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Identification and estimation of stevioside in the commercial samples of stevia leaf and powder by HPTLC and HPLC

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Abstract

Stevioside, a major sweet component of leaves of *Stevia rebaudiana* (Bertoni), is about 300 times sweeter than sucrose. Highly refined stevia leaf extracts and stevioside are officially used as low calorie sweeteners in many countries. The present study describes a method for rapid identification and estimation of stevioside in commercial samples using HPTLC and HPLC. Identification of stevioside in samples was done by HPTLC. The separation was achieved on a pre-coated silica gel $60F_{254}$ plate with mobile phase; ethyl acetate: methanol: water (75:15:10 v/v/v). Densitometric scanning was perfomed at 510 nm after visualization with a solution of acetic anhydride: sulphuric acid: ethanol (1:1:10 v/v/v). The identity of the peak corresponding to stevioside was further confirmed by spectral analysis. HPLC was used for the estimation of stevioside content. The calibration curve was linear in the concentration range from 0.1 to 1 mg/ml. The detection limit for stevioside was 0.05 mg/ml (1 µg per injection). The percentage stevioside content of the samples i.e. stevia powder and stevia leaf was found to be; 8.859 % and 3.703 % respectively. The method allows rapid identification and quantification of stevioside in different samples and can be used for routine analysis of stevioside in commercial samples.

Key-Words: Stevioside; *Stevia rebaudiana* (Bertoni); HPTLC; Stevia powder; HPLC.

Introduction

Stevia rebaudiana (Bertoni), often referred to as ''the sweet herb of Paraguay'', is a perennial shrub belonging to Asteraceae (Compositae) family. It is native to certain regions of South America like Paraguay and Brazil. The major sweet component present in the leaves of *Stevia rebaudiana* (Bertoni), Stevioside, tastes about 300 times sweeter than sucrose (0.4% solution).[1] The plant as well as its extract has been used since long time as a sweetener in various regions viz. South America, Asia, Japan and China. The leaves are found to contain a complex mixture of eight sweet diterpene glycosides, including stevioside, steviolbioside, rebaudiosides (A,B,C,D,E) and dulcoside A.[2] Stevia leaves, stevioside and its highly refined extracts are officially used as a low calorie Sweetener in countries such as Brazil, Korea and Japan.[3,4] Several studies have reported the antihyperglycaemic, insulinotropic, glucagonostatic and antihypertensive effects of stevia glycosides.[5,6,7,8]

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Many studies have also been conducted to determine the toxicological effects of stevia extract and stevioside[9,10], however no significant toxicity has been reported with either stevioside or stevia extract. JECFA (Joint FAO/WHO Expert Committee on Food Additives) in 2004, established an ''Acceptable Daily Intake" or ADI of 0–2 mg/kg body weight/day of total steviol glycosides in a finished product on a temporary basis.[11] Various methods have been described for the estimation of stevioside content in stevia leaves including enzymatic determination, near infrared reflectance spectroscopy (NIRS) and HPLC.[12-17] All these methods are either time consuming or require highly sophisticated and costly instruments. The present study describes a method for rapid identification and estimation of stevioside content in commercial samples using HPTLC and HPLC.

Material and Methods

Stevioside standard was obtained from Sigma Aldrich, USA. Commercial stevia leaf powder and stevia powder were purchased from Maruti chemicals company, Ahmedabad, India. Acetonitrile (ACN) (HPLC grade) was from Merck, Germany, water (for

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HPLC) from Qualigens fine chemicals, India and HPTLC plates from E. Merck, Germany. All the other chemicals and solvents used were of analytical grade.

Preparation of standard solution (for HPTLC)

The standard solution was prepared by accurately weighing 5 mg of stevioside, transferring it to a 5 ml volumetric flask and making up the volume using methanol in order to make 1 mg/ml solution.

Extraction of sample (for HPTLC)

The sample was prepared by accurately weighing 1 gm of stevia leaf powder and extracting it with methanol (50 ml x 3) by sonication at 50±2^{0}C for 45 min. The extracts were filtered through filter paper, combined and dried under reduced pressure at 50±5^{0}C. Extract thus obtained was defatted with hexane (10 ml x 3). Hexane solution was removed and the residue obtained was again dried under reduced pressure to remove traces of hexane. This residue was used to make 5 mg/ml solution of sample for further analysis.

