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Note

Effect of different drying methods on the composition of steviol glycosides in *Stevia rebaudiana* Bertoni leaves**

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A b s t r a c t. Drying techniques can modify the composition of certain plant compounds. Therefore, the aim of the study was to assess the effect of different drying methods on steviol glycosides in *Stevia rebaudiana* Bertoni leaves. Four different drying methods were applied to *Stevia rebaudiana* Bertoni leaves, which were then subjected to aqueous extraction. Radiation or convection drying was performed in stoves at 60ºC, whereas shade or sun drying methods were applied at 29.7ºC and 70% of relative humidity. Stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, dulcoside A, and steviolbioside were quantified by a validated HPLC method. Among steviol glycosides, the content (g 100 g^{-1} dry basis) of stevioside, rebaudioside A, rebaudioside B, and rebaudioside C varied according to the drying method. The total glycoside content was higher in sun-dried samples, with no significant differences compared to shade or convection drying, whereas radiation drying adversely affected the content of rebaudioside A and rebaudioside C ($p < 0.01$) and was therefore a method lowering total glycoside content. The effect of the different drying methods was also reflected in the proportion of the sweetener profile. Convection drying could be suitable for modern food processing industries while shadow or sun drying may be a low-cost alternative for farmers.

K e y w o r d s: *Stevia rebaudiana* Bertoni, drying methods, steviol glycosides, sweetener profile

INTRODUCTION

Stevia rebaudiana Bertoni is a plant representing one of the family Asteraceae genera, which is native to tropical and subtropical regions of Central and South America (Goyal *et al.,* 2010). In recent years, the food industry has focused its interest on the use of natural sweeteners, and *Stevia rebaudiana* Bertoni has been highlighted as it contains steviol glycosides that are 200-300 times sweeter than sugar (Goyal *et al.,* 2010; Jackson *et al.,* 2009). Steviol glycosides have no calories and are generally recognized as safe (GRAS) by the Food and Drug Administration in the United States of America (FDA, 2008). In Europe, the European Commission granted the authorization of the use of steviol glycosides as a food sweetener in 2011 (EU, 2011).

In generally cultivated varieties of *Stevia rebaudiana* Bertoni, the main steviol glycosides found in the leaves are stevioside, rebaudioside A, and rebaudioside C; other glycosides, including dulcoside A, stevolbioside, rubusoside, rebaudioside B, D, E, and F, are present in smaller amounts (Goyal *et al.,* 2010; Jackson *et al.,* 2009; Wöelwer-Rieck *et al.,* 2010).

Leaves containing a large amount of initial moisture are highly susceptible to rapid degradation (Chua and Chou, 2003). Drying is a very common practice to extend the shelf life of products since moisture reduction prevents the growth of microorganism (Shen-Dun *et al.,* 2011) and allows longer periods of storage, maintaining quality and stability of product (Lemus-Mondaca *et al.,* 2015), while it minimizes packing, transport, handling, and distribution requirements (Kwok *et al*., 2004).

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The use of *Stevia rebaudiana* Bertoni in the food industry requires post-harvest drying processing in order to maintain the stability of the product during transport and storage. However, it is widely recognized that different drying techniques can modify the composition of certain compounds in the product.

Drying *Stevia rebaudiana* Bertoni up to 50ºC increases the content of steviol glycosides without differences at temperatures between 60 to 80ºC (Lemus-Mondaca *et al.,* 2015); however, it is adversely affected at 180ºC (Periche *et al.,* 2015). Moreover, the effect of temperature on other compounds also present in the leaves, such as phenols, vitamin C, and flavonoids, are quite different.

Among different drying methods, air-drying is an ancient process where a constant hot stream of air causes moisture evaporation (Ratti, 2001). Dehydrated products obtained by air-drying have a long shelf life but the quality could be changed in the original product, depending on the conditions applied (Ratti, 2001). Vacuum freeze-drying is a method with high-quality final products; however, is the most expensive process for manufacturing a dehydrated product (Chua and Chou, 2003).

One the other hand, sun drying is one of the low-cost drying methods and therefore a common practice in farming and agricultural processing in many developing countries, including some regions of Mexico, where the outdoor temperature reaches 30°C or higher (Chua and Chou, 2003).

In a previous work, traditional and industrial drying methods were compared, and it was demonstrated that different drying methods modify the antioxidant capacity accompanied by changes in the luminescence of *S. rebaudiana* leaves (Moguel-Ordóñez *et al.,* 2015). Therefore, the aim of the study was to assess the effect of different drying methods on the composition of steviol glycosides in *Stevia rebaudiana* Bertoni leaves.

