International Journal of Biotechnology and Microbiology
www.biotechnologyjournals.com
Online ISSN: 2664-7680, Print ISSN: 2664-7672
Received: 02-01-2023, Accepted: 18-01-2023, Published: 04-02-2023
Volume 5, Issue 1, 2023, Page No. 16-23

Purification and drying of steviol glycosides (Sweetener) from *Stevia rebaudiana* bertoni

Dilip B Jadhao[1], Ravindra S Dhande[2*], Bhaskar N Thorat[1]
[1] Advanced Drying Laboratory, Department of Chemical Engineering, Institute of Chemical Technology (Formerly UDCT), Mumbai, Maharashtra, India
[2] Department of Botany, Shri R.R. Lahoti Science College Morshi, District Amravati, Maharashtra, India

Abstract
In the present study, the purification of Steviol glycosides from the leaves of *S. rebaudiana* was carried out using different physical separation techniques such as extraction and crystallization followed by vacuum drying. The extraction of the Steviol glycosides from the leaves of *S. rebaudiana* was carried out using hot water. The effect of Steviol glycosides to water ratio on extraction efficiency was studied. It was found that the Steviol glycosides to water ratio of 1:35 gave higher percentage recovery of Steviol glycosides. The extract clarification is an important stage as it imparts better visual quality to the final product and this was done using decolouring and flocculating agent such as calcium hydroxide followed by the bleaching treatment using bleaching gas. Subsequent to extract clarification, the techniques of evaporation and crystallization were effectively employed for further purification of Steviol glycosides with a recovery of 95% Steviol glycosides of high purity and reasonably high percentage of rebaudioside A in the product. The process of crystallization was studied in terms of supersaturation (sweeter component). In order to get steviol glycosides crystals in the dry form, vacuum drying was carried out and it was found that steviol glycosides gives crystals in the range 30-70 µm after drying. A new method for HPLC analysis was also developed for the analysis of Steviol glycosides.

Keywords: crystallization, natural sweetener, medicinal plant, anti-diabetic

Introduction

Natural compounds derived from plants are part of our daily diet. In recent years, there has been an increased focus on healthy way of life along with the search for new natural products. Amongst them are specific sweeteners, such as Steviol glycosides, now in great demand globally. Stevia rebaudiana, the nature's sweetest gift belongs to the family Asteraceae. The main source of sugar globally comes from cane sugar and beet. These sugars along with sweetening qualities contribute to high calories, which can lead to obesity, a risk factor for some chronic diseases such as Diabetes mellitus, Hypertension, Cardiovascular diseases and so on. Hence, craving for sweetness led man to discover several forms of alternative intense sweeteners, which have made possible to offer consumers sweet taste sans calories. Major Steviol glycosides such as stevioside and rebaudioside A are the major sweet components isolated from the leaves of *Stevia rebaudiana*. It is about 300 times sweeter than sucrose and noncaloric [1]. Therefore, it is gaining popularity as a sweetener in Asia and South America and has been used as a dietary supplement in United States [2]. Steviosides can be degraded to its major metabolite, steviol, by intestinal bacterial microflora of human being [3, 5]. The chemical structures of steviol and major steviol glycosides such as stevioside and rebaudioside A are as shown in Figure 1. Steviol glycosides have been reported to possess therapeutic value as, antihypertensive or antihyperglycemic agent [6, 10]. The available data indicate that Steviol glycosides are nontoxic, non-mutagenic and no carcinogenic in various mammalian species [11, 12]. It has been reported that, the leaf has several sweetener glycosides such as stevioside, rebaudioside A, B, C, D, E and dulcosides A and B. Those present in larger quantity are stevioside (5-10%), rebaudioside A (2-4%) and the others are present in smaller concentrations [13]. Crystallization is the process of forming a solid phase from a supersaturated solution. It is an important solid-liquid separation technique used in chemical, pharmaceutical, and food industry. The newly formed solid phase may have distinct physical and chemical properties, such as solubility, dissolution rate, density, thermal and chemical stability. Searching of the desired form of a polymorph of the crystalline product is one of the major activities in pharmaceutical industry. Consequently, this technique has been widely used for the isolation and purification of various natural products [14]. It has been reported that Steviol glycosides is capable of forming silky needles arranged in tufts after crystallization with methanol and water [15]. To select the appropriate solvent for crystallization, solubility is one of the major parameters to be investigated. Supersaturation, which is a function of solubility, is a kinetic and thermodynamic parameter that influences the crystal size distribution, morphology and polymorphic formation. Thus, knowing the solubility is very essential. There are few reports available in the literature on the solubility of Steviol glycosides in alcohol and water [16, 18]. However, the data is insufficient and requires detailed solubility study of Steviol glycosides in water in order to look into yield based technical as well as economic feasibility of the process from commercial point of view.

Several methodologies for herbal extraction such as ultrasonic extraction [19], microwave assisted extraction [20], pressurized hot water extraction [21] have been mentioned in the literature. However, these techniques were found to be time consuming, expensive and tedious. Keeping these points in mind, in this study, we have developed an effective and industrially feasible method for extraction of steviol glycosides from the leaves of *Stevia rebaudiana* which uses,

hot water system as the extractant. Several processes of extraction of stevia leaves presented in literature follows approximately the same methodology. First, the extraction from the leaves of *S. rebaudiana* is carried out with water or alcohols (ethanol or methanol); the obtained extract is in the form of a solution loaded with colloidal particles of dark brown colour, containing all the active principles, pigments of the leaf, soluble polysaccharides and other impurities.

