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Influence of Extraction Methods on the Yield of Steviol Glycosides and Antioxidants in *Stevia rebaudiana* Extracts

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Abstract

This study evaluated the application of ultrasound techniques and microwave energy, compared to conventional extraction methods (high temperatures at atmospheric pressure), for the solid-liquid extraction of steviol glycosides (sweeteners) and antioxidants (total phenols, flavonoids and antioxidant capacity) from dehydrated *Stevia* leaves. Different temperatures (from 50 to 100 ºC), times (from 1 to 40 min) and microwave powers (1.98 and 3.30 W/g extract) were used. There was a great difference in the resulting yields according to the treatments applied. Steviol glycosides and antioxidants were negatively correlated; therefore there is no single treatment suitable for obtaining the highest yield in both groups of compounds simultaneously. The greatest yield of steviol glycosides was obtained with microwave energy (3.30 W/g extract, 2 min), whereas, the conventional method (90 ºC, 1 min) was the most suitable for antioxidant extraction. Consequently, the best process depends on the subsequent use (sweetener or antioxidant) of the aqueous extract of *Stevia* leaves.

Keywords: microwave energy, ultrasound technique, antioxidants, phenols, flavonoids, steviol glycosides.

Introduction

Stevia rebaudiana Bertoni is a perennial herb of the family Asteraceae, from Brazil and Paraguay. The main characteristic of *Stevia* leaves is its high sweetness (250-300 times greater than sucrose) which is due to the presence of diterpenes, specifically steviol glycosides [1]. The Joint Expert Committee on Food Additives (JECFA) has established regulations for the extraction and purification conditions of steviol glycosides (steviolmonoside, rubusoside, steviolbioside, dulcoside A, stevioside, rebaudioside B, rebaudioside A, rebaudioside C, rebaudioside F) and their maximum daily intake. Steviol glycoside extracts must have a purity of at least 95%. The acceptable daily intake (ADI) is 4 mg per kg bodyweight and day [2]. Stevioside was reported to be the most abundant steviol glycoside (4-13%) found

in plant leaves, followed by rebaudioside A (2-4%), rebaudioside C (1-2%) and dulcoside A (0.4-0.7%). Steviolbioside, rebaudioside B, D, E, F and 19 other compounds were also identified in leaf extracts, but only as minor constituents [3].

The most common use of *Stevia* leaves is the extraction and purification of steviol glycosides to obtain a non-caloric natural sweetener as a sugar substitute or as an alternative to artificial sweeteners [4]. The sweetening properties of those glycosides, however, differ from one to another. Whereas stevioside exhibits a significant bitter aftertaste, Rebaudioside A has a sweet taste, which has been attributed to the presence of an extra "glucose moiety" in the Rebaudioside A structure [5].

Apart from the sweetening power of *Stevia*, its leaves have important therapeutic properties which are responsible for the increasing interest in the consumption of this aqueous extract. *Stevia* leaves are rich in compounds with anti-inflammatory, diuretic antihypertensive, antihyperglycemic, antidiarrehic, antitumor and antioxidant properties [6]. Flavonoids and phenolic compounds present in *Stevia* leaves are responsible for the high antioxidant capacity [7, 8]. Therefore the direct intake of dried *Stevia* leaf infusions or their addition in different food formulations such as juices, biscuits, jams, confectionery products, etc. could enhance the functional properties of these products. The EFSA (European Food Safety Authority) recognized the safety of purified steviol glycosides for use in food and beverages as a food additive/sweetener in November 2011 [9], although its use was authorized in different Asian and American countries decades ago. Japan was the first country to commercialize steviol glycosides as a sweetener in food and drugs in 1968 [10].