MATERIALS AND METHODS

Standards of steviolbioside (ASB-00019349), dulcoside A (ASB-00004949), rebaudioside C (ASB-00018228), rebaudioside B (ASB-00018227), and rebaudioside D (ASB-00018229) were purchased from Chromadex (Irvine, CA, USA); standards of rebaudioside A (01432) and stevioside (S3572) were purchased from Sigma-Aldrich (USA). Acetonitrile and water (HPLC grade) were purchased from J.T. Baker (Phillipsburg, NJ). Glycoside standards were lyophilized to increase the stability and precision of standard curves; lyophilization was performed under vacuum pressure of 133×10^{-3} Bar and a temperature of −40°C (Labconco, Kansas City, MO), then the samples were mixed with HPLC water, filtered through 0.45 µm, and stored at −20°C prior to use.

A variety of *Stevia rebaudiana* Morita II was grown and collected from Southeast México. The plantation had a crop management according to the production methodology described by Ramírez *et al.* (2011). Samples were obtained from the first cut of the plot at an age of three months. The leaves were harvested manually using stainless steel scissors. The cut was made at 9 a.m. when the morning dew was evaporated to avoid cutting wet leaves. The leaves (1 kg) were dispersed in a stainless steel tray and 3 replicates were performed for each type of drying.

Four drying methods were applied to *Stevia rebaudiana* B. leaves as previously described (Moguel-Ordóñez *et al.,* 2015): radiation, convection, shade, and sun drying. A stove with air circulation (convection) and a regular stove (radiation) were used at 60ºC. For the shade and sun drying methods, temperature (29.7ºC) and relative humidity (70%) were monitored constantly. At the beginning of every drying method, the leaves were weighed every 4 h (radiation and convection) or 24 h (shade and sun); the drying methods were applied until there was no significant weight loss. Three samples of each drying method were obtained, codified with consecutive numbers (1-12), and independently processed during further experiments.

Dried material was milled to obtain a particle size of 1.0 mm and kept in the dark until analysis. Extracts were prepared according to literature procedure (Wöelwer-Rieck *et al.,* 2010). Briefly, 500 mg of dried homogenized leaves were weighed and extracted three times with 5 mL of water each time in a boiling water bath at 100°C for 30 min. The extracts were cooled to room temperature and centrifuged for 10 min (2500 × g, 10°C). The aqueous phases were transferred to a 25 ml volumetric flask and filled to capacity after the last extraction. The solution was filtered through a membrane filter (0.45 µm) to remove any solid residue before HPLC analysis. Each sample (n = 12) of the drying method was independently extracted in triplicate and subjected to HPLC analysis.

The high-performance liquid chromatography method (Aranda-González *et al.,* 2014; 2015), previously validated in the laboratory, was performed according to JECFA (2010). The chromatographic analysis was carried out on a Luna C18 (2) (length: 250 mm; inner diameter: 4.6 mm, particle size: 5 µm) column (Phenomenex Co., Ltd., CA, USA) without temperature control. The mobile phase was a 32:68 (v/v) mixture of acetonitrile and 10 mmol l^{-1} sodium phosphate buffer (pH 2.6) at a constant flow rate of 1 ml min^{-1}. The analysis was carried out in an Agilent 100 HPLC system with a UV-Vis detector set to a wavelength of 210 nm. Chromatographic analysis was performed with Clarity software 2.7.3.498 version (2009). Each extract was analysed in triplicate with an injection volume of 20 µl.

Standard curves were prepared by dilution of the stock solution with HPLC water to reach concentration ranges of 100-500 µg ml^{-1} for rebaudioside A and stevioside and 25-150 µg ml^{-1} for steviolbioside, dulcoside A, rebaudioside, rebaudioside B and rebaudioside D and analyzed by

HPLC in triplicate. A plot of peak area as a function of the analyte concentration was developed and the linear regression was calculated by the method of least squares.

One-way ANOVA followed by HSD Tukey *post-hoc* was used to study the effect of the drying methods on the composition of steviol glycosides.

RESULTS AND DISCUSSION

Standard calibration curves were made using standard solutions with a final concentration of 100, 200, 300, 400, and 500 μg ml^{-1} for rebaudioside A and stevioside, and a concentration of 25, 50, 75, 100, 125, and 150 μg ml^{-1} for minor glycosides. The linearity response and linear regression equation for each glycoside is presented in Table 1. A good correlation coefficient and coefficient of determination was obtained as expected, given that the method used was previously validated (Aranda-González *et al.,* 2014); however, they are presented to establish the conditions of quantification.