The sample of commercial stevia powder was prepared by directly dissolving accurately weighed quantity of powder in methanol to make 1 mg/ml and 5 mg/ml solutions respectively.

Preparation of standard and sample solutions for HPLC

The same procedures were followed for the preparation of standard and sample solutions for HPLC, except that, all the solutions were made by dissolving samples in HPLC grade ACN.

HPLTC procedure

HPTLC was performed on a pre-coated silica gel 60 F_{254} (10cm×10 cm) plate of uniform thickness (0.2mm) using a CAMAG HPTLC system (CAMAG, Switzerland) equipped with LINOMAT-V automatic sample applicator, CAMAG TLC Scanner-3, CAMAG Reprostar-3 video documentation system and integrated win CATS software. The plate was prewashed with methanol and dried at 105^{0}C for 30 min. for activation. Spots of both standard and samples were applied (in volumes of 2 and 4 µl), on the plate in the form of 6 mm wide bands using Hamilton syringe (100 µl capacity, USA) with the help of LINOMAT-V automatic sample applicator (Camag, Switzerland), under N_2 gas. The distance from bottom of the plate was 10 mm, from side 15 mm and the distance between two tracks was 10 mm. The chromatogram was developed in a Camag twin trough chamber which was previously saturated with the mobile phase *viz.* ethyl acetate: methanol: water (75:15:10 v/v/v). The mobile phase was allowed to run upto a distance of 9 cm from the base. The plate was then removed and air dried for 15 min. The spots were visualized by dipping the plate in a solution of acetic anhydride: sulphuric acid: ethanol (1:1:10 v/v/v) followed by heating the plate at 110^{0}C for 5 min.[18] The plate was then allowed to cool and then scanned at 510 nm in reflectance-absorbance mode using CAMAG TLC scanner-3. CAMAG Reprostar-3 video documentation system was used to take images of the TLC plate under white light as well as under UV at 366 nm.

HPLC analysis

The HPLC was performed by using Shimadzu, SCL 10 A vp system (Japan) equipped with Rheodyne- 7725i injection valve (20 µL) (Rheodyne , U.S.A.), LC-10 AT vp pumps and SPD-10 A vp UV-Vis detector along with the integrated CLASS- VP software. Analysis was performed on a Luna C 18 (2) reverse phase column (Phenomenex), steel packed, 250 x 4.60 mm with internal diameter of 5 µ. The isocratic elution of acetonitrile: water (80:20) was used as a mobile phase. The flow rate was 1 ml/min and the detector was set at 210 nm. The injection volumes were 20 µL throughout the experiment. All the solvents and samples were sonicated and filtered through 0.45 µm filter (Millipore) before use in HPLC. The identification and quantitation of stevioside in samples was done by retention time, UV spectra and by comparing the peak area of sample with that of the standard. Calibration curve for stevioside was constructed in the concentration range of 0.1 to 1 mg/ml.

Results and Conclusion

HPTLC identification

The presence of stevioside in the samples was confirmed by HPTLC analysis of the developed chromatograms. Figure 1 shows the 3-D display of all the tracks at 510 nm. The individual chromatograms of the standard as well as samples are displayed in Figures 2-9. The band of stevioside was confirmed by comparing the R_f value of the standard (0.59) with that of the samples (between 0.56-0.58; Table 1). The identity of the peak was further confirmed by spectral analysis. Figure 10 shows the Overlay spectra of the selected band in standard as well as sample tracks confirming its identity and purity. The photos of the TLC plate showing all the eight tracks after development under white light and UV at 366 nm are shown in Images 1 and 2 respectively.

In our study, previously reported mobile phase for the estimation of stevioside, ethyl acetate: ethanol: water (80:20:12 v/v/v) [18] did not give satisfactory results (Tailing and dragging of spots was observed) and hence a modified mobile phase *viz.* ethyl acetate: methanol: water (75: 15: 10 v/v/v) was used which gave more clear separation.

Two different concentrations of sample and the standard (2 and 4 µl) were applied on the same plate to rule out any possibility of error.

