

ISOLATION AND PURIFICATION OF STEVIOSIDE FROM STEVIA LEAVES

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Stevia rebaudiana Bertoni which has gained industrial and scientific interests is a suitable nutritional alternative to sucrose as a sweetener. Recently, there have been studies which show the extraction of this phytochemical substance from stevia leaves and purification methods by several alcohols and chromatographic methods. However, these methods are not cost-effective. Therefore, an attempt was made to extract and purify ST using inexpensive, scalable and simple techniques where different steps like extraction, electrocoagulation, ion exchange, activated charcoal, vacuum evaporation and butanol wash were used as purification steps. The present study established a new improved technology of extraction of ST from stevia leaves using water as a solvent followed by various purification steps. 496 mg of Stevioside extracted in the form of crystals was obtained from 100 g of leaves which is 10 times more than the reported yield of 54 mg from 100 g stevia leaves in literature. This methodology can be scaled up at the industry level for future large production to meet the huge demand for natural sweeteners.

Keywords: ST, rebaudioside-A, *Stevia rebaudiana* Bertoni, conventional extraction

1. INTRODUCTION

Sweetness is one of the most loved taste sensations known in humans for ages. Natural sweeteners contain mainly sucrose, glucose and fructose coming from natural resources (Crammer and Ikan, 1977). Glucose, sucrose and fructose are natural sweeteners having high calorific values and are not suitable for human health and body weight issues. Sucrose is one of the compounds which is not desired for human health as it may cause obesity, type 2 diabetes, particular cancers, cardiovascular disease and hypertension (Chranioti et al., 2016). Therefore, health aware people want to move towards alternative sweeteners which are sought for (Goyal et al., 2010). There are many alternative synthesized sweeteners available in the market such as saccharin, aspartame, cyclamates etc. However, they also pose some health issues due to their toxic effects in long term use (Anton et al., 2010; Weihrach and Diehl, 2004). Thus, a search for non – sucrose and natural sweeteners is carried out, including low calorific value and non-toxic effect for prolonged use.

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There are over hundreds of species of the genus *Stevia* but only SR provides the sweetest taste (Megeji et al., 2005; Savita et al., 2004; Yadav et al., 2011). *Stevia* sweetener extractives are prompt to exert helpful effects on human health, together with the medicament (Lee et al., 2001), inhibitor (Xi, 1998), anti-human reovirus activities (Ghosh et al., 2008; Takahashi et al., 2001). Because of the rising health awareness in India, *stevia* has been successfully cultivated in recent years in many areas of India such as Rajasthan, Maharashtra, Kerala and Orissa (Goyal et al., 2010). There are two important natural sweeteners present in *Stevia Bertoni* namely ST (4–13%) and Reb A (2–24%). Other compounds such as Rebaudiana C, Ducoside A, Steviolbioside, Rebaudioside B, D E, F are also present in the leaves in amounts of less than 1% by weight (Singh and Rao, 2005). From many glycosides, ST is found to be the most useful *Stevia* glycoside and is known for its sweetness which is more than 200 times that of sucrose, while Reb-A is more soluble in water and gives an essence. *Stevia* leaves contain more steviol glycoside (SGs) than other parts of the *stevia* plant. Hence, extracting glycosides from *stevia* leaves is more preferable to other parts of the plant. Extraction is the first step to separate the desired phytochemical compounds from the raw materials. Most of the extraction methods include solvent extraction, distillation, pressing and sublimation according to the extraction principle. Among these, solvent extraction is the most widely used method. The extraction of natural products from the plant material has the following stages:

- 1) diffusion of the solvent from bulk to the solid matrix;
- 2) dissolution of the solute in the solvent;
- 3) diffusion of solute from the solid matrix to the bulk;
- 4) collection of extracted solutes as crystals.

To choose a right solvent for edible substance extraction is always trivial. The major concerns in selection are selectivity, solubility, cost and safety. Methanol and ethanol are the most common solvents used for extraction. Extraction is supposed to be better with similar molecules and non-miscible characteristics. The major parameters during extraction to be monitored are raw materials, the solvent-to-solid ratio, the effect the extraction efficiency (Lee et al., 2001; Megeji et al., 2005; Savita et al., 2004; Xi, 1998; Yadav et al., 2011). The extraction efficiency is improved by small particle size due to large surface which comes in contact with solvents and more mass transfer can take place. However, very fine particle size results in difficulty of filtration after extraction. Although, increasing temperature favours the increased solubility of the solvent and high rate of diffusion but high temperature may cause loss of solvent by evaporation and phyto-components may also be degraded at elevated temperatures. The extraction efficiency increases with the increase in extraction time up to equilibrium. The greater the solvent-to-solid ratio, the higher the extraction yield. However, a solvent-to-solid ratio that is too high requires excessive extraction solvent and a long time for concentration (Hidayat and Wulandari, 2021).

