Description of New Methods

Variation in steviol glycoside contents of stevia (*Stevia rebaudiana*) leaves under various leaf drying processes

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Abstract

Stevia (*Stevia rebaudiana*) leaves contain valuable sweet compounds, called steviol glycosides (SVglys.). The quantity and quality of these sweet compounds may be changed during the leaf drying process. The present study was conducted to survey variations in SVglys. contents of stevia leaves dried through different methods. Stevia leaves were harvested before flowering and were submitted to different drying methods, including oven (45 ℃ and 75 ℃), shade, infrared, microwave, sun, and freeze-dry, and thereafter the SVglys. contents were assessed. Results showed that the SVglys. content and its compositions such as stevioside (Stev.) and rebaudioside A (Reb. A) were significantly affected by leaf drying methods. The highest contents of steviol glycosides and stevioside were obtained using infrared, microwave, sun, and freeze-dry methods. while the other methods resulted in lower SVglys. and Reb. A contents. A significantly high content of Reb. A was recorded by freeze-drying and sun methods. Our results conclusively showed that freeze-dry and sun drying methods were more effective than other drying methods in retention of SVglys. contents of leaves and recorded the lowest SVglys. losses during the drying process.

Keywords: freeze-dry, infrared, microwave, stevioside; Rebaudioside A

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Introduction

Today, natural sweeteners are highly interesting for researchers, due to their useful roles in regard to blood glucose and diabetic problems. The sweeteners derived from stevia (*stevia rebaudiana*) leaves contain the lowest calories constituents that are used as food additives. Stevia is a perennial plant natively grown in Paraguay recently widespread in some other countries (Angelini et al., 2018; Clemente et al., 2021; Shock, 1982) and is well known as a new crop. However, some limitations, such as low germination, have brought severe challenges to the promotion of stevia cultivation (Afshari et al., 2020). The sweet compounds of this species belong to terpenes and, owing to their steviol base and attached glucose units, are known as steviol glycosides (SVglys.), which are produced in leaves (Brandle and Telmer, 2007). It has been reported that SVglys. are sweeter than sugar (Kennelly, 2001) while they have a low gastrointestinal absorption, and

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therefore cannot interfere with the blood glucose (Momtazi-Borojeni et al., 2017). This property increases the importance of SVglys., and consequently the stevia cropping has been on the growth. Stevia leaves are rich in SVglys. and among SVglys., stevioside (Stev.) and rebaudioside A (Reb. A) are the most important SVglys. compositions (Singh and Rao, 2005).

The SVglys. are biosynthesized in leaf mesophyll cells and stored in vacuoles (Brandle and Telmer, 2007). SVglys are usually extracted from stevia dry leaves by several procedures. The fresh leaves can be dried through several methods which may affect the quality and quantity of the SVglys. as well as other useful compounds (Gasmalla et al., 2014). Furthermore, it has been shown that SVglys. contents of stevia leaves were increased by drying temperatures up to 50 ℃ and thereafter decreased (Lemus-Mondaca et al., 2016). There are few documents dealing with drying effects on stevia leaf constituents; thereby, the effects of drying processes have been evaluated and the most suitable method for stevia leaf processing and consequently for SVglys. extraction have been recommended. In a study using four drying methods, microwave drying method was suggested as a preferred drying method, which did not show any alteration in Stev. and Reb. A contents of the leaves, maintaining their sweetening properties (Halim et al., 2019). Furthermore, three drying methods have been evaluated about SVglys. production and antioxidant capacity, and it was concluded that the ideal method for drying stevia leaves depends on their final use (sweetener or antioxidant); however, finally hot air drying at 180 ℃ was recommended (Periche et al., 2015).

The response of SVglys. to drying and high temperatures have not been fully investigated and sometimes contradictory results have been observed in the literature. Although a few scholars discussed some effects of the drying methods on stevia leaf compositions, they did not address all available methods and also did not provide a conclusive answer in regard to the effects of drying process of stevia fresh leaves and their compositions. In addition, the quality of SVglys. was hardly considered in the literature. Accordingly, the effects of various drying methods were assessed in the present study with regard to the changes in the SVglys. contents of stevia leave in order to determine a suitable drying method for retention of SVglys. from dried leaves.