The active principles of fresh or dehydrated leaves are traditionally extracted by means of an aqueous extraction at high temperature and atmospheric pressure (conventional method) [11]. However, some authors have shown that other techniques such as ultrasound or microwave energy can maximize or improve the extraction of active compounds [12]. The application of ultrasound could be a good choice because this technique induces greater penetration by the solvent into the cellular matrix, an alteration of the structure and therefore an improvement in the mass transfer [13]. In fact, the ultrasound technique has been used successfully to extract steviol glycosides from *Stevia* leaves [14, 15]. In addition, it has been used to extract antioxidant compounds from other plants: polyphenols and antioxidant capacity from olive leaves [16], polyphenols from grape seeds [17] and flavonoids from *Citrus aurantium* [18]. Another possible extraction technique is microwave energy. The friction resulting from molecular movement contributes to the rapid heating of the vegetable matrix, with the advantage of a great reduction in the time required for extraction compared to the

conventional method. This technique, which has been developed rapidly in the last decade, has been widely used in the extraction of organic compounds. Wang et al. [19] successfully applied microwave energy to the extraction of phenolic compounds from Chinese herbs. Jaitak et al. [14] and Teo et al. [20] also applied microwave energy to the extraction of steviol glycosides.

This study aimed to evaluate the effect of the application of ultrasound and microwave energy compared to the conventional method in the solid-liquid extraction of antioxidants (total phenolic content, flavonoids and antioxidant capacity) and steviol glycosides from dehydrated *Stevia* leaves. The influence of time and temperature was also studied. The chromatographic procedure used to identify and quantify the steviol glycosides compounds was validated in order to ensure the suitability of the method.

Material and Methods

Stevia samples and extraction procedure

Organically produced dried leaves of *Stevia rebaudiana* Bertoni (Raab, Vitalfood, Rohrbach, Germany) were used in this study. One gram of dried *Stevia* leaf powder (ground in a grinding mill, A11 Basic, IKA, Germany) was dispersed in 100 mL of water. Aqueous extracts of dried *Stevia* leaves were obtained at atmospheric pressure (conventional method) using a thermostatic bath (JP Selecta Precisdig, Spain) heated to different temperatures (50, 70, 90 and 100˚C) for different times (1, 5, 20 and 40 minutes); ultrasonic energy (US) in a thermostat bath (Ultrasounds-H, JPSelecta, Spain) at different temperatures (50, 70 and 90°C) and for different times (1, 5 and 20 minutes) and applying microwave energy (MW) (Samsung, GW72N) at a relative power of 1.98 W/g extract for 1, 2, 3 and 5 minutes and 3.30 W/g extract for 1 to 2 minutes. When this last power was applied, it was not possible to last longer than 2 minutes because it caused the boiling and overflow of the sample. Subsequently, the aqueous extracts were filtered through filter paper and cooled before the analytical determinations were made. All the analyses were performed in triplicate.

Standard compounds and reagents

HPLC-grade acetonitrile and methanol were purchased from VWR (Fontenay-sous-Bois, France) and analytical grade ethanol and ammonium acetate were purchased from Scharlab (Barcelona, Spain). The standards Rebaudioside A, Rebaudioside C, Dulcoside A, Stevioside and Steviolbioside (purity > 98%) were obtained from Chromadex (CA, USA). De-ionized water from MilliQ (Millipore Corp., Bedford, MA) was used throughout the procedure. Solid-phase extraction

(SPE) was carried out on a vacuum manifold system (Lichrolut, Merck, Darmstadt, Germany) using StrataC18-E cartridges (500 mg, 3 mL, 55 µm, 70 Å) from Phenomenex (Torrance, CA) for the determination of steviol glycosides. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany) were used to determine the total antioxidant activity. Sodium nitrite, (+)-catechin, sodium hydroxide (Sigma-Aldrich, Germany) and aluminum chloride hexahydrate (Fluka, Germany) were used to analyse the flavonoids. Sodium carbonate, gallic acid and Folin-Ciocalteu reagent (all purchased from Sigma-Aldrich, Germany) were utilized for phenolic determination.