For many years, to extract phytochemicals from plant materials, conventional extraction (CV) techniques based on maceration and thermal extraction have been used. However, these extraction techniques have lower efficiency and lengthy processing. Thus, new emerging green extraction technologies such as supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) (Gomez et al., 2020) have been developed. These green technologies provide better yield with reduced energy and solvent consumption compared to CV. The green extraction techniques require less solvent and time compared to conventional methods. However, these techniques are not easily scalable at industrial setting. Even after extraction by appropriate solvent, along with SGs, other compounds such as chlorophylls, carotenoids and tannins also get extracted from *stevia* which results in dark brown coloured crude extract having a bitter-taste and foul-smelling. Therefore, successive purification is necessary for developing a product of commercial quality (90% purity or more). Purification of STs often involves processes such as inorganic salt treatment, ion exchange separation, column chromatography, ultrafiltration, nano-filtration, crystallization, etc. (Akashi et al., 1975; Ishizone, 1979). Therefore, some attempts have been made to extract and purify ST using simple, easy and scalable techniques. In this work, the effect of various parameters in the extraction step such as time, temperature and solid to liquid ratio on the yield of SGs have been studied.

2. MATERIALS AND METHODS

Stevia leaves which is 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 grinding to 500-micron size. Dry leaves contained 6–7% ST and 1–2% of Reb A. The standard HPLC grade ST (90%) and Reb-A (98%) were purchased from TCI Chemical, India.

The powdered sample of 50 g was used for extraction with 750 ml of water as a solvent for 4 h at 78 °C. The aqueous extract was cooled, filtered under vacuum (600–620 mm Hg) and processed further for electrocoagulation to remove chlorophylls which gave a green colour to the extract. In this step, direct current (15 V, 0.8–1.2 A) was passed for 1 h via two pairs of the aluminium plate as electrodes and 15 g NaCl was added as an electrolyte. The resulting mixture was again vacuum filtered and the same process of electrocoagulation was repeated once more to remove all chlorophyll successfully. The resulted 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, P from the solution. After this operation to filter the solution celite was used as filter aid which was found more effective to be used for filtration. For further removal of impurities, butanol was used as a solvent and liquid-liquid extraction was done where aqueous phase was concentrated to achieve crystals of ST. Later, crystals of ST were filtered and dried and analysis of ST was done using UV-spectrophotometer. The content of SGs in an aqueous solution was estimated using UV-spectrophotometer by [Kaur et al. \(2009\)](#). The aqueous extracts were hydrolysed with 5N HCl at 70 °C for 1 hour. The glucose units liberated from the ST upon hydrolysis took part in the Dubois reaction with 5% phenol and 95% sulphuric acid (H₂SO₄). The intensity of orange-brown colour was read at 490 nm. The concentration of glucose was measured against glucose standard and was multiplied by a factor of 1.64 (based on molecular weight) to calculate ST content. The equation of calibration was obtained as Eq. (1) with an R^2 of 0.9953.

$$y = 0.0033x + 0.1313 \quad (1)$$

where, y is the absorbance in nm and x is the concentration of the solution in g/ml.

3. RESULTS AND DISCUSSION

Several experiments were performed to achieve the desired recovery of ST and Reb A with increasing and decreasing different solvents, other process parameters and adding more operations in the process. The first trial run was done as per Table 1 where for extraction water was selected as a solvent due to its low viscosity and non-toxicity. ST and Reb A are natural components and degrade at higher temperature (100 °C). Thus, the temperature for extraction was selected at 78 °C. At a higher temperature, the viscosity of the solvent further decreased and diffusivity of phytochemicals in the solvent enhanced. The resulting solution and residue of leaves were separated by filtration. To obtain ST and Reb A in the form of crystals, vacuum evaporation was performed to achieve supersaturation but due to higher amount of solvent, supersaturation could not be maintained and crystals were not found at all. For further trial, the solvent amount was reduced to 500 ml. Additionally to achieve nucleation, anti-solvent such as butanol was also used in the final step to remove impurities. However, no crystals were yielded. It may have been due to less solvent being available than required. So in further trial, 750 ml of water as a solvent was selected, still no crystals were obtained. In spite of increasing and decreasing solvent, other observations were also encountered like: the colour of the extract was dark brown which indicates the presence of the chlorophyll and carotenoid pigments in the extract. To increase ST and Reb A concentration chlorophylls and carotenoids pigments need to be removed from the solution either by salt treatment Ca(OH)₂ or electrocoagulation ([Huang et al., 2010](#); [Jumpatong et al., 2006](#)). These techniques can remove impurities by 90%.

