents

A EZ-Faast amino acid kit (Phenomenex, Torrance, CA, USA) was used to carry out the amino acid analyses. This kit, in addition to chloroform, hydrochloric acid, isooctane, n-propanol, sodium carbonate and sodium hydroxide, contains the following amino acid standards: alanine (Ala), asparagine (Asn), aspartic acid (Asp), cistine (C-C), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), valine (Val) and norvaline (Nor) ; all 99+% purity.

6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich) were used to determine the total antioxidant activity. Sodium nitrite, (+)-catechin, sodium hydroxide (Sigma-Aldrich) and aluminum chloride hexahydrate (Fluka) were used for

flavonoid analysis. Sodium carbonate, gallic acid and Folin-Ciocalteu reagent (all purchased from Sigma-Aldrich) were utilized for phenolic determination.

Determination of Free Amino Acids.

The free amino acid content of the *Stevia* infusion was measured using the derivatization technique for GC-MS [21] with the before mentioned EZ-Faast amino acid kit. The derivatized amino acids were extracted with isooctane/chloroform (100 μL) and analyzed using the 6890 GC-MS Agilent system. An aliquot of the derivatized amino acid solution (10 μL) was injected into a 10 m x 0.25 mm Zebron ZB-AAA capillary column (250 ºC in split mode, 5:1).The oven temperature was 110 ºC for 1 min, then increased at 30 ºC/min to 320 ºC, and held at 320 ºC for 2 min. The transfer line was held at 320 ºC, and the carrier gas flow rate was kept constant throughout the run at 1.1 mL/min. The ion source was maintained at 220 ºC and the electron impact mode was 70 eV.

In order to calculate the amount of each amino acid in the infusions, a calibration curve (50, 100, 200, 350, 500, 700 nmol/mL) was plotted for each amino acid using the amino acid standard mixtures solution (200 nmol/mL). The area of each amino acid was measured relative to the area of internal standard, norvaline (m/z 158 ion).

Determination of total phenolic content

The total phenolic content was determined spectrophotometrically by the modified Folin-Ciocalteu method [22]. Absorbance was measured at 760 nm using a spectrophotometer (JASCO V-630). The quantification was made considering a standard curve of gallic acid and the results were expressed as mg of gallic acid equivalent per gram of *Stevia* (dry matter).

Determination of total flavonoid content

Total flavonoid content was determined using the modified colorimetric method described by Dewanto et al. [23]. Absorbance was measured at 510 nm. The quantification was made considering a standard curve of of (+)-catechin and the results were expressed as mg of (+)-catechin equivalent per gram of *Stevia* (dry matter).

Determination of total antioxidant activity

The antioxidant activity (AA) of the extract was analyzed on the basis of the scavenging activities of the stable 2,2-diphenyl-1-picrylhydrazyl free radical as described by Shahidi et al. [24] with some modifications. Absorbance of the sample was measured at 515 nm using methanol as a blank. The quantification was made considering a standard curve of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the results were expressed as mg of Trolox equivalent per gram of *Stevia* (dry matter).

Statistical analysis

A multifactor ANOVA (with LSD test and $\alpha = 0.05$), using the Statgraphics Centurion program, was applied to study the effect of temperature and time on the amino acids, total phenols, flavonoids and antioxidant activity. The interaction between both factors was also considered. A Principal Component Analysis (PCA), with the software Unscrambler X.10., was also applied to describe the relationships between the treatments and the variables analysed.

Results and Discussion

Free amino acids in Stevia infusions: Influence of time and temperature conditions.

The average values and the standard deviations of the eleven free amino acids (Ala, Asn, Asp, Glu, Ile, Leu, Phe, Pro, Ser, Tyr and Val) quantified in dried *Stevia* leaves are shown in Table 1. The data corresponding to these compounds in the infusions (obtained at different temperatures: 50, 70, 90ºC and times: 1, 5, 20, 40 minutes) are available in Online Resource 1. In addition, Table 1 shows the multifactor ANOVA results (homogenous groups, F-ratios for time and temperature factors and the interactions of both factors) for each of the analyzed compounds.