T a b l e 1. Parameters calculated from linear regression model of steviol glycose standard solutions

Steviol glycoside standard solution	Correlation coefficient (r)	Coefficient of determination (R^2)	Linear regression model
Rebaudioside A	0.999	0.998	y = 3.705x+117.5
Stevioside	0.997	0.995	y = 5.176x+81.632
Dulcoside A	0.997	0.994	y = 6.257x–45.573
Steviolbioside	0.998	0.996	y = 0.987x–3.542
Rebaudioside B	0.995	0.991	y = 2.105x–27.44
Rebaudioside C	0.995	0.990	y = 4.353x–8.360
Rebaudioside D	0.997	0.995	y = 2.073x+9.591

Data presented were obtained from standard curves analyzed by triplicate.

The average values and the standard deviation of the steviol glycosides are shown in Table 2. All values are expressed in g 100 g^{-1} of dry weight. The drying curves obtained with the methods applied have been published previously (Moguel-Ordóñez *et al.,* 2015) and are available for consulting. Briefly, moisture was monitored during 24 h for radiation or convection drying and during 96 h for shade or sun drying; the equilibrium in the moisture content was reached at 8, 20, and 48 h upon radiation, convection, and sun or shade drying, respectively. For each sample, drying was stopped when there was no significant weight loss.

It has been reported that all steviol glycosides have a different sweetness degree (Goyal *et al.,* 2010) *eg* rebaudioside B is 150 times sweeter than sugar, rebaudioside D is 200-300 times sweeter, whereas rebaudioside C is only 30 times sweeter than sugar (Prakash *et al.,* 2012). In the food industry, the content of rebaudioside A and stevioside are particularly important because they both have a high sweetening capacity and are found in greater quantities in leaves of *Stevia rebaudiana* Bertoni (Goyal *et al.,* 2010). However, although stevioside is 250-300 times sweeter than sugar, it also has a bitter aftertaste, while rebaudioside A is even sweeter than stevioside (350-450 sweeter than sucrose) and has no bitter aftertaste (Goyal *et al.,* 2010).

As shown in Table 2, the drying method had an effect on the content of four of the seven glycosides evaluated. Glycosides whose content varied according to the drying method were stevioside, rebaudioside A, rebaudioside B, and rebaudioside C.

The total glycoside content was higher in samples dried in the sun, with no significant differences compared to shade and convection drying; however, the method with lower content of total glycosides was the radiation drying, which was significantly lower ($p<0.01$) compared to sun drying.

T a b l e 2. Steviol glycosides content in leaves of *Stevia rebaudiana* Bertoni Morita II subjected to different types of drying

Steviol glycoside standard solution	Drying method (g 100 g^{-1})			
	Radiation	Convection	Sun	Shade
Rebaudioside A	7.76 ± 0.22b**	9.33 ± 0.19ab	10.53 ± 0.91a**	9.28 ± 0.62ab
Stevioside	4.03 ± 0.29ab	4.14 ± 0.34a*	3.57 ± 0.12ab	3.43 ± 0.09b*
Dulcoside A	0.14 ± 0.02a	0.14 ± 0.02a	0.13 ± 0.02a	0.12 ± 0.01a
Steviolbioside	0.3 ± 0.08a	0.34 ± 0.12a	0.44 ± 0.06a	0.38 ± 0.05a
Rebaudioside B	0.38 ± 0.04ab	0.36 ± 0.05b*	0.45 ± 0.01a*	0.39 ± 0.01ab
Rebaudioside C	0.78 ± 0.02b**	0.84 ± 0.03b**	1.05 ± 0.02a**	0.8 ± 0.03b**
Rebaudioside D	0.93 ± 0.01a	0.87 ± 0.35a	1.11 ± 0.15a	1.14 ± 0.13a
Total	14.34 ± 0.43b**	16.03 ± 1.02ab	17.29 ± 0.98a**	15.8 ± 1.29ab

Data are mean ± SD of steviol glycoside content of three different samples of each drying method analyzed in triplicate by HPLC. Rows with different letters denote significant differences at *$p<0.05$ or **$p<0.01$ by Tukey *post hoc*.

Radiation drying adversely affected the content of rebaudioside A and rebaudioside C ($p < 0.01$), which was significantly lower than in sun drying; this explains the difference in the total content of steviol glycosides.

Comparing the industrial drying methods (radiation *vs*. convection drying), no significant differences in total or individual glycosides were found. However, among the traditional drying methods (sun *vs*. shade), there was a significant difference ($p < 0.01$) in the content of rebaudioside C, which was higher in the sun drying.