Materials and Methods

Stevia leaves were collected from well-grown plants before flowering stage (65 days from transplanting into the soil). The leaves were selected based on their similarity in size, color, and location on the stem. All stevia (*stevia rebaudiana*) plants were grown in similar conditions regarding soil, irrigation, radiation, and fertilizers, in the greenhouse of Shahrekord University. Then, ten leaves were collected from each plant in the middle of the day. The harvested leaves were completely mixed and divided into seven groups and each group had three subgroups as experiment replication. Each group of fresh leaves was dried by a different method including oven (45 ℃ and 75 ℃), shade, infrared, microwave, sunlight, and freeze-dry as detailed below:

The oven method was applied using two hot ovens (GCA, Parsazma) at two temperatures (45±1 and 75±1 ℃) for 48 h. The leaves were put in paper pockets and placed into the oven while the pocket tops were completely open in order to provide normal ventilation.

Shade drying was applied for 5 days and the temperature under the shade varied between 16 - 25 ℃ during the day.

Infrared source for drying was prepared by an electric machine equipped with our faculty. The temperature applied by radiation was 40 ℃ for 45 min.

The microwave drying method was done using a Samsung digital microwave (2450 MHz, 1000W) for 60 Sec. Before the experiment, some preliminary microwave records were conducted to find the desirable method of applying microwave radiation for drying stevia leaves.

Direct sunlight was used for drying leaves for 3 days and the samples were dried by daylight and ambient temperature (daily mean temperature:

Table 1
Analysis of variance for changes in the metabolites of stevia (*stevia rebaudiana*) leaves as affected by various drying processes

Source of variation	df	Means of Squares (MS)				
		Reb A	Reb C	Stev	Total SVglys.	Reb. /Stev. Ratio
Drying methods	6	3**	0.07**	5.7**	16**	0.026**
Error	14	0.58	0.015	1.23	3.5	0.004
Coefficient of variation (CV)	-	20	7.8	17.5	16	11.3
Coefficient of determination (R^2)	-	0.68	0.66	0.65	0.66	0.72

* and ** show significant at P≤0.05 and P≤0.01, respectively.

30 ℃). Totally, the samples were directly exposed to sunlight for 36 h.

A laboratory freeze dryer (VaCo 5-45, ZiRBUS) was used for drying treatment of the leaves for 48h, at a vacuum pressure of $9.1x10^{-1}$mm Hg and -55 ℃.

In all samples, the dried weight (after drying process) reached below 23% of the fresh weight. The dried leaves were powdered in order to carry out the SVglys. analysis.

The SVglys.. extraction was conducted using ethanol as described below (Baroni-nezhad et al., 2021; Zamani et al., 2021). For SVglys. extraction, 10 ml ethanol 70% was added to 100 mg of the powdered leaves and adequately mixed. The mixture was homogenized using a shaker for 24 h. The solution was filtered by a syringe-attached filter (0.45 µm) and kept at 4 ℃ for SVglys. analysis using high performance liquid chromatography (HPLC).

In order to make SVglys. assay, a Knauer HPLC equipped with an NH_2 column (Kromasyl, 5 µm, 100 Å, 25 cm × 4.6 mm) and diode-array detector (DAD) were used (Baroni-nezhad et al., 2021). The mobile phases included distilled water and acetonitrile under isocratic conditions which started from 20:80 of water:acetonitrile (pH:3) in a flow rate of 1 mm/min. The pure Stev. and Reb. A (Sigma, CAS number: 57817-89-7 and 58543-16-1, respectively) were used as standards. The SVglys. absorbance was recorded at 210 nm. The chromatograms area was calculated and converted to SVglys. content based on a pure standard and expressed as leaf dry matter percentage.

The variance of the data was analyzed using SAS 9.4 and the means were compared using the least square differences (LSD). The compared means were presented with graphs prepared from Sigma Plot 14.0.

