

Evaluation of Stevioside and Rebaudioside A in Sri Lankan Food and Beverages, Using a Validated HPLC Method

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DOI: <https://doi.org/10.51583/IJLTEMAS.2025.140400100>

Received: 01 May 2025; Accepted: 05 May 2025; Published: 17 May 2025

Abstract: Currently, there is a trend of using non-caloric high-intense sweeteners, as substitutes for sugars in food, carbonated and non-carbonated beverages to acquire the required sweet taste. Steviol glycosides have been recognized as a non-caloric natural sweetener bearing European food additive number of E960. Stevioside is the major component in steviol glycosides followed by rebaudioside A. The aim of the study was to evaluate the stevioside and rebaudioside A in different food and beverages matrixes found in the market of Sri Lanka using validated reverse phase HPLC method. validation of the method performed using USP reference standard. The Limit of detection for stevioside and rebaudioside A were 2.6 mg/kg and 3.9 mg/kg respectively. Linearity range for stevioside and rebaudiosideA was 2 to 500 mg/kg and 5 to 500 mg/kg with a correlation of coefficient of 0. 9999. Accuracy as the percentage of recovery for both analytes were between 80.7% - 118.7% for different spiked levels. In conclusion, the developed analytical procedure was sensitive, simple, accurate to determine stevioside and rebaudioside A in food, beverage and their commercial preparations and it was found that most of products were within the acceptable range.

Key words: Steviol glycosides, Rebaudioside A, Stevioside, beverages, food

I. Introduction

Consumption of high levels of sugar is one of the foremost factors in gaining overweight and obese which negatively affects the health of human beings [1]. The World Health Organization (WHO) has promoted lowering sugar consumption to be lower than 5% of total energy intake from free sugars. Many countries have taken steps to regulate the level of added sugar in their popular food items. Even in developing countries, market demand for food and beverage products with alternative nutritional characteristics such as zero caloric or sugar-free food and soft drinks to fulfill the low sugar requirements of the consumer to maintain good health [2]. Presently, health awareness has increased in beverages and their commercial preparations and the demand for food products that sustain better health with a greater variety of low-calorie products.

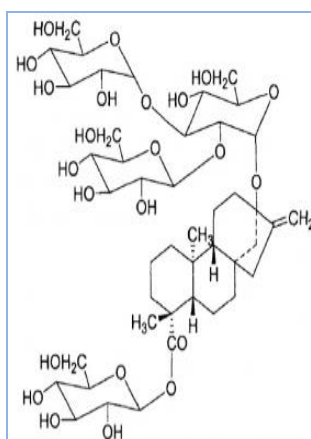
The Health Ministry of Sri Lanka has introduced a traffic light system for food drinks enabling consumers to identify them as low, medium, or high added sugar. However, these regulatory influences and the wide awareness among the public of the hazardous health effects of sugar consumption have forced food manufacturers to come up with low-sugar products [3].

As a result, there is a trend of using non-caloric high-intense sweeteners, as substitutes for sugars in the production of food, carbonated and non-carbonated beverages, and tabletop sweeteners in order to maintain the sweet taste by duplicating sugar taste but with a low level of energy. Steviol or steviol glycosides are extracted from leaves of *Stevia rebaudinana Bertoni* which is a flowering plant native to South America, and it is known as a non-caloric natural sweetener bearing a European food additive number of E960[4]. Stevioside and rebaudioside A (reb A) are the major derivatives of Steviol glycosides followed by rebaudioside C, rebaudioside B, rebaudioside D, rebaudioside E, steviol bioside, rebaudioside and Dulcoside A with traces [5]. Fig.1 gives the chemical structures and formula for rebaudioside A, and stevioside.

Relative sweeteners range from 180-350 times sweeter than that of sucrose in a 2.5–10% aqueous solution, and as such only small amounts of stevia sweeteners are needed to match the sweetness provided by sugar [6]-[7]. Unlike many of non-caloric sweeteners that steviol glycosides is sufficiently thermally and hydrolytically stable for food use, including acidic beverages, under normal conditions of processing and storage [8]-[9] Stevia sweeteners can be used by food and beverage manufacturers as an ingredient in beverages like diet sodas, light or low-sugar juices and flavored waters, canned fruits, condiments, and dairy products such as ice cream, flavored milk, and yogurt. As stevia sweeteners (stevioside and reb A) are reasonably thermally stable under high temperatures, can be used in processing of foods such as baked goods, cereals, chocolate, and other confections and syrups without undergoing browning or caramelization when heated [10].

