Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 5, 2014

Original Article

A RAPID AND EFFICIENT HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC (HPTLC) METHOD FOR SIMULTANEOUS ANALYSIS OF STEVIOSIDE AND REBAUDIOSIDE-A IN *STEVIA REBAUDIANA*

MONICA SAIFI[1], ATHAR ALI[1], MANU SAINI[1, 2], NAZIMA NASRULLAH[1], SHAZIA KHAN[1], M. Z. ABDIN[1*]

Centre for Transgenic Plant Development, Department of Biotechnology, Faculty of Science, Jamia Hamdard, Hamdard Nagar, New Delhi 110062, India[1], Department of Radiation Biology, Institute of Nuclear Medicine and Allied Sciences, Brig. S.K. Mazumdar Marg, New Delhi 110054, India[2]. Email: mzabdin@rediffmail.com

Received: 28 Mar 2014 Revised and Accepted: 01 May 2014

ABSTRACT

Objective: To develop a simple, rapid, sensitive and validated high performance thin layer chromatography method (HPTLC) for simultaneous identification and quantification of stevioside and rebaudioside-A, two major steviol glycosides in *STEVIA REBAUDIANA* leaves extract prepared using filtration method.

Methods: The separation was achieved on silica gel 60 F_{254} HPTLC plates using chloroform: methanol: water (60:32:4, v/v/v) as the mobile phase. Densitometric analysis of stevioside and rebaudioside-A were carried out using absorption-reflection mode at 400nm.

Results: Densitometric analysis of leaf extract gave compact spots for stevioside (R_f 0.33±0.03) and rebaudioside-A (0.24±0.04). The linear regression analysis of data for the calibration plots showed a good linear relationship with r=0.995 and 0.999 for stevioside and rebaudioside-A, respectively. The average recovery of stevioside and rebaudioside-A was 98.97 and 97.68%, respectively indicating the good reproducibility. Instrument precision and repeatability of the method was found to be 1.02 and 1.27 for stevioside and 0.95 and 1.50 for rebaudioside-A. Statistical analysis of the data showed that the method is reproducible.

Conclusion: These results prove that this method is rapid, precise, accurate and reproducible. Hence, it can be employed for evaluating the concentrations of steviol glycosides in the leaf samples of *STEVIA REBAUDIANA* and quality control of single as well as polyherbal formulations containing stevioside and rebaudioside-A by pharmaceutical industries and government agencies.

Keywords: *STEVIA REBAUDIANA* Bertoni, Stevioside, Rebaudioside-A, High Performance Thin Layer Chromatography.

INTRODUCTION

STEVIA REBAUDIANA Bertoni, the sweet herb of Paraguay, is fast becoming a major source of high potency sweetener, which produces sweet taste but has no calorific value [1]. Crude sweetener extracts from *Stevia's* leaves, have been used from decades to sweeten soft drinks, soju, soy sauce, yogurt, and other foods in Japan, Korea and Brazil [2]. The dry extract from the leaves of *S. rebaudiana* also contains flavonoids, alkaloids, water-soluble chlorophylls, xanthophylls, hydroxycynnamic acids, neutral water-soluble oligosaccharides, free sugars, amino acids, lipids and essential oils [3].

Stevioside and rebaudioside-A are the predominant steviol glycosides found in *S. rebaudiana*. Commercial intrest in steviol glycosides as sweeteners has been high for a long time. These have been used as food and medicine (ethnobotanical) for many years, in many countries. Stevioside and rebaudioside-A have been suggested to exert beneficial effects on human health, including anti-hyperglycemic [4], anti-cancerous [5], anti-hypertensive [6, 7], anti-rotavirus and cardiovascular actions [8], antioxidant [9], and anti-inflammatory [10]. Their contents in the leaves of stevia are however, varied from cultivar to cultivar and also from region to region [11]. With the ever increasing use of herbal medicines worldwide and the rapid expansion of the global market for these products, the safety and quality of medicinal plant materials and finished herbal medicinal products have become a major concern for health authorities, pharmaceutical industries and the public [12]. Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines, since they might represent appropriately the "chemical integrities" of the herbal medicines and therefore, be used for authentication and identification of herbal products [13].