Out of these, some processes report the removal of greases from the leaves with solvents, such as chloroform or hexane, a preliminary elimination of essential oils, chlorophyll and other a polar principle [22, 23]. The second stage consist of the clarification of the extract, which is usually carried out using chromatography; especially, the high-speed counter-current chromatography, Resins, metallic ions, ultrafiltration and organic solvents have been widely used for the separation and purification of steviol glycosides and various complex natural products [24, 32]. After clarification, all methods practically process the extract in similar manner. The solution is concentrated and dissolved again in methanol for Steviol glycosides crystallization. The process of clarification is being practiced industrially by the treatment of extract with metallic ions associated with organic solvents. New processes using advanced technology are being tested. Tan *et al* [16] have developed a process of extraction from Stevia leaves with supercritical CO_2 in the presence of methanol, extracting stevioside and rebaudioside A [33]. A similar work has been done by Erkucuk *et al*. [33], extracting first the greases and pigments of stevia leaves by supercritical extraction followed by conventional extracting the sweetening principles [17]. The extract clarification is an important stage because it results into a better visual quality of the final product. However, usual clarification processes have some disadvantages: organic solvents and metallic ions leave residues which are harmful to health and thus are forbidden according to ICH guidelines. Ultrafiltration membranes and other advanced technologies are associated with high capital as well as high operating cost. The present study focuses on the cost effective, economical process of extraction and purification of Steviol glycosides. The techno-commercial aspects are expected to be in the favour of the proposed separation and purification technology.

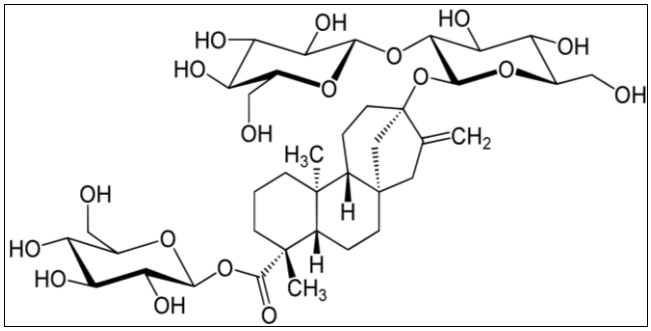


Fig 1: Molecular structure of stevioside

Material and method

1. Chemicals

Steviol glycoside (≥ 98%) was kindly provided by GreenRev Agro Pvt. Ltd. Mumbai, India. Chemical reagent and solvent such as sodium sulphite, hydrochloric acid, orthophosphoric acid and methanol was were from S.D. Fine Chemicals, India.

2. Extraction

Leaves of *S. rebaudiana* were purchased from the local market in Mumbai. Stevia glycosides are freely soluble in water and hence water was chosen for extraction. The leaves were ground to coarse powder and extracted at 65ºC temperature of water for 2 h at 110 rpm. The extraction of Steviol glycosides from the ground powder was carried by mixing coarse powder and water using glass jar reactor equipped with baffles and a stirrer. The solvent was removed and the process was repeated for one more time to remove the final traces of Steviol glycosides from the ground powder of stevia leaves. The extracts were then combined and concentrated by evaporating the water. The obtained extract was used for further studies. The effect of Steviol glycosides to solvent ratio on the extraction efficiency was also studied by varying the Steviol glycosides to solvent ratio.

3. Clarification/Purification of Extract

The crude greenish dark brown extract was treated with calcium hydroxide till the pH was attained to 12-14 and stirred for 20 minutes at 60°C temperature, in order to remove unwanted plant colouring matters such as chlorophyll and carotenoids. It is known that Steviol glycosides remains stable over a wide range of pH and heat [18]. This leads to precipitation which was then filtered followed by washing with fresh water in order to recover the glycoside effectively. The washings were added to the aqueous filtrate followed by reductive bleaching treatment using bleaching gas. Bleaching was carried for 10-20 min by passing bleaching gas till the pH was attained to 4. This leads to the precipitation of residual salt along with colouring matters thus leading to the further removal of impurities.

The bleaching gas was obtained by reacting hydrochloric acid with sodium sulphite. During the bleaching process, pH was meticulously monitored, since, Stevia glycosides starts degrading below pH 4. The precipitate was removed by filtration and the process was repeated for one more time until the clear off-white solution was obtained. The filtrate was used for further study. Steviol glycosides content in each of the recovery process was quantified. The solid content, pH, chlorophyll and carotenoids were determined to check the level of purity of Steviol glycosides extract.

4. Determination of solubility of Steviol glycosides in water

The solubility of Steviol glycosides in water was measured at different temperatures. To the 10 mL of solvent, excess quantity of Steviol glycosides was added. Subsequently, the liquid-solid suspension was constantly agitated at 120 rpm for 2h in REMI Shaker to achieve uniform mixing. The clear solution was then removed using syringe filter and dried in the vacuum oven at 50ºC. The obtained solids were weighed and the solubility was reported as mg of Steviol glycosides per ml of solvent. The same procedure was repeated at different temperatures in order to get solubility curve.

5. Crystallization of Steviol glycosides

The isolation of white Steviol glycoside from glycosides mixture of *S. rebaudiana* was carried out by using evaporation and then slow cooling crystallization technique. The supersaturation of the solution is the driving force for

both crystal growth and nucleation. To achieve supersaturation, the evaporation of water was carried out resulting into the increased concentration of solids. The process of evaporation was carried out at a fixed temperature by heating a solution. Glycoside extract which was obtained after clarification process was concentrated by slowly evaporating the water at 80-90ºC till the total solid content was attained to above 6% and above. The hot, clear solution was then filtered and the filtrate was allowed to cool slowly at room temperature. During cooling, the solution becomes turbid and later on further cooling, the solids are expelled from the solution. The formation of turbid solution can be referred to the appearance of supersaturation and thereafter, crystal formation. The mother liquid was decanted and the crystals were collected and dried in a vacuum dryer for 3-4 hrs. By carrying out crystallization repeatedly for couple of times, more refined, high purity Steviol glycosides could be obtained. Moreover, the supersaturation is the concentration difference between that of the supersaturated solution in which the crystals are growing and that of a solution in equilibrium with the crystal.