Steviol glycosides analysis

Steviol glycosides extraction was carried out as described by Woelwer et al. [21], but using different extraction volumes. The aqueous extract (0.5 mL) was diluted with water (2.5 mL) and this solution was subjected to solid-phase extraction (SPE). The resulting solution was loaded on a 3 mL Strata SPE cartridge pre-activated with methanol (3 mL) and water (3 mL). The SPE cartridge was then sequentially washed with 3 mL of water and 3 mL of acetonitrile/water (2:8 v/v); and then air dried for 2 minutes; the steviol-glycosides were eluted from the cartridge using 5 mL of 80% acetonitrile in water. The eluate was subjected to LC-MS-MS analysis.

The chromatographic analysis was performed on an Agilent 1200 Series HPLC system coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies Inc., CA, USA) with an ionization source electrospray type. A LC-MS-MS method was used in the present study for the analysis of the steviol glycosides. Chromatographic separation was carried out in gradient mode by Zorbax SB-C18 column (50mm x 2.1mm, 1.8 µm) maintained at 40ºC, with a mobile phase consisting of 10 mM aqueous ammonium acetate (A) and acetonitrile (B). Binary gradient conditions were used: initial, 7% B, held for 0.2 min: linear gradient to 20% B at 0.3 min and then to 48% B at 5 min; sudden increase to 100% B at 5.1 min and hold until 7 min; followed by a linear gradient to initial condition at 7.1 min and a final hold at this composition until 9 min. The flow-rate and the injection volume were 0.4 mL/min. and 5 µL, respectively. The electrospray was operated in negative ion mode. Choi et al. [22] stated that negative ion mode is 10 times more sensitive than positive ion mode. The conditions used in the ionization source were: temperature of the drying gas (N_2) 325ºC to 11L/min, nebulizer pressure of 50 psi and the capillary voltage of 4000 V. Identification and quantification of steviol glycosides in the samples and the standards was performed using the multiple reaction monitoring (MRM) mode.

106 The stock standard solution of steviol glycosides was prepared by weighing the appropriate amount of the pure standard
107 and diluting it with methanol to obtain a final concentration of 1 mg/mL. The working standard solution was obtained at a
108 concentration of 0.01 mg/mL in water. The stock standard solution was stored at -20ºC and the working standard
109 solution was at +4ºC. Quantification was performed by means of calibration curves obtained from standard solutions
110 (0.5-10 µg/mL). Samples were spiked to verify the absence of a matrix effect in the analysis. In order to ensure the
111 quality of the results and evaluate the stability of the proposed method, an internal quality control (a standard solution)
112 was injected in the equipment as a first step before each batch of the sample.

113 *Validation of the steviol glycosides analysis method*

114 The guidelines established by EU Commission Decision [23] were followed in order to validate the steviol glycosides
115 analytical methodology. For this purpose, several parameters were studied: linearity, accuracy and precision
116 (repeatability and reproducibility). The accuracy of the method was established through recovery studies and the
117 precision was verified by repeatability or intraday precision (RSD_r) and reproducibility or interday precision (RSD_R).
118 LODs (limit of detection) and LOQs (limit of quantification) were determined through the analysis of standard solutions.
119 These values were defined as the amount of analyte for which signal-to-noise ratios (S/N) were higher than 3 and 10
120 respectively.

121 *Determination of total phenolic content*

122 The total phenolic content was determined spectrophotometrically using the modified Folin-Ciocalteu method [24].
123 Absorbance was measured at 760 nm using a spectrophotometer (JASCO V-630). The quantification was made
124 considering a standard curve of gallic acid and the results were expressed as mg of gallic acid equivalent per gram of
125 *Stevia* (dry matter).

126 *Determination of total flavonoid content*

127 Total flavonoid content was determined using the modified colorimetric method described by Dewanto et al. [25].
128 Absorbance was measured at 510 nm. The quantification was made considering a standard curve of (+)-catechin and
129 the results were expressed as mg of (+)-catechin equivalent per gram of *Stevia* (dry matter).