A method that yielded higher content of stevioside was the convection drying, but it was significantly different only compared to shade drying ($p < 0.05$); sun dried leaves had higher content of rebaudioside A and rebaudioside B, compared to radiation ($p < 0.01$) and convection ($p < 0.05$) drying, respectively. However, rebaudioside C was a glycoside characterised by high content, which was significantly higher in the sun drying and different compared to the other three treatments ($p < 0.01$).

The biosynthetic pathway of steviol glycosides has not been fully elucidated. It is known that after the formation of steviol, a series of glycosylation takes place in the cytosol, leading to formation of a large family of steviol glycosides. Glycosylation of steviol at the C-13 hydroxy group produces steviolmonoside, which is glycosylated at C-2' to form steviolbioside. A further glycosylation of the C-4 carboxylic acid moiety of steviolbioside yields stevioside, followed by a glycosylation of the C-3' to form rebaudioside A (Ceunen and Geuns, 2013). Rebaudioside B in leaf extracts has often been regarded as being mostly produced during extraction due to partial hydrolysis of rebaudioside A or stevioside (Ceunen and Geuns, 2013).

The biosynthesis of dulcoside A and rebaudioside C is unknown; however, *in vitro* it involves alternative glycosylation of steviolmonoside to form dulcoside A and further glycosylation of dulcoside A at C-3' to form rebaudioside C (Ceunen and Geuns, 2013).

Taking this into consideration, it is possible that *Stevia rebaudiana* Bertoni leaves has steviol within the cytosol, which chemically reacts during drying, continuing the biosynthetic pathway. This hypothesis is based on the content of the highest amount of rebaudioside A and rebaudioside C found in sun drying, given that both glycosides are at the end of the biosynthetic pathway and sun drying is the method closer to the natural habitat of the plant. However, more studies are needed.

Although there were no significant differences in the content, it was possible to quantify dulcoside A, rebaudioside D, and steviolbioside in the four drying methods, as previously published (Aranda-González *et al.,* 2014).

The rebaudioside A to stevioside ratio is an accepted measure of sweetness quality (Ceunen and Geuns, 2013; Ceunen *et al.,* 2012; Dacome *et al.,* 2005; Rajasekaran *et al.,* 2007; Yadav *et al.,* 2011). However, this measure does not consider other glycosides found in *Stevia rebaudiana* leaves in smaller amounts but with also a sweetening capacity. To assess which drying method yields a better sweetener profile (sweetness/bitterness), the authors propose a measure of sweetness quality including the rest of the glycosides in addition to rebaudioside A (sweet steviol glycosides/stevioside ratio). Hence, the proportion of sweet steviol glycosides was calculated as the sum (g 100 g^{-1}) of glycosides: dulcoside A, steviolbioside, rebaudioside A, rebaudioside B, rebaudioside C, and rebaudioside D/stevioside. As seen in Fig. 1, the sun and shade drying had a higher proportion of steviol glycosides without the bitter aftertaste, whereas the 60ºC drying negatively affected this ratio. These data are explained by variations in the main glycosides stevioside and rebaudioside A (Table 2), which affect not only the total content of glycosides but also the overall sweetness profile.

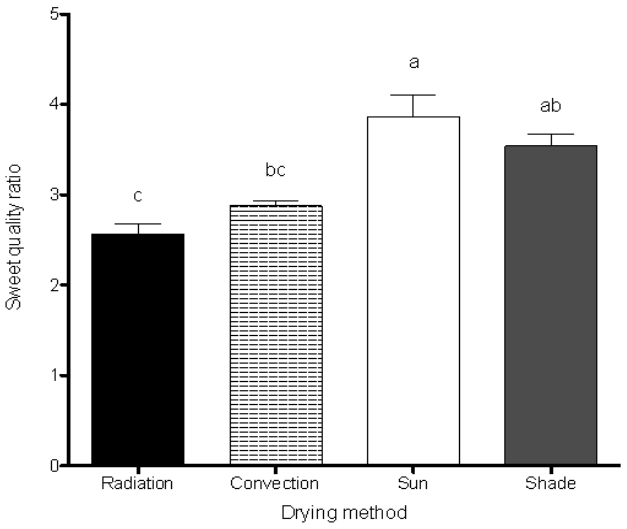

Fig. 1. Data are mean ± SD of the sweet quality ratio. Sweet quality ratio was calculated as the sum of the content (g 100 g^{-1}) of all glycosides except stevioside/stevioside of three samples of each drying method analyzed in triplicate by HPLC. Columns with different letters denote significant differences at $p<0.05$ by Tukey *post hoc*.

