Effect of Different Drying Methods on the Phenolic, Flavonoid and Volatile Compounds of *Stevia rebaudiana* Leaves

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Abstract

Different drying methods (hot air drying, freeze drying and shade drying) were evaluated to discern the optimal conditions for the preservation of flavonoid, phenolic and volatile compounds in stevia leaves. All the methods applied affected the antioxidant and volatile compounds in dried stevia leaves differently. 2-Hexenal, hexanal and α-pinene were the most abundant volatile compounds produced by freeze drying and shade drying (21.1-19.7; 14.2-10 and 19.4-5.04 μg/g, respectively); and furan tetrahydro and α-pinene (3.2 and 3.1 μg/g, respectively) by air drying. While chlorogenic acid, coumaric acid and sinapic acid were the most abundant phenolic compounds produced by all the drying treatments (with values that ranged between 88.6-191.8; 41.7-91.3 and 33.2-178.5 mg/100g dry weight of stevia, respectively). The content of volatile compounds was higher with shade drying, whereas most flavonoids and phenolic acids had higher concentrations following freeze drying, although some flavonoids and phenolic acids exhibited a higher increment with air drying. There is no best drying treatment, however, freeze drying results in an extract with satisfactory antioxidant properties and good aromatic characteristics.

Keywords: volatile compounds, freeze-drying, shade-drying, HPLC-DAD, GC-MS.

Introduction

Stevia rebaudiana is a perennial herb, native to Paraguay, which has economic value due to its high content in sweeteners [1]. In fact, its dried leaves have been used as a sweetener in

South America for centuries, and nowadays extracts of steviol glycosides are consumed all over the world [2]. These extracts are 300 times sweeter than sucrose, with the advantage of having: zero calories, zero carbohydrates, and not causing spikes in blood sugar levels [3]. The European Food Safety Authority [4] recognized the safety of stevia leaf extracts for alimentary use in November 2011. Stevia leaves are more and more consumed as infusions due to their antioxidant properties, which stem from their high content in flavonoid and phenolic compounds [5-9]. In addition, their leaves have important therapeutic properties, are rich in compounds with anti-inflammatory, diuretic, anti-hypertensive, antihyperglycemic, antidiarrehal, antitumor and immunomodulatory effects [10].

Stevia leaves, like other herbal teas or medicinal plants, need to be dried for conservation and consumption purposes. The drying process has two principal effects: preventing the growth of microorganisms and facilitating storage and transportation [11]. At the same time, drying herbs can give rise to other alterations which affect herb quality, such as changes in appearance and alterations in aroma caused by losses in volatiles or the formation of new volatiles as a result of oxidation reactions or esterification reactions [12]. Different methods can be applied to dehydrate plants. The simpler, cheaper ones include letting the leaves dry in the shade [13] or using hot air to accelerate the process [14,15]. An innovative technique using freeze drying [11] has been proven to better preserve the quality of medicinal plants [16]. It should be noted that different drying techniques influence the characteristic of the different compounds present in herbal teas. There is a great discrepancy about the extraction of active compounds from herbal teas according to the different drying techniques applied [17]. Different studies have reported changes in the antioxidant capacity of some herbal teas according to the drying method used [11, 12, 15]. In this line, Di Cesare et al. [18] and Diaz-Maroto et al. [19] observed changes in colour and volatile compounds of the aromatic herbs as a consequence of drying.

As far as the authors know, there is no research related to the influence of different drying methods on phenolic and volatile compounds of stevia leaves. For this reason, the aim of this study was to evaluate how the drying method (shade drying, hot air drying and freeze drying) affects phenolic and volatile compounds in stevia leaves, in order to optimize the drying method which maximizes the presence of these compounds.

Material and Methods

Stevia samples and drying conditions

Organically produced *Stevia rebaudiana* Bertoni leaves from Valencia (Spain) were used in this study. Three different drying conditions were used: shade drying at 20ºC for 30 days, hot air drying at 180ºC for 3 minutes in a convective drier, and freeze drying at a vacuum pressure of $9.5\text{x}10^{-1}$ mm Hg for 24 hours.

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 of apigenin, caffeic acid, catechin, chlorogenic acid, cinnamic acid, coumaric acid, 4-methoxybenzoic, 4-methylcatechol, quercetin, rutin and sinapic acid (purity > 98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). De-ionized water from MilliQ (Millipore Corp., Bedford, MA) was used throughout the procedure.