130 *Determination of total antioxidant capacity*

131 The antioxidant activity (AA) of the extract was analyzed on the basis of the scavenging activities of the stable 2,2-
132 diphenyl-1-picrylhydrazyl free radical as described by Shahidi et al. [26], 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 analysis of variance (ANOVA) (with Statgraphics Centurion) was used to study the influence of method, temperature and time required during the extraction treatments on the steviol glycosides, antioxidant, phenols and flavonoids. Furthermore, a Principal Component Analysis (PCA) was also performed using the software Unscrambler X.10 to describe the relationships between the treatments and the variables analysed.

Results and Discussion

Validation of the steviol glycosides analytical methodology

The results from the steviol glycosides validation procedure are available in Online Resource 1. In order to obtain the linearity value an external standard calibration curve was made using standard solutions with final concentration levels of: 0.5, 1, 2, 5, 7 and 10 mg/L (ppm). Six replicates were made for each level. The calibration curves were obtained by plotting the peak area of the compound at each level versus the concentration. The linearity response observed from 0.5 to 10 mg/L was good because the correlation coefficient between peak areas and injected nominal concentrations was $R^2 \geq 0.995$.

The recovery studies were performed by adding known quantities of steviol glycosides to a sample (1, 5 and 10 mg/L). Six replicates of all the spiked sample levels were analyzed using the HPLC method. The method used permitted recovery of steviol glycosides between 70.5 and 105.6 % for the concentration range studied. The relative standard deviation (RSD) corresponding to recovery values was less than 20% in all cases (ranging from 4.0 to 18), confirming that the analytical method was accurate.

Repeatability (RSD_r) was evaluated by performing the assay on six replicates of fortified *Stevia* samples, at the same levels (1, 5 and 10 mg/kg), and was carried out by the same operator on the same day. In order to evaluate reproducibility (RSD_R) the experiment was performed by 2 different operators on 3 consecutive days. The results were expressed as the percentage of relative standard deviation.

Intra-day precision (RSDr) ranged from 1.7% to 14.6%, inter-day precision (RSD_R) from 5.2% to 16.5% (Online Resource 1). These RSD values are in complete agreement with EU Commission Decision [23] requirements since they

were always lower than 20% for all the concentration levels assayed. The LOD (limit of detection) ranged between 0.04 and 0.14 and the LOQ (limit of quantification) ranged between 0.15 and 0.49. Therefore, it can be concluded that the method used in this work has good precision.

The results of the validation prove that the analytical procedure carried out appropriately guarantees the quantitative values of steviol glycosides obtained in the samples analyzed.

Steviol glycosides in Stevia extracts: Influence of extraction treatment, time and temperature

Figure 1 is presented in order to facilitate the comparison of variability patterns between the different conditions applied to obtain the extracts. It shows the average values and the standard deviation of the 4 steviol glycosides (Dulcoside A, Rebaudioside A, Rebaudioside C and Stevioside) identified and quantified in the extracts obtained using different methods: conventional (CV), ultrasound (US), and microwave (MW), at different temperatures: 50, 70 and 90ºC and times: (1, 2, 3, 5, 20 and 40 minutes). Additionally, this figure shows the homogenous groups of the ANOVA carried out for a single factor "treatment" (method-temperature-time), which means a total of 27 treatments. The F-ratio values ranged between 11.58 and 26.86 in all cases. Unlike the results found by other authors [27], in this study steviolbioside was not found in any sample. This finding is not considered to be surprising because there is evidence that rebaudioside B and steviolbioside are not native constituents of *Stevia rebaudiana*, but rather can be formed by partial hydrolysis during the extraction process, and are consequently artifacts of the extraction procedure [28, 29].

Considering Figure 1, it is obvious that the conventional treatment (CV) had a lower yield in the extraction of the steviol glycosides than the other two treatments (US and MW). In the conventional treatment, maximum extraction occurred at 5 minutes, at longer extraction times the yield was lower at all temperatures studied.