According to the review of the regulatory process carried out by the U.S. Food and Drug Administration (FDA), high-purity steviolglycosides have been Generally Recognized as Safe (GRAS). European Food Safety Authority (EFSA) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) have also accepted, that steviol glycosides are safe to be used and declared the acceptable daily intake (ADI) level as 4 mg /kg of body weight [11]-[13]. It has also been reported that stevia metabolism is the same in healthy children as it is in healthy adults [14]. As such, the FDA and JECFA have concluded that high-purity stevia sweeteners are safe for children to consume within the ADI. However, at present stevia sweeteners are acceptable for

use in most countries [15] including Sri Lanka [16] with the approved daily intake within the range of food and beverage (carbonated and non-carbonated) and tabletop sweeteners. As a result, use of stevia can be observed significantly in the market of

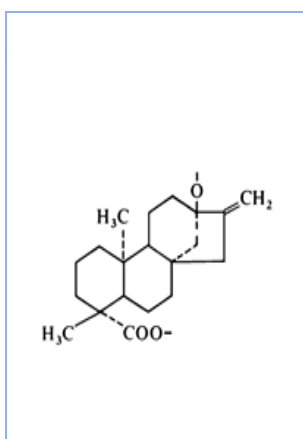


(A)

Chemical Name : rebaudioside A

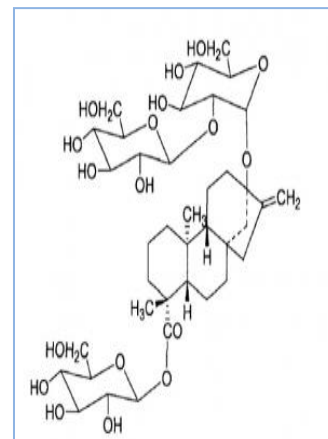
Molecular Formula $C_{44}H_{70}O_{23}$

Molecular Weight : 967.01 g/mol



(B)

Steviol backbone



(C)

Chemical Name : stevioside

Molecular Formula : $C_{38}H_{76}O_{18}$

Molecular Weight : 804.87g/mol

Fig. 1 (A)Rebaudioside A, (B)Steviol backbone and (C) Stevioside

Sri Lanka. However, concerning the intake safety, it is essential to verify the targeted sweetener amount if it is complying with the regulations. Therefore, having a simple, reliable, suitable validated analytical method is required extremely. There are several methods described in the literature for the analysis of steviol glycosides in food and beverage and tabletop sweeteners after extracted into the mixture of acetonitrile and ultra-pure water or with 0.01% formic acid in acetonitrile with 0.01% of formic acid in ultra-pure water. There are several techniques have been discussed in the literature for the detection of steviol glycosides including Ion chromatography, HPLC, UPLC, UPLC-MS, and LC-MS-MS [17,18]. However, HPLC method is the most convenient and sensitive enough among the available methods[19,20,21].The aim of the present study was to develop a simple precise method with optimizing available data including easy and less time-consuming sample preparation to evaluate the stevioside and rebA, which were present in more than 95% of total steviolglycosides[22]as steviol equivalent, of alcoholic beverages(carbonated and non-carbonated), commercial sweeteners, and cereal-based baked food items available in the Sri Lankan market.

II. Materials and Methods

Samples of carbonated and non-carbonated beverages, sugar free biscuits and tabletop sweeteners were selected to cover the scope of validation.

Non-alcoholic beverages including carbonated beverages (n=15), non-carbonated beverages (flavored, fruit drinks and nectars) (n = 10) were purchased from supermarkets belongs to four and five reputed companies respectively. It was also obtained few cereals based baked items(n=5) and tabletop sweeteners(n=5) which contain the sweetener, steviol glycoside. Steviol glycosides USP reference standard with purity > 98% and HPLC grade acetonitrile were purchased from sigma Aldrich, whereas analar grade potassium di hydrogen ortho phosphate which was purchased from BDH.