HPLC quantification

High performance liquid chromatography, being more sensitive and accurate, was used for the estimation of stevioside content in the samples. A calibration curve was plotted in the concentration range of 0.1 to 1 mg/ml to assess the linearity (Figure 11) and it was found to be linear (r^2=0.998). The detection limit for stevioside was found to be 0.05 mg/ml (1 µg per injection). The identification and quantification of stevioside content in the samples was done by comparing the retention time and peak area of sample with that of the standard. The retention time (RT) of stevioside in standard (0.1 mg/ml and 1 mg/ml), commercial stevia powder and stevia leaf extract was found to be 10.500, 10.683, 10.717 and 10.075 min. respectively (Figures 12, 13, 14, 15). The percentage stevioside content of the samples, commercial stevia powder and stevia leaf extract was found to be 8.859 % and 3.703 % respectively (Table 2).

HPTLC is an indispensible tool for identification of plants and their constituents. Hence HPTLC was used in the present study to identify the presence of stevioside in the leaf powder and commercial sample. Quantitation of phytoconstituents by HPLC is highly accurate and dependable. The marketed samples often are adulterated with cheaper synthetic sweeteners and to estimate the stevioside content rapidly and economically, no proper methods were described. The present study was, therefore an effort to develop a rapid and an economical method for identification and estimation of stevioside in a sample, which was achieved.

Acknowledgements

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ISSN: 0976-7126

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Table 1: R_f values of standard and samples

Track no.	Track ID	Application volume	R_f value of stevioside
1.	Stevioside Standard (1 mg/ml)	2 µl	0.59
2.	Stevioside Standard (1 mg/ml)	4 µl	0.59
3.	Stevia Powder (1 mg/ml)	2 µl	0.58
4.	Stevia Powder (1 mg/ml)	4 µl	0.57
5.	Stevia Powder (5 mg/ml)	2 µl	0.57
6.	Stevia Powder (5 mg/ml)	4 µl	0.56
7.	Stevia Leaf extract (5 mg/ml)	2 µl	0.56
8.	Stevia Leaf extract (5 mg/ml)	4 µl	0.55

Table 2: HPLC analysis of samples

S/No.	Sample	Retention time (RT)	Peak height	Peak area	% content
1.	Stevioside Standard (0.1 mg/ml)	10.500	79373	212376	100%
2.	Stevioside Standard (1 mg/ml)	10.683	510553	16254502	100%
3.	Stevia Powder (5 mg/ml)	10.717	272648	7200172	8.859%
4.	Stevia leaf extract (5 mg/ml)	10.075	95464	3009823	3.703%

ISSN: 0976-7126

Figure 1: 3-D display of all the tracks at 510 nm

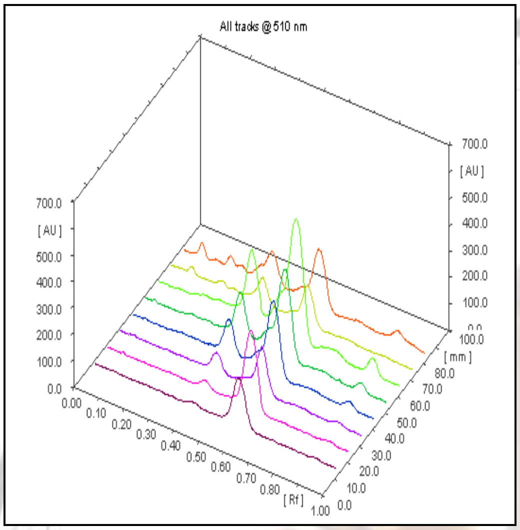


Figure 2: Track 1, Chromatogram of standard Stevioside 1 mg/ml, application volume - 2µl

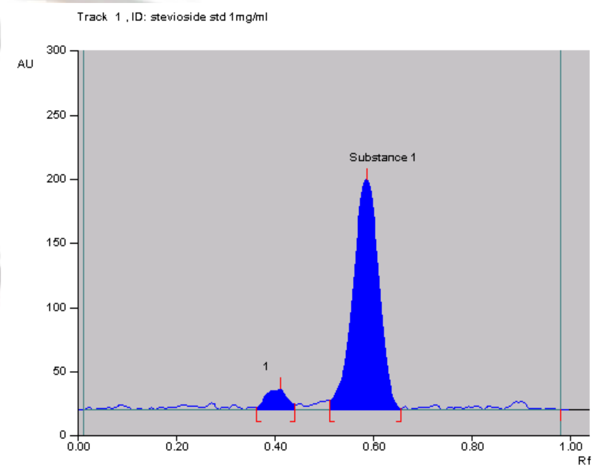


Figure 3: Track 2, Chromatogram of standard Stevioside 1 mg/ml, application volume- 4µl

