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Extraction and isolation of stevioside and rebaudiana A from *Stevia Bertoni* leaves

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Stevia rebaudiana Bertoni leaves are a natural source of diterpenic glycosides and various bioactive compounds. Over the past two decades, this plant has garnered substantial scientific and industrial interest as a potential nutritional alternative to sugar. Notably, stevia contains glycosides, particularly stevioside (ST) and rebaudioside A (Reb-A), which are of significant interest for their applications in the production of sweeteners, nutraceuticals, and functional foods. This study systematically examines the influence of key extraction parameters, including plant material size, solvent type, solid-to-liquid (S/L) ratio, and agitation, on the extraction efficiency of ST and Reb A. The optimized extraction methodology developed in this study yielded 42.07 % of the total extract from stevia dry leaves. These findings provide valuable insights for enhancing the efficiency of the extraction and isolation processes, facilitating their potential scale-up for industrial applications.

Keywords: Stevia, stevioside, rebaudiana-A, extraction, isolation, purification.

INTRODUCTION

Stevia Rebaudiana Bertoni, a sweet hub from South America, is being cultivated around the world and used as a low-calorific natural sweetener in food and beverage industries as it is 200-300 times sweeter than sugar [1]. Its sweetness is mainly due to two steviol glycosides, namely stevioside (ST) and rebaudiana A (Reb A) [2]. There are various conventional or green extraction methods reported to extract ST and Reb A from stevia leaves [3-7]. In a recent publication, different green methods such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and supercritical fluid extraction (SFE) were used to extract ST and Reb A from stevia leaves [8]. In green extraction technology, a solvent such as water, methanol, isopropyl alcohol, or glycerol was used to yield the key components in the extract solution [4, 6, 9-11]. In recent publications, researchers have also used a binary solution of different alcohols for better extraction yield [3, 5, 6, 12-14]. The highest steviol glycoside extracted in the extract phase reported for green extraction is 26.91 g/100g stevia leaves using pressurized liquid extraction using 70% ethanol as a solvent at process conditions of L/S = 30 mlg^{-1}, 60 min, 125 ^{o}C and 10.0 MPa [15]. Most of the green extraction methods used for extraction of ST and Reb A reported in literature haven't used any further unit operation to get steviol glycoside in crystal form which is essential, as it is used as a natural sweetener in the food industry. Most of the authors of research work related to green extraction methods haven't included any isolation or purification steps to get the product in the crystal form. However, many researchers have used the conventional extraction method followed by various isolation steps to get steviol glycoside in the crystal form [16-20]. Moreover, all the green extraction methods require high initial capital costs and can't be operated continuously and hence are difficult to be scaled up at industrial level [21].

After extraction of steviol glycosides from stevia a crude extract was obtained which was foul-smelling, bitter-tasting, and dark brown, unable to be used directly in food products. Therefore, successive purification is necessary for developing a product of commercial quality (90% purity and up). Purification of stevioside often involves processes such as inorganic salt treatment, ion exchange separation, columns, solvent liquid extraction, ion exchange, ultra-membranes, nanofiltration, crystallization and fractional distillation [22-28]. There are few publications involving conventional extraction and modern purification steps which were based on size difference (membrane separation), charges (ion exchange), solubility (crystallization)

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and polarity (chromatography) available in the literature. [16, 19, 20, 29-33]. In a recent publication, 4.15 g of dry extract of steviol glycoside was obtained from 10.5 g initially present in 100 g of stevia leaves which have 39.52 % overall efficiency [33]. The extraction and isolation of pure steviol glycosides, as reported in the literature, are often labor-intensive, costly, and energy-demanding. Certain isolation methods for steviol glycosides generate harmful residues and bitter alkaloids, which can negatively impact the quality and taste of the sweet glycosides [20, 34]. Despite the significant advancement in extraction and purification, the production of pure steviol glycosides that are scalable, is still complicated [19]. Thus, there was a need to have a combination of extraction and isolation steps to get ST and Reb A from stevia leaves which are scalable at an industrial scale. In this work, the combination of extraction and isolation steps was developed to get the ST and Reb A in crystal form.

