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Do the different drying conditions affect the sweet compounds in Stevia rebaudiana?

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Abstract
The major steviol glycosides (stevioside and rebaudioside-A) content in *Stevia rebaudiana* leaves, dried under three different conditions (sun, shade and oven drying) were investigated. Leaf samples were refluxed four times with methanol, followed by extract colour removal through warming it with dichloromethane followed by subsequent cooling. Estimation of stevioside and rebaudioside-A were performed through HPLC analysis of dichloromethane insoluble leaf extract. There was a non-significant difference in stevioside and rebaudioside-A content of the leaves dried under such conditions. However, maximum stevioside content (8.25 %) was observed in leaves dried under sun and minimum (8.04%) under oven drying conditions. Whereas, highest rebaudioside-A (7.50%) was found under oven drying and minimum (7.06%) in shade drying conditions.

Keywords: stevioside, rebaudioside-a, drying, HPLC, stability

1. Introduction
Sugar is the commonly used natural sweetener in almost every sweetening product. Despite being a natural sweetener, excessive intake of sugar leads to development of certain chronic diseases mainly diabetes, obesity and cardiac diseases etc. Sugar plays a major role in the production of thousands of food products from cured meats, frozen fruits to confectioneries. Besides having some merits as a food preservative, sugar has demerit of decomposition on heating at pH of 9 or more. So there is a need for an alternative to sugar, which can reduce the calorie intake to support good health. Stevioside and rebaudioside-A are such known alternative sweetening compounds extracted mainly from the leaves of *Stevia rebaudiana* Bertoni, a perennial shrub indigenous to Northeastern part of Paraguay belonging to Asteraceae family (Grembecka, 2015) [1]. In addition to stevioside and rebaudioside-A, few other diterpenoid glycosides have also been isolated from the plant viz. steviolbioside, rebaudioside-B-F, dulcoside-A and B and rubusoside (Staratt *et al*., 2002; Chaturvedula *et al*., 2011; Lorenzo *et al*., 2014) [2, 4]. Stevioside and rebaudioside-A are the two major steviol glycosides, which are responsible for imparting low calorie sweetening property to the shrub. Stevioside is major sweet compound but having bitterness and is about 300 times sweeter than sucrose (Giuffre *et al.,* 2013) [5]. Whereas, rebaudioside-A is present in lower amount with more sweetness (1.2 to 1.6 times of stevioside) comparative to stevioside (Kinghorn and Soejarto, 1985) [6]. Moreover, rebaudioside-A had been generally recognised as safe (GRAS) by US FDA since 2008.
Medicinal plants after being harvested at their proper growth and development stage are required to be carefully dried and stored under controlled conditions till the stage of processing. Drying and storage become necessary because many times the herb cannot be immediately processed due to the processing site being either far off or because of heavy rush of raw material at the processing unit. Drying is an important post-harvest technique for almost all the herbs to be converted into useful products. It is the simple and basic approach to maintain quality of medicinal and aromatic plants by increasing their shelf life (Rocha *et al*., 2011) [7]. In some previous reports by Stafford *et al*., 2005 [8] and Lim and Murtijaya, 2007 [9] drying have been reported to influence chemical composition and bioactive compounds of the plants. In *Valeriana* species, root (economic part) drying at low temperature with forced air supply has been recommended to avoid decline in valepotriates content (Lutomaski and Turowska, 1973) [10]. Similarly, immediate drying of *Hypericum perforatum* leaves after

collection was suggested at 40-60ºC to avoid loss of hypericin content (Bomme, 1997) [11]. In some studies, drying was observed to increase bioactive chemical of certain herbs (Yousif *et al*., 1999; Diaz-Maroto *et al*., 2002) [12, 13]. In case of *Stevia rebaudiana*, fresh stevia leaves contain about 80-90 per cent of moisture and will deteriorate fast if not dried properly. Moreover, active chemical content may undergo degradation during drying, storage and processing of plant material due to enzymatic or microbial reactions (Patil and Laloraya, 1983) [14], moisture, light and heat etc. So, the objective of the present study was to investigate the effects of different drying conditions (sun, shade and oven drying) on the stevioside and rebaudioside-A content of *S. rebaudiana* leaves.

