Research Article

Modified Extraction method and Characterization of sweet compounds from *Stevia Rebaudiana*

Jyoti Srivastava[1] , Mahroz[2], Padma S Vankar[3]

Abstract

Stevia is a natural sweetener obtained from the leaves of *Stevia rebaudiana* (Bertoni), belonging to the *Asteraceae* family. The objective of the present study was to develop a rapid and effective methodology for the extraction of stevioside and rebaudioside A from Stevia leaves and to compare the yields with different extraction techniques. Modified method of extraction was done with water finally the glycosides were precipitated by methanol-water solvent (0.25:1.0 v/v). The solvent from the extract was removed by lyophilization. Extracted product was analyzed by HPLC-PDA followed by mass-spectrometric (MS) detections. The decolorized extract was separated on an NH_2 column using PDA detection with excitation at 210 nm. The mobile phase consisted of acetonitrile/water (80:20 v/v) with a flow rate of 1 ml/min. Evaluative criteria for stevioside and rebaudioside yield in this new method was high. An improved analytical method was developed which may be applied to quality control of stevioside content in dried leaves of Stevia before processing.

Keywords: *Stevia rebaudiana, Asteraceae*, Lyophilization, HPLC-PDA, Stevioside, Rebaudioside a

Introduction

Stevia is widely used as a sugar substitute[1,2]. There are two major components- Stevioside and Rebaudioside A, of stevia which attribute to it being low calorie natural substance. They are glycoside of diterpene steviol however, other glycosides of the same aglycone are also found in the plant[3]. They are widely used as natural sweeteners[4] for diabetic patients, but the most challenging problem encountered is long extraction procedures that are required for the optimization of product yield. Stevia leaves have a taste that is unique and very sweet with a slight licorice aftertaste. Products extracted from the herb *S. rebaudiana* are used commercially as an important natural non-caloric sweetener in an attempt to substitute synthetic sweeteners[5]

Efficient commercial processing of stevia leaves has been carried out by research groups in Japan and China and there are several patents describing methods for the extraction of steviol glycosides. Resershers[6,7] categorized the extraction patents based on solvent, solvent plus a decolorizing agent[8], adsorption chromatography[9]ion exchange[10] and selective precipitation of individual glycosides[11]. The most efficient and commonly practiced extraction processes involve four steps[12] : aqueous or solvent extraction, ion exchange, precipitation or coagulation with filtration, then crystallization and drying. New methods based on ultra-filtration have been introduced recently[13].

Once the sweet components are separated from the other biotic materials of stevia, there are a wide range of analytical tools to assess the distribution and levels of sweet diterpenoid glycosides in the individual plantations. Some of the earlier methods were thin layer chromatography[14] over pressure layered chromatography[15] Stevioside levels were also determined by the use of enzymes[16] , however the most common method has been high pressure liquid chromatography where amino bonded columns have been used extensively[17].

In this paper we are summarizing a simple, practical method for the extraction and purification of stevioside, rebaudioside

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A in a relatively pure form from stevia leaves. The extraction method used where ferrous sulphate has been used[18]. It was observed that addition of ferrous sulphate caused darkening of the leaves extract and thickening of the solution. As a result the extracted final product always had yellow tinge, which was very hard to remove in order to get colorless stevioside. But in our procedure the decolorization has proved to the most efficient and high purity Stevioside was obtained which was better suited for human consumption under its intended conditions of use as a general purpose sweetener. It is a diterpenoid glycoside[19] comprising an aglycone (steviol) and three molecules of glucose. In addition to stevioside, several other sweet compounds such as steviobioside, rebaudioside A, B, C, D, E were isolated from stevia leaf and identified by HPLC-PDA. Although rather high concentrations of stevioside and stevia extracts were shown to reduce the growth of some bacteria, the concentrations used for sweetening purposes are rather low. Therefore, the beneficial effect of the use of stevioside would rather be due to the substitution of sucrose in the food by a non-carcinogenic substance[20].

Materials and methods

Materials

Stevia leaves were harvested from the plantation fields in Lucknow, India. The leaves were dried at room temperature for 5 days and stored at the cold storage room at +4◦C. Before the experiment runs, the plant material was blend (the mean size was 500µm), packed in plastic bags.