Table 1. Different process schemes experiments run

Sr. No	Process steps	Process condition	Result
1	(a) water extraction, (b) vacuum filtration, (c) vacuum evaporation	extraction: 50 g stevia leaves, 1500 ml water, 4 h, temp. = 78 °C,	no crystals got pigments
2	(a) water extraction, (b) vacuum filtration, (c) vacuum evaporation, (d) butanol wash	extraction: 50 g stevia leaves, 500 ml water, 4 h, temp. = 78 °C, butanol wash: 100 ml aqueous extract and 40 ml butanol	no crystals got pigments
3	(a) water extraction, (b) vacuum filtration, (c) vacuum evaporation, (d) butanol wash	extraction: 50 g stevia leaves, 750 ml water, 4 h, temp. = 78 °C, butanol wash: 220 ml aqueous extract and 88 ml butanol	no crystals got pigments
4	(a) water extraction, (b) treatment with Ca(OH) ₂ , (c) vacuum filtration, (d) adsorption with activated charcoal, (e) vacuum evaporation, (f) butanol wash	extraction: 50 g stevia leaves, 750 ml wa- ter, 4 h, temp. = 78 °C, Ca(OH) ₂ , 50 ml saturated solution, adsorption: 7.36 g activated charcoal, 1 h, butanol wash: 180 ml aqueous extract and 88 ml butanol	no crystals got pigments
5	(a) water extraction, (b) treatment with Ca(OH) ₂ , (c) vacuum filtration, (d) adsorption with activated charcoal, (e) vacuum evaporation, (f) MeOH wash	extraction: 50 g stevia leaves, 750 ml wa- ter, 4 h, temp. = 78 °C, Ca(OH) ₂ 50 ml saturated solution, adsorption: 7.36 g activated charcoal, 1 h, butanol wash: 175 ml aqueous extract and 88 ml MeOH	no crystals got pigments
6	(a) water extraction, (b) treatment with Ca(OH) ₂ , (c) vacuum filtration, (d) adsorption with activated charcoal, (e) vacuum evaporation, (f) EtOH wash	extraction: 50 g stevia leaves, 750 ml wa- ter, 4 h, temp. = 78 °C, Ca(OH) ₂ 50 ml saturated solution, adsorption: 7.36 g activated charcoal, 1 h, butanol wash: 175 ml aqueous extract and 88 ml EtOH	no crystals got pigments
7	(a) water extraction, (b) treatment with Ca(OH) ₂ , (c) vacuum filtration, (d) adsorption with activated charcoal, (e) vacuum evaporation, (f) IPA wash	extraction: 50 g stevia leaves, 750 ml wa- ter, 4 h, temp. = 78 °C, Ca(OH) ₂ 50 ml saturated solution, adsorption: 7.36 g activated charcoal, 1 h, butanol wash: 174 ml aqueous extract and 88 ml IPA	no crystals got pigments
8	(a) water extraction, (b) vacuum filtration, (c) treatment with Ca(OH) ₂ , (d) vacuum filtration, (e) adsorption with activated charcoal, (f) vacuum evaporation, (g) extraction with butanol, (h) butanol phase for vacuum evapora- tion	extraction: 50 g stevia leaves, 750 ml wa- ter, 4 h, temp. = 78 °C, Ca(OH) ₂ 50 ml saturated solution, adsorption: 7.36 g activated charcoal, 1 h, butanol extraction: 180 ml butanol for 1 h	no crystals got pigments

Continued on next page

Table 1 [cont.]