Volatile compounds analysis

Volatile compounds were analyzed following the method purge and trap thermal desorption described by Escriche et al. [20] with the only exception that 200 mg dried powder of stevia leaf and 100 μL of the internal standard 2-pentanol (10 μg/mL H_2O) were used in each analysis. This mix was shaken for several minutes to guarantee total homogenization. Samples were placed in a purging vessel flask and left in a water bath at 45 °C for 20 min.

Purified nitrogen (100 mL min^{-1}) was forced through a porous frit placed at the bottom of the vessel. Volatile compounds were trapped in Tenax (TA, 20-35 mesh), thermally desorbed (TurboMatrix TD, Perkin ElmerTM, CT-USA) and GC-MS analysed (Finnigan TRACE TM MS TermoQuest, Austin, USA) using a DB-WAX capillary column (SGE, Australia) (60 m length, 0.32 mm i.d., 1.0 μm film thickness). The analyses were carried out in triplicate.

The volatile compounds were tentatively identified using their mass spectra and their Kovats retention indices (alkanes: C8–C20 by Fluka Buchs, Schwiez, Switzerland) [20]. The data were expressed in μg/g dry weight of stevia leaf, assuming a response factor equal to one [21].

Flavonoids and phenolic acids analysis

The stevia leaves were ground in a grinding mill (A11 Basic, IKA, Germany), and 200 mg of the dried powder were shaken in 30 mL of methanol/water (1:1 v/v) for 5 minutes. The mixture was sonicated for 10 minutes and then centrifuged at 3000 x g for 5 minutes. An aliquot of the extract was injected in the HPLC, after being filtered through filter paper (0.45 μm pore size).

Analyses of the extracts were carried out using HPLC-Alliance 2695, with a 2996 photodiode array detector (Waters, USA). Flavonoids and phenolic compounds were separated on a Brisa LC2, C18 column (250 x 4.6mm x 5 μm) (Teknokroma, Spain). The binary mobile phase consisted of solvent A (ACN) and solvent B (water and formic acid, 99:1). Binary gradient conditions were used: initial, 90% B, linear gradient to 40% B at 25 min and then to 20% B at 26 min; holding until 30 min; followed by a linear gradient to initial condition at 35 min and a final hold at this composition until 40 min. The column was maintained at 30ºC. The flow-rate and the injection volume were 0.5 mL/min. and 10 μL, respectively.

Chromatograms were recorded at three wavelengths (290, 320 and 360 nm). Flavonoids and phenolic acids were identified by comparison of chromatographic retention times and UV spectral characteristics of unknown analytes with authentic standards. Calibration curves were constructed via least squares linear regression analyses of the ratio of the peak area of each representative compound versus the respective concentration. Quantitative results were expressed as mg of component per 100g dry weight of stevia.

The pure standard of flavonoids and phenolic acids were diluted with methanol to obtain a final concentration of 1 mg/mL for the stock standard solution. The working standard solution was obtained at a concentration of 100 ng/mL in water. The stock standard solution was stored at -20ºC and the working standard solution at +4ºC.

Calibration curves obtained from standard solutions (0.5-10 ng/mL) were used to perform the quantification. Samples were spiked to verify the absence of a matrix effect in the analysis. An internal quality control (a standard solution) was injected into the equipment as a first step, before each batch of the sample, in order to ensure the quality of the results and evaluate the stability of the proposed method.

*Validation of flavonoids and phenolic acids analysis method***.**

The guidelines established by the EU Commission Decision [22] were followed in order to validate the analytical methodology employed to analyse the flavonoids and phenolic acids. For this purpose, several parameters were studied: linearity, accuracy and precision (repeatability and reproducibility). The accuracy of the method was established through recovery studies and the precision was verified by repeatability (intraday precision) and reproducibility (interday precision).

Statistical analysis

An analysis of variance (ANOVA) ($\alpha = 0.05$) with least significant difference (LSD) test using Statgraphics Plus 5.1 was performed on the data from flavonoids and phenolic acids as well as the volatile compounds. In addition to this, the data were analyzed using multivariate techniques, applying the software Unscrambler version 9.7 (CAMO, 2005). The variables were weighted with the inverse of the standard deviation of all objects in order to compensate for the different scales of the variables. A Principal Components Analysis (PCA) was applied to describe the relationship between the flavonoids and phenolic compounds together with the volatile profile.

Results and Discussion

Influence of drying method on the phenolic and flavonoid compounds.