MATERIALS AND METHODS

Materials

Stevia leaves which wre a raw material for the process were purchased from local farmers in Gujarat, India. Leaves were dried in shade for two days and moisture content was kept close to zero before being grounded to 400-500 µm. The standard HPLC grade ST (85 %) and Reb-A (98%) were purchased from TCI Chemical, India. The HPLC-grade solvents such as water, butanol, methanol and isopropyl alcohol have been purchased from Alpha Chemika, India.

Effect of different solvents on extraction

A 5 g sample of dried grounded stevia leaves was taken in a 100 mL beaker with solvent as per the required solid-to-liquid ratio (1:10, 1:15, 1:20 and 1:25.). It was kept for maceration for 24 h. After 24 hours the sample was filtered by vacuum filtration. After experimenting with extraction using different solvents, the 2 mL sample was taken and sent for HPLC analysis.

ST and Reb A content in stevia leaves

The content of ST and Reb A in stevia leaves was determined by the reflux extraction method. The stevia powder of 50 g was extracted with 750 mL of water using the reflux extraction method for 8 h. After extraction, the mixture was cooled, and filtered by vacuum and 5 mL of the sample was analyzed by HPLC analysis.

Effect of agitation on extraction

The extraction was performed in a glass beaker of 1 L capacity filled with 750 mL of water as a solvent. An overhead stirrer was used at 100 rpm. The temperature was controlled through an automatic PID temperature-controlled water bath (15 L) for extraction and kept at 80 °C. 50 g of dried stevia powder with a size of 400-500 µm was added to the beaker. The extraction was performed for 4 h and the mixture was cooled and filtered using vacuum filtration unit. A sample of 2 mL was taken for HPLC analysis. The procedure above was repeated at different speeds (100 rpm, 200 rpm, 300 rpm, & 400 rpm) to study the effect of agitation on extraction.

Extraction technology

A powdered sample of 130 g was used for extraction with 1950 mL of water as a solvent at 80 °C for 4 h. The aqueous extract was cooled, filtered under vacuum (600-620 mm Hg) and further processed by electrocoagulation to remove chlorophylls which give a green color to the extract. In this step, a direct current (15 V, 0.8-1.2 A) was passed for 1 h *via* two pairs of aluminium plates as electrodes and 15 g NaCl was added as an electrolyte. The resulting mixture was again vacuum filtered and the process of electrocoagulation was repeated once more to remove all chlorophyll. The resulting solution was passed through 7.36 g of activated charcoal. Further, cation and anion resins were used to remove dissolved ions like Na, K, Ca Mg, and P from the solution. After this operation to filter the solution celite was used as a filter aid, which was found more effective to be used for filtration. The extract was preconcentrated by vacuum evaporation. For crystallization, butanol was used as an anti-solvent to obtain crystals. After each process step, samples were taken and sent for HPLC analysis. A material balance was performed in each stage. The extraction recovery of the extraction technology was calculated using the following equation:

$$\%Yield\ (Extraction\ Recovery) = \frac{content\ of\ ST\ and\ Reb\ A\ in\ crystal\ in\ gram}{content\ of\ ST\ and\ Reb\ A\ stevia\ dried\ leaves\ in\ gram} \times 100. \quad (1)$$

HPLC method

The chromatographic method was carried out on a C18 (2) (length: 250 mm; inner diameter: 4.6 mm, particle size: 5 µm) column without temperature control with a UV-Vis detector set to a wavelength of 210 nm. The mobile phase was a 32:68 (v/v) mixture of acetonitrile: water and 10 mmol/L sodium phosphate buffer (pH 2.6) at a flow rate of 1 mL/min.

The sample injection volume was 20 µL and HPLC analysis was performed [35].