Results

The results clearly showed that the SVglys. content of stevia leaves was significantly affected by drying methods, as presented in Table 1 (P≤0.05). In addition, it was found that the SVglys. compositions also varied under the drying processes. The highest content of Reb. A was obtained in the freeze-drying method, which did not show a significant difference from the sun drying method (Fig. I. A). The lowest content of Reb. A was observed in the samples dried by oven, showing no significant variation in comparison with those of shade, infrared, and microwave methods. The variation caused by the drying methods showed a different trend in Reb. C, as oven (45 ℃) and shade showed remarkable reduction in Reb. C contents in comparison with the other drying methods (Fig. I. B). Variations in Stev. contents were close to those of Reb. A variation, responding to the drying method treatments. Similarly, it was observed that the oven and shade treatments had the lowest Stev. contents while freeze-dry, sun, microwave, and infrared drying methods kept high content of Stev. in stevia leaves (Fig. I. C). The total detected SVglys. content was significantly affected by drying methods and remarkable contents of the total detected SVglys. were observed in drying treatments with freeze-dry, sun, microwave, and infrared methods in this order while the oven and shade methods resulted in the reduced SVglys. contents of stevia leave approximately by 30%, in comparison with the other drying methods (Fig. I. D).

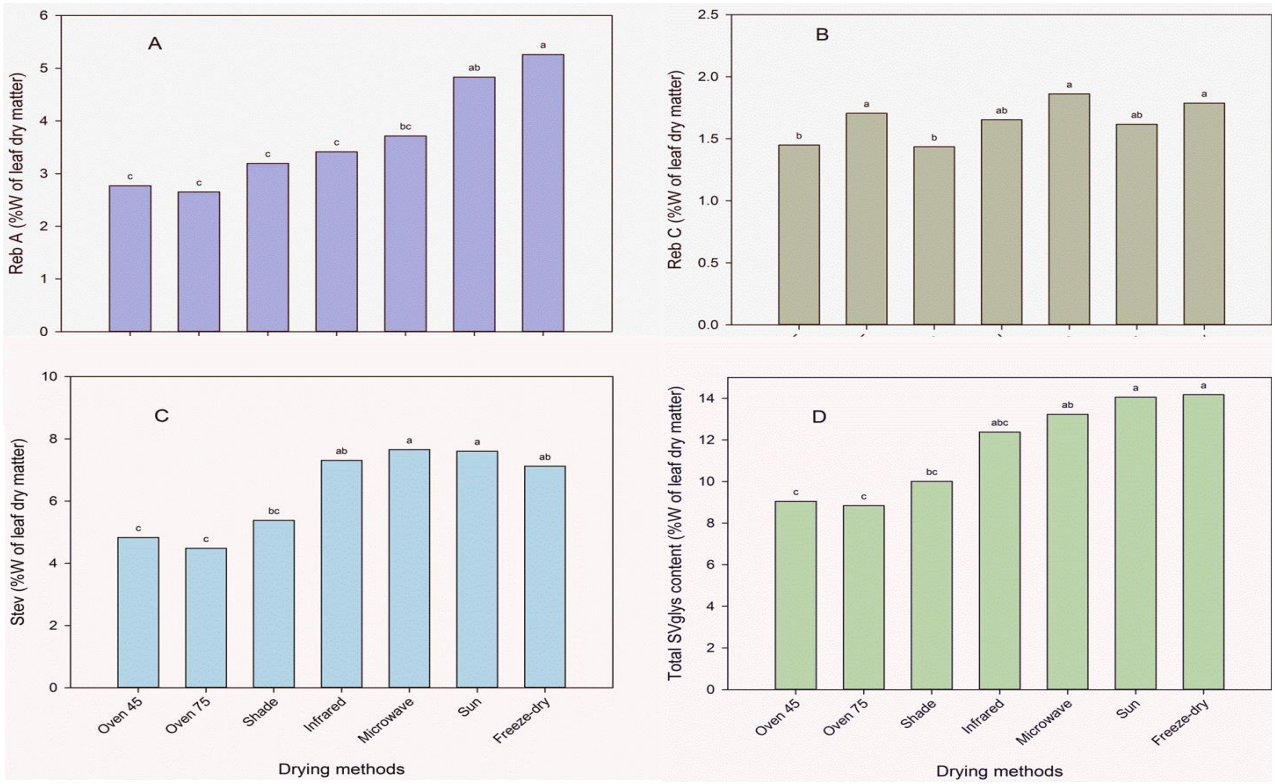

Fig. I. The variation of the Rebaudioside A (A) , Rebaudioside C (B), Stevioside (C), and total steviol glycosides (D) under different drying methods; The comparison of means has been done using least significant difference (LSD) method and the same letters show a statistically non-significant difference among treatments.

The ratios of SVglys. compositions, especially Reb. A to Stev., were changed by the drying methods, as presented in Reb. A/Stev. ratio (Fig. II). A desirable value of Reb. A/Stev. ratio was found by leaf drying treatments through freeze-dry and sun drying methods, which significantly was higher than Reb. A/Stev. ratios in oven, shade, infrared and microwave drying methods. The lowest ratios of Reb. A/Stev. were observed in oven (45 ℃), infrared, and microwave drying methods.

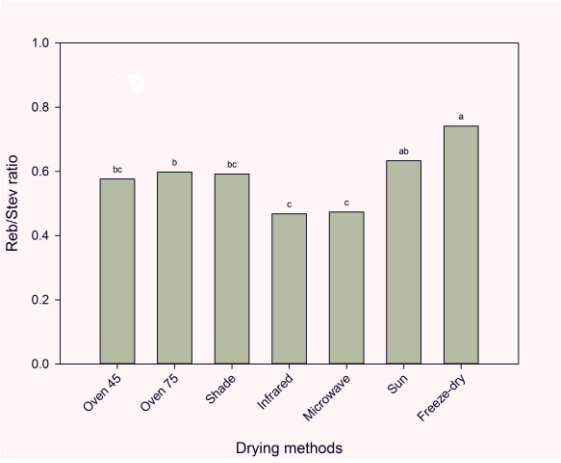

Fig. II. The variation of the Rebaudioside A/Stevioside (Reb A/Stev) ratio under different drying methods. The compare means have been done using least significant difference (LSD) method and the same letters statistically show a non-significant difference among treatments.

Discussion