With respect to the ultrasound treatment (US), the highest extraction of steviol glycosides was observed at the lowest temperature (50°C) and shortest time (1 min) (2 mg Dulcoside A/g, 14.12 mg Rebaudioside A/g, 6.25 mg Rebaudioside C/g, 39.06 mg Stevioside/g). These results are in agreement with the findings of Liu et al. [15] who reported that extraction assisted by ultrasound increased the yield 1.5 times in comparison with the classical extraction method as long as low temperature and short times were applied.

Microwave treatment (MW) led to the extraction of the greatest amount of steviol glycosides, similar to US at 50ºC, 1 min. The highest yield (2.03 mg Dulcoside A/g, 17.03 mg Rebaudioside A/g, 6.6 mg Rebaudioside C/g, 46.48 mg Stevioside/g) being reached when applying the highest power (3.3 W/g) for 2 minutes of extraction. Teo et al. [20]

obtained values of Stevioside (14.07-21.37 mg/g) lower than in this study using microwave extraction and confirmed the improved efficacy of this method as compared to extraction with hot pressurized water. Likewise, Jaitak et al. [14] obtained an increase in the yield of Rebaudioside A and Stevioside by means of microwave extraction, in comparison with conventional cold extraction and ultrasound.

Total phenols, flavonoids and antioxidant capacity in Stevia extracts: Influence of extraction treatment, time and temperature

Figure 2 shows the average values and the standard deviation of total phenols (mg gallic acid equivalent/g *Stevia*), flavonoids (mg of catequin equivalent/g *Stevia*) and total antioxidants (mg Trolox equivalent/g *Stevia*) quantified in the extracts obtained using different methods (conventional, ultrasound and microwave), temperatures (50, 70, 90 and 100ºC) and times (1, 2, 3, 5, 20 and 40 minutes). Furthermore, homogenous groups obtained in the ANOVA are also represented by letters in the figure, showing statistical differences between components in the treatments studied (α=99%) with the following F-ratios: 18.77 (phenols), 52.15 (flavonoids) and 24.72 (antioxidants).

The conventional extraction method achieved the highest efficiency in all cases in comparison with the results obtained with extraction by means of ultrasound technique and microwave energy. However, previous studies reported that ultrasound [16] and microwave [20] improved phenol extraction in olive leaves and Chinese herbs, respectively. Zhang et al. [30] also obtained successful results using ultrasonic and microwave techniques for the extraction of flavonoids in medicinal plants.

In this work efficiency was especially important when conventional extraction was carried out at 90 °C for phenols (93.41 mg gallic acid/g) and total antioxidants (131 mg trolox/g *Stevia*). An increase in temperature beyond 90°C did not improve extraction; on the contrary it had a negative effect on the phenolic compounds and total antioxidants, probably due to their degradation at boiling point. Liazid et al. [31] observed that some phenolic compounds were no longer stable at 100ºC and Inglett et al. [32] reported the instability of antioxidant compounds at these temperatures. However, in the case of flavonoids the highest yield in this work was achieved when conventional extraction was carried out at 100 ºC (52.92-69.18 mg catechin/g) without significant differences at times greater than 5 min. For phenol compounds the greatest yield was also found at 100 ºC, however only at longer extraction times (20 and 40 min) and also at 90 ºC for all the extraction times studied. On the other hand, for total antioxidants the greatest effectivity was observed at intermediate temperatures, 70 and especially 90ºC.

When the extractions were carried out with ultrasound technique, the best results were obtained with long treatment times without significant differences between the different temperatures studied. At a temperature of 50ºC, the greatest yield occurred after 20 min of treatment: total phenols (80 mg gallic acid equivalent/g *Stevia*), flavonoids (43 mg of catequin equivalent/g *Stevia*) and total antioxidants (81 mg Trolox equivalent/g *Stevia*). However, at 70 and 90ºC, the maximum yield was obtained after 5 min without significant differences to the result obtained after 20 min.