Chemicals

Water, Acetonitrile (CH_3CN) and Methanol (CH_3OH) were of HPLC grade and purchased from Merck (Darmstadt, Germany) and were used in the analysis. Distilled water (Direct Q-UV 3, Millipore) used in the analysis, Calcium Oxide, Alum, Ammonium Chloride (S.D. fines).

Extraction of stevia leaves

Liquid extraction is the procedure for isolation of stevioside from stevia leaves on a pilot scale mostly prevalent[21] 1L of hot water (80◦C) was used to extract 500 g dried and ground stevia leaves.

Ultra sound assisted Extraction

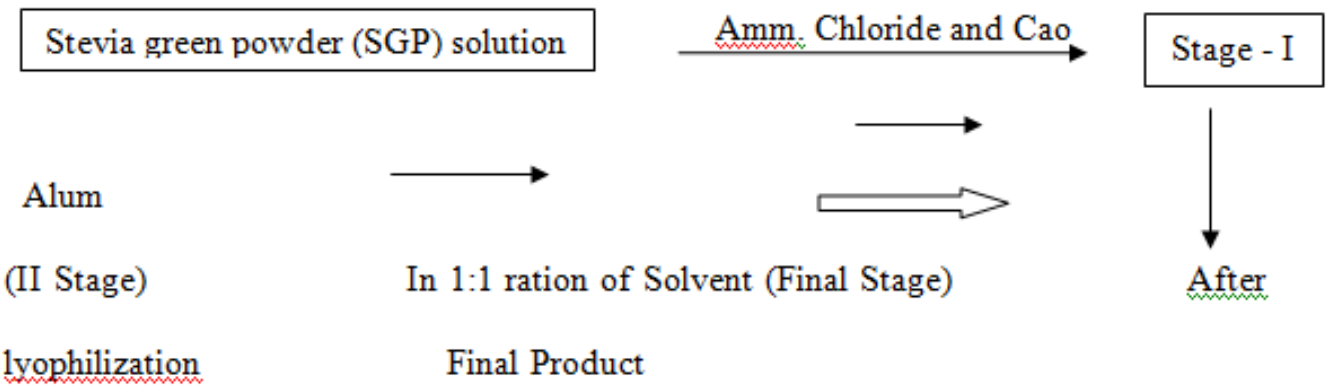
The above solution of green stevia powder was taken in a flask which was immersed in ultra-sonicator (Julabo, USR 3) for 30 mins.

Centifugation

Further on the solution of stevia was centrifuged in (Hermil-Z 323-C) apparatus at 10,000g for 15 min at 4◦C and allowed to stand. To the supernatant solution, 1 g of Ammonium Chloride (NH_4Cl) and 2 g Calcium oxide (CaO) were added. This caused rise in pH from 6.6 to 9. In the next step 5 g of Alum ($AlK(SO_4)_2.12\ H_2O$) was added to the solution which brought down the pH from 9 to 7. The solution was stirred properly. The extract was precipitated from the light green solution, apparent white precipitate settled down. The solution at this stage was filtered and to the filtrate 1:1 water and methanol mixture was added. The extract re-precipitated and this time it was kept for 5-6 hrs to settle down. The volume of the filtrate was reduced by evaporation at 70◦C in vacuum by rotary evaporator (Buchi, R 3000).

Lyophilization

Lyophilization means freeze drying, widely used in the biopharmaceutical industry for stabilizing labile biomolecules, by removing the greater proportion of the water present to leave behind a dried solid. During primary drying, while the majority of water is being removed by sublimation, this temperature should not be exceeded in the product if structural collapse of the cake is to be avoided and a pharmaceutically elegant appearance is to be maintained to yield 10-15% pure stevioside. This has been accepted to being the best technique. Recently, methods was developed a pressurized hot water extraction method for stevioside from *S. rebaudiana* there by establishing a "Green" method of isolation[22].



HPLC analysis of the extracts and residues

Sample preparation

Evaporated and dry extract (20 mg) was dissolved in 5 ml HPLC grade methanol in order to determine the glycoside composition of the raw material and all the sample solutions were passed through 0.45µm nylon membrane (Ultipore-6,6) filters to remove non-dissolved particles.