The average value of phenolic compounds (mg /100g dry weight of stevia) quantified in the stevia leaves obtained using different drying methods (shade drying, freeze drying and air drying), as well as the ANOVA F-ratio and homogenous groups for each of the analyzed compounds are shown in Table 1. Eleven compounds were identified in all samples: apigenin, caffeic acid, catechin, chlorogenic acid, cinnamic acid, coumaric acid, 4-methoxybenzoic, 4-methylcatechol, quercetin, rutin and sinapic acid.

With regard to the validation parameters, good linearity was obtained, with R^2 values ranging from 0.991 for 4-methoxybenzoic to 0.999 for quercetin, catechin and 4-methylcatechol. The range of the average recoveries varied from 90% for caffeic acid to 117% for sinapic acid. The RSD_r (repeatability standard deviation) for all compounds was less than 9% and the RSD_R (reproducibility standard deviation) was always less than 13%. In both cases the values were below 20%, and therefore in agreement with the requirements of the Commission Decision [22].

The highest F-ratio in Table 1 shows that coumaric and sinapic acid were most influenced by the drying method. The concentrations of other compounds such as apigenin, quercetin and cinnamic acid showed practically no differences as a result of applying the three treatments.

The majority of the compounds analyzed reached their maximum values with the freeze drying method. Compounds such as chlorogenic acid, coumaric acid and sinapic acid exhibited a higher concentration after freeze drying (191.84, 91.35 and 178.56 mg/100g stevia leaf, respectively) and air drying (167.56, 70.36 and 165.14 mg/100g stevia leaf, respectively) than shade drying (88.60, 41.71 and 33.21 mg/100g stevia leaf, respectively). However, the values obtained for 4-metoxybenzoic following freeze drying (7.48 mg/100g stevia leaf) were lower than those for the other treatments (air drying-26.28 mg/100g stevia leaf and shade drying-15.39 mg/100g stevia leaf).

Many antioxidant compounds have been identified in stevia leaves by different authors, but their conclusions with respect to both the specific compounds and the concentration levels are very different and even contradictory. This can be explained by the fact that the drying methods employed were different in each case. However, in some studies it was not even mentioned. Different flavonoids (flavonols and flavones) have been identified: quercetin and its derivatives, apigenin and its derivatives, kaempferol-3-O-rhamnoside, luteolin and their derivatives [23,24,25] in stevia dried leaves. Karaköse et al. [26] identified 24 chlorogenic acids using LC-ESI-MS. Muanda et al. [8] identified (at room temperature) the same phenolic and flavonoid compounds in stevia dried leaves as in the present work, with the exception of 4-methoxybenzoic acid, 4-methylcatechol and sinapic acid. Kim et al. [27] identified 6 phenolic acids: pyrogallol, 4-methoxybenzoic acid, 4-methylcatechol, sinapic acid, coumaric acid and cinnamic acid (at 40ºC for 12h). All of them were identified in the present study, with the exception of pyrogallol. It is important to highlight that the values obtained by Kim et al. [27] were lower than those reported by Muanda et al. [8].

Considering other medicinal herbal teas, Lin et al. [11] claimed that freeze drying was the best method for preserving the higher contents of caffeic acid derivatives and total phenolics in *Echinacea Purpurea* leaves. Ferreira and Luthria [28] obtained lower levels of antioxidant capacity for shade drying than hot air drying in *Artemisia annua* L. leaves.

Influence of drying method on the volatile compounds.

Thirty volatile compounds were tentatively identified. Table 2 shows the mean concentration values of the quantified volatile compounds (expressed as μg/g dry weight of stevia leaf) as well as their standard deviations (SD) for the three drying methods.

The most abundant compounds produced by shade drying and freeze drying were 2-hexenal (21.09 and 19.78 μg/g), hexanal (14.23 and 10.02 μg/g) and α-pinene (19.40 and 5.04 μg/g), respectively. The most abundant compounds produced by air drying, were furan tetrahydro (3.25 μg/g) and α-pinene (3.14 μg/g).

In contrast to the phenolic and flavonoid compounds, shade drying better preserves the volatile fraction of stevia leaves in comparison with freeze drying and air drying.

There are a few studies about the volatile fraction of stevia leaves and all of them analyzed the volatile compounds in the essential oils in stevia. Muanda et al. [8] identified 34 volatile compounds, Moussa et al. [29] found 22 compounds, Turko et al. [30] reported 23 compounds and Zygadlo et al. [31] identified 41 compounds, only 5 of them (α-pinene, hexanal, limonene, 1-octen-3-ol, caryophyllene) were identified in this study, which is logical because in the present work the analysis was performed directly on the stevia dried leaves and not on the essential oil.