In the case of microwave energy extraction a power of 1.98 W/g led to a slight increase in all the compounds analyzed when the time of extraction reached 3 min. Therefore, the highest yield could be obtained using a microwave power of 1.98 W/g extract, for 3 minutes: total phenols (81 mg gallic acid equivalent/g *Stevia*), flavonoids (45mg of catequin equivalent/g *Stevia*) and total antioxidants (96 mg Trolox equivalent/g *Stevia*).

Comparing the results obtained by other authors in relation to the influence of microwave and ultrasound on the antioxidant capacity of the extracts; Inglett et al. [32] report that they obtained lower values of antioxidant activity by extraction with microwave energy, as this technique could degrade some antioxidant compounds. Ya-Quin et al. [33] determined that temperatures over 40ºC in extraction assisted by ultrasound did not improve the extraction of some phenols in citrus peel due to the induced instability of phenolic compounds at high temperatures. However, Ahmad-Qasem et al. [16] registered higher antioxidant levels in olive leaves using ultrasound methods.

Global behavior of antioxidant properties and steviol glycosides

Once the individual behaviour of steviol glycosides and antioxidant compounds were analyzed, a PCA was used to assess the overall effect of the conditions (method, time and temperature) used to obtain the *Stevia* extracts. Figure 3 shows the PCA biplot (scores “treatments” and loading “variables”) obtained. The first two components explained 93 % of the total variance (PC1, 65 % and PC2, 28 %). The proximity of the treatments: ultrasound for 1 min. (US_50_1) and microwave for 2 min (MW_b_2) (placed at the far end of the left axis in the figure) denotes that both treatments promote a greater extraction of these compounds than the other treatments.

On the contrary, the samples at 90ºC situated on the opposite side on the top (right axis), had the highest level of the antioxidant properties analyzed. Moreover, the steviol glycosides were negatively correlated with the antioxidants properties. Therefore the treatments with low values of antioxidants showed high values of steviol glycosides.

Conclusions

The extraction method used to obtain aqueous extracts of *Stevia* dehydrated leaves (conventional at atmospheric pressure, ultrasound and microwave), has a great effect on the yield of steviol glycosides and antioxidants. Due to the fact that both groups of compounds are negatively correlated there is no single treatment suitable for obtaining the best yield in both groups of compounds simultaneously. High microwave power (3.30 W/g extract) and long microwave treatment time (2 min) was found to be the most recommendable for obtaining the maximum amount of steviol glycosides, followed by ultrasound at a low temperature (50ºC) and short treatment time (1 min). On the contrary, ultrasound and microwave energy degraded the antioxidant compounds of aqueous extracts of *Stevia*, for this reason, the conventional treatment was the most suitable for obtaining the greatest amount of phenols, flavonoids and total antioxidants. In this case, 90ºC and short treatment times (1 min) maximized the yield of these compounds. Therefore, the optimum solid-liquid extraction conditions would depend on whether the aqueous extraction of *Stevia* leaves is used for sweetening or for antioxidant purposes.

It can be assumed that the results of this study, using *Stevia rebaudiana* Bert. can be extrapolated to other varieties and genotypes of *Stevia*, however, this should be confirmed in further studies.

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Conflict of Interest The authors declare that they have no conflict of interest.

References

1.Lemus R, Vega A, Zura L, Ah K (2012) *Stevia* rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. Food Chem*,* 132:1121-1132.

2. JECFA. Joint FAO/WHO Expert Committee on Food Additives (2008) Steviol glycosides. In Compendium of Food Additive Specifications, 69th Meeting, FAO/WHO Monographs 5, Rome, Italy.

3. Wölwer-Rieck U. (2012) The Leaves of Stevia rebaudiana (Bertoni), Their Constituents and the Analyses Thereof: A Review. J Agric Food Chem, 60:886-895.