Sr. No	Process steps	Process condition	Result
9	(a) water extraction, (b) vacuum filtration, (c) electrocoagulation, (d) vacuum filtration, (e) extraction with butanol, (f) butanol phase for adsorption with activated charcoal, (g) butanol phase vacuum evaporation	extraction: 50 g stevia leaves, 750 ml water, 4 h, temp. = 78 °C, electrocoagulation: 15 g NaCl added, $V = 12 \text{ V}$, $I = 0.8\text{--}1.2 \text{ A}$, 2 h, butanol extraction: 180 ml butanol for 1 h, adsorption: 7.36 g activated charcoal	no crystals got pigments
10	(a) water extraction, (b) vacuum filtration, (c) electrocoagulation, (d) vacuum filtration, (e) treatment with $\text{Ca}(\text{OH})_2$, (f) vacuum filtration, (g) extraction with butanol, (h) butanol phase for adsorption with activated charcoal, (i) butanol phase vacuum evaporation	extraction: 50 g stevia leaves, 750 ml water, 4 h, temp. = 78 °C, electrocoagulation: 15 g NaCl added, $V = 12 \text{ V}$, $I = 0.8\text{--}1.2 \text{ A}$, 2 h, $\text{Ca}(\text{OH})_2$ 50 ml saturated solution, butanol extraction: 180 ml butanol for 1 h, adsorption: 7.36 g activated charcoal	3.8 g crystals/100 g stevia leaves, no sweet taste, no pigments
11	(a) water extraction, (b) vacuum filtration, (c) electrocoagulation, (d) vacuum filtration, (e) adsorption with activated charcoal, (f) extraction with butanol, (g) aqueous phase for adsorption with activated charcoal, (h) aqueous phase vacuum evaporation	extraction: 50 g stevia leaves, 750 ml water, 4 h, temp. = 78 °C, electrocoagulation: 15 g NaCl added, $V = 12 \text{ V}$, $I = 0.8\text{--}1.2 \text{ A}$, 2 h, butanol extraction: 180 ml butanol for 1 h, adsorption: 7.36 g activated charcoal	19.44 g crystals/100 g stevia leaves, sweet taste, brown in colour
12	(a) water extraction, (b) vacuum filtration, (c) electrocoagulation, (d) vacuum filtration, (e) adsorption with activated charcoal, (f) adsorption with cation resin, (g) adsorption with anion resin, (h) filtration with celite, (i) vacuum evaporation, (j) butanol wash (k) aqueous phase filtration	extraction: 50 g stevia leaves, 750 ml water, 4 h, temp. = 78 °C, electrocoagulation: 15 g NaCl added, $V = 12 \text{ V}$, $I = 0.8\text{--}1.2 \text{ A}$, 2 h, adsorption: 7.36 g activated charcoal, butanol wash: 40 ml butanol for 50 ml ex- tract	496.4 mg ST/100 g stevia leaves

Therefore, for further experiments salt treatment was selected and different solvents such as butanol, methanol, ethanol and isopropyl alcohol were applied as an anti-solvents after vacuum evaporation. It was noticed that the salt treatment and adsorption step with extract was not sufficient to remove all impurities, and hence extraction with butanol was also included in the next trial (trial 8). However, no crystals and no pigments were observed. For further improvement in methodology, salt treatment was replaced with electrocoagulation. This process showed that impurities were not removed completely from the water extract. So in the next trial electrocoagulation and salt treatment were applied together. The impurities of

the extract were removed up to a substantial amount. However, after crystallization, crystals of $\text{Ca}(\text{OH})_2$ were obtained instead of ST because $\text{Ca}(\text{OH})_2$ was added as salt after electrocoagulation.

Later experiments were performed removing impurities by electrocoagulation only. However, due to the presence of cation and anion impurities, brown coloured crystals were obtained. Another experiment was performed to remove ion impurities by cation and anion adsorption on resin. This trial was quite successful and this methodology resulted in white coloured and sweet tasting crystals. The block diagram of the methodology is given in Fig. 1. The effect of each step or unit operations play an important role in extraction and purifications. In a single operation, a total of 1.1 g of the solid extract was obtained from 100 g of dry stevia leaves. This process scheme yielded 496.3 mg of ST which is the highest yield obtained till now. All the published research work has been summarized in the form of recovery yield of