Table 1.HPLC profile of glycoside in *Stevia rebaudiana*

Peak no.	RT(min)	Molecular (m/z)	Fragments (m/z)	Formula	Compound
1	16.518	804	822	$C_{38}H_{60}O_{18}$	Stevioside
2	21.484	967	984	$C_{44}H_{70}O_{23}$	Rebaudioside A

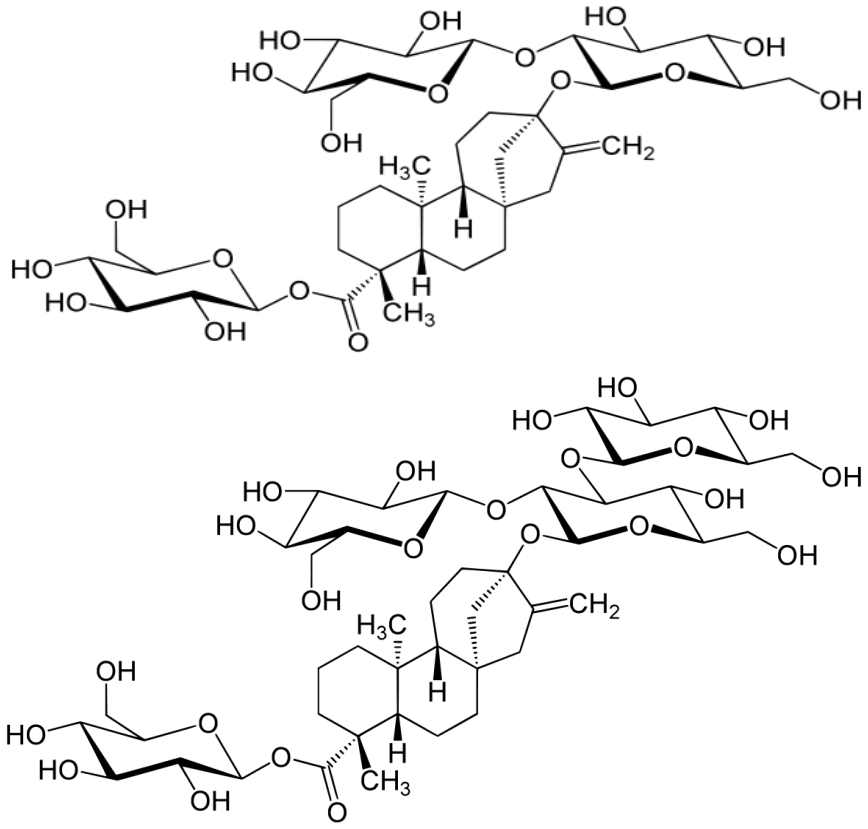


Figure 1.The structure of Stevioside and Rebaidioside

HPLC-UV conditions

HPLC-PDA analyses were performed on a HPLC equipped with WATERS, 515 pump unit,

PDA detector (waters 2998) and manual injector. A Dynamax NH_2 (4.6mm×15mm×21. 4mm; 5 µm particle size) column was used[23]. The mobile phase comprised water (A) and acetonitrile (B). Isocratic elution was performed starting with 20A/80B, pH 5 adjusted with acetic acid, at a flow rate of 1.0 ml/min, with 10 µl injections and a run time of 15 min. The UV detector was set to monitor at 210 nm and the column temperature was ambient. Column was equilibrated for 10 min with the initial conditions prior to the next injection.

Use of NH_2columns was reported by[24,25,26,27] then MS has been discussed by reachers[28]. Rebaudioside A (13-[(2-O-b-D-glucopyranosyl-3-O-b-D-glucopyranosyl-b-D-glucopyranosyl) oxy] kaur-16-en-18-oic acid b-D-glucopyranosyl ester) and stevioside (13-[(2-O-b-D-glucopyranosyl-b-D-glucopyranosyl) oxy] kaur-16-en-18-oic acid b-D-glucopyranosyl ester) were the two major diterpene steviol glycoside isolated.