Of the eleven amino acids found in this study, four of them (Ile, Leu, Phe and Val) are recognized by the FAO as indispensable. The total amino acid concentration, considering the sum of all the compounds, was higher in the dried leaves (11.70 mg/g) than in infusions; in which the maximum values for the majority of the compounds analyzed was achieved with the treatment 90 °C, 5 minutes. Compounds such as Glu, Phe, Ser and Tyr showed greater concentration in the dried leaves (0.95, 0.30, 8.10 and 0.12 mg/g, respectively) than in infusions, as the average values obtained for these compounds considering all treatments were: 0.38, 0.06, 4.43 and 0.05 mg/g, respectively.

The amount of Ala, Asp, Leu and Pro in dried leaves (0.34, 0.38, 0.08 and 0.55 mg/g, respectively) was lower than in infusions (average values considering all treatments: 0.56, 0.43, 0.11 and 0.97 mg/g, respectively). However, the values obtained for Leu in the less aggressive treatments (50°C during 1 and 5 minutes) were almost identical to those for the dried leaves. Other compounds such as Asn, Ile and Val showed practically no differences between the concentration obtained for the dried leaves (0.36, 0.26 and 0.29, respectively) and for the infusions (average values: 0.37, 0.22 and 0.26, respectively).

Considering the abundance of the different compounds, Serine on its own accounts for 68.8% of the total amino acids quantified in dry leaves and 56.4 % of the total amino acids quantified in infusions (average value of those obtained in all the treatments). In addition to Serine, other amino acids were also quite abundant in dried leaves: Asp (3.2%), Glu (8.1%) and Pro (4.7%), which represented 16.1% of the total. Likewise, with respect to the infusions, after Serine the next three most abundant compounds were Ala (7.1%), Asp (5.5%) and Pro (12.4%), accounting for 25.1% of total.

With respect to the influence of temperature and time on the evolution of the different compounds, the ANOVA showed that both factors had practically no influence on the concentration of the compounds. Of the 11 compounds quantified, only three of them presented significant differences for temperature (Val, Leu and Ile) and four of them for time (Val, Leu, Ser and Asn). However, it is important to note that although the statistical analysis revealed significant differences, the observation of the values shows that actually these were very small.

There is little information relating to free amino acids in *Stevia*. Rafiq et al. [17] only identified eight of them in *Stevia* leaves (Ala, Ile, Ser, Pro, Asp, Glu, Tyr and Lys). All of these amino acids were found in this study, with the exception of Lys. However, Val, Leu, Phe, and Asn, found in this study, were not found by the above authors. Li et al. [25] and Abou-Arab et al. [18] identified 15 amino acids (Glu, Asp, Lys, Ser, Ile, Ala, Pro, Tyr, Arg, His, Phe, Leu, Val, Thr and Gly) in *Stevia* leaves previously subjected to protein hydrolysis. Besides these, the latter authors found Met and Cys, as well. All the amino acids present in *Stevia* in this study (with the exception of Val), are also found in green and black tea [15] with the total quantity ranging between 1.19 and 6.98 mg/g, depending on the tea variety.

These data were somewhat lower than those found in this work for *Stevia* infusions (6.84 and 9.11 mg/g). However, for other varieties of green tea, Ding et al. [26] reported higher values, between 24.70 and 33.50 mg/g.

Antioxidant activity, total phenols and flavonoids in Stevia infusions: Influence of time and temperature conditions.

Table 2 shows the average values and the standard deviations of total phenols, flavonoids and antioxidant activity found in dried *Stevia* leaves, as well as the multifactor ANOVA results (homogenous groups, F-ratios for the factors time and temperature, and the interactions of both factors). Higher concentrations of the three parameters were found in the infusions than in the dried leaves.

The time/temperature interaction (95% LSD interval), obtained from the ANOVA, is included in Fig. 1 in order to facilitate the comparison of variability patterns between factors. Fig. 1 shows that the evolution of these parameters depends on the temperature and the time conditions applied to obtain the infusions. For the three parameters analyzed, the higher the temperature the greater the aqueous extraction. Although both factors had a significant influence, temperature had greater impact than time, which is reflected by the F-ratio values (F ratio of temperature ranged from 323.74 to 490.57 and F ratio of time ranged from 2.52 to 21.97) on the three parameters analyzed.