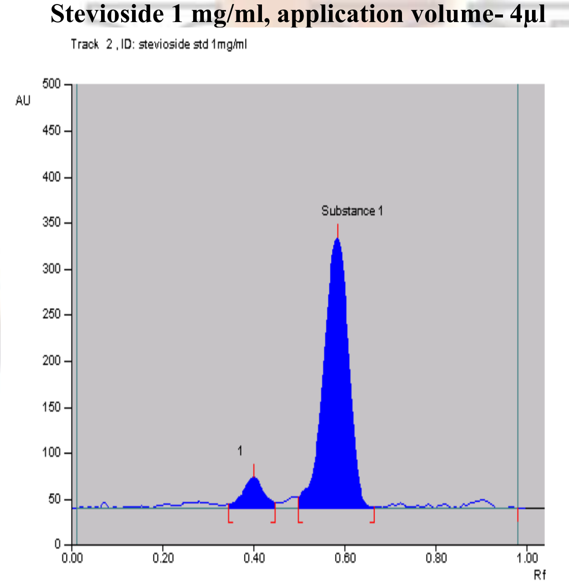


Figure 4: Track 3, Chromatogram of Stevia powder 1 mg/ml, application volume- 2µl

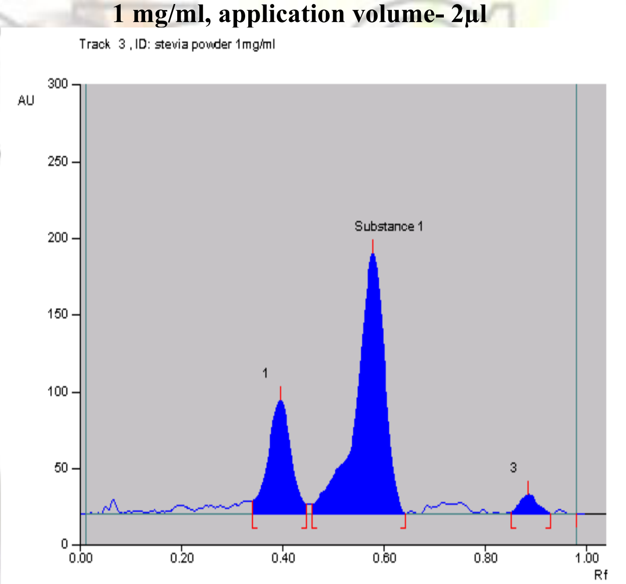


Figure 5: Track 4, Chromatogram of Stevia powder 1 mg/ml, application volume- 4µl

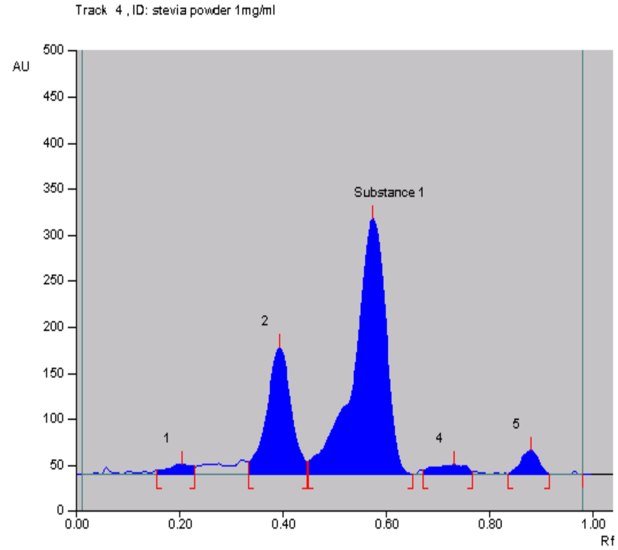


Figure 6: Track 5, Chromatogram of Stevia powder 5 mg/ml, application volume- 2µl