Drying is the first step in processing stevia leaves for SVglys. extraction. Therefore, the drying method can affect the SVglys. yield and extraction, as demonstrated in our results. Migration of water from cells and tissues due to the drying process induces changes in pH, oxidation-reduction potential, and ionic strength (Lewicki, 1998), which may change the reactions and metabolites. Also, cell disruption (shrinkage) caused by drying and undesirable temperatures is an already known phenomena, and the disruption may depend on the drying time period and intensity. Hence, under a slow drying process, enzymatic reactions can be continued during the drying process (Lewicki, 1998), which enable it to modify the leaf metabolites. Some methods such as freeze-dry seize the opportunity for biochemical reactions and somehow stabilize the cell metabolites. In this

study, freeze-drying seems to have inhibited the adverse biochemical reaction on the harvested leaves and resulted in a high yield of SVglys. and components. The solid state of water during freeze-drying protects the primary structure and the shape of the products with a minimal volume reduction. In addition, the lower temperatures in the process allow maximal nutrient and bioactive compound retention (Bhatta et al., 2020). Considering the literature and also our results, it seems that these processes have been properly occurred for stevia dried leaves by the freeze-drying method. Furthermore, the effects of freeze-drying on fresh leaves can be attributed to lowering the cell respiration (Haard, 1984) which may lead to a loss inhibition of the carbohydrates of leaf. In fact, respiration losses of metabolites can be inhibited. It should be noticed that most of the small carbohydrates such as glucose are involved in the SVglys. structure. The slow drying process slows down respiration, but does not eliminate the respiration completely (Haas et al., 1974). In a similar experiment, it has been reported that freeze-drying maintained the SVglys. contents of the dried leaves of stevia in an acceptable value (Periche et al., 2015). Contrary to freeze-dry and sun-drying methods, in our study it was clearly found that oven drying and shade drying methods were inefficient in SVglys. retention from stevia leaves. This finding was more remarkable, especially in Reb. A, as similarly reported by Halim et al (Halim et al., 2019), who found that freeze-drying and sun-drying resulted in the highest values of Reb. A compared to drying through microwave and oven (80 ℃). The main reason for the inefficiency in SVglys. yield under oven and shade methods is not clear, but the indirect sunlight, ambient humidity, and probably the activities of microorganisms can be involved. In a similar study, freeze-drying of stevia leaves was found significantly more efficient than the shading method in regard to Stev. content (Periche et al., 2015).