Results and discussion

Since the sweeteners from Stevia leaves contain glycosides such as Stevioside, which is 250-300 times and rebaudioside A 350-450 times more powerful as sweetening agent with respect to sucrose it has widespread use in foods, beverages and medicines. The response factor for a stevioside standard was used to determine the amount of the other glycosides after correcting for differences in molecular weight. Methanol and water showed better extraction ability for isolation of stevioside from Stevia leaves. However, water represents the green alternative to methanol. The sequential organosolvent procedure

designed for both analytical and purification purposes apparently indicated an exceptional yield of glycosylated diterpenes for the methanolic step.

UV-Vis spectra

It was reported that the absorption peak at the wavelength of 420 nm resulted from dark (pale) yellow pigments, and the absorption at the wavelength of 680 nm resulted from green chlorophyll. The steviol-glycosides were previously identified on the basis of their UV spectrum in different stages. As the solution got decolorized from green to muddy to offwhite and then to clear solution, the peaks at 420 and 680 nm show diminishing order as shown in Fig-2.

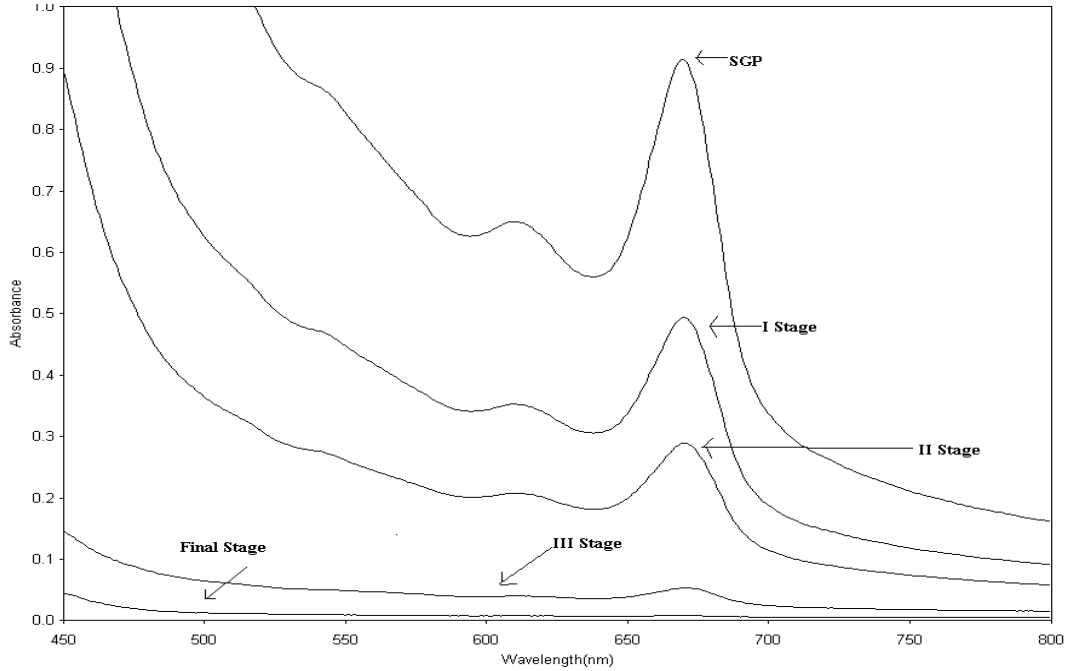


Figure 2.UV-Vis spectra at 680 nm

Optimization of HPLC-MS conditions for the evaluation of stevia

An investigation of the chromatographic behavior of the derivatives was carried out shows the chromatograms obtained from the separation of the derivatives. For HPLC analysis of the diterpenoid glycosides, aliquots were filtered (0.45mm) and applied to a Dynamax NH_2 (4.6mm×15mm×21. 4mm; 5 µm particle size) column using isocratic elution was performed starting with 20A/80B, pH 5 adjusted with acetic acid, at a flow rate of 1.0 ml/ min, with 10 µl injections and a run time of 15 min. Typical retention times observed for the diterpenoid glycosides were: stevioside (16.518 min) and rebaudioside A (21.484) on the HPLC-PDA chromatogram (Fig. 3). The analysis of the content of diterpenic glycosides