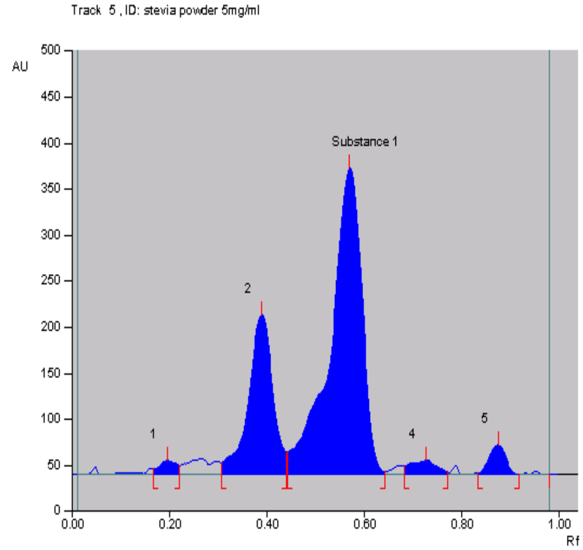


Figure 7: Track 6, Chromatogram of Stevia powder 5 mg/ml, application volume- 4µl

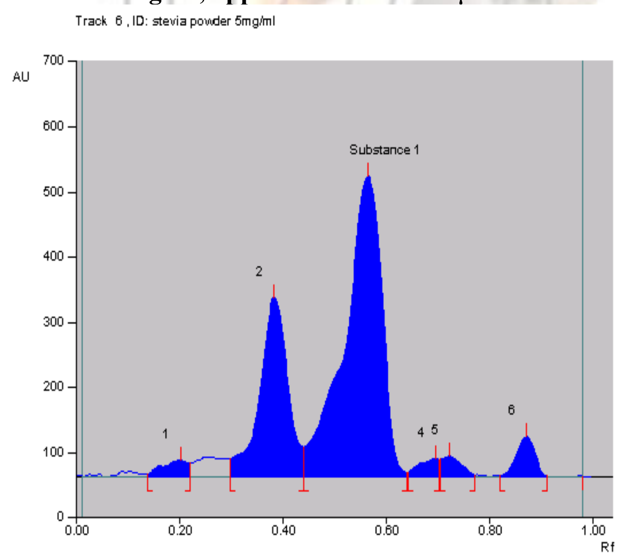
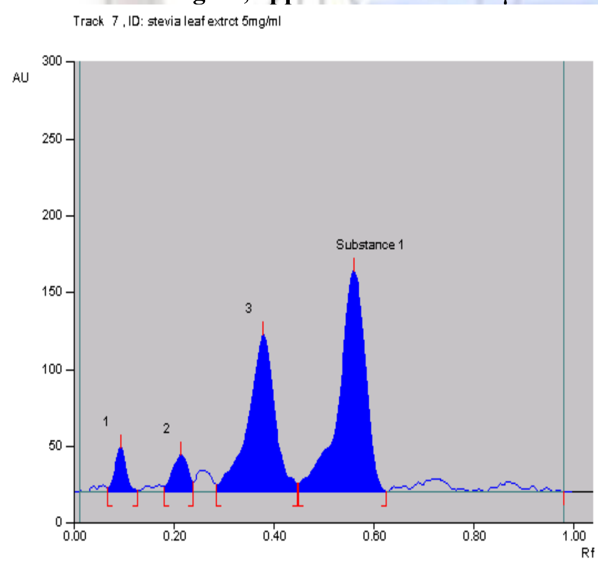


Figure 8: Track 7, Chromatogram of Stevia leaf extract 5 mg/ml, application volume- 2µl



ISSN: 0976-7126

Figure 9: Track 8, Chromatogram of Stevia leaf extract 5 mg/ml, application volume- 4µl