The supersaturation can be defined by equations (1) and (2).

$$\Delta y = y - y_s \quad (1)$$

The supersaturation ratio α is defined by,

$$\alpha = \frac{y}{y_s} \quad (2)$$

Where,
Δy = Supersaturation, mass fraction of solution
y = mass fraction of solute in solution
y_s = mass fraction of solute in saturated solution.

6. Vacuum Drying
The cake obtained obtained by the filtration was subjected to vacuum drying. The dryer (Salvia Lab, Switzerland) consisted of electrically heated drying chamber connected to vacuum pump. The drying temperature of 50^0C was maintained with the corresponding vacuum of 50m abr. The total drying time of 6 hrs was sufficient to obtained dried powder of stevioside.

7. HPLC Analysis of Steviol glycosides
The Agilent (Germany) HPLC system, consisting of a model G1329A standard auto-sampler, model G1316A thermostat column, model G1322 A vacuum degasser, quaternary pump, model G1314B variable wavelength detector, was used. The separation was achieved on a stainless-steel silica based Zorbax Eclipse XDB–C18 column (ϕ4.6 mm×150 mm, 5 µm). The column temperature was maintained at 30ºC. Steviol glycosides were eluted using mobile phase consisting of methanol and 0.1% v/v H_3PO_4 (70:30) at the flow rate of 1 ml/min. The eluent was monitored at 219 nm. The standard curve was obtained by analysing known concentration of Steviol glycosides. The standard curve was plotted between the concentration of stevioside and the area under the curve. This plot was used for the determination of concentration of the Steviol glycosides in the unknown solution. All the samples were prepared in the methanol of 10 mg/l concentration and filtered through 0.22 µm filter to remove any suspended particles. The amount of sample injected in the column was kept constant at 10 µl. The filtered solvents were sonicated for 10 min to remove any dissolved gases.

8. SEM of dried steviol glycosides
Electron micrographs of Steviol glycosides crystals were obtained using a scanning electron microscope (Leica Cambridge S360, UK) operating at 5 kV. The specimens were mounted on plasma coated with JEOL-JFC-1600 AUTO FINE COATER.

Result and discussion
1. Extraction
The extraction of glycoside from the leaves of *S. rebaudiana* was performed using hot water as a solvent and glycoside to different water ratios. The effect of glycoside to solvent ratio on the extraction efficiency was studied by varying these ratios. Figure 2 shows the plot of extracted Steviol glycosides verses dry feed to solvent ratio. It can be seen that the extraction increases until the feed to solvent ratio of 1:35, there-after, there is no significant effect of increasing the amount of solvent on the extraction efficiency. Similar observations have been reported by Abou-Arab *et al* [13] who obtained 93-98% of extraction efficiency using the equivalent quantity of water. Figure 3 shows the reduction in soluble solids when excess solvent is used during the process. This is interesting observations which tell us about the preferential solubilization of Steviol glycosides as compared to other soluble matter which consists of polysaccharides and other such impurities. From these two figures, it can be said that the 1:35 ratio would be an ideal from the process economic point of view as illustrated by extraction of about 7.5% (w/w) concentration of total Steviol glycosides in the aqueous solution.

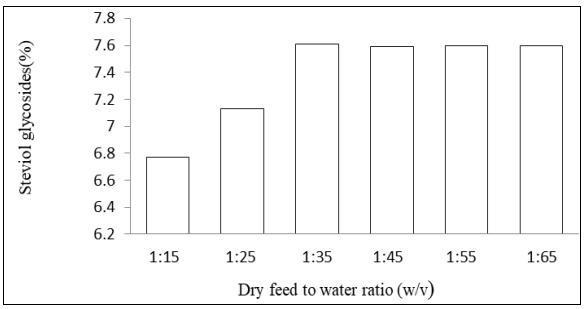


Fig 2: Effect of dry feed to water ratio on the percent yield of Steviol glycosides.

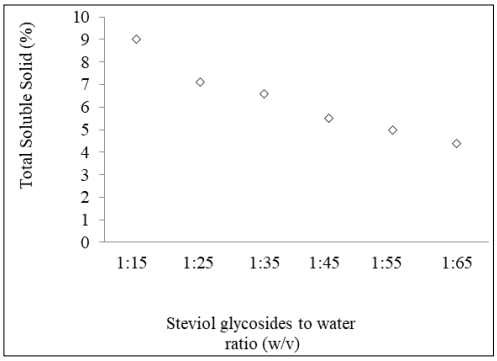


Fig 3: The percentage of solids extracted during extraction at Steviol glycosides to different water ratio.

2. Extract Clarification

The crude leaf extract was deep green to dark brown in colour due to the presence of pigments, chromospheres and other soluble components. Figure 4 is the chromatogram of the crude extract showing several peaks along with two major peaks of stevioside at the retention time 4.1 min and rebaudioside A at the retention time 8.1 min. The chromatogram shows that the pigments such as chlorophyll and carotenoids present in the crude extract affects to a great extent the purification process and therefore the purity of the Steviol glycosides. The crude green extract was treated with flocculent such as calcium hydroxide till the pH was attained to 12-14. This resulted into substantial removal of carotenoids and chlorophyll from the crude mixture as was seen by the lighter colour of the solution. The solution was then further treated with bleaching agent such as SO_2 until the pH reaches to about 4. Here, a transparent light colour was attained. It is known that the light is absorbed by certain chromophore present in the plant. C=C and C=O are essential building blocks which absorbs the UV and visible light thus producing the colour in organic matter. The bleaching treatment essentially converts double bond by reduction process and converts the coloured matter into colourless. More precisely, bleaching gas reacts with water to liberate nascent hydrogen. The nascent hydrogen so formed, then adds to the coloured matter. As a result, discoloration takes place to add the aesthetic value to the final desired product.