from the leaves of Stevia as determined by high pressure liquid chromatography (HPLC) indicated that the concentration of rebaudioside-A was greater than that of stevioside, No peak observed between stevioside and rebaudioside A. Besides the qualitative resolution of the main sweeteners of interest, this chromatographic procedure confirmed that the sum of rebaudioside-A + stevioside accounted for 14% of the crude extract, further increasing to 11% in the completely processed sample. Regarding MS conditions, the determinations were carried out in negative ion mode as the analytes signal was about 10 times higher than in positive ion mode. The higher sensitivity in negative mode was due to CH_2Cl_2 added to the mobile phase as source of chlorine. For all the steviol-glycosides, the LC-MS analysis showed the presence of molecular ion chlorine adduct $[M+Cl_{35}]-$ and $[M+Cl_{37}]-$, while in MS/MS fragmentation these compounds displayed a consecutive loss of sugar moieties and the constant presence, at higher collision energy, of a product ion with (m/z)− 317, corresponding to the aglycone steviol MS spectra of stevioside in negative mode, showed a molecular ion peak at *m/z* 803 [M]+ in the ESI mass spectrum corresponding to a molecular formula of $C_{38}H_{60}O_{18}$ *m/z* 641 is a fragment of stevioside (loss of one glucose unit) that refers to steviol bioside, and *m/z* 839 is an adduct of stevioside with chlorine. The fragmentation pattern of the SV at different collision energy (10–30 eV) is reported. Critically remarkable, for all steviol-glycosides an ion corresponding to [M−162]− was present even at lower cone voltage. MS also showed a quasi-molecular ion peak at m/z 822 [M+NH4]+. Similarly MS fragmentation of the rebaudioside showed a molecular ion peak at *m/z* 966 $[M+Na]^+$ in the ESI mass spectrum corresponding to a molecular formula of $C_{44}H_{70}O_{23}$ *m/z* 804 is a fragment of stevioside (loss of one glucose unit), and *m/z* 999 is an adduct of rebaudioside with chlorine. Fragmentation also showed a quasi-molecular ion peak at m/z 984 [M+NH4]+.