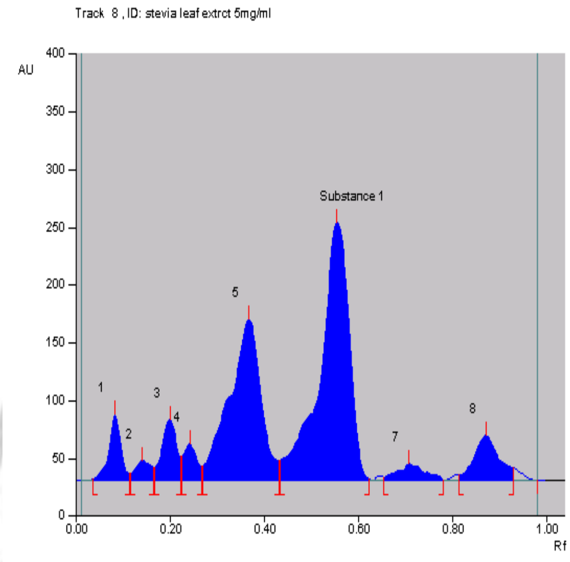


Figure 10: Overlay spectra of the band corresponding to stevioside in all tracks

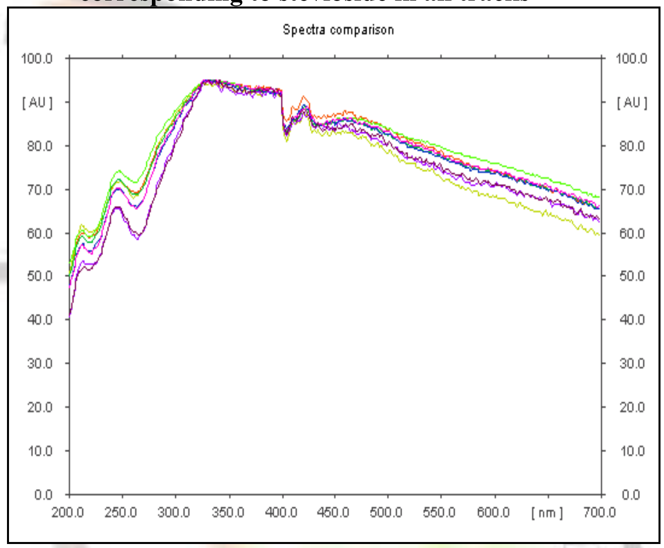


Figure 11: Calibration curve of stevioside

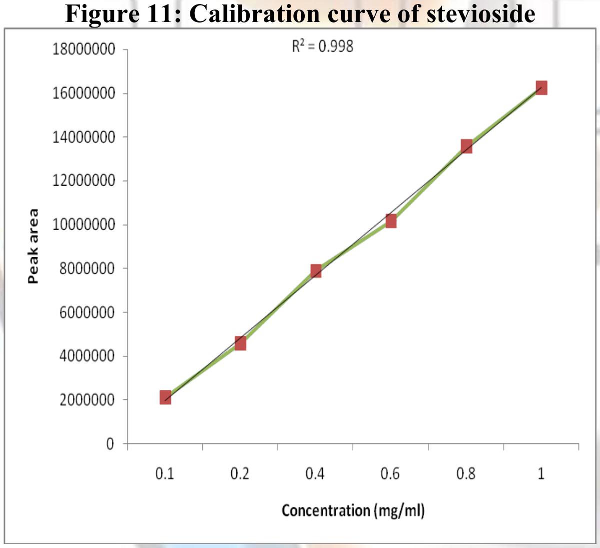
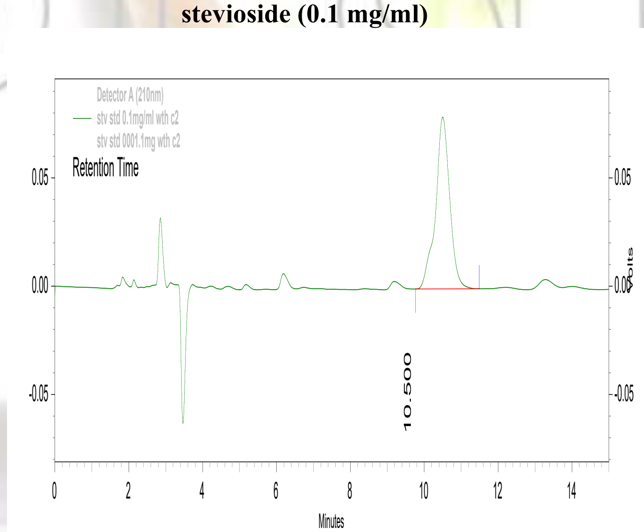


Figure 12: HPLC chromatogram of standard stevioside (0.1 mg/ml)



ISSN: 0976-7126

Figure 13: HPLC chromatogram of standard stevioside (1mg/ml)

