Estimation of steviol glycosides in food matrices by high performance liquid chromatography

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Abstract Steviol glycosides (SGs) are non-caloric, natural sweetener obtained from plant *Stevia rebaudiana* and are used as sugar substitute in foods. The level of SGs in foods should not exceed maximum permissible limit defined by regulatory agencies. Thus analytical methods are required for assay of stevioside (Stev) and rebaudioside A (Reb A), which are two major constituents of SGs, in foods. A method for extraction of Stev and Reb A from dairy viz., flavoured milk, flavoured yoghurt and non-dairy foods viz., carbonated water, jam, chewing gum and estimation of these by HPLC has been described. Extraction of SGs from dairy samples was achieved by treating samples with 20% acetonitrile in presence of Carrez solutions while these can be simply extracted with water from non-dairy samples. Separation and estimation of these two glycosides was achieved on C18 column (length: 4.6 × 250 mm, particle size: 5 µm) using isocratic mobile phase prepared by mixing of acetonitrile and 10 mM sodium phosphate buffer (pH 2.6) in ratio of 32:68 (v/v). Recovery of two SGs was quantitative. Separation and estimation of SGs by HPLC was robust. Limit of detection and limit of quantitation for Reb A in different food was in range from 1.057–1.834 to 3.525–6.114 mg kg⁻¹ while that of Stev was from 1.679–2.912 to 5.596–9.707 mg kg⁻¹, respectively. Neotame, an artificial sweetener can be used as internal standard for separation of SGs.

Keywords Steviol glycosides · Rebaudioside A · Stevioside · HPLC · Extraction · Dairy

Introduction

Steviol glycosides (SGs) are chemical compounds present in the leaves of the South American plant *Stevia rebaudiana* and are used as sugar substitutes in the food industry (Perez et al. 2016). Primary components responsible for the sweet properties of the plant extract are glycosides of diterpene ent-13-hydroxykaur-16-en-19-oic acid, collectively referred as steviol glycosides (Prakash et al. 2012). Leaves of the stevia plant contain at least 30 different glycosides (Aranda-Gonzalez et al. 2015), the major constituents being stevioside (Stev) and rebaudioside A (Reb A). Stev and Reb A impart sweet flavor. Stev is most abundant and accounts for 3–12% (w/w) of dried leaves and 90% of total glycosides (Moussa et al. 2003; Chat-sudthipong and Muanprasat 2009). It is 300 times sweeter than sucrose and is thus comparable to other non-nutritive commercially available sweeteners (Geuns 2003; Lemus-Mondaca et al. 2012). Reb A accounts for 2–4% of total glycosides (Yadav and Guleria 2012) and is 400 times sweeter than sucrose. It has been associated with some health promoting properties like antihyperglycaemic, insulinotropic, glucagonostatic and antihypertensive effects (Cardello et al. 1999; Kochhar et al. 2008). In its 69th meeting, Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established the safety of SGs and set an acceptable daily intake (ADI) of 4 mg kg⁻¹ body weight per day for SGs expressed as steviol (Benford et al. 2009). European Food Safety Authority (EFSA) has also endorsed the opinion of JECFA about the safety of SGs and accepted the proposed ADI (EFSA 2010, 2015). United

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States Food and Drug Administration (USFDA) has accorded the Generally Recognized As Safe (GRAS) status to high purity (95% minimum purity) SGs (USFDA 2016). Stevia as a sweeteners has been approved in many countries including Japan, China, Australia, New Zealand, Europe, USA (McQuate 2011; Perez et al. 2016) and India (FSSAI 2012).

Over the years, methods for extraction of SGs from stevia and their estimation have been developed. Extraction of SGs from stevia leaves with water and sample clean-up with solid phase extraction (SPE) are invariably employed prior to their separation and estimation by chromatographic methods which include reverse phase HPLC (Vanek et al. 2001; Minne et al. 2004; Woelwer-Rieck et al. 2010), liquid chromatographic-electrospray ionization mass spectrometry (Choi et al. 2002; Pol et al. 2007), capillary electrophoresis (Pavlíček and Tůma 2017), near infrared spectroscopy (Hearn and Subedi 2009; Yu et al. 2011) and high performance thin layer chromatography (HPTLC) (Chester et al. 2012). High instrumentation cost of mass spectrometry based estimation methods, complex nature of capillary electrophoresis and qualitative nature of HPTLC method limit their use in estimation methods. HPLC based method for SGs estimation is reference method (JECFA 2010) and is widely used for estimation of SGs from stevia. SGs are non-caloric sweetener permitted as food additive and are incorporated at various stage of food preparation. Complex nature of food and process conditions used in preparation of food necessitates need of development and validation of estimation methods applicable to different food matrices. The satisfactory extraction of the analyte of interest from the food matrix for their quantification by HPLC is usually studied by employing a suitable internal standard. Internal standard is a compound having similar nature as that of analyte yet different from the analyte, that is added to sample during analysis. Use of internal standard allows validation of quantitative extraction of analyte from food matrices. Complete recovery of internal standard indicates that the extraction conditions are correct. Usually, the internal standard should also match chemical and physical properties of the analyte(s) of interest to account for possible losses during extraction (Geuns et al. 2009). Further, the internal standard should elute in the chromatogram in an area where no other peak elutes and its peak should not interfere with the peak of analyte of interest.