2. Materials and methods

The experiment was carried out at Forest Products laboratory of Department of Forest Products, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan (Himachal Pradesh) situated between 30º 50' 30" to 30º52' 0" N latitude and 77º 8' 30" to 77º 11' 30" E longitudes and an altitude of 1200-1300 meters above msl.

2.1 Collection procedure and post-harvest approaches for plant material

Leaf samples of *S. rebaudiana* (pre-flowering stage) were collected from the plants raised in experimental farm of the Department of Forest Products, Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan (Himachal Pradesh). Fresh leaf samples of 100 grams were taken for each treatment. All the leaf samples were simultaneously kept for sun, shade and oven drying and their weights were recorded three times a day (10 AM, 1 PM and 4 PM). Recording of weights was continued till samples showed constancy in weights. Oven drying was carried out at 60ºC.

2.2 Chemicals and reagents used

Methanol and dichloromethane of analytical grade of S.D. fine chemicals and Thomas Baker brands were used for the extraction of stevioside and rebaudioside-A from the plant leaves. Acetonitrile and water in the ratio of 80:20 (CDH and SRL brands) were used for carrying out estimation of stevioside and rebaudioside-A. Reference stevioside and rebaudioside-A were procured from Life Technologies (India) Pvt. Ltd. located at Pitampura Delhi.

2.3 Soxhlet extraction of stevioside and rebaudioside-A

Dried leaf samples of each treatment were powdered. The powdered leaves (0.5 g) of *S. rebaudiana* were refluxed with methanol (25ml; 1 hr) on a water bath. The extract was filtered and residue so obtained again refluxed with methanol (25ml; 1hr). Same procedure was repeated for four times. After fourth refluxion, the residue was found non-sweet. The filtrate obtained after each refluxion was mixed together, methanol distilled off and residue finally vacuum dried. Further, the residue was mixed with dichloromethane (20 ml) and warmed on a water bath. The residue was allowed to cool for some time, contents were filtered. The process of mixing dichloromethane to residue followed by cooling was repeated till it become colourless. The dichloromethane insoluble residue was then completely dried under vacuum (Kumari *et al*., 2016) [15].

2.4 HPLC conditions

Analytical estimation of the steviol glycosides (stevioside and rebaudioside-A) was harmonized on a binary waters HPLC unit with waters HPLC pump 515 and dual absorbance detector 2487. Waters 5 µm amino column (4.6 mm x 250 mm) was used for separation of steviol glycosides. The detector was set-up at UV 210 nm. The data acquisition was done using a Empower software 2. Mobile phase comprised of HPLC grade acetonitrile: water (80: 20). Millipore filter papers were used for filtering mobile phase solvents (GVWPO 4700, 0.22 µm) and plant extracts (GVWPO 1300, 0.22 µm). After filtration the mobile phase solvents were separately degassed on a sonicator and then mixed (Acetonitrile: water: 80: 20).

2.5 HPLC analytical procedure

HPLC estimation of stevioside and rebaudioside-A was carried out as per the previous study by Kumari *et al*., 2016 [16]. Amino column was flushed with HPLC grade degassed acetonitrile: water (80:20) for 30 minutes. Dried dichloromethane insoluble extract was dissolved in acetonitrile: water (25ml), filtered through millipore filter paper and 20 µl volume was injected into amino column. However, before injecting the sample solution, standards (stevioside and rebaudioside-A) were injected to notice their retention time and thus to find the exact stevioside and rebaudioside-A peak on sample chromatogram.

Percentages of stevioside and rebaudioside-A were calculated using the following formula:

$$\textbf{Sweet compound (\%)} = \frac{\text{Test area}}{\text{Standard area}} \times \frac{\text{Weight of standard compound}}{\text{Standard compound dilution}} \times \frac{\text{Test sample dilution}}{\text{Test sample weight}} \times 100$$

2.6 Statistical Analysis

The experiment was laid out as complete randomized design (CRD) with seven replications. For statistical consideration and analysis of variance (ANOVA), SPSS software V-16 was used.