Considering treatment time, it can be stated that, practically, it does not influence the result of the total phenol content and flavonoids. However, time had a greater influence in the case of total antioxidant activity when compared to the other two parameters. For antioxidant activity, there was a significant increase in concentration from minute 5 (52 mg trolox/g) to minute 40 (82 mg trolox/g) at 50 ºC. For the other two temperatures (70 and 90ºC), the behavior with time was similar, with a maximum value at 20 min, and without significant differences with respect to 40 min. The difference with time for total antioxidant activity with respect to phenols and flavonoids could be due to the presence of other compounds with antioxidant capacity, which can contribute to total antioxidant activity [31].

Regarding the total phenol content, Tadhani et al. [27] reported lower average values (25.18 mg gallic acid/g) in dried *Stevia* leaf extracts (obtained with HCl 0.3N and methanol) than those obtained in this

paper (63.80 mg gallic acid/g). Shukla et al. [5, 27] reported a total phenol content of 56.74 and 61.50 mg of gallic acid/g in ethanolic and aqueous *Stevia* extracts respectively (obtained by a maceration process at room temperature). These values are similar to those obtained in this paper at 50 °C (average values: 65.20 mg gallic acid/g *Stevia*) but below those obtained at 70ºC and 90ºC (average values considering all treatments: 71.02 and 92.07 mg gallic acid/g), respectively.

With respect to the total flavonoids (Table 2), a level of 22.20 mg catechin/g dried leaves of *Stevia* was found in this study. Muanda et al. [6] obtained similar values (20.68 mg catechin/g) in dried *Stevia* leaf aqueous extracts, as well. Other authors reported values for the total flavonoids in this matrix but expressed in terms of other compounds: 21.73 mg gallic acid/g [27], and 0.83 mg quercetin/g [29]. In addition, the extracts were obtained using different solvents: HCl 0.3N and methanol by the first authors and ethyl acetate extract and methanol by the second ones. Kim et al. [30] obtained values of 15.64 mg quercetin per gram of *Stevia* in infusions (3 hour at 100 °C).

In relation to antioxidant activity, the average value obtained in this study was 48.17 mg trolox/g of dried *Stevia* leaves (aqueous extraction). This value was higher than reported by Tadhani et al. [27] (38.24 mg trolox/g).

The evolution of antioxidant activity, with temperature and time, observed in this study was similar to that described by other authors for tea infusions. Specifically, Samaniego et al. [14] reported that at 90 ºC, the extraction of polyphenol was faster and more effective than at lower temperatures, as long as time does not exceed 5 minutes. These authors highlight that higher times at this temperature may cause the loss of polyphenol compounds and, consequently, of antioxidant capacity.

Global behavior of antioxidant properties and amino acid composition

Once the individual behavior of amino acids and antioxidant compounds were analyzed, a PCA was used to assess the overall effect of the conditions (time and temperature) used to obtain the *Stevia* infusions. Fig. 2 shows the PCA biplot (scores "treatments" and loading "variables") obtained. The first two components explained 65 % of the total variance (PC1, 39 % and PC2, 26 %). The proximity between infusion treatments implies similar behaviour, while the proximity between variables implies the degree of correlation between them. Taking this into consideration, the infusion "90ºC for 5

minutes" (90_5) placed at the far end of the right axis had the most amino acids (except Asp, Ser and Tyr) and antioxidants. On the contrary, the samples (50_1 and 50_5) situated on the opposite side (left axis), had the lowest level of the majority of the analyzed variables.