Preparation of standard solutions

The 1000 mg/ L stock solution of steviol glycosides was prepared by dissolving the appropriate amount of pure standard in a mixture of ultra-pure water: acetonitrile (3:2) (extraction solution) and the appropriate calibration standard solutions were prepared by diluting the stock standard solution with the extraction solution to obtained 5, 25,50,100 and 200 mg/L. All prepared solutions were stored at 4°C until the HPLC analyses commence.

Sample preparation

Carbonated beverages were degassed using ultrasonic bath and the rest of the beverage products were well homogenized prior to analysis. Solid samples were ground into fine particles with a grinder. Steviol glycosides in the samples were extracted by a portion of 1.0-2.0 mL of degassed liquid samples and 1.0 -2.0 g of solid and semisolid samples. Finally, brought up to a total volume of 10.00 mL in a volumetric flask by adding a mixture of ultra-pure water: acetonitrile (3:2). The solution mixture was then shaken by using a vortex for three minutes and solid samples followed by centrifugation at 8000rpm for 4 minutes. Supernatant and liquid samples were then filtered through 0.45µm nylon micro filters prior to the injection to the HPLC system.

Instrumentation

The analysis was performed using an Agilent 1260 infinity II upgradeable HPLC system which consisted of quaternary pump, diode array detector and an auto sampler. The chromatographic separation was achieved using isocratic elution with 65% of mobile phase A and 35% of Mobile phase B on Agilent Pursuit 5 C₁₈ column (4.6 x 250mm, 5µm). The column was operated at 35°C and the flow rate was set at 0.8mL/min for 15 minutes. The sample injection volume was 10µL. The separated steviol glycosides were detected by diode array detector with wavelength of 210 nm. Agilent Chemstation software was used for data acquisition and analysis. The mobile phase of A contained 20 mmol/L K₂H₂PO₄ at the pH of 2.5 while 100% acetonitrile was used as the mobile phase B.

HPLC identified compounds were confirmed by the waters LC/MS having ACQUITIC Arc System of UHPLC with ACQUITY QD MS detector and waters 2998 PDA detector. The chromatographic separation was achieved using gradient elution with water with 0.1% formic acid as mobile phase A and acetonitrile with 0.1% formic acid, on Waters CORTECS[®] T3 (3x100mm, 2.7µm) column. The column was operated with the flow rate of 0.8 mL/min for 15 minutes and the sample injection volume was 10 µL.

Validation of the method

The method was validated according to the guidelines given by FDA (2012) [23] AOAC International (2002) [24] Eurachem guidelines (The Fitness for Purpose of Analytical Method Second Edition 2014) [25] Limit of detection (LOD), limit of quantification (LOQ), accuracy (recovery), precision (repeatability and reproducibility), and the linear dynamic range of the analytical technique were assessed intended for the method validation performance characteristics. LOD and LOQ values were evaluated by analyzing the sample blank; fortified with reference standard at the minimum detection levels in ten replicates. In order to find the accuracy and precision, samples were fortified with the analytes at three different concentration levels (125, 225 and 450 mg/kg) covering the entire working range.

Statistical analysis

All results were expressed as mean and standard deviation. Statistical significance ($p < 0.05$) of different brands was evaluated using independent t-test and ANOVA methods using SPSS statistical software version 21.

III. Results & Discussion

External calibrations were used to quantify, individual steviol glycosides using reference standard of steviol glycosides and peaks were identified according to the given system suitability study chromatogram (fig.2) and (fig.3) and even though there were nine number of glycosides were present, the HPLC method was validated for reb A and stevioside only as they were the most available components (95%), using the Steviol glycoside USP standard reference material. The method was validated against the parameters accuracy, precision (repeatability), reproducibility (ruggedness), linearity, working range, minimum detectable level and recovery of the analytical technique. The obtained LOD and LOQ values for stevioside were 2.6 mg/kg and 3.9mg/kg while the values for reb A were 3.1mg/kg and 4.6mg/kg respectively.