3. Results and Discussion

Analysis of *Stevia rebaudiana* leaf samples, dried in the sun, shade and oven showed non- significant difference in stevioside and rebaudioside-A content (Table 1). However, maximum stevioside content (8.25%) was observed in leaves dried under sun and minimum (8.04%) under oven drying conditions. Whereas, highest rebaudioside-A (7.50%) was found under oven drying and minimum (7.06%) in shade drying conditions. HPLC analysis of the standard compounds of stevioside and rebaudioside-A showed a retention time of 7.410 and 10.216, respectively (Figs. 1-2). HPLC chromatograms of leaf samples dried under sun, shade and oven conditions are presented in Figs. 3 to 5.

Table 1: Variation in stevioside and rebaudioside-A contents (%) in leaves of *S. rebaudiana* dried under different drying conditions

Drying conditions	Stevioside content (%)	Rebaudioside-A content (%)
Open sun	8.25(2.87)	7.12(2.67)
Shade	8.04(2.83)	7.06(2.66)
Oven	8.10(2.85)	7.50(2.73)
$CD_{0.05}$	*NS*	*NS*
SE±	*0.02*	*0.03*

Values in the parenthesis are transformed values using square root transformation

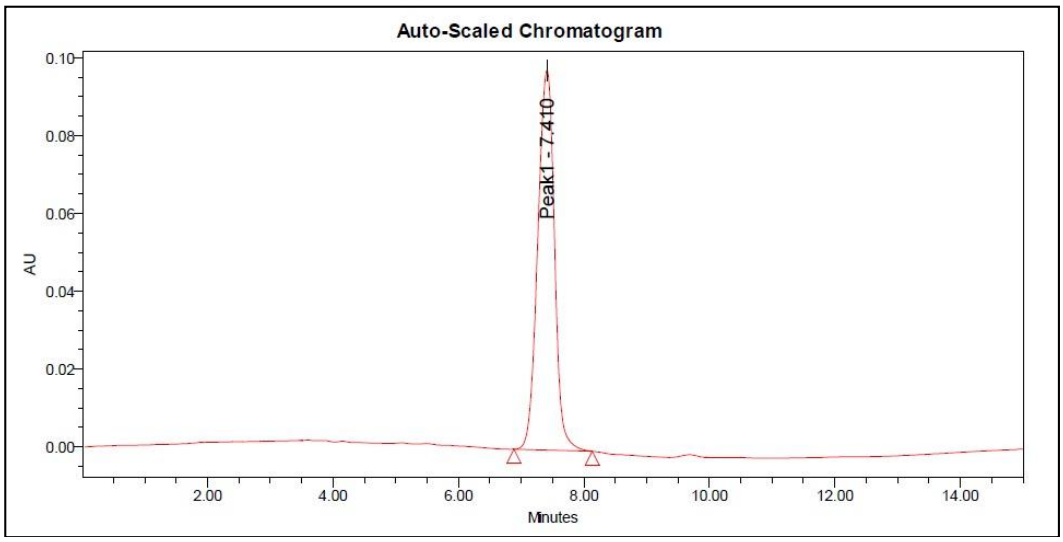

Fig 1: HPLC chromatogram of reference stevioside

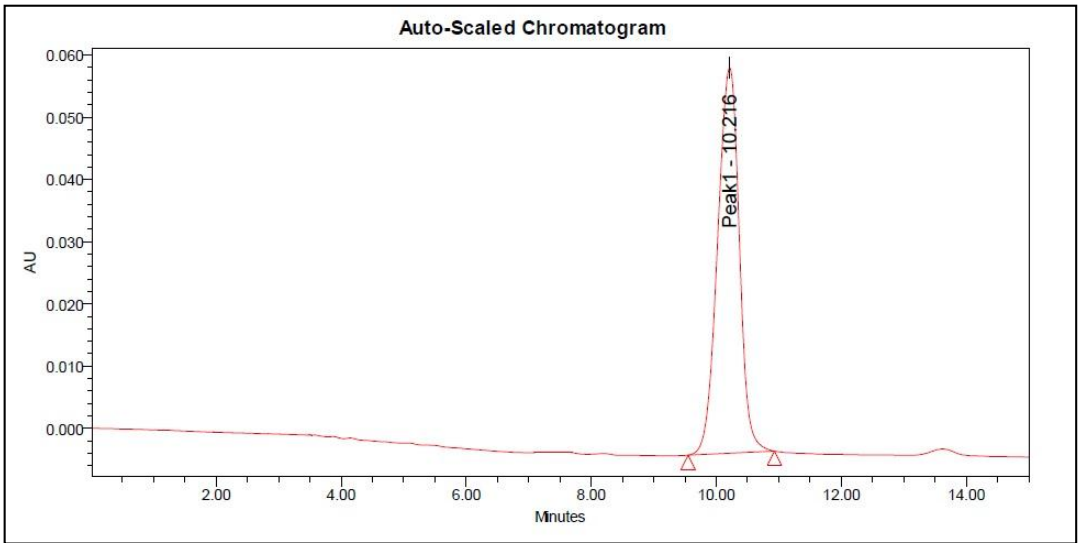

Fig 2: HPLC chromatogram of reference rebaudioside-A

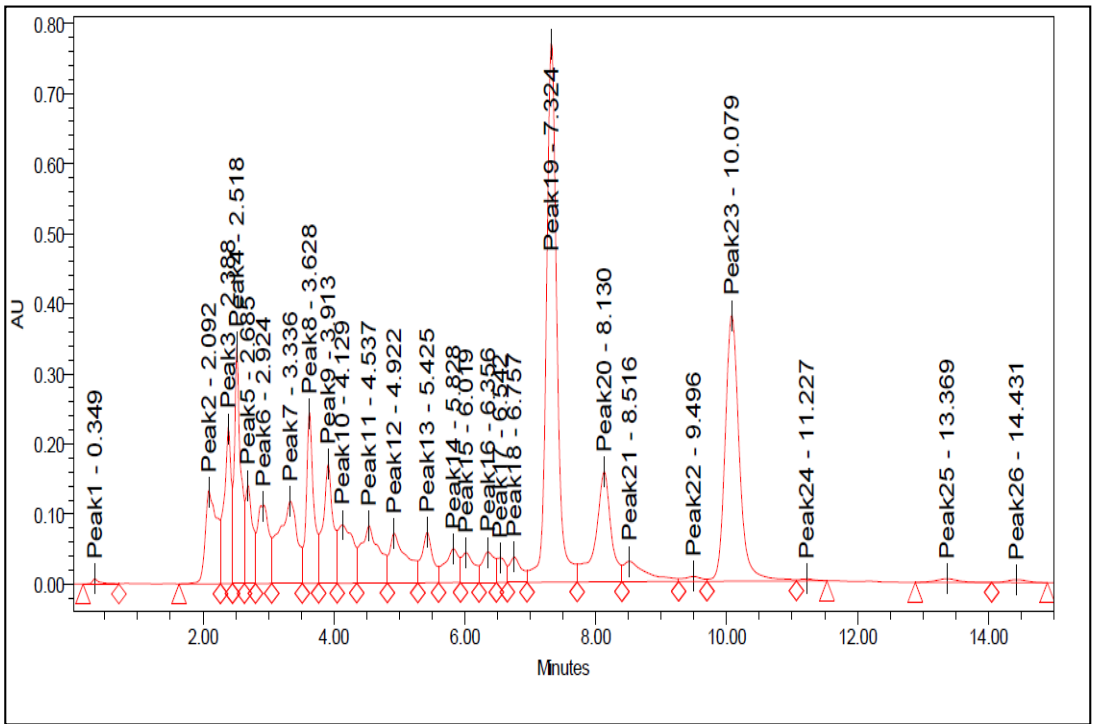

Fig 3: HPLC chromatogram of open sun dried leaf samples of *S. rebaudiana*

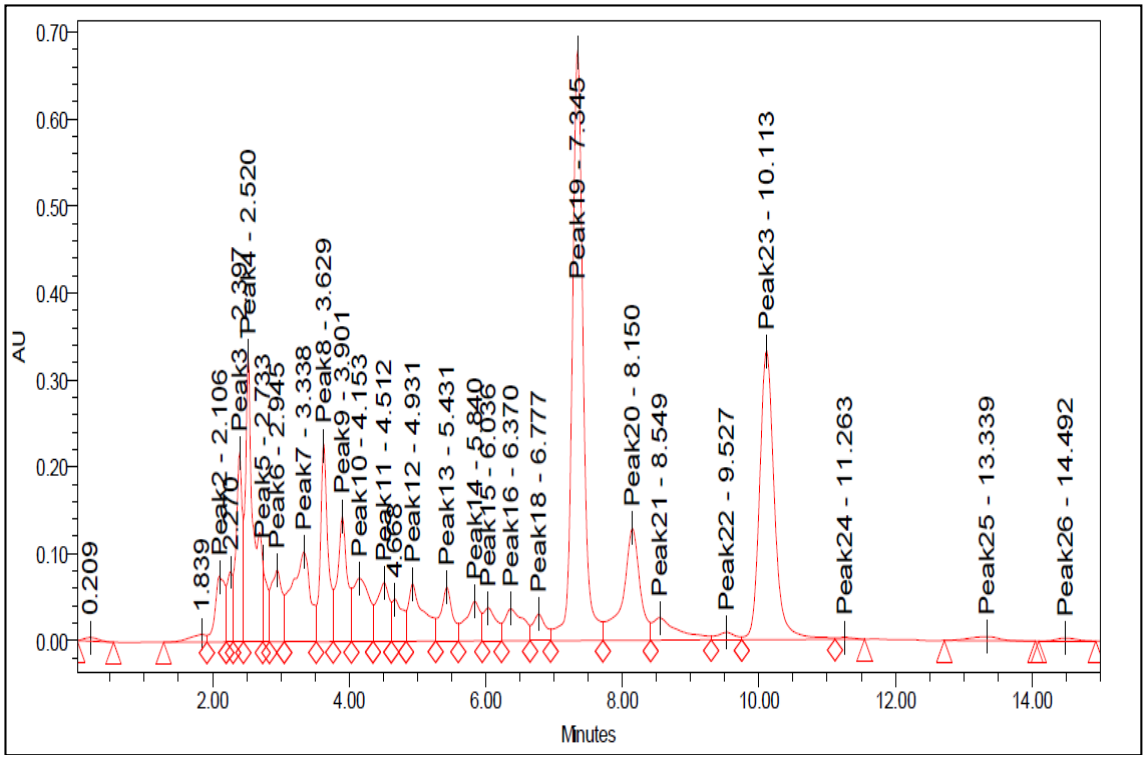

Fig 4: HPLC chromatogram of shade dried leaf samples of *S. rebaudiana*

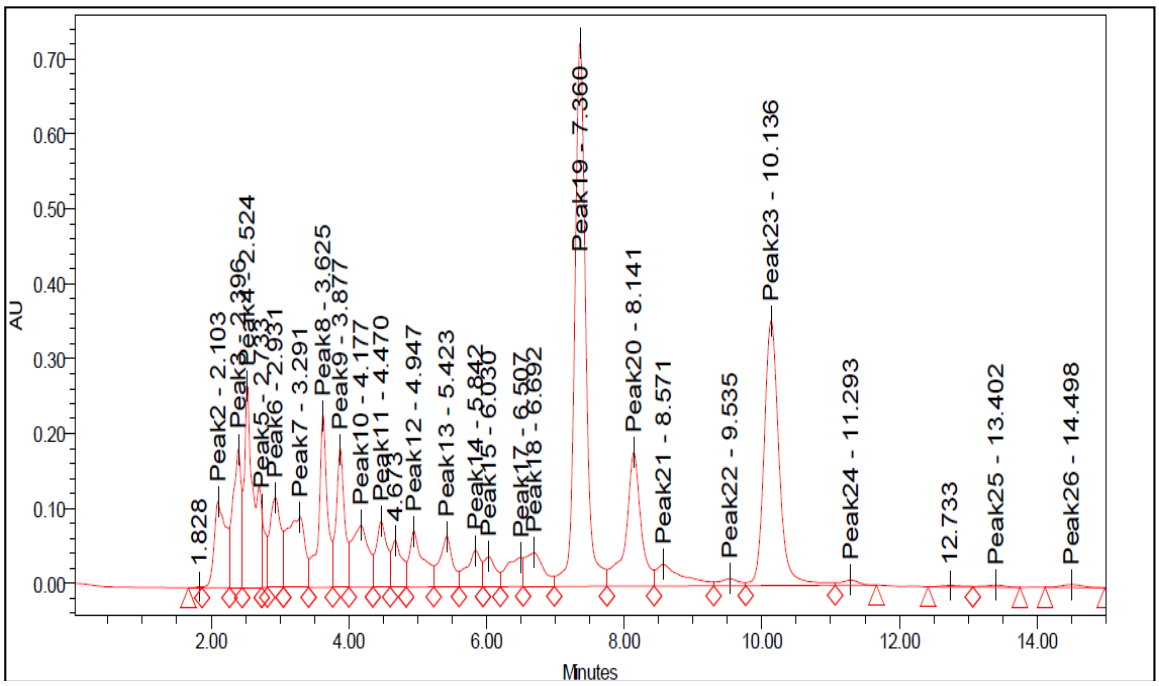

Fig 5: HPLC chromatogram of oven dried leaf samples of *S. rebaudiana*

The reason behind the non-significant difference in stevioside and rebaudioside-A content appears to be photo-stability (Clos *et al*., 2010) [17] and thermo-stability of stevioside and rebaudioside-A at an elevated temperature ranging from 100-200 °C (Abou