Coloured matter + Nascent hydrogen ⟶ Colourless matter (3)

The extent to which the decolourization takes place is as shown in Figure 5. Almost 50% reduction in pigments is seen after the calcium hydroxide treatment. Thereafter, a substantial reduction can be seen when treated with the bleaching reagent. The pigment content reduces by about 90% both in terms of chlorophyll and carotenoids as shown in this figure.

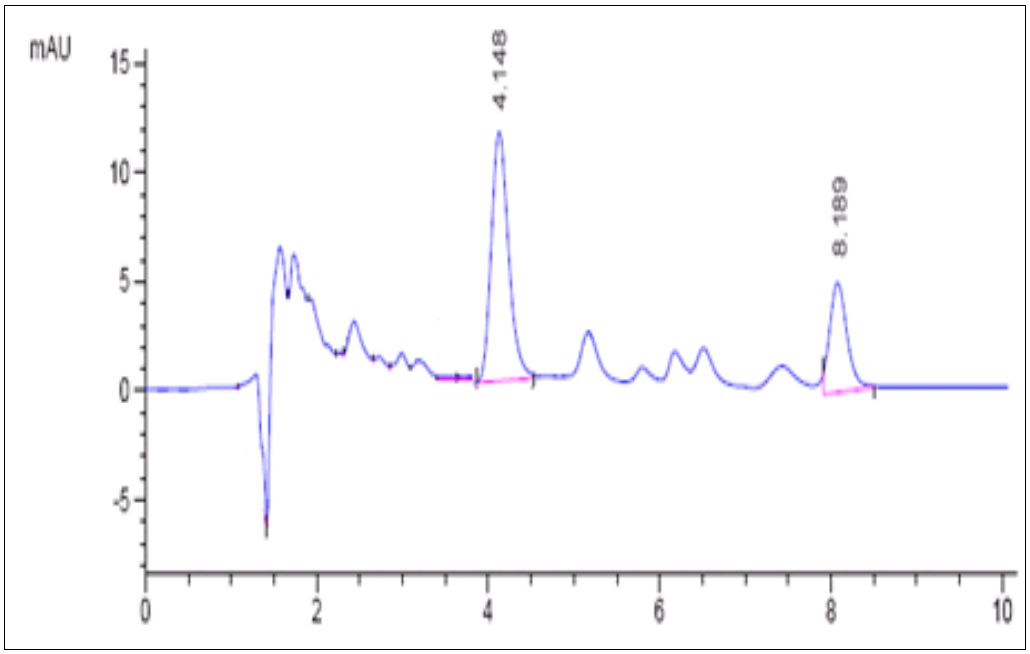


Fig 4: Chromatogram of initial extract of *S. rebaudiana* leaves

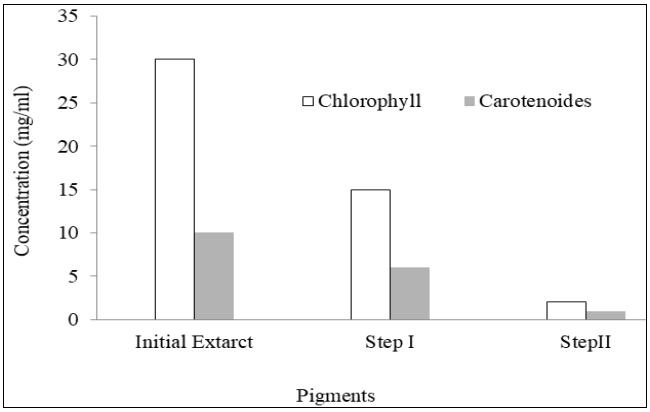


Fig 5: Total pigments content after each step of Purification

3. Crystallization

To select the appropriate solvent for a crystallization process, solubility and the ICH guidelines are the key aspects of the process. As the Steviol glycosides are freely soluble in water, the crystallization was carried out in the same medium. Figure 6 shows the solubility profile of Steviol glycosides in water as a function of temperature.

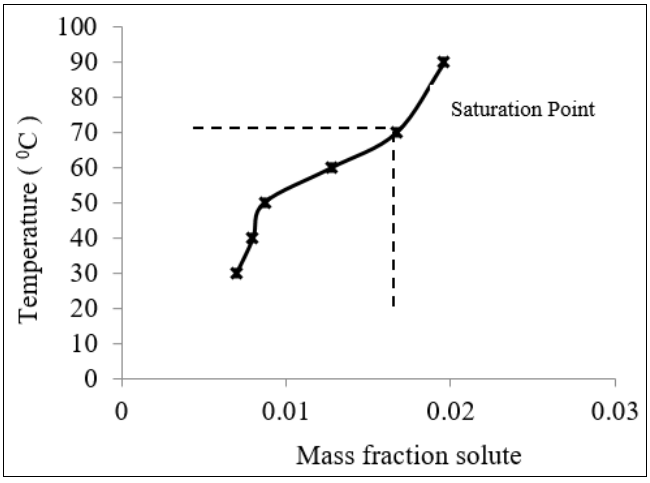


Fig 6: Equilibrium relationship for bulk Steviol glycosides crystals (Solubility curve)

The solubility remarkably increases from 50^{0}C to 70^{0}C, almost by a factor of two. Before 50^{0}C and post 70^{0}C, the change in the solubility is small as compared to 50-70^{0}C temperature range. Equilibrium is reached when the solution is saturated and the equilibrium relationship indicates the significant crystallization point where, maximum recovery of white crystallized bulk sweetest product of steviol glycosides was obtained which is as shown in Table 1**.** Such type of curve (Fig. 6) is an ideal one for cooling crystallization, where by the supersaturation by means of cooling brings about the separation of two phases rather easily. Figure 7 shows the plot of supersaturation as a function of temperature difference and it can be seen that approximately 0.0003 mass fraction of solute was the degree of supersaturation and the corresponding supersaturation ratio given by equation (2) was found to be in the range of 1.18.