The linearity of standard curves was expressed in terms of the regression coefficient (R^2). and for both stevioside and reb A, R^2 value of the curve was 0.9998. This showed a good linear dynamic range of the analytical method which capable of producing good sensitivity with the working range of 2 to 500 mg/kg for stevioside and 5 to 500 mg/kg for reb A. It was demonstrated that good performance in terms of accuracy as percentage of recovery, laid between 86.8% - 102.4% for stevioside and 80.7% - 118.7% for reb A, after spiking matrixes of food and beverage with three levels of concentrations of 25mg/kg, 100mg/kg and 300mg/kg, which implies that the recoveries of both analytes considered under this method are satisfactory for the beverages and food products in the market.

The repeatability for stevioside showed a relative standard deviation (RSD) of 3.2% for the lowest concentration, 2.8% for the medium concentration while for the highest concentration 2.4. and for reb A it was 2.6 %, 2.5% and 1.6%. All these three RSD values were within the IUPAC recommendation suggesting good precision of the method for stevioside and reb A.

The measurement of uncertainty for both glycosides which were calculated as the percentage of expanded uncertainty [24], considering the coverage factor of 2 ($k=2$) were 4.9 and 3.9 for reb.A and stevioside respectively.

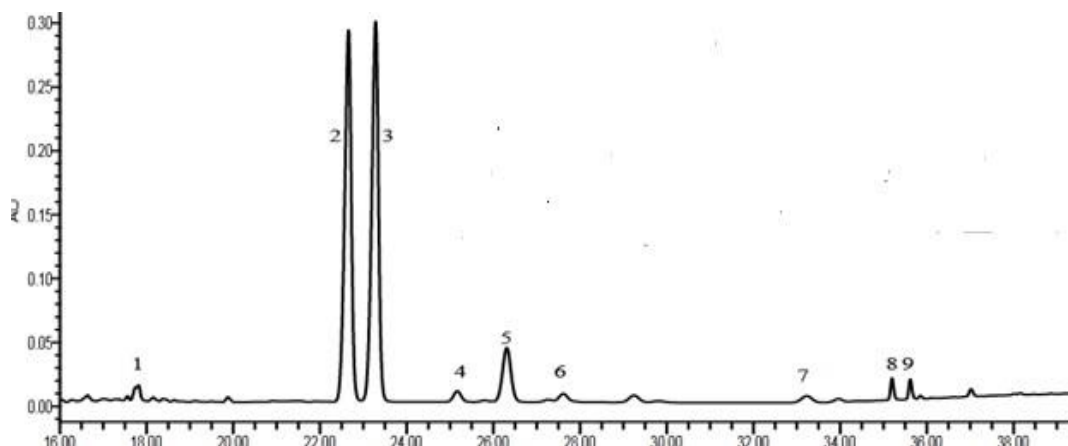


Figure:02 Steviol glycoside USP reference standard system suitability chromatogram given by the supplier 1. Rebaudioside D 2. Rebaudioside A 3. Stevioside 4. Rebaudioside F 5. Rebaudioside C 6. Dulcoside A 7. Rubusoside 8. Rebaudioside B 9. Steviolbioside

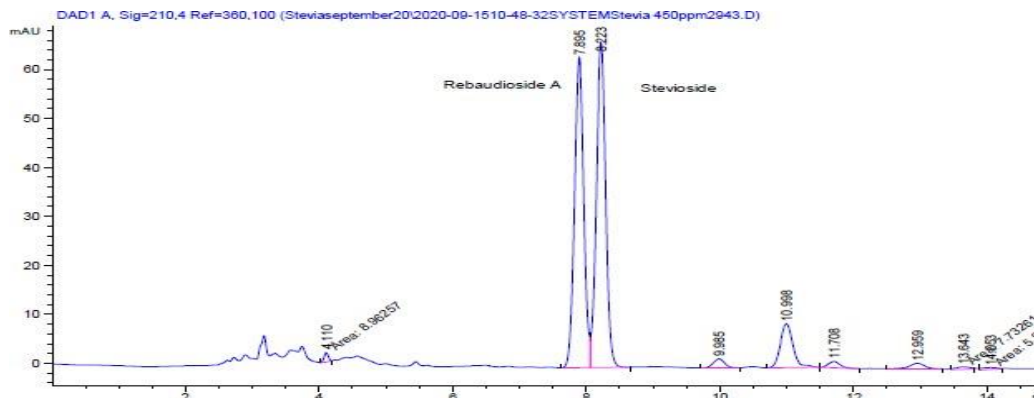


Figure :03 Chromatogram of spiked sample with steviol glycosides, USP reference standard