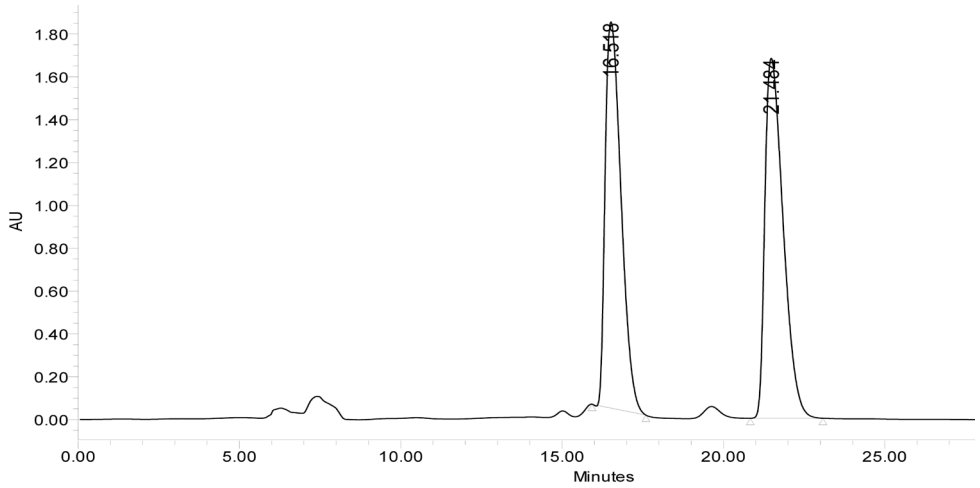
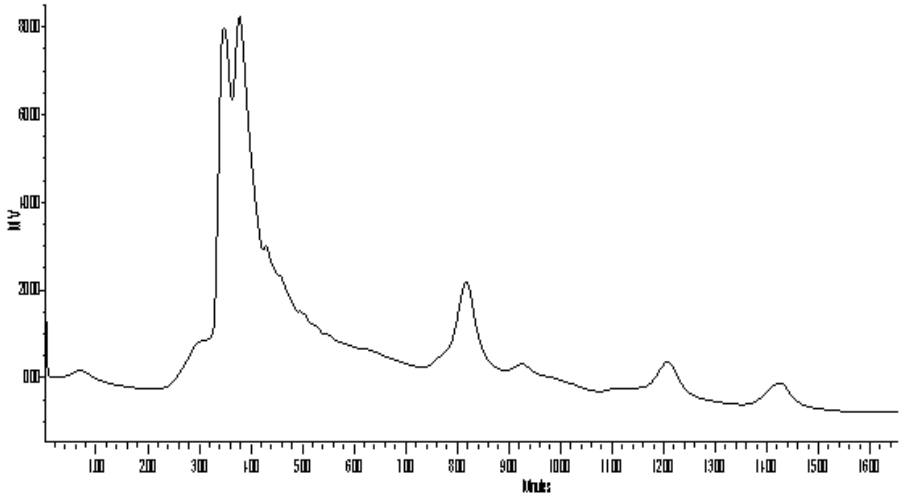
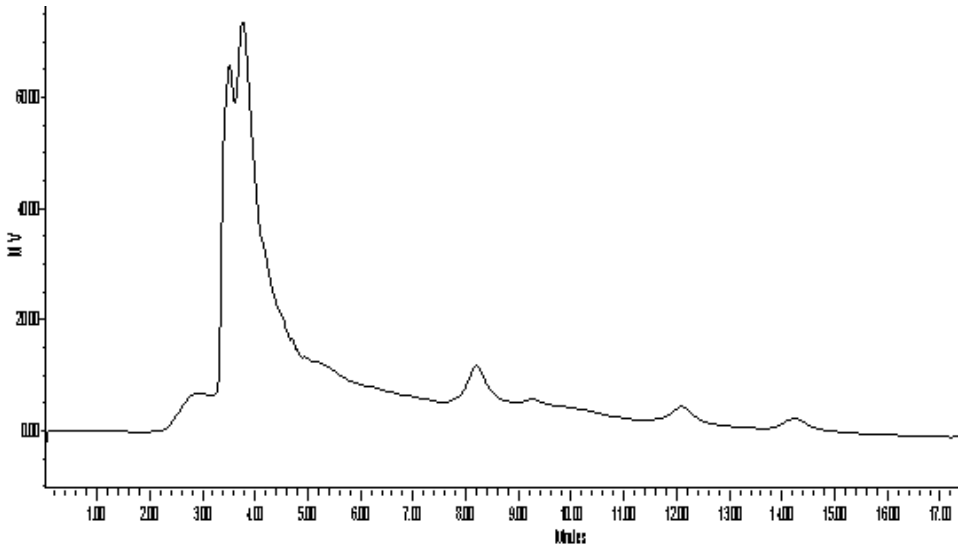