arab *et al*., 2010; Serio et al., 2010; Mondaca *et al*., 2012) [18-20]. However, on the contrary, stevioside have been reported more stable than rebaudioside-A in carbonated beverages (Chang and Cook, 1983) [21]. Later on, the study was repeated since outcome was unexpected pertaining to almost same chemical structure of stevioside and rebaudioside-A (Clos *et al*., 2010) [17]. Clos and his co-workers found no degradation of either stevioside or rebaudioside-A in cola and lemon-lime beverages on exposure to sunlight. Moreover, stevioside content in stevia leaves was reported to decrease on oven drying of leaves of three varieties (Spanti, Egy1 and China1) of *Stevia rebaudiana* at 60°C (Khalil *et al*., 2015) [22]. As such, we cannot compare our findings with that of Chang and Cook (1983) [21], Clos *et al*. (2010) [17] and Khalil *et al*. (2015) [22], as the material used were different in all the cases. Former two studies worked on finding out the stability of stevioside and rebaudioside-A content in long termed stored beverages and rebiana (>97% pure rebaudioside A) sweetened beverages respectively. Whereas, Khalil *et al*., 2015 [22] observed the effect of open air, microwave and oven drying on the stevioside content of three varieties (Spanti, Egy1 and

China1) of *Stevia rebaudiana*. However, Findings of the work done by Clos *et al*. (2010) [17] supported the study in terms of stability of stevioside and rebaudioside-A in carbonated beverages on their exposure to sunlight.

4. Conclusion

Different drying methods have a non-significant effect on the stevioside and rebaudioside-A content of *S. rebaudiana*. As per stevioside is concerned, it was maximum in sun dried leaves and minimum in oven dried leaves. While, rebaudioside-A was found maximum in oven dried leaf samples and minimum in sun dried leaves.

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