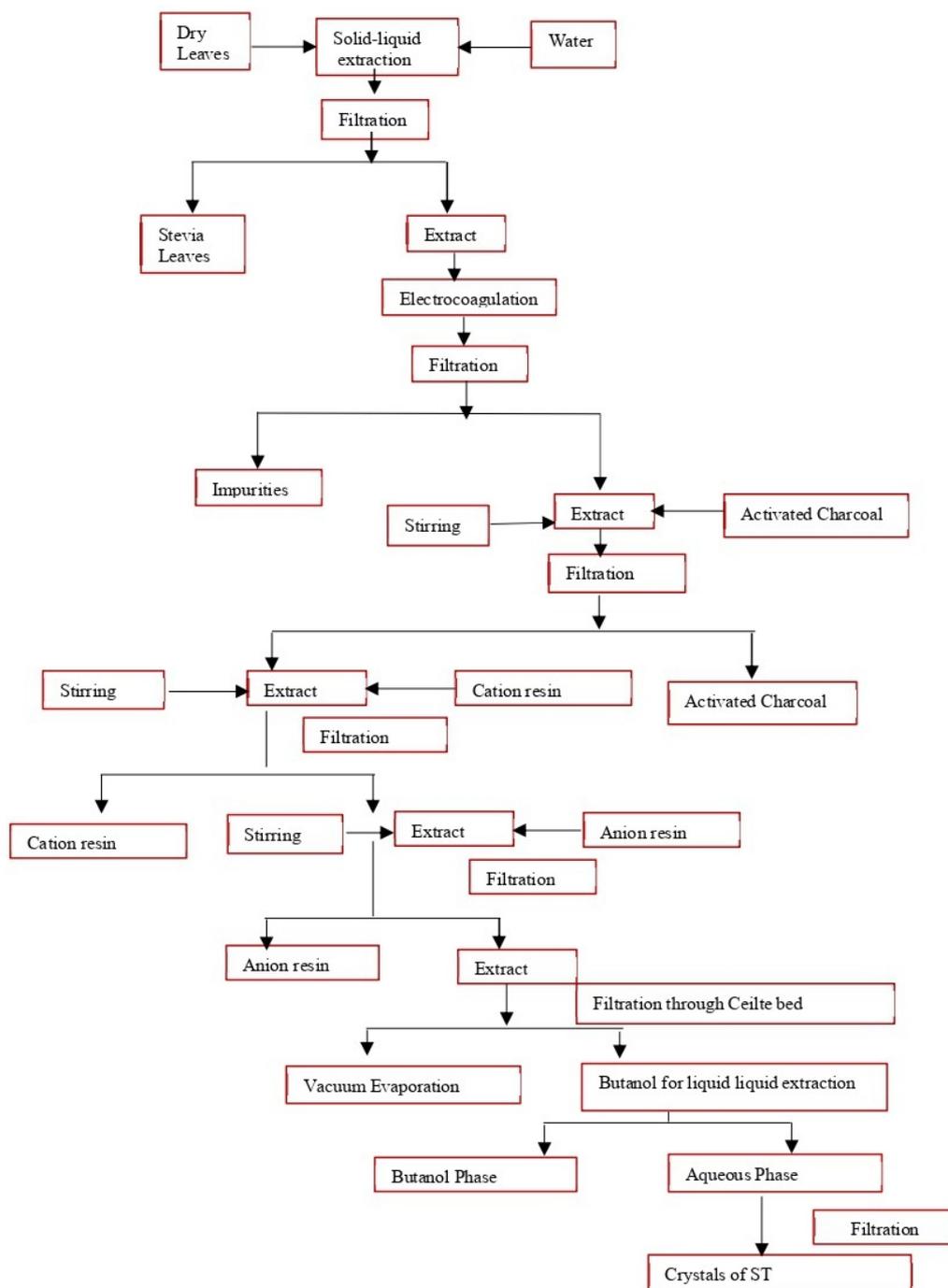


Fig. 1. Overall methodology of the final process for extraction of ST from stevia leaves

steviol glycosides and purity in Table 2 which shows maximum extraction of 54 mg from 105 g or 250 g of dry stevia leaves (Huang, Fu et al. 2010; Kumari, Rana et al. 2017). In another study where leaves were pretreated with ethanol, 27 mg of ST were obtained from 100 g of leaves (Formigoni et al., 2018). Other process parameters are given in Table 2.