4. Anton S, Martin C, Han H, Coulon S, Cefalu W, Geiselman P et al. (2010) Effects of *Stevia*, aspartame, and sucrose on food intake, satiety and postprandial glucose and insulin levels. Appetite 55: 37–43.

5. De Oliveira BH, Packer JF, Chimelli M, De Jesus DA (2007) Enzymatic modification of stevioside by cell-free extract of Gibberella fujikuroi. J Biotechnol, 131: 92-96.

267 6. Chatsudthipong V, Muanprasat C (2009) Stevioside and related compounds: Therapeutic benefits beyond sweetness.
268 Pharmacol Therapeut 121:41-54.
269 7. Muanda F, Soulimani R, Diop B, Dicko A (2011) Study on chemical composition and biological activities of essential
270 oil and extracts from *Stevia rebaudiana* Bertoni leaves. LWT-Food Sci Technol 44:1865-1872.
271 8. Periche A, Koutsidis G, Escriche I (2014) Composition of antioxidants and amino acids in Stevia leaf infusions. Plant
272 Foods Hum Nutr 69: 1-7.
273 9. European Food Safety Authority (EFSA) (2011) Revised exposure assessment for steviol glycosides for the proposed
274 uses as a food additive. EFSA J 9 (1):1972. doi:10.2903/j.efsa.2011.1972.
275 10. Kroyer J (2010) Stevioside and Stevia-sweeter in food: application, stability and interaction with food ingredients.
276 Journal of Consumer Protection and Food Safety. doi:10.1007/s00003-010-0557-3.
277 11. Quispe C, Viveros-Valdez E, Schmeda-Hirschmann G (2012) Phenolic Constituents of the Chilean Herbal Tea
278 *Fabiana imbricata* R. et P. Plant Food Hum Nutr 67: 242–246.
279 12. Dorta E, Lobo MG, González (2013) Improving the efficiency of antioxidant extraction from Mango Peel by using
280 microwave-assisted extraction. Plant Food Hum Nutr 68:190-199
281 13. Toma M, Vinatoru M, Paniwnyk L, Mason TJ (2001) Investigation of the effects of ultrasound on vegetal tissues
282 during solvent extraction. Ultrason Sonochem, 8: 137-142.
283 14. Jaitak V, Bandna BS, Kaul VK (2009) An efficient microwave-assisted extraction process of stevioside and
284 rebaudioside-A from *Stevia* rebaudiana (Bertoni). Phytochem Anal, 20: 240-245.
285 15. Liu J, Li JW, Tang J (2010) Ultrasonically assisted extraction of total carbohydrates from *Stevia* rebaudiana Bertoni
286 and identification of extracts. Food and Bioprod Process, 88: 215-221.
287 16. Ahmad-Qasem MH, Cánovas J Barrajón-Catalán E, Micol V, Cárcel JA, García-Pérez JV (2013) Kinetic and
288 compositional study of phenolic extraction from olive leaves (var. Serrana) by using power ultrasound. Innov Food Sci
289 Emerg, 17: 120-129.
290 17. Da Porto C, Porretto E, Decorti D (2013) Comparison of ultrasound-assisted extraction with conventional extraction
291 methods of oil and polyphenols from grape (Vitis vinifera L.) sedes. Ultrason Sonochem, 20: 1076-1080.
292 18. Yang L, Cao YL, Jiang JG, Lin QS, Chen J, Zhu L (2010) Response surface optimization of ultrasound-assisted
293 flavonoids extraction from the flower of Citrus aurantium L. var. amara. Engl. J Sep Sci, 33: 1349-1355.

19. Wang JX, Xiao XH, Li GK (2008) Study of vacuum microwave-assisted extraction of polyphenolic compounds and pigment from Chinese herbs. J Chromatogr A, 1198-1199, 45-53.

20. Teo CC, Yong JWH, Tan SN, Ong ES (2009) Validation of green-solvent extraction combined with chromatographic chemical fingerprint to evaluate quality of *Stevia* rebaudiana Bertoni. J Sep Sci, 32: 613-622.