Conclusions

Infusions of Stevia leaves have higher levels of both antioxidants (total phenols, flavonoids and antioxidant activity) and certain amino acids such as Ala, Asp, Leu and Pro, in comparison with dry Stevia leaves. Temperature has a greater effect than time in the case of the three antioxidant parameters, so the higher the temperature, the greater the aqueous extraction. Minimum values for these parameters were obtained at 50 °C and maximum at 90 °C. At this last temperature there were no important improvements in the extraction of these compounds after 5 min. With respect to the majority of amino acids, no important differences were observed in their concentrations as a consequence of varying the temperature and time conditions. However, the infusion treatment 90ºC for 5 min can be considered the most appropriate since it promoted a small increment in the concentration of 8 of the 11 compounds. Therefore, with respect to the compounds analyzed in this study, the best conditions for obtaining Stevia leaf infusions are the most similar to the domestic culinary process, very hot water (slightly below boiling) for a short time. Given that infusions have been shown to be efficacious in the extraction of antioxidants, it would be interesting to investigate the profile of specific antioxidants further.

Acknowledgments

The authors thank the Research and Development Support Program, "Ayuda a Estancias de personal docente e investigador de la UPV en Centros de Investigación de Prestigio (PAID-00-12)" of the Universidad Politécnica de Valencia (Spain).

References

1. Goyal SK, Samsher R, Goyal RK (2010) Stevia (*Stevia rebaudiana*) a bio-sweetener: A review. Int J Food Sci Nutr 61: 1–10.

2. Hanson JR, De Oliveira BH (1993) Stevioside and related sweet diterpenoid glycosides. Nat Prod Res 10: 301-309.

41 3. Anton S, Martin C, Han H, Coulon S, Cefalu W, Geiselman P et al. (2010) Effects of *Stevia*,
42 aspartame, and sucrose on food intake, satiety and postprandial glucose and insulin levels. Appetite
43 55: 37–43.
44 4. Chatsudthipong V, Muanprasat C (2009) Stevioside and related compounds: Therapeutic benefits
45 beyond sweetness. Pharmacol Therapeut 121:41–54.
46 5. Shukla S, Mehta A, Bajpai V (2009) In vitro antioxidant activity and total phenolic content of
47 ethanolic leaf extract of *Stevia rebaudiana* Bert. Food Chem Toxicol 47: 2338-2343.
48 6. Muanda F, Soulimani R, Diop B, Dicko A (2011) Study on chemical composition and biological
49 activities of essential oil and extracts from *Stevia rebaudiana* Bertoni leaves. LWT-Food Sci
50 Technol 44:1865-1872.
51 7. Yao L, Jiang Y, Datta N, Singanusong R, Liu X, Duan J, Raymont K, Lisle A, Xu Y(2004) HPLC
52 analyses of flavanols and phenolic acids in the fresh young shoots of tea (Camellia sinenesis) grown
53 in Australia. Food Chem 84: 253-263.
54 8. Dugo P, Cacciola F, Donato P, Assis R, Bastos E, Mondello L (2009) High efficiency liquid
55 chromatography techniques coupled to mass spectrometry for the characterization of mate extracts. J
56 Chromatogr A 43:7213-7221.
57 9. Biswas AK, Chatli MK, Sahoo J (2012) Antioxidant potential of curry (Murraya koenigii L.) and
58 mint (Mentha spicata) leaf extracts and their effect on colour and oxidative stability of raw ground
59 pork meat during refrigeration storage. Food Chem 133:467-472.
60 10. Atoui K, Mansouri A, Boskou G, Kefalas P (2005) Tea and herbal infusions: Their antioxidant
61 activity and phenolic profile. Food Chem 89, 27-36.
62 11. Gorjanovic S, Komes D, Pastor F, Belscak A, Pezo L, Hecimovic I, Suznjevic D (2012)
63 Antioxidant Capacity of Teas and Herbal Infusions: Polarographic Assessment. J Agric Food Chem
64 60: 9573-9580.
65 12. Quispe C, Viveros-Valdez E, Schmeda-Hirschmann G (2012) Phenolic Constituents of the Chilean
66 Herbal Tea *Fabiana imbricata* R. et P. Plant Food Hum Nutr 67:242–246.

13. Büyükbalci A, Nehir El S (2008) Determination of In Vitro Antidiabetic Effects, Antioxidant Activities and Phenol Contents of Some Herbal Teas. Plant Food Hum Nutr 63:27–33.