Table 2. Different process schemes experiments run

Sr. No	Process steps	Process condition	Ref.
1	(a) hot water extraction, (b) adsorption, (c) vacuum evaporator (d) extraction with ethyl acetate, (e) adsorption, (f) high-speed counter-current chromatography	ST: 54 mg/105 gm leaves, Reb A: 36 mg/105 gm leaves	Huang et al. (2010)
2	(a) refluxion with methanol, (b) refluxion with chloroform, (c) washed with methanol, (d) distilled off methanol, (e) added water (200 ml), (f) extracted with n-butyl alcohol, (g) vacuum evaporation, (h) column chromatography	ST: 54 mg/250 gm leaves, Reb A: 36 mg/250 gm leaves,	Kumari et al. (2017)
3	(a) ethanolic pretreatment, (b) aqueous extraction, (c) filtration, (d) ultrafiltration, (e) nanofiltration, (f) cationic column, (g) anion column, (h) adsorption column, (i) drying, (j) final product	ST: 0.274 mg/gm dry extract, Reb A: 0.13 mg/gm dry extract	Formigoni et al. (2018)

4. SELECTION OF OPERATIONS AND SOLVENTS

(i) *Extraction*: The stevia plant materials such as leaves contain polar as well as non-polar compounds (Kumari et al. 2017). Since ST and Reb-A are polar in nature, extraction steps were carried out by polar solvents like water, ethanol or methanol. Alcohol such as ethanol can extract SGs more clearly than water. However, alcohols cannot eliminate impurities such as fat, non-polar substances etc.

Moreover, if a large amount of organic solvent is used in extraction, the organic solvent ends up as more problematic volatile and hazardous chemical waste. Hence, this work avoids hazardous organic solvents and water was used as a solvent for extraction.

(ii) *Electrocoagulation*: Even after the extraction by suitable solvent, non-polar impurities such as chlorophylls, carotenoids and polar undesirable components such as tannins also get extracted in an aqueous

solution. This problem is more prominent with extracted components from plant leaves (Jumpatong et al., 2006). The presence of tannins, essential oils and flavonoids in an aqueous solution is responsible for the bitter after taste. 90% of these impurities can be reduced by dechlorophyllation. Besides eliminating the impurities, electrocoagulation steps also increased the pH from 6 to 8 which also aided to enhance the rate of crystallization in the final step.

(iii) *Activated charcoal and ion exchanger*: The activated charcoal adsorbed pigments along with approximately 10% of ST and Reb A from the aqueous solution which made the solution quite clear rather than brown. During extraction, various minerals such as K, Ca, Mg, P also got extracted with ST and Reb A in solvent. To remove these minerals, cation and anion resins were used. The aqueous extract was passed through a strong cation resin which adsorbed the salt cations and cationic organic impurities from the aqueous extract. After that, anion resin was used to adsorb anions and anion organic impurities from the solution.

(iv) *Filtration with celite bed*: For further removal of pale yellow colour due to the presence of tannins in the aqueous solution, filtration with the celite bed was done. The resulting solution became light yellow as celite adsorbed tannins from the extract.

(v) *Vacuum evaporation and butanol wash*: To enhance the super-saturation level at a lower temperature, vacuum evaporation was performed. To produce crystals, butanol was added as an anti-solvent after evaporation. Since butanol is immiscible in water, the separation of butanol and aqueous phase was performed. The crystals were formed in the aqueous phase and were separated by filtration.

5. EFFECT OF SOLID TO LIQUID RATIO ON EXTRACTION OF ST

In the extraction, it was found that the amount of solvent had a significant effect on ST extraction. A few experiments have been performed to investigate the effect along with agitation. Fig. 2 shows the extraction of ST with varying time. It was observed that the effect was insignificant. In general, 1:2 leaf to solvent ratio is not sufficient to extract phytochemicals from plant material. However, a large ratio between the two has to be maintained for effective extraction. In the present work 1:15 and 1:30 ratio was maintained for extraction. It can be seen from Figure 2 that 1:30 leaf to solvent ratio is quite high although the extraction of ST was reduced. It also shows that agitation does not control the diffusion of ST extract from solid to liquid phase and was found to be insignificant. This concludes that it may be due to the mass transfer