There is little information on the yield of steviol glycosides depending on the method of drying. Comparison of the information reported by different authors about the content of glycosides can be challenging for several reasons:

- it depends mainly on the variety of *Stevia* used, which is often unreported (Jarma-Orozco *et al.,* 2011);
- it is known that the growing conditions can modify the content of steviol glycosides (Jarma-Orozco *et al.,* 2011);
- different extraction conditions are found elsewhere, including the use of solvents with different polarity i.e. chloroform, methanol, or water (Lorenzo *et al.,* 2014; Kolb *et al.,* 2001) or different methods such as supercritical fluid extraction (Pól *et al.,* 2007), microwave (Jaitak *et al.,* 2009), ultrasonic and even enzymatic extraction (Jie *et al.,* 2010; Puri *et al.,* 2012); and to a lesser extent;

– different analytical techniques (Tada *et al.,* 2013; Wöelwer-Rieck *et al.,* 2010).

Therefore, the results presented in this paper highlight an additional factor to be considered: the method of drying, taking into account that the same variety of *Stevia rebaudiana* Bertoni, extraction condition, and analytical technique were used. Moreover, the variety used in the present study (Morita II) is widely used in the food industry, and therefore it is important to control all the factors that yield better sweetness.

To our knowledge, there are only two reports addressing the effect of drying conditions on the content of glycosides (Lemus-Mondaca *et al.,* 2015; Periche *et al.,* 20015). However, there are many methodological conditions that do not allow comparison with the results obtained in the present work, such as: the drying method (different temperature and time), weather and soil condition of the plant (Spain and Chile), extraction method (different solvents), and for the content of glycosides the use of a different *S. rebaudiana* variety can be inferred. However, it is noteworthy that both reports also found significant differences due to the type of drying, which confirms the findings. Periche *et al.* (2015) demonstrate that the content of phenols, flavonoids, and antioxidants is higher in drying at 180°C compared to 100°C, while the glycoside content is higher when the extraction is conducted at 100°C instead of 180ºC. Their results demonstrate that drying affects the antioxidant capacity and the content of glycosides oppositely; that is, a method of drying that yields more antioxidant compounds will result in lower content of glycosides (Periche *et al.,* 20015) and vice versa. On the other hand, Lemus-Mondaca *et al.* (2015) evaluate drying at different temperatures including those near the evaluated here (30 and 60°C); however, the duration was too short (9.5 and 3 h, respectively). Nevertheless, the authors concluded that both the drying temperature and time have a significant effect on the bioactive compounds.

Using exactly the same drying methods presented here, Moguel-Ordóñez *et al.* (2015) demonstrate that sun dried leaves have more luminescence, chlorophyll-associated green colour, and total pigments but lower antioxidant capacity compared to convection drying, which yields the greatest antioxidant capacity but lower luminosity and green colour.

Traditional drying (sun or shade) requires at least 48 h, compared with radiation (8 h) or convection (20 h) (Moguel-Ordóñez *et al.,* 2015) and its use may involve contamination of leaves and dependence on weather conditions; however, it can be a good alternative for small producers given its good yield of glycosides and low cost and accessibility. At the industrial level, radiation drying would be the fastest method although at the cost of a reduced glycoside yield and lower sweetness profile, so its use should not be discouraged.

In a previous paper, convection and shadow drying resulted in a product with enhanced colour and total pigment content (Moguel-Ordóñez *et al.,* 2015). The data presented here show that both methods yield a higher content of glycosides, and therefore are the most recommended for use by the industry and by small producers, respectively.

Taking in consideration all these data, it is likely that the speed of loss of moisture is a very important factor not only for the appearance of the leaves but their antioxidant and sweetener capacity, and should be mentioned in any report about the bioactive compounds in *Stevia rebaudiana* Bertoni dried leaves.

CONCLUSIONS

1. The different drying methods have an effect on the composition of steviol glycosides in *Stevia rebaudiana* Bertoni leaves.

2. Glycosides whose content varied according to the drying method were rebaudioside A, B, D, and stevioside, whereas rebaudioside D, dulcoside A, and steviolbioside remained unchanged.

3. The effect of the different drying methods was reflected in the total content of glycosides and the sweetener profile (sweetness/bitterness) that could affect the overall sweetener capacity.

4. Convection drying was a drying method that yielded higher total steviol glycosides with a sweetener profile and, therefore, it is suitable for modern food processing industries.

5. Small producers can use either shadow or sun drying, obtaining a product with a high level of total steviol glycosides and sweetener profile.

Conflict of interest: The Authors do not declare conflict of interest.

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