A number of analytical as well as biochemical methods have been described in scientific literature for characterization and estimation of steviosides in *S. rebaudiana* leaves. These include enzymatic hydrolysis determination and chemical detection [14], TLC [15, 16], densitometry [17], high performance liquid chromatography (HPLC) [18], ultra-high performance liquid chromatography-mass spectroscopy (UHPLC/MS) [19], liquid chromatography-mass spectroscopy (LC/MS), liquid chromatography/electrospray ionization-mass spectrometry (LC-MS-ESI) [20], gas chromatography-mass spectroscopy (GC/MS) [21, 22], desorption electrospray ionization mass spectrometry (DEIMS) [23], and two-dimensional high performance liquid chromatography (2DHPLC) [24]. These methods except TLC are, however, time consuming, expensive, complicated and need high purity samples. In case of TLC, the detection limit is very high. Hence, there is a need for developing a method which should be rapid, sensitive, specific and cost effective. Recently, we have also developed a HPTLC method for quantification of esculin in different fractions of *Cichorium intybus* leaf extract [25]

The aim of the present study was, therefore, to develop a simple, rapid, precise, accurate and validated HPTLC method for simultaneous quantification of two pharmaceutically active steviol glycosides, namely stevioside and rebaudioside-A (Fig.1) in the leaves of *S. rebaudiana* from different geographical origin and grown at Herbal Garden of Jamia Hamdard, New Delhi, CSIR-IHBT, Palampur and FRI, Dehradun, India.

MATERIAL AND METHODS

Chemicals and reference compounds

All the chemicals, including solvents, were of analytical grade and procured from E. Merck (Darmstadt, Germany). The HPTLC plates coated with Silica gel, 60F254 were purchased from E. Merck (Darmstadt, Germany). The standards, stevioside and rebaudioside-A were purchased from Sigma-Aldrich, Poole, Dorset, UK.

Plant materials

The fresh leaves from different cultivars of *S. rebaudiana,* which were grown in Herbal Garden of Jamia Hamdard, New Delhi, India (serial no. 1-4, Table 1), were collected. The leaf samples of the herbs were also procured from CSIR-IHBT, Palampur, H.P. (serial no. 5, 6 and 7, Table 1) and FRI, Dehradun, India (serial no. 8, Table 1). All the samples were collected in the month of December at flowering stage and washed with water to remove soil particles. Thereafter, these were dried in shade at 25-30˚C and stored at 25±3˚C in air tight containers till further use.

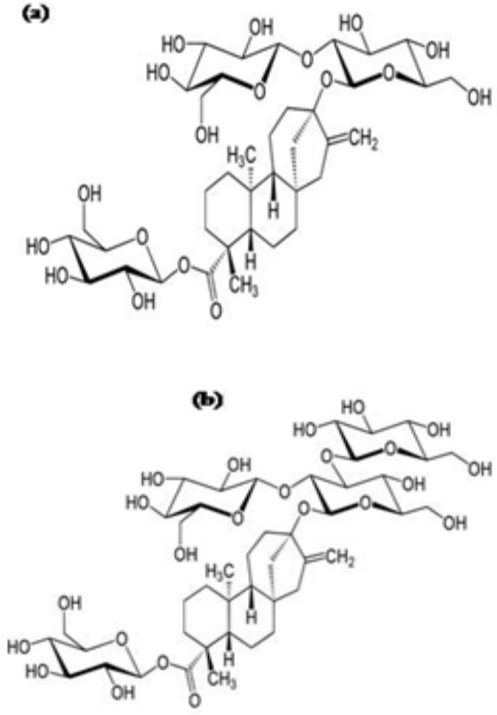

Fig. 1: Chemical Structures of Steviol Glycosides, Stevioside (a) and Rebaudioside-A (b)

Preparation of sample extract

The dried leaf sample (1g) of *S. rebaudiana* was crushed in a mortar pestle and passed through a sieve to obtain a powder of particle size of approximately 1mm. The leaf powder was then transferred into a volumetric flask of 50ml and mixed with 20ml of 95% ethanol. The mixture was heated with reflux in a boiling water bath for 15min, and filtered through whatman filter paper number 0.45. All the samples were subjected to this process and the ethanol extracts obtained were used for estimation of stevioside and rebaudioside-A.