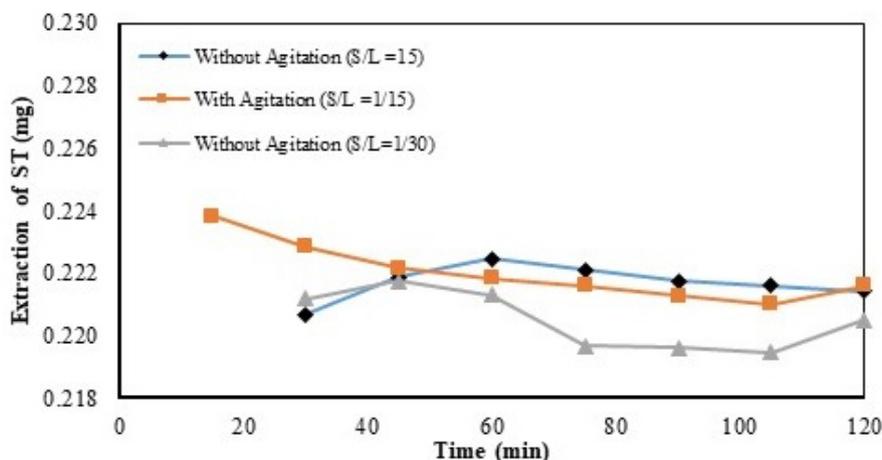


Fig. 2. Effect of time on ST concentration for runs with and without agitation for different solid to liquid ratios

within the solid being a rate-limiting step. In the extraction steps, the concentration of ST in an aqueous solution was around 0.22% (w/w), the yield obtained only from the extraction step was around 3.3 g from 100 g of stevia leaves using water as a solvent.

6. COST ANALYSIS

Stevia leaves were bought for less than \$2 from local farms and the solvent used was water. In later stage of purification butanol was used which can also be recovered and recycled up to 99%. So the overall cost to produce 1 gm of ST crystals was 10 cents which is the cheapest price of the extract. The combination of steps involved in purification makes the technology viable for scale up. This also makes the process much cheaper.

7. CONCLUSION

Stevia rebaudiana Bertoni is becoming more commercially attractive because of its nutritional benefits. Thermal classical extraction techniques are more often replaced with advanced non-thermal extraction procedures as higher yields are obtained in shorter times, which reduces solvent and energy consumption. However, in this study, along with the conventional extraction method, a number of unit operations have been combined for better extraction and isolation of ST. The process developed is novel and not presented anywhere in literature. The yield of ST was enhanced from 54 mg to 496.3 mg using this process. The novel technique used here for extraction and isolation of ST from stevia leaves is a simple, inexpensive and green process which is also cost-effective for scale-up.

REFERENCES

- Akashi H., Yokoyama Y., Osada M., 1975. Extraction and purification of steviosides. *Japan Kokai*, 75(24), 300.
- Anton S. D., Martin C.K., Han H., Coulon S., Cefalu W.T., Geiselman P., Williamson D.A., 2010. Effects of stevia, aspartame, and sucrose on food intake, satiety, and postprandial glucose and insulin levels. *Appetite*, 55, 37–43. DOI: [10.1016/j.appet.2010.03.009](https://doi.org/10.1016/j.appet.2010.03.009).
- Chranioti C., Chanioti S., Tzia C., 2016. Comparison of spray, freeze and oven drying as a means of reducing bitter aftertaste of steviol glycosides (derived from *Stevia rebaudiana* Bertoni plant) – Evaluation of the final products. *Food Chem.*, 190, 1151–1158. DOI: [10.1016/j.foodchem.2015.06.083](https://doi.org/10.1016/j.foodchem.2015.06.083).
- Crammer B., Ikan R., 1977. Properties and syntheses of sweetening agents. *Chem. Soc. Rev.*, 6, 431–465. DOI: [10.1039/CS9770600431](https://doi.org/10.1039/CS9770600431).
- Formigoni M., Milani P.G., da Silva Avíncola A., dos Santos V.J., Benossi L., Dacome A.S., Pilau E.J., da Costa S.C., 2018. Pretreatment with ethanol as an alternative to improve steviol glycosides extraction and purification from a new variety of stevia. *Food Chemistry*, 241, 452–459. DOI: [10.1016/j.foodchem.2017.09.022](https://doi.org/10.1016/j.foodchem.2017.09.022).
- Ghosh S., Subudhi E., Nayak S., 2008. Antimicrobial assay of *Stevia rebaudiana* Bertoni leaf extracts against 10 pathogens. *Int. J. Integr. Biol.*, 2(1), 27–31.
- Gomez L., Tiwari B., Garcia-Vaquero M., 2020. Chapter 9 - Emerging extraction techniques: Microwave-assisted extraction, In: Torres M.D., Kraan S., Dominguez H. (Eds.), *Advances in green and sustainable chemistry. Sustainable seaweed technologies*, 207-224. Elsevier. DOI: [10.1016/B978-0-12-817943-7.00008-1](https://doi.org/10.1016/B978-0-12-817943-7.00008-1).
- Goyal S.K., Samsher G.R., Goyal R.K., 2010. Stevia (*Stevia rebaudiana*) a bio-sweetener: a review. *Int. J. Food Sci. Nutr.*, 61, 1–10. DOI: [10.3109/09637480903193049](https://doi.org/10.3109/09637480903193049).