Global behavior of phenolic and volatile compounds.

A PCA was applied in order to appreciate the overall effect that the drying method has on phenolic and volatile compounds together. The corresponding bi-plot obtained (scores "treatments" and loading "variables") is shown in Fig. 1 (PC1 explained 59 % of the total variance and PC2, 20 %). The proximity between variables indicates the correlation between them, and in the case of drying treatments similar behavior. In general, this figure shows opposing behavior between the two groups of variables (phenols and volatiles) with respect to the effect of the drying treatments applied.

The shade drying treatment is placed at the far end of the right axis in the figure, which corresponds to the highest values of the volatile compounds and the lowest of the phenolic compounds. On the contrary, freeze drying and air drying are placed on the opposite side (left axis), which corresponds to the highest content of phenolic compounds. The only exceptions to this general pattern are apigenin and quercetin which are placed with the volatile compounds even though they are antioxidant compounds.

Apparently, some volatile compounds could be generated as a result of oxidation and degradation reactions involving the phenolic and acid compounds [32], so perhaps freeze drying helps to preserve them, whereas drying in the shade favors degradation processes.

Conclusions

All the drying methods applied (freeze drying, shade drying and air drying) affected the antioxidant and volatile compounds in the dried stevia leaves. The two types of compounds reacted differently; the content of volatile compounds was higher with shade drying whereas most flavonoids and phenolic acids had higher concentrations when freeze drying was applied. However, some flavonoids and phenolic acids exhibited a higher increment with air drying. Therefore there is no ideal drying treatment which can be chosen, although freeze drying is the most recommendable if an extract with sufficient antioxidant properties and satisfactory aromatic characteristics is desired.

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Table 1. Mean and standard deviation of flavonoid and phenolic compounds quantified in the three drying methods (mg/100 g dry weight of stevia leaf).

mg/100g stevia leaf	Freeze drying	Air drying	Shade drying	Anova F-ratio
apigenin	0.24(0.04)^{a}	0.25(0.02)^{a}	0.39(0.02)^{a}	1^{ns}
caffeic acid	1.22(0.02)^{b}	0.71(0.04)^{a}	0.75(0.03)^{a}	350***
catechin	8.35(0.38)^{c}	6.18(0.33)^{b}	4.38(0.42)^{a}	55**
chlorogenic acid	191.84(0.7)^{c}	167.56(0.12)^{b}	88.60(3.19)^{a}	1621***
cinnamic acid	0.27(0.07)^{ab}	0.34(0.02)^{b}	0.19(0.02)^{a}	7^{ns}
coumaric acid	91.35(0.16)^{c}	70.36(0.30)^{b}	41.71(0.48)^{a}	10616***
4-methoxybenzoic	7.48(0.39)^{a}	26.28(0.43)^{c}	15.39(0.2)^{b}	1394***
4-methylcatechol	2.49(0.02)^{b}	2.99(0.56)^{b}	0.73(0.07)^{a}	26*
quercetin	0.33(0.03)^{a}	0.28(0.02)^{a}	0.39(0.06)^{a}	5^{ns}
rutin	20.07(0.13)^{c}	15.08(0.22)^{b}	7.05(0.02)^{a}	4174***
sinapic acid	178.56(0.7)^{c}	165.14(1.53)^{b}	33.21(0.23)^{a}	13544***

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns: non significant

Table 2. Semiquantification of volatile compounds (µg/g dry weight of stevia assuming a response factor equal to 1) in stevia dried leaves (n = 3).