Preparation of standard solution

The standard stock solution (1mg/ml) of stevioside was prepared by accurately weighing 1mg of stevioside, and dissolving in 1.0ml of methanol. Further dilutions were prepared by diluting this stock solution with methanol. Standard stock solution (1mg/2.5ml) of rebaudioside-A was prepared by weighing 1mg of rebaudioside-A, and dissolving in 2.5ml methanol. Further dilution was prepared by diluting this stock solution with methanol. The two standard solutions were then mixed in 1:1 ratio for further use.

Chromatography

The HPTLC was performed on 20x10cm aluminum foil plate coated with a 200µm layer of silica gel $60F_{254}$ (E. Merck, Germany). The plate was activated for 30 minutes at 110°C in hot air oven. A 2µl of each sample solution was applied as 6mm band by means of a Camag (Switzerland) Linomat 5 applicator fitted with a 100µl syringe. A constant application rate of 150nl/s was used. Linear ascending development using the mobile phase, chloroform: methanol: water (60:32:4v/v/v) was performed in a glass twin-trough chamber (Camag) previously saturated with mobile phase for 20min (optimized saturation time) at room temperature. Reference marker compound was also applied on the TLC plate along with samples to confirm the presence of stevioside and rebaudioside-A, respectively in test samples. The development distance was 90mm. Subsequent to the development, the TLC plate was air dried for 5-10min. The dried plate was then sprayed with a spraying reagent consisting of acetic anhydride: sulphuric acid: ethanol (1:1:4), followed by heating at 110°C for 3-5min. The densitometric analysis was performed at 400nm using Camag TLC Scanner 3 in absorption-reflection mode, winCATS software (v. 1.4.3.6335). Slit dimension was 6.00 x 0.45mm with scanning speed of 20mm/s.

Calibration curve of steviol glycosides

Different volumes of standard mix solution (1, 2, 3, 4, 5, 6µl) containing stevioside (200, 400, 600, 800, 1000, 1200ng/spot) and rebaudioside-A (100, 200, 300, 400, 500, 600ng/spot) were then applied to TLC plates to prepare six point linear calibration curve. The calibration curves of stevioside and rebaudioside-A were obtained by plotting peak area versus concentration of stevioside and rebaudioside-A applied.

Quantification of stevioside and rebaudioside-A in test samples

2µl each of sample extracts were applied in triplicate on a TLC plate with a Linomat V applicator. The plate was developed and scanned as mentioned above and peak areas were recorded. The amounts of two steviol glycosides in all sample extracts were calculated using the calibration curves of stevioside and rebaudioside-A, respectively.

Method validation

The method was validated for repeatability, precision and accuracy. Repeatability of the method was affirmed by multiple measurements (n=9) of stevioside and rebaudioside-A after application on the TLC plate (200ng/spot and 100ng/spot, respectively) under the same analytical and laboratory conditions. Intermediate precision of the method was studied by analyzing aliquots of standard solution of stevioside and rebaudioside-A (200, 800 and 1200ng/spot of stevioside and 100, 400 and 600ng/spot of rebaudioside-A, respectively) on the same day (intra-day precision) and on different days (inter-day precision). The results were expressed as relative standard deviation (%RSD) between different days.

Accuracy of the method was tested by performing the recovery studies. To the pre-quantified *S. rebaudiana* leaf extract, known amounts of stevioside and rebaudioside-A (100ng each) in methanol were added and estimated as described above.

RESULTS AND DISCUSSION

In the present study, an HPTLC method was developed for quantification of two major steviol glycosides found in the leaves of *S. rebaudiana*, stevioside and rebaudioside-A.