When the solubility of steviol glycosides increases appreciably with temperature, the supersaturation can be expressed as an equivalent temperature difference instead of mass fraction difference. The relation between these driving potential is shown in Fig.7 which contains a small section of the solubility curve of steviol glycosides in mass fraction solute. The field above the line at 70^{0}C represents the unsaturated solution and that below the line, supersaturated solutions. Point A refers to a saturated solution at temperature T_c, which is the temperature of the growing crystal, and point D to the supersaturated solution at temperature T. Since, the heat is evolved by the crystal as it grows, T_c is slightly larger than T, providing the driving force of ΔT_h for heat transfer from crystal to the liquid. The supersaturation α is normally based on the bulk temperature and, as shown by the difference in point E and D. Point B refers to a saturated solution of the same composition as the supersaturated solution in which the crystals are growing. It would be at temperature T_s, where $T_s > T$. Point C refers to temperature T_c and the concentration equal to that of supersaturated solution.

Using Equation (1), the supersaturation potential can be represented by the line segment A^C. The equivalent temperature driving potential can be shown by line segment *BC*. Segment *AB* of the solubility curve can be considered linear over the small concentration spanned by the line *AC* and the temperature potential defined by

$$\Delta T_c = T_s - T_c \quad (4)$$

T_s = Supersaturation Temperature, T_c = Saturation Temperature

From the above equation, the temperature potential (ΔT_C) was found to be 20°C which was slightly smaller than the actual difference in temperature, T of the solution and the corresponding saturation temperature T_s.

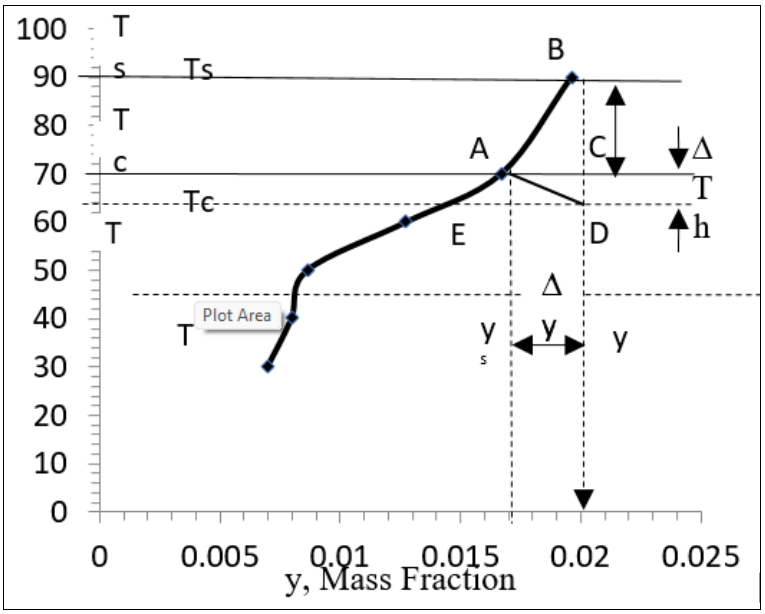


Fig 7: Supersaturation and Temperature Potential

The recovery of the content of Steviol glycosides in the product is as shown in Table 1 using evaporation and crystallization followed by recrystallization. The content of Steviol glycosides in the crystallized product increases with subsequent crystallization. There was found to be slight variation in the melting point of crystallized and recrystallized product. The superior or better quality of Steviol glycosides shows slightly higher range of melting point as shown in Table 1.

Table 1. Isolation of Steviol glycosides after Crystallization and Re-crystallization

Crystals procured from different operations	Melting point ºC		Steviol Glycoside (%)	Recovery or Overall Yeild (%)
	Stevioside	Reb A		
Crystallization by evaporation	192- 198	226-235	93.78	95.6
Recrystallization	195- 198	228-238	96	92.76

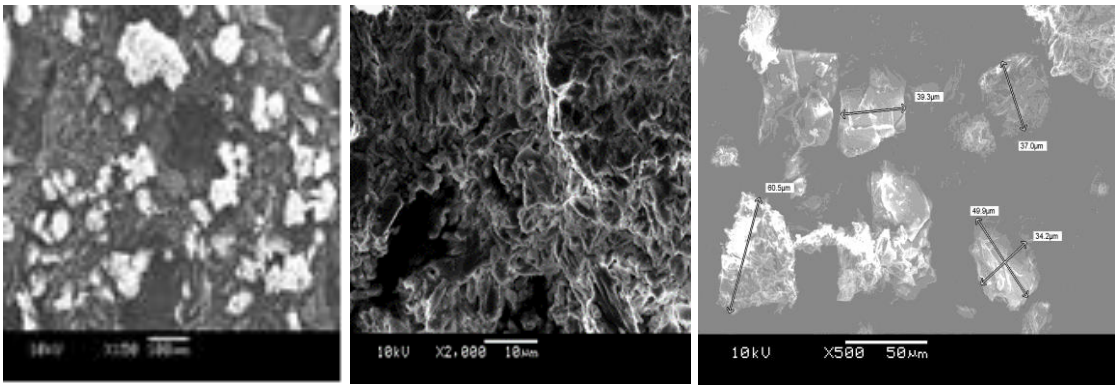
The overall yield of 90% was quite satisfactory from further scale up point of view.