Volatile compounds	**Shade drying**	**Air drying**	**Freeze drying**	**KI cal**	**ID**	**Anova F-ratio**
Alcohols						
1-penten-3-ol	0.60(0.09)^{a}	0.15(0.01)^{a}	1.96(0.02)^{b}	774	MS;KI	14.8*
1-pentanol	0.42(0.01)^{a}	0.63(0.03)^{a}	0.33(0.04)^{a}	704	MS;KI	2.43^{ns}
1-octen-3-ol	5.85(0.08)^{c}	0.68(0.02)^{a}	2.37(0.08)^{b}	980	MS;KI	149***
3,7dimethyl-1,3 octadien-3-ol	5.50(0.07)^{c}	0.93(0.01)^{a}	2.35(0.10)^{b}	1110	MS;KI	99***
2 ethyl-1-hexanol,	0.88(0.02)^{b}	0.18(0.01)^{a}	0.27(0.02)^{a}	1028	MS;KI	46**
Aldehydes						
2-ethyl-butanal	2.64(0.12)^{b}	0.29(0.02)^{a}	0.33(0.05)^{a}	662	MS;KI	23.7**
3-methyl-butanal	3.01(0.20)^{b}	0.11(0.01)^{a}	0.78(0.06)^{a}	676	MS;KI	10.87*
pentanal	2.25(0.17)^{b}	1.26(0.02)^{a}	1.22(0.09)^{a}	780	MS;KI	2.03*
hexanal	14.23(0.53)^{b}	0.86(0.01)^{a}	10.02(0.26)^{b}	860	MS;KI	27.4**
heptanal	0.41(0.02)^{b}	0.11(0.01)^{a}	0.08(0.01)^{a}	896	MS;KI	14.3*
2-hexenal	21.09(0.66)^{b}	0.83(0.08)^{a}	19.78(0.96)^{b}	768	MS;KI	24.9**
2-heptenal	2.63(0.02)^{b}	0.28(0.01)^{a}	0.64(0.07)^{a}	932	MS;KI	121***
2-4 heptadienal	3.29(0.05)^{c}	0.36(0.02)^{a}	1.69(0.08)^{b}	1015	MS;KI	75***
octanal	0.34(0.01)^{a}	0.20(0.01)^{a}	0.29(0.02)^{a}	1004	MS;KI	3.7^{ns}
nonanal	2.34(0.07)^{a}	1.28(0.08)^{a}	1.73(0.13)^{a}	1106	MS;KI	3.5^{ns}
decanal	0.68(0.02)^{a}	0.74(0.01)^{a}	1.06(0.08)^{a}	1204	MS;KI	1.9^{ns}
Hydrocarbons						
benzene	0.44(0.07)^{a}	0.19(0.03)^{a}	0.12(0.01)^{a}	662	MS;KI	1.15^{ns}
1-heptene	1.92(0.08)^{b}	0.05(0.01)^{a}	0.81(0.05)^{a}	690	MS;KI	21.08**
1-octene	2.37(0.12)^{b}	0.11(0.01)^{a}	1.06(0.14)^{ab}	790	MS;KI	11.12*
Ketones						
3-buten-2-one	0.50(0.01)^{a}	0.69(0.11)^{a}	0.49(0.05)^{a}	707	MS;KI	0.2^{ns}
4-hydroxy-2-butanone	0.74(0.07)^{a}	0.58(0.06)^{a}	0.48(0.04)^{a}	720	MS;KI	0.4^{ns}
6 methyl-5-hepten-2-one	0.29(0.01)^{a}	0.22(0.02)^{a}	0.29(0.03)^{a}	987	MS;KI	0.4^{ns}
Terpenes						
α-pinene	19.40(0.70)^{b}	3.14(0.10)^{a}	5.04(0.52)^{a}	912	MS;KI	25.7**
limonene	0.72(0.01)^{a}	0.54(0.02)^{a}	0.45(0.05)^{a}	1024	MS;KI	2.2^{ns}
caryophyllene	8.24(0.08)^{b}	1.68(0.15)^{a}	2.36(0.28)^{a}	1432	MS;KI	47**
Nitriles						
2-hydroxy-2-methyl-propanenitrile	3.41(0.17)^{b}	0.56(0.02)^{a}	0.53(0.03)^{a}	752	MS;KI	19.5**
Furanes						
tetrahydro furan	2.61(0.31)^{a}	3.25(0.15)^{a}	1.34(0.16)^{b}	628	MS;KI	1.26*
Sulfur compounds						
dimethyl sulfide	1.02(0.01)^{b}	0.18(0.01)^{a}	0.39(0.04)^{a}	741	MS;KI	41.36**

* $p<0.05$, **$p<0.01$,*** $p<0.001$, ns: non significant
KI cal: Kovats retention indices calculated.
ID: method of identification, MS (comparison with mass spectrum from NIST library) and KI (comparison of Kovats index with the literature [20]).

Figure captions

Fig. 1 Bi-plot of Principal Components Analysis for the drying treatments (black diamond ♦) and the analysed variables: phenolic, flavonoid and volatile compounds (white diamond ◊).