21. Woelwer-Rieck U, Lankes C, Wawrzun A, Wüst M (2010) Improved HPLC method for the evaluation of the major steviol glycosides in leaves of Stevia rebaudiana. Eur Food Res Technol 231:581-588.

22. Choi YH, Kim I, Yoon K, Lee SJ, Kim CY, Yoo KP, Choi YH, Kim J (2002) Supercritical fluid extraction and liquid chromatographic-electrospray mass spectrometric analysis of stevioside from Stevia rebaudiana leaves. Chromatographia, 55:617-620.

23. Commission Decision 2002/657/EC of 12 August (2002). Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, OJEC L221, 8-36. Brusels, Belgium.

24. 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.

25. Dewanto V, Wu X, Adom KK, Liu RH (2002) Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agri Food Chem 50:3010-3014.

26. Shahidi F, Liyana-Pathirana CM, Wall DS (2006) Antioxidant activity of white and black sesame seeds and their hull fractions. Food Chem 99: 478-483.

27. Cacciola F, Delmonte P, Jaworska K, Dugo P, Mondello L, Rader J (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.

28. Kennelly E (2002) Sweet and non-sweet constituents of Stevia rebaudiana (Bertoni) Bertoni. Stevia, the Genus of Stevia, Medicinal and aromatic plants- industrial profiles. Ed. Taylor and Francis, London, ISBN: 0-415-26830-3, 68–85.

29. Prakash I, DuBois GE, Clos JF, Wilkens KL, Fosdick LE (2008) Development of rebaudiana, a natural, non-caloric sweetener. Food Chem Toxicol 46:75−82.

30. Zhang G, He L, Hu M (2011) Optimized ultrasonic-assisted extraction of flavonoids from Prunella vulgaris L. and evaluation of antioxidant activities in vitro. Innov Food Sci Emerg, 12: 18-25.

321 31. Liazid A, Palma M, Brigui J, Barroso CG (2007) Investigation on phenolic compounds stability during microwave-
322 assisted extraction. J Chromatogr A, 1140: 29–34.
323 32. Inglett GE, Rose DJ, Chen D, Stevenson DG, Biswas A (2010) Phenolic content and antioxidant activity of extracts
324 from whole buckwheat (Fagopyrum esculentum Möench) with or without microwave irradiation. Food Chem, 119:
325 1216-1219.
326 33. Ya-Qin M, Jian-Chu C, Dong-Hong L, Xing-Qian Y (2009) Simultaneous extraction of phenolic compounds of citrus
327 peel extracts: Effect of ultrasound. Ultrason Sonochem 16: 57-62.

328 **Figure captions**

329 **Fig. 1** Average values of Dulcoside A, Rebaudioside A, Rebaudioside C and Stevioside in the extracts of *Stevia* leaves
330 obtained applying different methods: conventional (CV), ultrasound (US), and microwave (MW), at different
331 temperatures: 50, 70 and 90ºC and times: 1, 2, 3, 5, 20 and 40 minutes. Letters in bars indicate homogenous groups. In
332 legend of MW: a: 1.98 W/g extract and b: 3.30 W/g extract.

333 **Fig. 2** Total phenols (mg galic acid equivalent/g *Stevia*), flavonoids (mg of catequin equivalent/g *Stevia*) and total
334 antioxidants (mg Trolox equivalent/g *Stevia*) quantified in the extracts obtained at different methods: conventional (CV),
335 ultrasound (US) and microwave (MW); temperatures (50, 70, 90 and 100 ºC) and times (1, 2, 3, 5, 20 and 40 minutes).
336 Letters in bars indicate homogenous groups. In legend of MW: a: 1.98 W/g extract and b: 3.30 W/g extract.

337 **Fig. 3** Bi-plot of Principal Components Analysis for the treatments (white rhombus ◊) and the variables (black rhombus
338 ♦)