5. Drying and moisture absorption

The wet cake of steviol glycoside contains about 16.5% w/w moisture content, which was brought down to <10 % in an vacuum dryer. The colour of crystals changes from pale yellow to white on drying.

6. SEM Analysis of Dried Crystals

Figure 8 shows the crystal images of steviol glycosides and the crystal size was found to be in the range of 30-70 µm after recrystallization.



a b c

Fig 8: SEM images of Steviol glycosides after recrystallisation and drying. (a) Low resolution at 150X (b) High resolution at 2000X and (c) Particle size determination at resolution 500X

HPLC analysis

The chromatograms of Steviol glycosides from the leaves of *Stevia rebaudiana* were compared with standard Steviol glycosides and the percent purity of Steviol glycosides was found to be 96% with high unexpected percentage of rebaudioside A as compared to stevioside. Figures 9 and 10 shows the chromatograms of the standard Steviol glycosides and the crystals obtained in this study, respectively. The presence of stevioside at 4.18 min retention time and rebaudioside A at 8.19 min retention time, clearly shows the intrinsic advantage of crystallization in attaining more sweeter component as a substantial fraction in the extracted purified product.

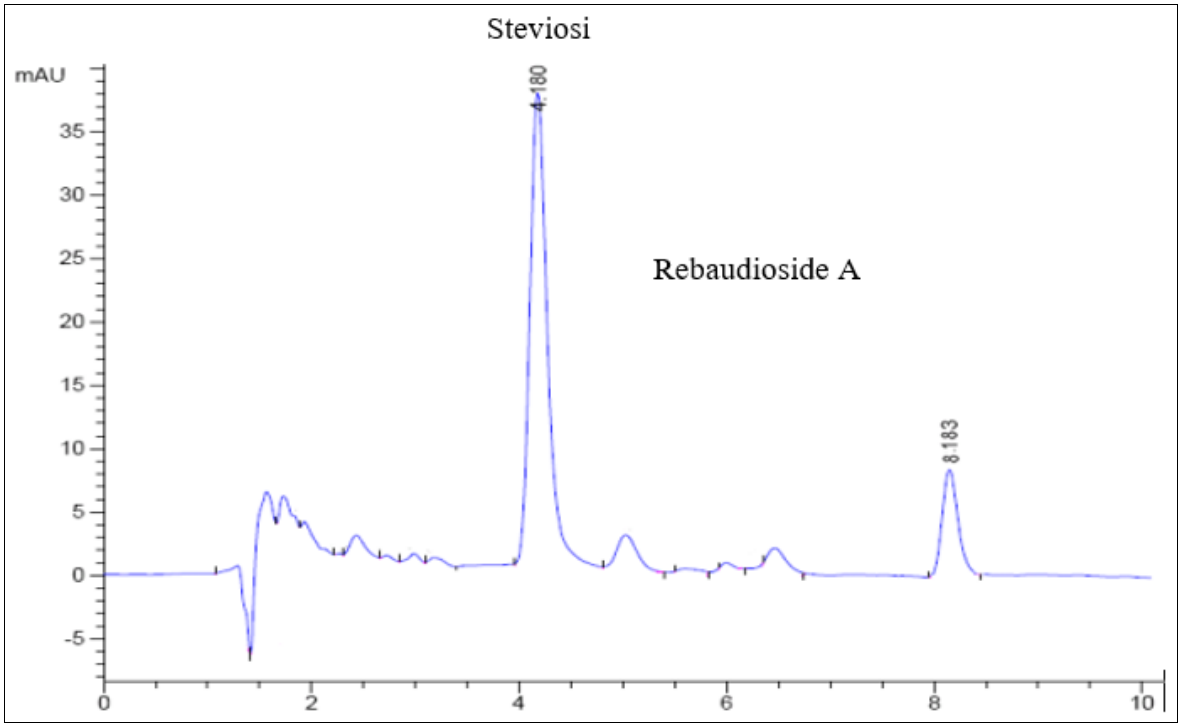


Fig 9: Chromatogram of standered Steviol glycosides.