As a one of reference standard, belongs to the calibration is used to calculate the steviol equivalence of a particular stevia extract the proportions of the various components must be known. Steviol equivalent concentration is the product of the conversion factor and the concentration of the specific steviol glycoside concerned. The EU-approved conversion factors are shown in Table 0. [25,27] They occasionally differ to a minor degree from those applied by other countries such as Australia and New Zealand.

Table 1 EU approved conversion factors for different steviols

Glycosides	Regulatory* conversion factor to steviol equivalents
Steviol	1.00
Stevioside	0.40
Rebaudioside A	0.33
Rebaudioside B	0.40
Rebaudioside C	0.34
Rebaudioside D	0.29
Rebaudioside E	0.33
Rebaudioside F	0.34
Rebaudioside M	0.25
Dulcoside A	0.40

When consider the market of non-alcoholic drinks (carbonated and non-carbonated) of energy reduced or stated as with no added sugar, it is not allowed to have more than the maximum usable dose (80mg/L) as steviol glycosides specified in the food act regulation.

It was found that most of carbonated beverages available in the market included steviol glycosides as the sugar alternative (Table 2). Reb A and stevioside obtained significantly high in concentration while other glycosides were in traces. It was also found that the amount of steviol glycosides as steviol equivalent of most of brands were complied with the regulation while few brands were deviated within the range of 1 to 20ppm.

Table 2: Amounts of steviol equivalent(mg/L) in carbonated beverages of the Sri Lankan market

Matrix	Sample	Rebaudioside.A (mg/kg)	Stevioside (mg/kg)	*Steviol glycosides (mg/kg)
Carbonated beverages	Brand1	42.8±0.6	43.3±1.6	86.1±1.5
	Brand 2	30.4±1.4	22.9±1.0	54.1±1.0
	Brand 3	81.1±0.5	20.5±1.3	101.6±1.3
	Brand 4	36.5±0.6	30.9±0.8	67.5±1.4
	Brand 5	46.0±0.1	14.6±0.4	60.6±0.3
	Brand 6	34.1±0.6	23.3±0.5	57.4±0.1
	Brand 7	45.4±0.1	12.1±0.1	57.5±0.2
	Brand 8	64.4±0.6	24.0±0.7	88.4±1.2
	Brand 9	17.6±0.2	20.9±0.1	38.5±0.3
	Brand 10	44.8±0.6	15.9±0.9	60.8±1.5
	Brand 11	49.3±0.1	31.8±0.2	81.1±0.3
	Brand 12	39.7±0.6	19.0±1.3	58.7±1.9
	Brand 13	33.2±0.1	4.1±0.6	37.2±0.7
	Brand14	34.0±0.5	13.1±0.8	47.3±1.2
	Brand 15	71.0±0.5	19.6±1.3	90.6±1.3

*steviol glycosides as steviol equivalent

Results are expressed as mean± standard error of mean

Results (Table 3) show that all the non-carbonated beverages were within the acceptable daily intake as

recommended regulations given by the food act of Sri Lanka as well as Codex Alimentarius and comparatively low quantity was found [16,29].

Table 3: Amounts of steviol equivalent (mg/L) in non-carbonated beverages (Fruit drinks (RTS), cordial and squeezes) of the Sri Lankan market.