RESULTS AND DISCUSSION

Effect of different solvents on extraction

Many popular solvents such as isopropyl alcohol, methanol, water and butanol were used for extracting ST and Reb A from stevia leaves in this study. The extraction was carried out at room temperature and the duration of extraction was 24 h. The % extraction recovery of ST and Reb A from stevia leaves using different solvents at different S/L ratios is shown in Fig. 1. For the lower S/L ratio, less amount of solvent for extraction was observed. It dissolved lesser phytochemicals from the plant material. With increasing S/L, the concentration gradient for the diffusion of solute from leaves to solvent increases and hence liquid extraction efficiency increases. The results, summarized in Table 1, demonstrate that methanol achieved the highest extraction recovery across different solid-to-liquid ratios, highlighting its superior solvent efficiency. However, methanol is costlier, more volatile compound than water. Since methanol has a boiling point of 64.7 ºC, Extraction cannot be operated beyond 60 ºC for methanol due to its low boiling point. Since ST and Reb A can remain stable up to 90 ºC for a large pH range[36], water can be used to extract more phytochemicals from the plant material at higher temperatures as rising in the temperature, the diffusivity of solute was increased in solvent for extraction.

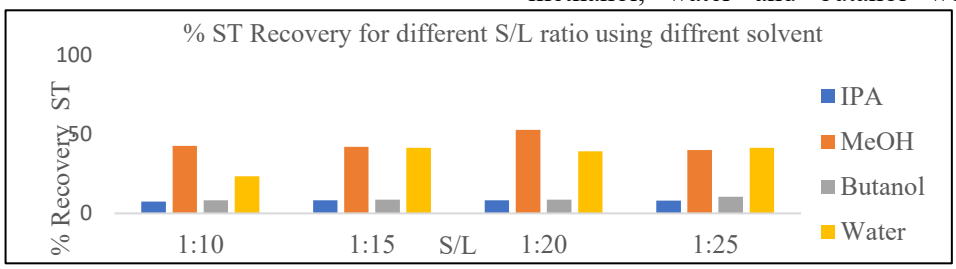


Figure 1. Extraction of ST using (A) IPA (B) n-butanol, (C) water (D) meOH

Table 1. Best S/L ratio for extracting ST and Reb A using the maceration method

Solvent	Best S:L ratio	% Reb A recovery	% ST recovery
IPA	1:15	5.61 %	8.32 %
n-butanol	1:25	7.29 %	10.47 %
Water	1:15	47.05%	41.27 %
Methanol	1:20	56.23 %	52.68 %

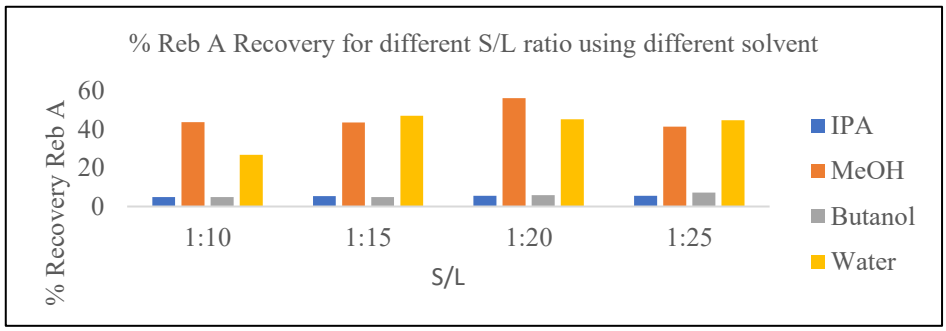


Figure 2. Extraction of Reb A using (A) IPA, (B) n-butanol, (C) water (D) MeOH

Effect of size of particle size on extraction

In this study, extraction experiments were carried out on different sieve sizes of the stevia leaves powder (120 µm,200 µm, 332 µm, 404 µm, 812.5 µm) and also for whole leaves while other extraction parameters remained the same (S/L=15, Temperature = 70 ºC, Time =3 h). In this work, the effect of different size of stevia leaves on the recovery extraction of ST and Reb A were studied using water as a solvent. Fig. 3 shows the effects of different particle sizes on extraction recovery. The result shows that the extraction yield sharply increases until the 200 µm size. Particle size below extraction recovery remains almost constant. The extraction recovery was not increased beyond 200 µm size. This suggests the internal mass transfer remains the rate-limiting step until the size of 200 µm. By reducing the size of the particle, the surface area can be increased Which reduces the diffusion path for mass transfer resulting in increased