- Hidayat R., Wulandari P.J.E.H.I., 2021. Methods of extraction: maceration, percolation and decoction. *Eureka Herba Indonesia*, 2, 68–74. DOI: [10.37275/ehi.v2i1.15](https://doi.org/10.37275/ehi.v2i1.15).
- Huang X.-Y., Fu J.-F., Di D.-L., 2010. Preparative isolation and purification of steviol glycosides from *Stevia rebaudiana* Bertoni using high-speed counter-current chromatography. *Sep. Purif. Technol.*, 71, 220–224. DOI: [10.1016/j.seppur.2009.11.025](https://doi.org/10.1016/j.seppur.2009.11.025).
- Ishizone H., 1979. Separation and purification of stevioside. *Japan Kokai Tokkyo Koho*, 79(12), 400.
- Jumpatong K., Phutdhawong W., Buddhasukh D.J.M., 2006. Dechlorophyllation by electrocoagulation. *Molecules*, 11, 156–162. DOI: [10.3390/11020156](https://doi.org/10.3390/11020156).
- Kaur G., 2009. *Biochemical composition and characterization of glycosides of stevia rebaudiana bertoni at different development stages*. MSc Thesis, Punjab Agricultural University, Ludhiana.
- Kumari N., Rana R., Sharma Y., Kumar S., 2017. Extraction, purification and analysis of sweet compounds in *Stevia rebaudiana* Bertoni using chromatographic techniques. *Indian J. Pharm. Sci.*, 79, 617–624. DOI: [10.4172/pharmaceutical-sciences.1000270](https://doi.org/10.4172/pharmaceutical-sciences.1000270).
- Lee C.-N., Wong K.-L., Liu J.-C., Chen Y.-J., Cheng, J.-T., Chan P., 2001. Inhibitory effect of stevioside on calcium influx to produce antihypertension. *Planta Med.*, 67, 796–799. DOI: [10.1055/s-2001-18841](https://doi.org/10.1055/s-2001-18841).
- Megeji N., Kumar J., Singh V., Kaul V., Ahuja P.S., 2005. Introducing *Stevia rebaudiana*, a natural zero-calorie sweetener. *Current Science*, 88, 801–804.
- Savita S., Sheela K., Sunanda S., Shankar A., Ramakrishna P., 2004. *Stevia rebaudiana* – A functional component for food industry. *J. Hum. Ecol.*, 15, 261–264. DOI: [10.1080/09709274.2004.11905703](https://doi.org/10.1080/09709274.2004.11905703).
- Singh S., Rao G., 2005. Stevia: The herbal sugar of 21st century. *Sugar Tech.*, 7, 17–24. DOI: [10.1007/BF02942413](https://doi.org/10.1007/BF02942413).
- Takahashi K., Matsuda M., Ohashi K., Taniguchi K., Nakagomi O., Abe Y., Mori S., Sato N., Okutani K., Shigeta S., 2001. Analysis of anti-rotavirus activity of extract from *Stevia rebaudiana*. *Antiviral Res.*, 49, 15–24. DOI: [10.1016/S0166-3542\(00\)00134-0](https://doi.org/10.1016/S0166-3542(00)00134-0).
- Weihrauch M., Diehl V., 2004. Artificial sweeteners – do they bear a carcinogenic risk? *Ann. Oncol.*, 15, 1460–1465. DOI: [10.1093/annonc/mdh256](https://doi.org/10.1093/annonc/mdh256).
- Xi Y., 1998. Antioxidant mechanism of *Stevia rebaudiana* extract and antioxidant activity of inorganic salts. *Nippon Shokuhin Kagaku Kogaku Kaishi*, 45, 317–322. DOI: [10.3136/nskkk.45.317](https://doi.org/10.3136/nskkk.45.317).
- Yadav A.K., Singh S., Dhyani D., Ahuja P.S., 2011. A review on the improvement of stevia [*Stevia rebaudiana* (Bertoni)]. *Can. J. Plant. Sci.*, 91, 1–27. DOI: [10.4141/cjps10086](https://doi.org/10.4141/cjps10086).

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