Optimization of chromatographic conditions

Detailed TLC studies revealed that the mobile phase comprising of chloroform: methanol: water in the ratio of 60:32:4 (v/v/v) had shown the highest selectivity for resolution of steviol glycosides. The bands of these compounds were well separated on HPTLC plate, at R_f 0.33±0.03 and 0.24±0.04 for stevioside and rebaudioside-A, respectively. The three dimensional patterns, obtained from standard and test samples, revealed that the peaks at R_f 0.33±0.03 (stevioside) and 0.24±0.04 (rebaudioside-A) for both the samples were super-imposable as shown in Fig 2.

Initial HPTLC fingerprinting study was performed on the pure marker compounds, stevioside and rebaudioside-A. The bands of marker compounds were scanned and their spectra were recorded at 400nm. Thus, for better quantitative analysis all the plates were scanned at 400nm. Fig 3 shows the chromatograms obtained at 400nm from the standard marker compounds separately and as standard mix.

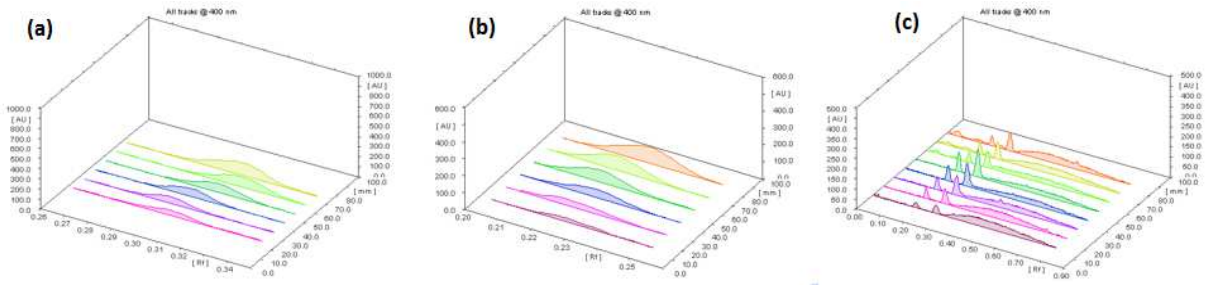

Fig. 2: 3-D chromatograms of Stevioside (a), Rebaudioside-A (b) and Stevioside and rebaudioside-A mix (c).

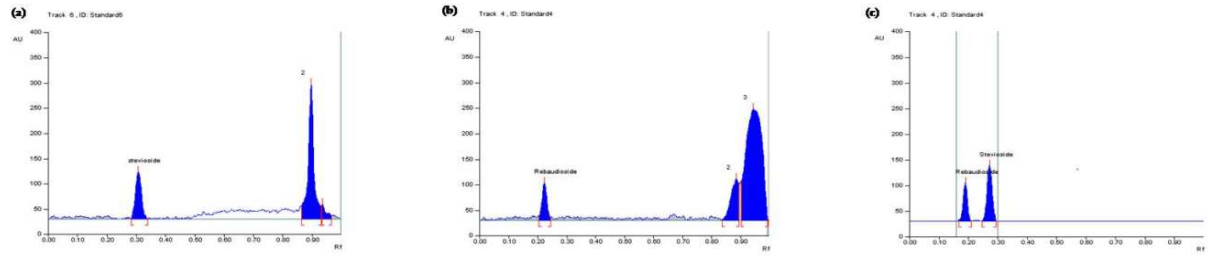

Fig. 3: HPTLC chromatograms of Stevioside (a), Rebaudioside-A (b) and Stevioside and rebaudioside-A mix (c).

Fingerprint patterns obtained from the test samples under identical conditions showed that, the amounts of two dominant steviol glycosides varied among different genotypes of Stevia collected from different geographical regions of India. These variations could either be due to the genotypic differences and/or genotypic and environmental interactions. In all the samples, the concentration of stevioside was found higher than rebaudioside-A (Table 1). HPTLC chromatograms obtained from all the samples showed the peaks corresponding to standard stevioside and rebaudioside-A marker compounds, representative chromatogram from one sample is shown in Fig. 4.