extraction. It also helps to rupture cells of the leaves and hence increases the efficiency of extraction. Fig. 3 also suggests that the extraction of unwanted phytochemical increases if size of the stevia leaves decreases. Similar results were found in the literature [37].

Effect of agitation on extraction

In this work, to study the effect of agitation on extraction, the extraction of stevioside and Reb A from stevia leaves to powder using water at the agitation of 300 RPM and without agitation, was performed and compared. During the extraction, in the absence of agitation, the settling of the stevia powder was observed during the experiment. The comparison of recovery of extraction for 300 RPM and without agitation is given in Fig. 4. From Fig. 4, it can be observed that the extraction recovery for 300 RPM is higher than extraction without agitation. During the extraction, the solute transfer was done in three steps. (1) washing (2) internal diffusion (3) external diffusion. In internal diffusion, solvent reaches through the pores of the plant material, dissolved the solute and diffused out near the solid surface.

However, during the external diffusion, solute was transferred from the surface of the solid to the bulk of the solvent. The experiment at 300 RPM was conducted to ensure the turbulent region outside the solid material (N_{Re} > 10,000). This agitation effect has created no resistance to external diffusion. Hence, the overall extraction efficiency has increased. However, extraction rates seem to remain highest at 300 rpm. Beyond 300 rpm, in this work, internal diffusion seems to be the controlling step.

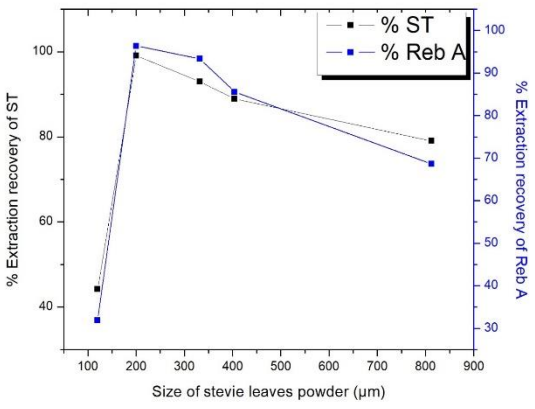
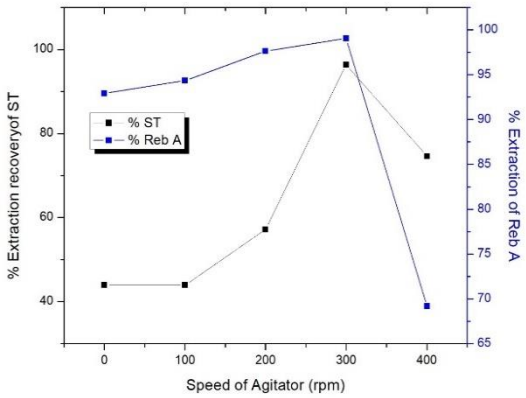


Figure 3. Effect of size of stevia leaves powder on the extraction of ST and Reb A at 80 °C

Effect of S/L ratio on extraction

For different S/L ratios, extraction kinetics studies were conducted to observe the effect of the duration of extraction. From Fig. 4, it can be observed that the best extraction recovery can be achieved by using S/L =15. At a lower S/L ratio, For S/L= 10 , all solvent evaporated during extraction studies and the extraction experiment was stopped at around 2 h.