Figure 3.Chromatogram of stevia leaves extract

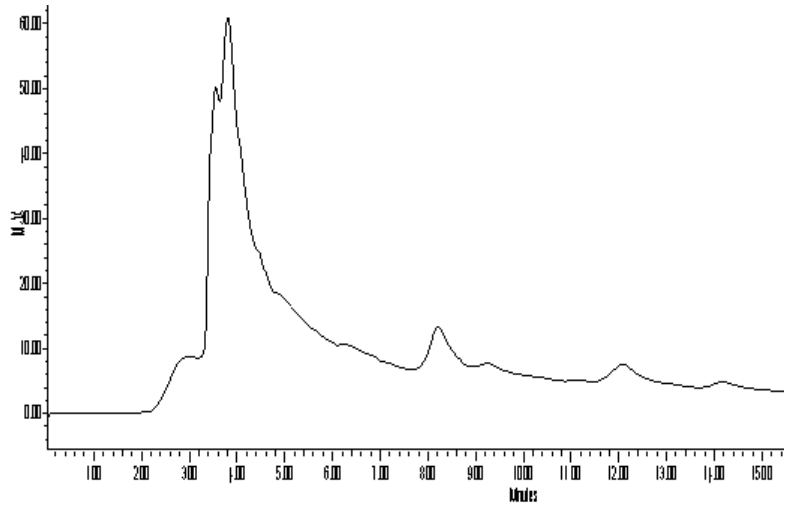
SPG



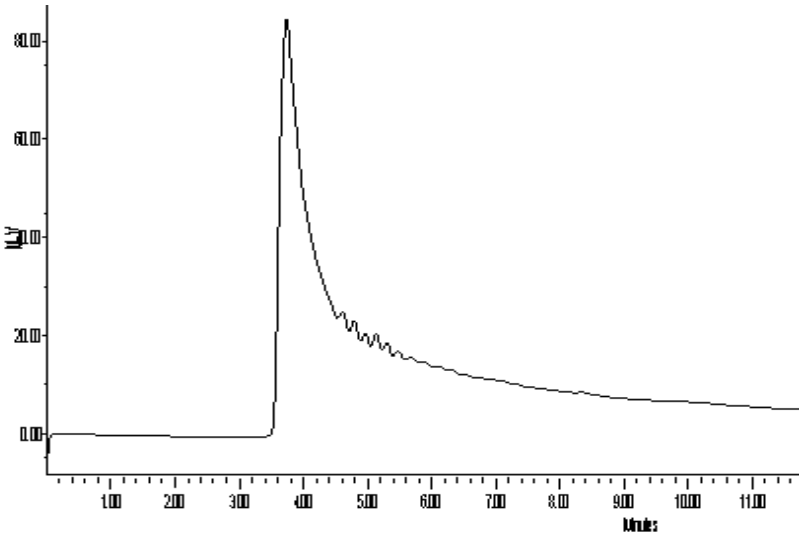
Stage- I



Stage- II



Final Stage



Final product

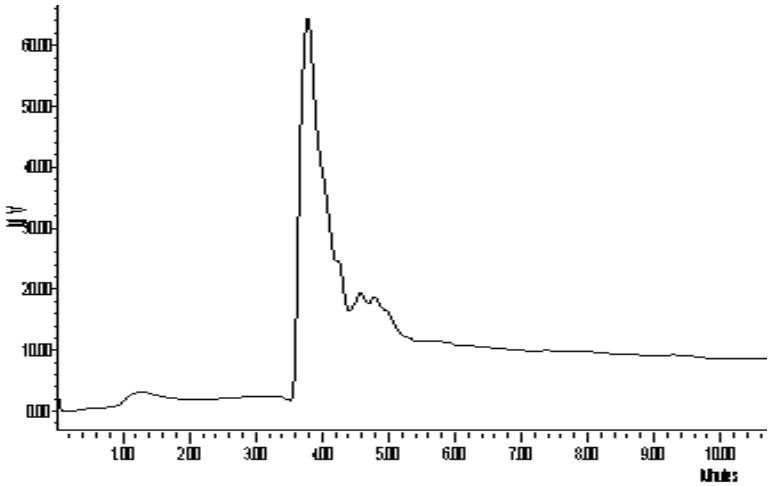


Figure 4.HPLC chromatogram of Stevia using UV–Vis detection at 200-800 nm

Stevioside

Yield 2.8 g (±5% per 100 g dry leaves), $C_{38}H_{60}O_{18}$, mp 202-207°C, IR spectrum (KBr, cm^{-1}): 3200-3600 (OH), 1729 (COO), 1656 (C=CH2), 1076, 1036 (C–OH). Mol. wt. = 804 g mol/1

Rebaudioside A

Yield 0.6 g (±1.2% per 100 g dry leaves), mp 235°C, IR spectrum (KBr, cm^{-1}): 3200-3600 (OH), 1728 (COO), 1646 (C=CH2), 1076, 1035 (C–OH). Mol. wt. = 966 g mol/1

Calibration Curve

A calibration curve for stevioside was constructed using the stevioside and the coefficient of regression was R^2 = 0.9935. The limit of detection (LOD), calculated from the calibration curves at S/N = 3, was 43.4 ng/g. The reproducibility of retention times in both dimensions was 0.1%. The reproducibility of peak areas was on average 4.5%.

Conclusions

A rapid and efficient method has been developed for the extraction of stevioside and rebaudioside-A in optimum yields using procedure. This method has the advantage of rapid extraction and fast screening of a large number of *S. rebaudiana* samples for assessment of planting material. In order to increase process productivity, yield and quality of products,

The purpose of the experiment was to compare the intensifications techniques with classical extraction.

Conflict of Interest: None

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