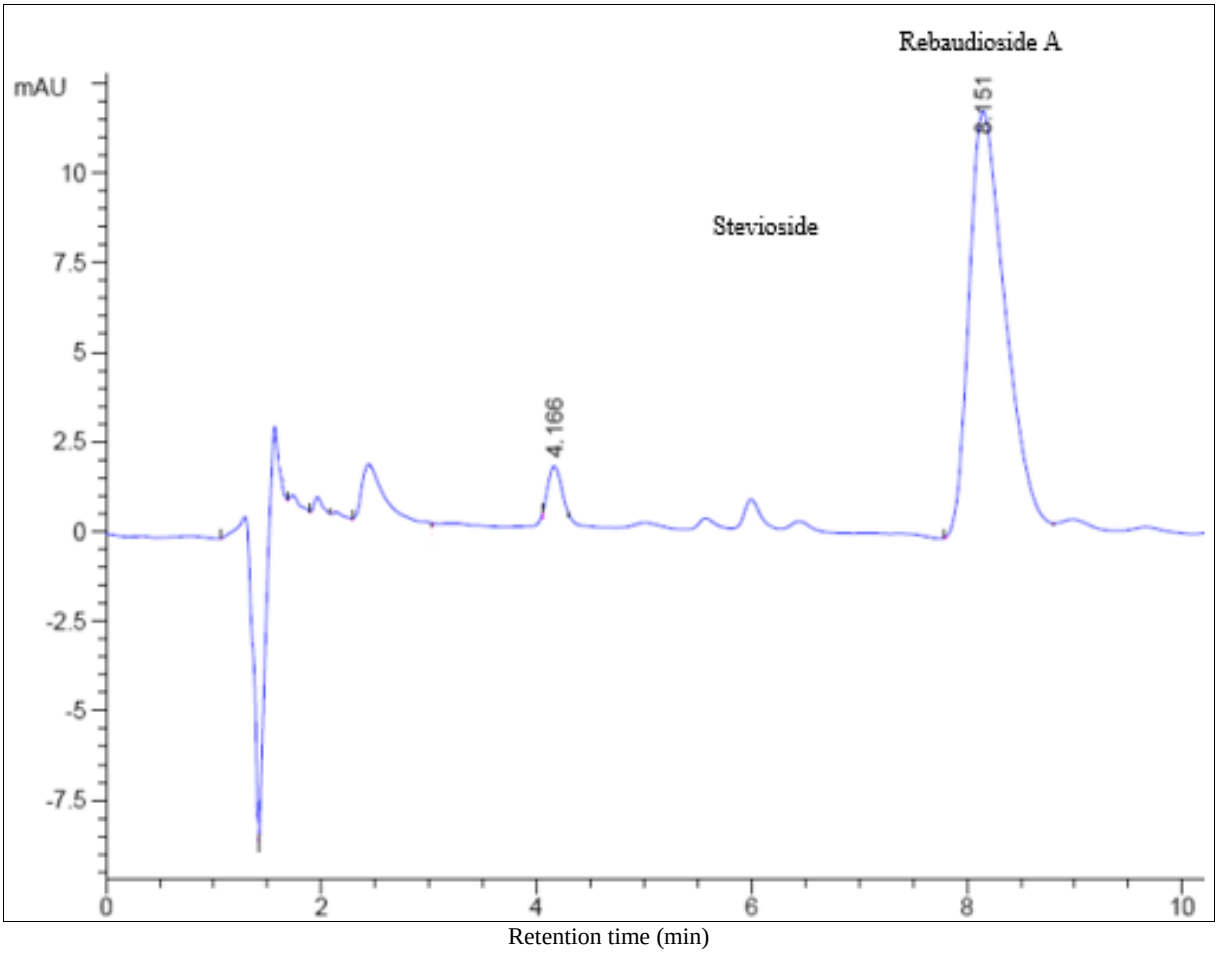


Fig 10: Chromatogram of obtained crystals of Steviol glycosides.

Conclusion

The extraction of Steviol glycosides from the leaves of *S. rebaudiana* using water as solvent was carried out. The optimized ratio of dried *S. rebaudiana* leaves to water was found to be 1:35. Followed by conventional extraction, the extract clarification was successfully carried out using flocculating and bleaching agent. Evaporation followed by cooling crystallization was effectively employed for the recovery of Steviol glycosides and it was found to be in the range of 90-95% of recovery of Steviol glycosides with 96% purity. The particle size of the recrystallized product was found to be in the range of 30-70 µm. Solubility pattern of Steviol glycosides was carried out in water. The process parameters of crystallization were studied in terms of supersaturation (Δy), supersaturation ratio (α) and temperature potential (ΔT_C). To obtain substantial yield of Steviol glycosides, 20°C super cooling was found to be sufficient practically. The simple and novel approach based on extraction followed by clarification of extract and crystallization suggested in the present work can be the most promising techniques for this kind of natural sweetener separation and purification.

Nomenclature

Δy = Supersaturation, mass fraction of solution

y = mass fraction of solute in solution

y_s = mass fraction of solute in saturated solution

T_s = Supersaturation Temperature,

T_c = Saturation Temperature

Greek letters

Supersaturation ratio	α
Supersaturation	Δy
Temperature potential	ΔT_C

Acknolwedment

The authors would like to thank profusely to Rajiv Gandhi Commission for Science and Technology, Government of Maharashtra, India for providing funding for this research work.

References

1. Bridel M, Lavieille R. The sweet principle of Kaa-he-e (Stevia rebaudiana). J. Pharm. Clin,1931:14:99-154.
2. Geuns JM. Stevioside. Phytochemistry,2003:64:913-921. Hutapea AM, Toskulkao C, Buddhasukh D, Wilairat P, Glinsukon T. Digestion of stevioside, a natural sweetener, by various digestive enzymes. J. Clin. Biochem. Nut,1997:23:177-186.
3. Gardana C, Simonetti P, Canzi E, Zanchi R, Pietta P. Metabolism of stevioside and rebaudioside A from Stevia rebaudiana extracts by human microflora. J. Agric. Food Chem,2003:51:6618-6622.
4. Koyama E, Sakai N, Ohori Y, Kitazawa K, Izawa O, Kakegawa K, Fujino A *et al*. Absorption and metabolism of glycosidic sweeteners of stevia mixture and their aglycone, steviol, in rats and humans. Food Chem. Toxicol,2003:41:875-883.
5. Chan P, Xu DY, Liu JC, Chen YJ, Tomlinson B, Huang WP *et al*. The effect of stevioside on blood pressure and