Method validation

Method validations was performed on parameters such as linearity, limit of detection (LOD) and quantification (LOQ), precision, accuracy, specificity and robustness.

Linearity

The linearity was obtained by analyzing different solutions of stevioside and rebaudioside-A with varying amounts in the linear range of 200-1200ng/spot and 100-600ng/spot, respectively and representative linear calibration curves of stevioside and rebaudioside-A, respectively as shown in Fig 5 a,b. The regression data, as shown in Table 2, indicated a good linear relationship between the concentrations and peak areas over the concentrations used.

LOD and LOQ

LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the baseline noise. The LOD was determined as the lowest amount of analyte that was reproducibly detected above the baseline noise. LOQ and LOD for stevioside and rebaudioside-A, respectively were determined using peak areas and given in Table 2.

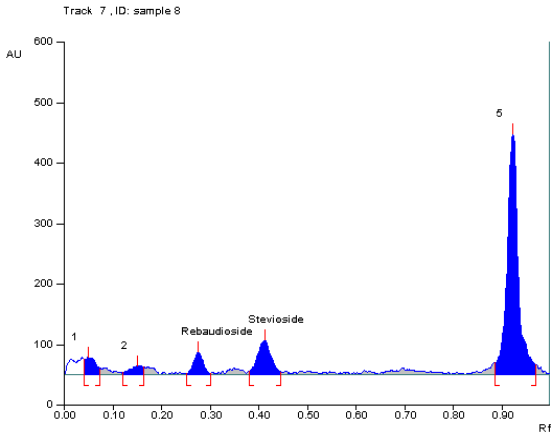

Fig. 4: HPTLC chromatogram of stevioside and rebaudioside-A in test sample.

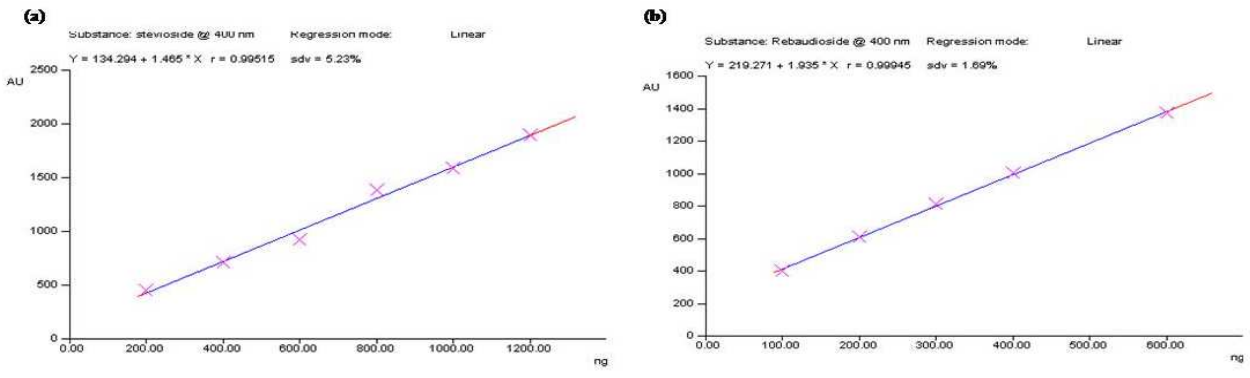

Fig. 5: Calibration curves of Stevioside (a) and Rebaudioside-A (b).