14. Samaniego C, Inurreta Y, Quesada JJ, Blanca R, Villalón M, López García H, López Martínez MC (2011) The influence of domestic culinary processes on the Trolox Equivalent Antioxidant Capacity of green tea infusions. J Food Compos Anal 24:79-86.

15. Wang L, Xu R, Hu B, Li W, Sun Y, Tu Y, Zeng X (2010) Analysis of free amino acids in Chinese teas and flower of tea plant by high performance liquid chromatography combined with solid-phase extraction. Food Chem 123: 1259–1266.

16. Gökmen V, Serpen A, Mogol BA (2012) Rapid determination of amino acids in foods by hydrophilic interaction liquid chromatography coupled to high-resolution mass spectrometry. Anal Bioanal Chem 403: 2915–2922.

17. Rafiq M, Dahot U, Mangrio SM, Naqvi HA, Qarshi IA (2007) In vitro clonal propagation and biochemical analysis of field established Steva rebaudiana Bertoni. Pak J Bot 39: 2467-2474. ISSN:2070-3368.

18. Abou-Arab AE, Abou-Arab AA, Abu-Salem MF (2010) Physico-chemical assessment of natural sweeteners steviosides produced from *Stevia rebaudiana* Bertoni plant. AJFS 4: 269–281. ISSN:1996-0794.

19. WHO (2007). Report of a Joint WHO/FAO/UNU Expert Consultation on Protein and Amino Acid Requirements in Human Nutrition (2002, Geneva, Switzerland). WHO Technical Report Series; No. 935.

20. European Food Safety Authority (EFSA) (2011) Revised exposure assessment for steviol glycosides for the proposed uses as a food additive. EFSA Journal 2011; 9 (1):1972. doi:10.2903/j.efsa.2011.1972

21. Husek P (2000) Method of preparing sample for amino acid analysis and kit for analyzing the same. Eur Patent Appl EP 1033576.

22. Sakanaka S; Tachibana Y; Okada Y (2004) Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). Food Chem 89: 569-575.

94 23. Dewanto V, Wu X, Adom KK, Liu RH (2002) Thermal processing enhances the nutritional value
95 of tomatoes by increasing total antioxidant activity. J Agri Food Chem 50:3010-3014.
96 24. Shahidi F, Liyana-Pathirana CM, Wall DS (2006) Antioxidant activity of white and black sesame
97 seeds and their hull fractions. Food Chem 99: 478-483.
98 25. Li G, Wang R, Quampah A, Rong Z, Shi C, Wu J (2011) Calibration and Prediction of Amino
99 Acids in *Stevia* Leaf Powder Using Near Infrared Reflectance Spectroscopy. J Agri Food Chem 59:
00 13065–13071.
01 26. Ding Y, Yu H, Mou S (2002) Direct determination of free amino acids and sugars in green tea by
02 anio-exchange chromatography with integrated pulsed amperometric detection. J Chromatogr A
03 928:237-244.
04 27. Tadhani M, Patel V, Subhash R (2007) In vitro antioxidant activities of *Stevia rebaudiana* leaves
05 and callus. J Food Compos Anal 20: 323–329.
06 28. Shukla S, Mehta A, Menta P, Bajpai V (2012) Antioxidant ability and phenolic content of aqueous
07 leaf extract of *Stevia rebaudiana* Bert. Exp Toxicol Pathol 64:807-811.
08 29. Ghanta S, Banerjee A, Poddar A, Chattopadhyay S (2007) Oxidative DNA damage preventive
09 activity and antioxidant potential of *Stevia rebaudiana* (Bertoni) Bertoni, a natural sweetener. J of
10 Agri Food Chem 55:10962–10967.
11 30. Kim I, Yang M, Lee O, Kang S (2011) The antioxidant activity and the bioactive compound content
12 of *Stevia rebaudiana* water extracts. LWT Food Sci Technol 44: 1328–1332.
13 31. Michiels JA, Kevers Cl, Pincemail J, Defraigne JO, Dommes J (2012) Extraction condicitons can
14 greatly influence antioxidant capacity assays in plant food matrices. Food Chem 130:986-993.