Pertaining to Stev. content, our findings lend support to the freeze-drying, sun drying, microwave, and infrared drying methods, in that order. This is a very important finding since Stev. is the most abundant type of SVglys. in stevia leaves. Formerly, freeze-dried leaves were reported to led to high Stev. Retention, and considering the composition of SVglys. as affected by the drying methods, it has been concluded that the least aggressive treatment was shade drying, which is compatible with our results (Halim et al., 2019). However, in the same study, drying the stevia leaves under microwave radiation (1200W) for three minutes was recommended for retention of sweeteners and antioxidant activity. This conclusion may be adopted due to high costs of freeze drying and also low volume of stevia crop. In addition, the microwave drying is much faster than freeze-dry method. In our study, the microwave drying method was the top method in total detected SVglys. contents and yield. It is believed that microwave drying allows for a low water activity (Chong and Lim, 2012), and the high content of SVglys. dried by this method can be attributed to the low water in the dried samples.

A general increase in the content of SVglys. has been observed by Lemus-Mondaca et al (Lemus-Mondaca et al., 2016) as the drying temperature was increased from 30 to 80 ℃, but this does not hold true in higher temperatures as well as in fast driers. In addition, the stability of Stev. under higher temperatures (up to 120 ℃ for 1 hour) has been observed in a study by Kroyer (2010), which justifies some Stev. behaviors in our trial. Meanwhile, there is a probability that the stability of Stev. in the harvested leaves might be affected by the drying time period and also biochemical reactions. For example, the transformation of Stev. to isosteviol through modification in pH and biochemical reactions has been frequently reported (Avent et al., 1990; Hsu et al., 2002; Karimi et al., 2014; Xu et al., 2007), which may change the SVglys. content and yield during the drying process. Likewise, the participation of SVglys. in antioxidant activities in water limited cells has also been reported (Javed et al., 2018; Karimi et al., 2016; Kovačević et al., 2018; Pandey and Chikara, 2015; Srivastava and Srivastava, 2014), and probably under a long drying time period it can consume the SVglys. derivatives in order to scavenge the radical oxygen species (Bender et al., 2015; Criado et al., 2014; Hajihashemi and Geuns, 2013). However, the post-harvest physiological study of stevia leaf can shed more light on the issue.

Our results demonstrated that the drying methods are able to change the SVglys. compositions and consequently change their quality, as Reb. A/Stev. ratio was affected by these treatments. Reb. A/Stev. ratio contributes to quality of the SVglys. as higher ratio means higher quality and sweetness. Therefore, this ratio is very important for stevia producers. In this regard, oven with lower temperature, microwave and infrared should be avoided in stevia leaf drying while freeze-dry and sun-drying can be preferred. The note is that Reb. A had a higher correlation with Reb. A/Stev. ratio, which means a high dependency of this ratio to Reb. A. In fact, Stev. was less affected by the drying methods, in comparison with Reb. A.

Conclusion

Our results clearly showed that the quality and quantity of the sweeteners of stevia leaves can be affected by the drying process. Although, the freeze-dry method showed the best function pertaining to stevia leaves drying and SVglys. retention, this method is applicable only on an experimental scale and is not easy for application on farm scales due to the relevant technology, costs, and the period of time required for drying. Accordingly, the sun drying method is concluded as a suitable method for stevia leaf drying, because it resulted in a negligible loss in the valuable sweet compounds in stevia leaves during the drying process. This method can also be recommended for economic purposes because some other methods like microwave and infrared incur more costs for stevia growers

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