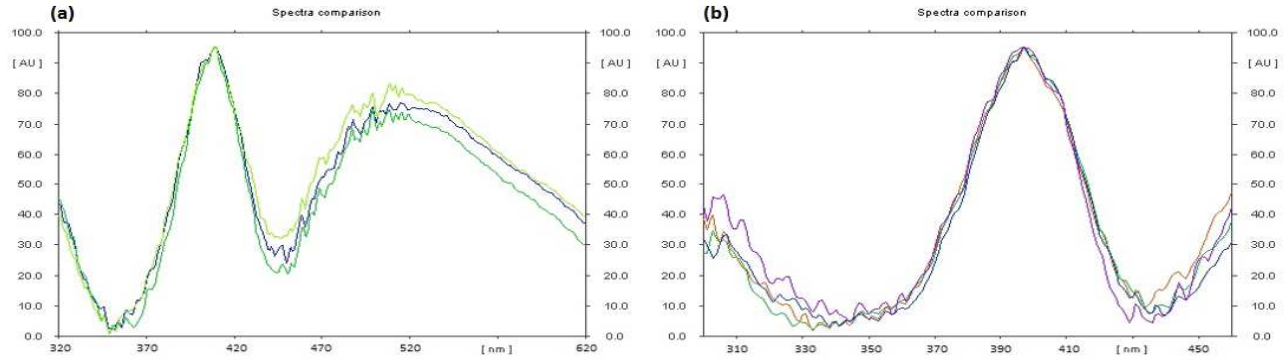

Fig. 6: Overlay of UV absorption spectra of marker compounds in the sample track along with standards, Stevioside (a) and Rebaudioside-A (b).

Precision

Six different volumes of same concentrations were spotted on a plate and analyzed by the proposed method to determine variation arising from the method itself and expressed as % R.S.D. Intermediate precision (inter-day and intra-day) was investigated by repeating the same experiment on the same and three different days. The instrument, method and intermediate precision data are summarized in Table 3a and 3b respectively.

Accuracy

To the pre-analyzed sample, 100ng each of stevioside and rebaudioside-A, respectively were added and the mixture was analyzed by the proposed method. The experiment was conducted in triplicate to check recovery and accuracy of the system. The results are summarized in Table 4, showing the accuracy (expressed in recovery) of the method as the mean values of stevioside and rebaudioside-A, respectively.

Specificity

Specificity of the method was ascertained by analyzing the standard and sample solutions. To ascertain the purity of the peak of stevioside and rebaudioside-A in the test samples (leaf extracts), its *insitu* absorbance spectra were compared with that of the standards i.e. stevioside and rebaudioside-A, respectively and found to be super imposable, thus confirming the peak purity (Fig 6).

Robustness

The standard and test solutions were spotted on HPTLC plates and several slightly different combinations of the three solvents were used to assess robustness. The modified mobile phase of chloroform: methanol: water (60:32:4v/v), afforded good resolution with R_f 0.33-0.36 and 0.24-0.26 for stevioside and rebaudioside-A, respectively [26, 27].

Table 1: Amount of steviol glycosides present in the leaves of *S. rebaudiana* collected from different locations (n=3)

S. No	Biological origin	Stevioside		Rebaudioside-A	
		Average (%)	R.S.D. (%)	Average (%)	RSD (%)
1.	Madhya Pradesh, India	4.57	4.53	1.60	4.40
2.	Haryana, India	3.63	3.34	1.56	1.74
3.	Himachal Pradesh, India	5.21	3.90	1.79	1.80
4.	Kashmir, India	7.80	1.30	1.32	3.09
5.	Himachal Pradesh (CSIR-IHBT), India	7.50	2.71	2.17	2.32
6.	Brazil	3.24	1.29	1.60	3.43
7.	U.S	3.77	2.67	1.55	2.96
8.	Uttarakhand, India	3.77	2.13	1.52	1.94

Table 2: The linearity, limit of detection (LOD) and limit of quantification (LOQ) data

Steviol glycosides	Equation y=ax+b	Correlation coefficient (r)	LOD (ng)	LOQ (ng)
Stevioside	134.294 + 1.465 * X	0.99515	100	200
Rebaudioside-A	219.271 + 1.935 * X	0.99945	40	100

Table 3a: Instrument and method precision of *STEVIA REBAUDIANA* (n=6)