15 **Figure Caption**

16 **Fig. 1** Time-temperature interaction (95% LSD interval) of the antioxidant parameters: total phenols
17 (a), total flavonoids (b) and antioxidant activity (c), obtained from the ANOVA

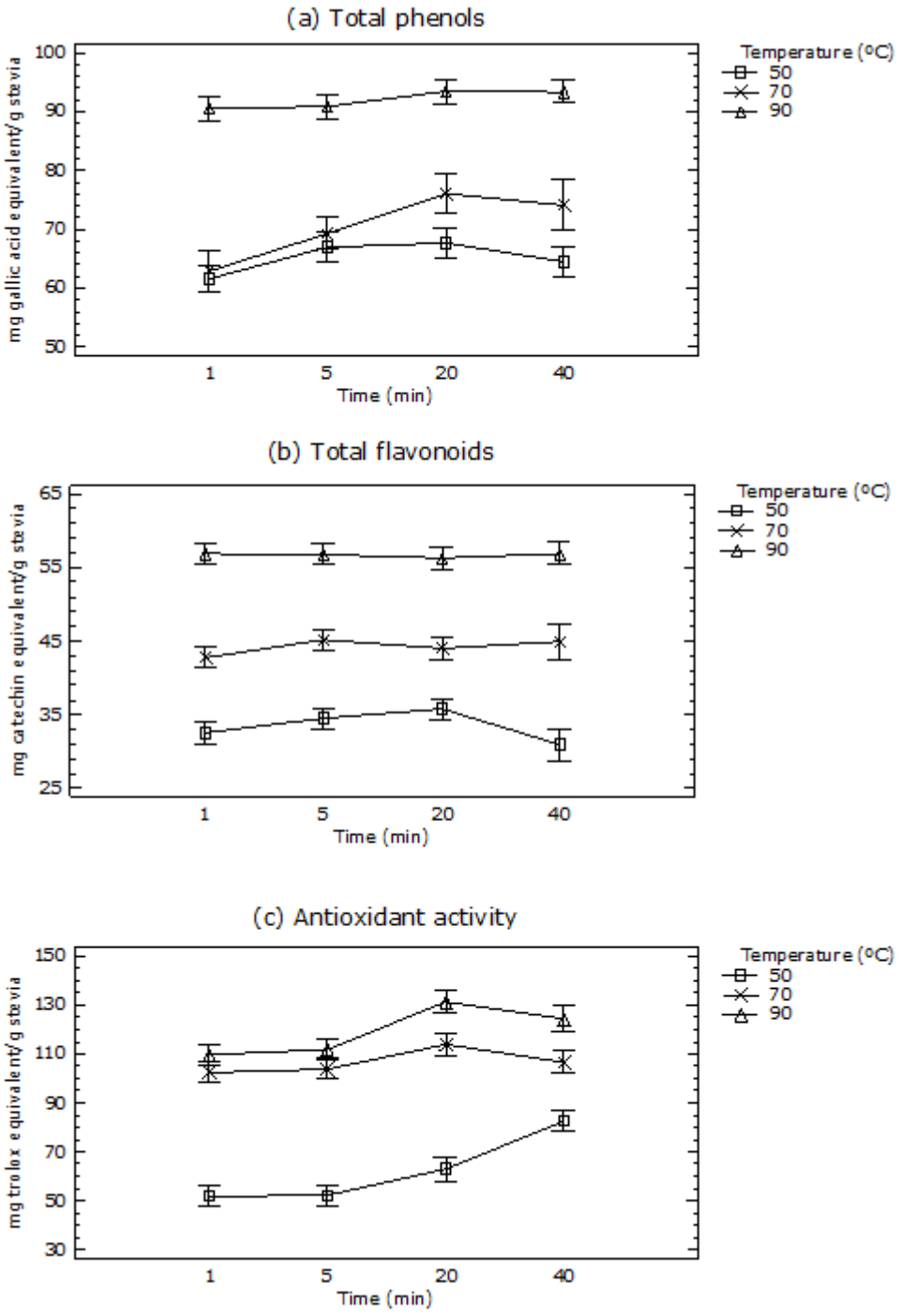

Fig. 2 Bi-plot of Principal Component Analysis for the infusion treatments (white diamond ◊) and the analysed variables: amino acids and antioxidant parameters (total phenols, total flavonoids and antioxidant activity) (black diamond ♦).