plasma catecholamines in spontaneously hypertensive rats. Life Sci,1998:63:1679-1684.
6. Chan P, Tomlinson B, Chen YJJ, Liu C, Hsieh MH, Cheng JT. A double-blind placebo-controlled study of the effectiveness and tolerability of oral stevioside in human hypertension. Br. J. Clin. Pharmacol,2000:50:215-220.
7. Gregersen S, Jeppesen PB, Holst JJ, Hermansen K. Antihyperglycemic effects of stevioside in type 2 diabetic subjects. Metabolism,2004:53(1):73-76.
8. Liu JC, Kao PK, Chan P, Hsu YH, Hou CC, Lien GS, *et al*. Mechanism of the antihypertensive effect of stevioside in anesthetized dogs. Pharmacology,2003:67(1):14-20.
9. Jeppesen PB, Gregersen S, Rolfsen SE, Jepsen M, Colombo M, Agger A, Xiao J *et al.* Antihyperglycemic and blood pressure-reducing effects of stevioside in the diabetic Goto-Kakizaki rat. Metabolism,2003:52(3):372-378.
10. Xili L, Chengjiany B, Eryi X, Reiming S, Yuengming W, Haodong S *et al*. Chronic oral toxicity and carcinogenicity study of stevioside in rats. Food Chem. Toxicol,1992:30:957-965.
11. Toskulkao C, Chattered L, Temcharoen P, Glinsukon T. Acute toxicity of stevioside, a natural sweetener, and its metabolite, steviol, in several animal species. Drug Chem. Toxicol,1997:20:31-4.
12. Esmat Abou-Arab, Abou-Arab A Azza, Abu-Salem M. Ferial. Physico-chemical assessment of natural sweeteners stevioside produced from Stevia rebaudiana bertoni plant. African Journal of Food Science,2010:4(5):269-281.
13. Wen Lin Xu, Yi Bo Huang, Jun Hong Qian, Ou Sha, Ya Qiong Wang. Separation and purification of stigmasterol and β-sitosterol from phytosterol mixtures by solvent crystallization method. Sep. Purif. Technol,2005:41:173–178.
14. Prakash. Stevioside polymorphic and amorphous forms, methods for their formulation, and uses, US Patent, 2008, No. 0292764.
15. Tan S. Isolation of sweetner from *Stevia rebaudiana* (Mitsui Petrochemical Industries); Japan Kokai Tokkyo Koho JP Pat. 63,177,764 ((88 177,764) (CLA23L1/22)), 1988, 7
16. Ashurst PR. Chemistry and technology of soft drinks and fruit juices, Wiely- Blackwell publication, 2005, 82-83.
17. Morals, Élida de Paula, Regina Nádia Machado, Camargo Fernandes. Clarification of *Stevia rebaudiana* (Bert.) Bertoni extract by adsorption in modified zeolites. Acta Scientiarum Maringá,2001:23(6):1375-1380.
18. Sun-Ja Kim, Hosakatte Niranjana Murthy, Eun-Joo Hahna, Hyung Lae Lee, Kee-Yoeup Paek. Parameters affecting the extraction of ginsenosides from the adventitious roots of ginseng (Panax ginseng C.A. Meyer). Sep. Purif. Techno,2007:56::401-406.
19. Jaitak V, Bandana Singh B, Kaul VK. An Efficient Microwave-assisted Extraction Process of Stevioside and Rebaudioside-A from Stevia rebaudiana (Bertoni). Phytochem Analm,2009:20:240-245.
20. Teo CC, Tan SN, Yong JWH, Hew CS, Ong ES. Validation of green- solvent extraction combined with chromatographic chemical fingerprint to evaluate quality of Stevia rebaudiana Bertoni. J Sep Sci,2009:32:612-622.
21. Tanaka O. Preparation of sweetening agents. (Ajinomoto Co. Inc.). Japan Kokai. Patent No,1977:77:41, 275.
22. Kodaka K. Purification of sugar solution from Stevia dry leaves. Japan. Kokai. Pat. Appl. 76/27,634 (Cl. A23L1/22), 1977, 3.
23. Huang X, Fang-Fu J, Di Duo-Long. Preparative isolation and purification of steviol glycosides from Stevia rebaudiana Bertoni using high-speed counter-current chromatography. Sep Purif Technol,2010:71:220-224.
24. Li FW, Lin Y, Wang X, Geng Y, Wang D. Preparative isolation and purification of capsaicinoids from Capsicum frutescens using high-speed counter-current chromatography, Sep. Purif. Technol,2009:64:304-308.
25. Liu Z, Sun Y, Wang H, Zhu H, Zhou J, Hu J Wang. Preparative isolation and purification of acetophenones from the Chinese medicinal plant Cynanchum bungei Decne. by high-speed counter-current chromatography, Sep. Purif. Technol,2008:64:247-252.
26. Wu T, Lin JB, Yang Y, Abdulla R, Chen J, Aisa HA. Preparative isolation of three flavonoids from Flos Gossypii by high-speed counter-current chromatography, Sep. Purif. Technol,2009:66:295-298.
27. Ling JY, Zhang GY, Lin, Cui ZJ, Zhang CK. Supercritical fluid extraction of cordycepin and adenosine from Cordyceps kyushuensis and Purification by high-speed counter-current chromatography, Sep. Purif. Technol,2009:66:625-629.
28. Chaoyang Maa, Guangjun Taoa, JianTanga, Zaixiang Loua, HongxinWanga, Xiaohong Gua, Liming Hub *et al*. Preparative separation and purification of rosavin in Rhodiola rosea by macroporous adsorption resins. Sep. Purif. Technol,2009:69(1):22-28.
29. Antonio S, Dacome A, Cleuza C, da Silva a, Cecı´lia E.M. da Costa c *et al*. Sweet diterpenic glycosides balance of a new cultivar of Stevia rebaudiana (Bert.) Bertoni: Isolation and quantitative distribution by chromatographic, spectroscopic, and electrophoretic methods. Process Biochemistry,2005:40:3587-3594.
30. Shou-qin Zhang, Hui-min Bi, Chang-jiao Liu. Extraction of bio-active components from Rhodiola sachalinensis under ultrahigh hydrostatic pressure. Sep. Purif. Technol,2007:57:277-282.
31. Hua-Neng Xu, Chao-Hong He. Extraction of isoflavones from stem of Pueraria lobata (Willd.) Ohwi using n-butanol/water two-phase solvent system and separation of daidzein. Sep. Purif. Technol,2007:56:85-89.
32. Erkucuk IH, Akgun O, Yesil-Celiktas. Supercritical CO2 extraction of glycosides from Stevia rebaudiana leaves: Identification and optimization. J. of Supercritical Fluids,2009:51:29–35.