Sample	Conc. (ng/spot)	Instrument precision			Method precision		
		Mean area	SD	%RSD	Mean area	SD	%RSD
Stevioside	200	467.65	9.96	2.13	460.39	13.58	2.95
Rebaudioside-A	50	111.14	1.33	1.20	110.22	2.55	2.32
Stevioside	400	843.50	12.65	1.52	848.32	12.72	1.52
Rebaudioside-A	100	178.23	1.85	1.04	167.25	2.57	1.54
Stevioside	600	1259.83	10.07	0.80	1250.33	13.7	1.10
Rebaudioside-A	150	219.16	2.08	0.95	210.42	3.15	1.50
Stevioside	800	1874.15	13.4	0.72	1879.06	13.15	0.78
Rebaudioside-A	200	312.52	2.50	0.80	308.67	3.08	1.00
Stevioside	1000	2325.51	11.62	0.53	2311.22	13.63	0.59
Rebaudioside-A	250	428.92	4.07	0.95	432.62	5.36	1.24
Stevioside	1200	2807.61	11.23	0.42	2800.31	14.56	0.52
Rebaudioside-A	300	469.56	4.08	0.87	475.59	7.37	1.55

Table 3b: Intermediate precision of *STEVIA REBAUDIANA* (n=6)

Sample	Conc. (ng/spot)	Intraday precision			Interday precision		
		Mean area	SD	%RSD	Mean area	SD	%RSD
Stevioside	200	455.61	12.57	2.76	452.42	13.07	2.89
Rebaudioside-A	50	101.82	1.07	1.06	121.61	2.29	1.89
Stevioside	400	848.42	13.15	1.55	832.11	13.23	1.59
Rebaudioside-A	100	167.62	2.09	1.25	181.22	3.38	1.87
Stevioside	600	1240.12	14.75	1.17	1230.83	14.76	1.22
Rebaudioside-A	150	202.22	2.06	1.02	229.13	2.56	1.12
Stevioside	800	1860.52	15.07	0.81	1880.32	14.10	0.75
Rebaudioside-A	200	320.69	2.56	0.80	320.16	3.42	1.07
Stevioside	1000	2319.66	13.45	0.58	2305.42	14.06	0.61
Rebaudioside-A	250	448.62	4.71	1.05	419.62	4.11	0.98
Stevioside	1200	2795.31	14.53	0.52	2798.32	15.95	0.57
Rebaudioside-A	300	470.08	5.54	1.18	471.66	7.2	1.54

Table 4: Recovery study of Stevioside and Rebaudioside-A (n=3) using HPTLC method

Steviol glycosides	Amount present (mean ng)	Amount added (ng)	Amount detected (mean ng)	Average recovery (%)	%RSD	SD
Stevioside	472.1	100	566.54	98.97	0.0021	0.21
Rebaudioside A	131.94	100	285.17	97.68	0.0032	0.32

CONCLUSION

In the present study, HPTLC method was developed for quantification of two dominant steviol glycosides, mainly stevioside and rebaudioside-A in the leaves of *S. rebaudiana*, which is traditionally used as an alternative to artificial sweetener and known for its anti-diabetic properties. Jaitak *et al*. [28] have also quantified steviol glycosides using high performance thin layer chromatography (HPTLC) procedure with densitometric detection, but the method of extraction used by them was quite laborious and time consuming. On the contrary, the method developed by us is efficient in terms of time, yield and is also simple, specific, rapid, reproducible and cost effective. It is also superior to the reported UV and HPLC methods. It was suitably developed and validated for precision, accuracy, specificity, ruggedness and robustness. This method has revealed significant differences in steviol glycosides (stevioside and rebaudioside-A) among the genotypes investigated and these variations could either be due to differences at genotypic level and/or because of interactions between genotypes and environmental conditions. The method will be suitable for the quality control of single and polyherbal formulations of traditional medicines containing *S. rebaudiana* leaves. It can also be used to quantify the amount of these glycosides in the plant leaves to be used for commercial extraction. The scientists working in varietal improvement programs of *S. rebaudiana* can also use this method to evaluate these glycosides in germplasm and improved varieties.

ACKNOWLEDGEMENT

The research facilities developed from UGC-SAP (DRS-1) grant sanctioned to the Department of Biotechnology and used in this study are thankfully acknowledged. The Research Fellowship awarded to A. Ali and N. Nasrullah is gratefully acknowledged.

CONFLICTS OF INTREST

Authors declare no conflicts of interest.

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