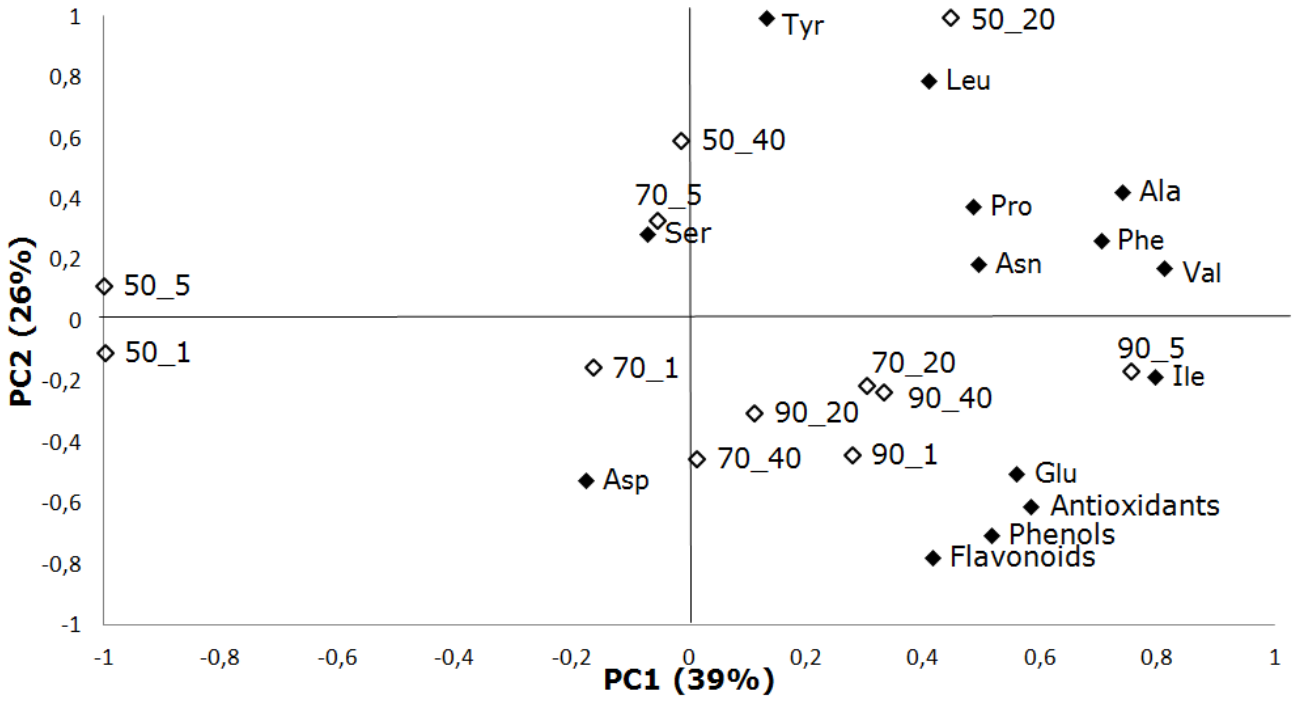

Table 1. Amino acids (mg /g stevia) in dried leaves (mean and standard deviation) and multifactor ANOVA results for these amino acids in stevia infusions obtained at different temperatures and treatment times. F-ratios for each of the two factors (temperature and time) and their interactions are included.