Figure 4. Effect of agitation speed on extraction at 80 °C and S/L =15



Purification of extract

The extraction technology for extraction and isolation of ST and Reb A from stevia leaves was developed. This schematic diagram of the methodology is given in Fig. 5. The detailed compound material balance of ST and Reb A at each stage is given in Table 2. The extraction technology developed in our previous work has been used for the material balance of ST and Reb A[38]. In this extraction technology, a study has been carried out to find the importance of each unit operation step in this technology. Before the extraction step, the content of the ST and Reb A in stevia leaves was determined using the reflux extraction method as per the previous discussion. The extraction step recovered 86 % of ST and 92.50 % of Reb A from the stevia leaves. In the extract impurities such as pigments, chlorophylls were also present. For isolation and purification of ST and Reb A electrocoagulation, adsorption by activated charcoal, cation, anion, and vacuum evaporation were used. In isolation and purification steps, 60.47 % of ST and 24.66 % Reb A were recovered.

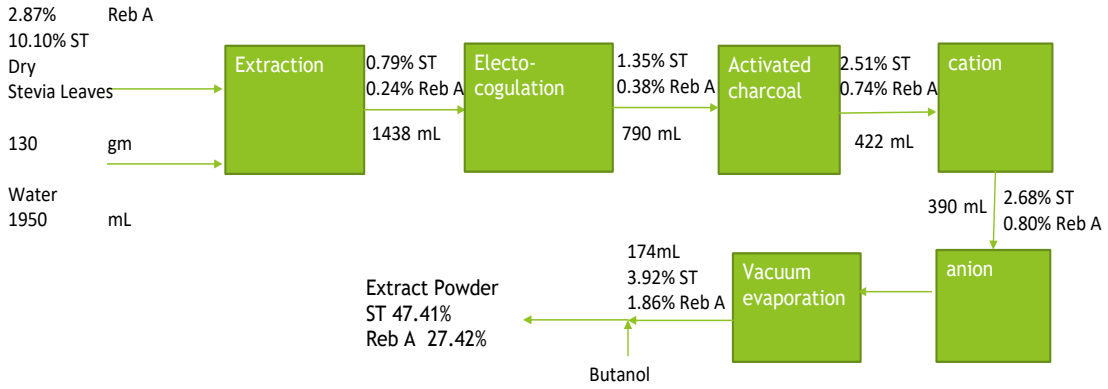


Figure 5. Extraction and isolation of ST and Reb A from stevia leaves (prepared in this work)

Table 2. Material balance of ST and Reb A from 130 stevia leaves to powder and 1950 mL of water

Sr. No	Unit operation	Input ST (g)	Output ST (g)	Input Reb A (g)	Output Reb A (g)	Conc. of ST	Conc. of Reb A
1	Extraction	13.13	11.36	3.73	3.45	0.79%	0.24%
2	Electro-coagulation	11.36	10.66	3.45	3.00	1.35%	0.38%
3	Activated charcoal	10.66	10.59	3.00	3.12	2.51%	0.74%
4	Cation	10.59	10.45	3.12	3.12	2.68%	0.80%
5	Anion	10.45	9.69	3.12	2.93	2.91%	0.88%
6	Vacuum Evaporation	9.69	6.82	2.93	3.24	3.92%	1.86%
7	Extract power	6.82	4.49	3.24	2.60	47.41%	27.42%

Table 3. Yield/ Extraction recovery of ST and Reb A using extraction and isolation method

S. No	Compound	In 130 g leaves (g)	In extract powder (g)	Yield of the purifiction
1	ST	13.13	4.49	34.23%
2	Reb A	3.73	2.60	69.67%
3	Total extract (mixture of ST and Reb A)	16.86	7.09	42.07%

Table 4. Summery of recent studies of UAE

Power	Time	Temp	Tip dia	Solvent	Result	Optimi-zation	Ref.
360 W	18 min	30 ^{0}C	20 mm	Isopropyl alcohol (60%)	35.61 mg/ 100 g (Reb A)	NA	[13]
330 W	1 min	50 °C	Ultrasonic bath	Water	14.12 mg/g (Reb A) 39.09 mg/g (ST)	NA	[10]
400 W	10 min	81.2 ˚C.	22 mm	Water	36.92 mg/g (Reb A) 96.48 mg/g (ST)	NA	[39]
480 W	18 min	30 ^{0}C	20 mm	Isopropyl alcohol (60%)	371 mg/ g (Reb A)	NA	[6]
360 W	6 min	30 ^{0}C	20 mm	EtOH (30 %)	338.5 mg/g (Reb A)	NA	[6]
360 W	6 min	30 ^{0}C	20 mm	Water	327.9 mg/g (Reb A)	NA	[6]