Matrix	Sample	Rebaudioside.A (mg/kg)	Stevioside (mg/kg)	*Steviol glycosides(mg/kg)
* NC Beverages	Brand 1	22.5±0.4	2.6±0.0	25.1±0.5
	Brand 2	19.6±0.2	5.4±0.4	25.0±0.6
	Brand 3	15.5±0.3	1.6±0.4	17.0±0.7
	Brand 4	13.2±0.4	11.7±0.3	24.9±0.7
	Brand5	19.9±0.6	25.0±1.4	45.4±1.6

	Brand 6	29.5±0.2	15.9±0.4	45.4±0.5
	Brand 7	19.0±0.5	48.4±1.4	67.3±2.0
	Brand 8	23.8±0.05	8.3±0.3	32.0±0.2
	Brand 9	29.8±0.05	9.0±0.3	38.8±0.3
	Brand10	21.5±0.38	5.4 ±0.7	26.9±1.1

*NC-Noncarbonated, *steviol glycosides as steviol equivalent

Results are expressed as mean± standard error of mean

When consider the tabletop sweeteners (Table 4), they come under the miscellaneous category, and maximum usable level of steviol glycosides steviol equivalents, various from 20mg/kg to 670mg/kg for energy reduced processed nuts, jam, jelly, food supplements supplied in a liquid or solid form and food supplements supplied in a syrup type. For chewable form, it allows up to 1800mg/kg. But there is no maximum suitable level has been declared for tabletop sweeteners and it allows as suitable quantity. However, with respect to the available tabletop sweeteners containing steviol glycosides, it was found as the lowest amount of 2.0 mg/kg and highest amount of 24.1mg/kg as steviol equivalent. when consider the food market under the desserts and

Table 4: Amounts of steviol equivalent(mg/kg) in tabletop

Matrix	Sample	Rebaudioside.A (mg/kg)	Stevioside (mg/kg)	*Steviol glycosides (mg/kg)
Tabletop sweeteners	Brand 1	-	2.0±0.3	2.0 ±0.3
	Brand 2	10.9±0.3	13.2±0.1	24.1 ±0.1
	Brand 3	5.6±0.1	-	5.6 ±0.1
	Brand 4	-	193.4±0.1	193.4±0.1
	Brand 5	-	213.4±0.2	213.4±0.2

Results are expressed as mean± standard error of mean when considering the food market under the desserts and similar products, tested as cereal based biscuits as energy reduced or with no sugar, only few products were to be found as baked items (Table 5) along with sweetener containing stevia. And it was found that the quantity is less than 100mg/kg which the regulations has been declared [28].

Table 5 Amounts of steviol equivalent (mg/kg) in baked items (biscuits) of the Sri Lankan Market

Matrix	Sample	Rebaudioside.A (mg/kg)	Stevioside (mg/kg)	Steviol glycosides (mg/kg)
Biscuits	Brand 1	10.3±0.1	5.8±1.9	16.1±0.6
	Brand 2	11.3±0.6	8.1±0.1	19.4±0.4
	Brand 3	10.0±0.7	6.2±0.6	16.2±1.3
	Brand 4	44.3±0.1	53.7±0.2	98.1±0.3
	Brand 5	24.5±0.2	8.1±0.2	32.7±0.0

. Results are expressed as mean± standard error of mean

As a summary, it was observed that steviol equivalent (Reb.A, stevioside, and steviol glycosides) content of different brands of each product matrixes had statistically significant differences ($p<0.05$). The resulted variation due to the manufacturer's formulation desires. The observed variation of total Steviol glucoside content of majority of brands are within the permissible levels.

IV. Conclusion

The developed analytical procedure is sensitive, simple, accurate and less time-consuming and a robust method. The method also complies with acceptance criteria of the guidelines of method validation. As such it can be successfully applied to estimate the levels of stevioside and rebaudioside A in routine analysis of food, beverage, baked items (cereal-based food) and table top sweeteners. It was found that most of a carbonated beverage has been sweetened with stevia. However, 1/3 of the tested carbonated beverages found to be more than the maximum permissible level as per the regulations (codex)[29]. And when consider the quantified steviol glycosides in non-carbonated fruit drinks was found approved dietary intake (ADI) to be lower than the upper level which leads to being no adverse effects in health, helps to ensure that human intake will be safe.

Conflict of interest statement

The authors wish to confirm that there are no known conflicts of interest associated with this publication.

V. Acknowledgement

Financial assistance by the Sri Lanka Treasury. The authors gratefully acknowledge the Industrial Technology Institute for the assistance in the research

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