Amino acids	Dried Leaves	ANOVA INFUSIONS									
		Temperature (T)				Time (t)					Interaction
	mean(SD)	50	70	90	F-ratio	1	5	20	40	F-ratio	T*t
Ala	0.34(0.02)	0.54^{a}	0.55^{a}	0.57^{a}	1.21^{ns}	0.53^{a}	0.56^{ab}	0.58^{b}	0.54^{ab}	1.81^{ns}	3.49^{*}
Asn	0.36(0.14)	0.35^{a}	0.35^{ab}	0.38^{b}	3.05^{ns}	0.33^{a}	0.39^{b}	0.38^{b}	0.33^{a}	5.7^{**}	2.19^{ns}
Asp	0.38(0.04)	0.42^{a}	0.44^{a}	0.42^{a}	0.49^{ns}	0.44^{a}	0.44^{a}	0.41^{a}	0.42^{a}	0.51^{ns}	1.52^{ns}
Glu	0.95(0.12)	0.34^{a}	0.38^{a}	0.42^{a}	1.59^{ns}	0.40^{a}	0.34^{a}	0.38^{a}	0.39^{a}	0.41^{ns}	0.73^{ns}
Ile	0.26(0.03)	0.20^{a}	0.21^{ab}	0.22^{b}	3.72^{*}	0.20^{a}	0.20^{a}	0.22^{a}	0.21^{a}	0.89^{ns}	1.75^{ns}
Leu	0.082(0.006)	0.11^{b}	0.11^{b}	0.10^{a}	17.51^{**}	0.09^{a}	0.10^{b}	0.12^{c}	0.12^{c}	27.92^{**}	28.47^{**}
Phe	0.30(0.06)	0.06^{a}	0.06^{a}	0.07^{a}	0.56^{ns}	0.06^{a}	0.06^{a}	0.06^{a}	0.07^{a}	0.89^{ns}	0.9^{ns}
Pro	0.55(0.04)	0.96^{a}	0.95^{a}	1.00^{a}	0.26^{ns}	0.92^{a}	1.00^{a}	1.03^{a}	0.91^{a}	0.98^{ns}	0.52^{ns}
Ser	8.1(0.7)	4.48^{a}	4.44^{a}	4.36^{a}	0.06^{ns}	4.17^{a}	5.08^{b}	4.55^{ab}	3.92^{a}	3.3^{*}	0.93^{ns}
Tyr	0.12(0.08)	0.05^{a}	0.05^{a}	0.06^{a}	2.3^{ns}	0.05^{a}	0.05^{a}	0.05^{a}	0.05^{a}	0.7^{ns}	0.9^{ns}
Val	0.29(0.02)	0.25^{a}	0.26^{b}	0.26^{b}	5.57^{*}	0.25^{a}	0.25^{ab}	0.27^{b}	0.27^{b}	4.47^{*}	5.69^{**}
Total	11.7	7.79	7.85	7.80	-	7.49	8.53	7.97	7.28	-	-

For each factor. different letters in each row indicate homogeneous groups (significant differences at 95% confidence level as obtained by the LSD test). ns=Not significant; $^{*}p<0.05$; $^{**}p<0.01$

Table 1. Total phenols (mg gallic acid equivalent/g stevia), flavonoids (mg catechin equivalent/g stevia) and antioxidant activity (mg Trolox equivalent/g stevia) in dried leaves (mean and standard deviation) and multifactor ANOVA results for these compounds in stevia infusions obtained at different temperatures and treatment times. F-ratios for each of the two factors (temperature and time) and their interactions are included.

Antioxidant parameters	Dried Leaves mean (SD)	ANOVA INFUSIONS								
		Temperature (T)				Time (t)				Interaction
		50	70	90	F-ratio	1	5	20	40	F-ratio	T*t
Phenols	63.8(1.3)	65.20^{a}	71.02^{b}	92.07^{c}	323.74^{***}	71.70^{a}	75.71^{b}	77.34^{c}	79.63^{c}	9.38^{**}	2.16^{ns}
Flavonoids	22.2(0.9)	32.47^{a}	47.53^{b}	56.72^{c}	490.57^{***}	44.64^{a}	45.93^{b}	45.77^{ab}	44.26^{a}	2.52^{*}	1.74^{ns}
Antioxidants	48(2)	62.45^{a}	106.68^{b}	119.12^{c}	344.71^{***}	87.96^{a}	89.26^{a}	102.64^{b}	104.48^{b}	21.97^{**}	5.67^{**}

For each factor, different letters in each row indicate homogeneous groups (significant differences at 95% confidence level as obtained by the LSD test). ns=Not significant; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$