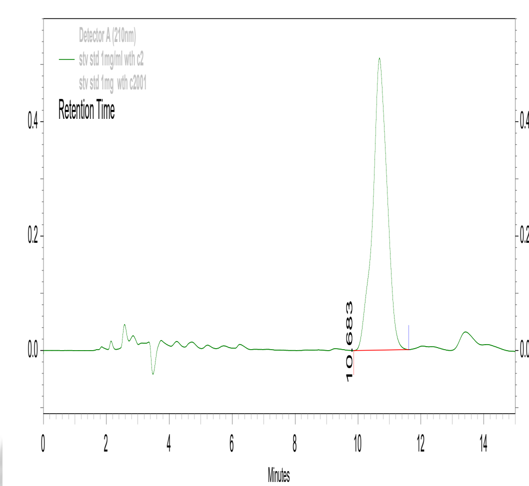


Figure 14: HPLC chromatogram of stevia powder sample (5mg/ml)

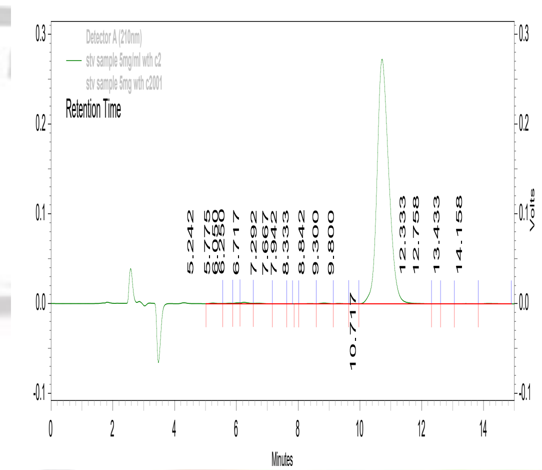
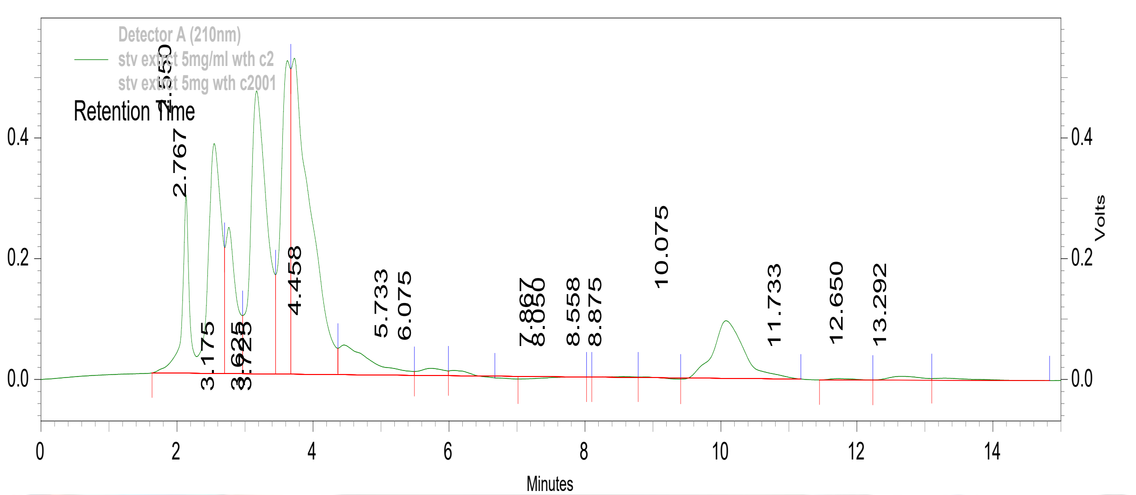


Figure 15: HPLC chromatogram of stevia leaf extract (5mg/ml)



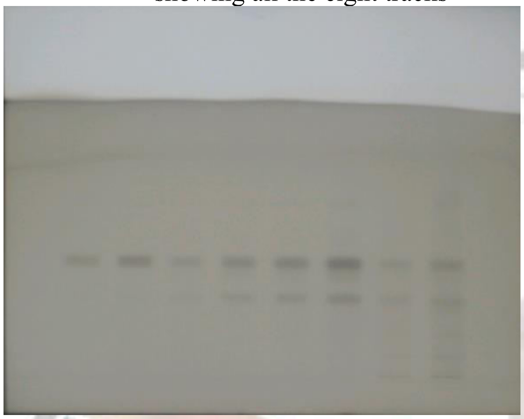


Image 1: Image of the TLC plate under white light showing all the eight tracks

Image 2: Image of the TLC plate at UV 366 nm