Table 5. Recent summery of MAE

Power	Time	Temp	Solid to liquid ratio	Solvent	Result	Optimi-zation	Ref
80 W	1 min	50 ^{0}C	100 mg/ 10 ml	Methanol :water (80 :20)	ST: 8.64 mg/g Reb A: 2.34 mg/g	NA	[9, 40]

200 W	120 sec	30 ^{0}C	1 g/10 ml	Water	ST: 76.58 mg/100 g	NA	[4]
3.30 W/gm	1 min	50 ^{0}C	1 g/100 ml	Water	ST: 46.48 mg/g, (Reb A): 17.03 mg/gm	NA	[10]
400 W	45 min	90 ^{0}C	1:10 g/ml	Ethanol	Reb A:4.21 mg/g , ST:17mg/g	RSM	[41]
160 W	4 min	50 ^{0}C	25 g/ 250 ml	75 % ethanol	ST:19.58 mg/g Reb-A 15.3 mg/g	ANN is better than RSM	[5]

Table 6. Summary of recent studies on SCF of ST and Reb A from stevia

Pressure	Temp	Solvent	Recovery	Remarks	Ref
211 bar	80 ^{0}C	17.4 % ethanol	ST: 36.66 mg/g Reb A: 17.79 mg/g	RSM (BBD)	[42]
225 bar	45 ^{0}C	40 % ethanol	ST: 98.56 mg/g Reb A: 65.07 mg/g	ANN is better than RSM (CCD)	[43]

The total extract (mixture of ST and Reb A) of 7.09 g in crystal form was recovered using this extraction technology. The yield of ST and Reb A was 34.23 % and 69.67 %, respectively and it is given in Table 3. The extraction method discussed in this paper were compared with literature and prsented in Tables 4-6. In this extraction work, the content of the ST and Reb A in the dry stevia leaves were 16.9 g and 5.02 g, respectively. The % of recovery of ST and Reb A from the stevia leaves was 97.40 % and 98.80 %, respectively.

Domestic farms supplied stevia leaves for under $2 per kg, and the main solvent used in the extraction process was water. This technology employs a recoverable organic solvent, which further increases cost effectiveness. The production cost for one gram of Steviol glycoside (ST and Reb A) crystals is about $0.10, which makes it the cheapest method. There is a possibility of selling the extract at $13 per gram.There is also a wide market for these extracts as they can be used in cosmetic manufacturing. The low set up costs are also due to an inability to use novel extraction techniques, as the process of purification in stamping does not involve any complex technologies. Furthermore, the combination of purification steps practiced makes this technology easily adaptable to high production volumes.

CONCLUSION

Stevia Rebaudiana Bertoni becomes an attractive natural sweetener in today's world because of its health benefits. To extract stevioside and Reb A from stevia leaves, the latter should be dried and ground before the extraction which can enhance the extraction yield. Moreover, from this study, water can be used instead of methanol because of its cost and lower hazard to the environment. In extraction, stevia leaves in powder form should be used to enhance extraction efficiency. In this work, crystals of 5.45 g of extract powder of steviol glycoside were obtained from 12.96 g initially present in the 100 g of stevia leaves which shows that our extraction methodology has 42 % extraction efficiency. In the work, various parameters such as duration, size of the leaves, solid-to-solvent ratio and temperature are reported that can affect the yield of the extraction steps. Thus, estimating the optimum conditions for extraction the overall yield of the entire extraction can be enhanced and the isolation methodology to get the product in crystal form.

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