In present investigation, extraction and estimation of Stev and Reb A from milk, yoghurt, chewing gum, jam and carbonated water has been described for their quantification by HPLC. The use of neotame, an artificial sweetener has been explored as internal standard.

Materials and methods

Materials

Reb A ($\geq 96\%$ HPLC), Stev ($\geq 98\%$ HPLC), salicin, aspartame and neotame were procured from Sigma Aldrich, St. Louis, Missouri, USA. Acetonitrile and methanol of HPLC grade were obtained from Merck Life Science Pvt. Ltd., Mumbai, India. O-phosphoric acid and sodium dihydrogen orthophosphate were acquired from Qualigens Fine Chemicals, Mumbai, India and formaldehyde was procured from sd fine-chem Limited, Mumbai, India. A mixed fruit jam (Kissan, Hindustan Unilever Limited), carbonated water (Mountain Dew, Pepsico) and chewing gum (Perfetti Van Melle India private Limited) were purchased from the local market. Chocolate brown and strawberry flavor were procured from Ajanta Food Products, Baddi, Himachal Pradesh, India. Color raspberry red powder (IH 7804) was procured from International flavor and Fragrances India Private Limited, Chennai, Tamil Nadu, India. Starter culture NCDC 144 containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus* was obtained from National Collection of Dairy Cultures (NCDC) at the Institute.

Preparation of dairy samples

Flavoured milk and yoghurt samples were prepared in the laboratory (De 1991) using pooled cow milk collected from the Livestock Research Center of Institute.

Flavoured milk was prepared from standardized milk (fat: 2.2% and solid-not-fat: 12%). After pasteurization of the milk, sugar (6%) and or SGs, colour (0.08%) and strawberry flavor (0.1%, w/v) were added in sequence. The final product was sterilized at 121 °C/15 min and stored at room temperature (20–25 °C).

Flavoured yoghurt was prepared from standardized milk (fat: 4% and solid-not-fat: 12%). After pasteurization of the milk, milk was subjected to two step homogenization (1st stage: 2500 psi, 2nd stage: 500 psi). Then, sugar (10%) and or SGs, colour (0.08%) and strawberry flavor (0.1% w/v) were added. The mixture was heated (90 °C, 10 min) and then cooled to 42 °C. The mixture was then inoculated with 2% starter culture and packed immediately in polypropylene cups. The cups were incubated at 42 °C/3 h. Prepared yoghurt was subsequently stored at 5 °C and was analysed within a week.

Preparation of spiked food samples

Flavoured milk and flavoured yoghurt

Reb A and Stev, individually at 200 mg kg^{-1} each or in combination at 100 mg kg^{-1} each were added during preparation of flavoured milk and yoghurt. The sugar concentration in such SGs-spiked samples was 6%.

Jam

Reb A and Stev were spiked individually at 360 mg kg^{-1} each or in combination of 180 mg kg^{-1} each SGs.

Carbonated water

Reb A and Stev were spiked individually at 200 mg kg^{-1} each or in combination of 100 mg kg^{-1} each SGs.

Chewing gum

Reb A and Stev were spiked individually at 3500 mg kg^{-1} each or in combination of 1750 mg kg^{-1} each SGs.

Extraction of SGs from food matrices

Extraction of SGs from the dairy products was adopted from the method developed by Sharma et al. (2009) for extraction of sugars from soymilk. The method in brief is described here. Two millilitres of flavoured yoghurt or flavoured milk were taken in 15 ml centrifuge tube and then mixed with 1.5 ml distilled water using vortex mixer. The contents were incubated at 60°C for 10 min and then cooled to room temperature. Then, 0.25 ml Carrez I (500 mM aqueous potassium ferrocyanide), 0.25 ml Carrez II (500 mM aqueous zinc acetate) and 1 ml of acetonitrile were added in sequence. After mixing, the contents were kept undisturbed for 1 h at room temperature and then centrifuged (10,000g, 8 min, 20°C). The supernatant was collected and passed through $0.25 \mu\text{m}$ syringe filter prior to HPLC analysis.

Extraction of SGs from jam was done according to the method described by Bartholomees et al. (2016). Two grams jam sample was weighed in 15 ml centrifuge tube and mixed with 3 ml water using vortex mixer. After placing sample in ultrasonic bath for 15 min at 30°C , contents were centrifuged (8000g, 15 min, 20°C) and supernatant was collected. Extraction was repeated two times each with 3 ml water. Supernatants were pooled and then centrifuged (10,000g, 5 min, 20°C). Supernatant was passed through $0.25 \mu\text{m}$ syringe filter prior to HPLC analysis.

Chewing gum (2 g) was crushed using pestle and mortar till the entire content of chewing gum including central jelly is mixed. Ten ml of water (40°C) was added and the contents were vortexed. After placing sample in ultrasonic bath for 15 min at 30°C , the contents were centrifuged (8000g, 15 min, 20°C) and supernatant was collected. Extraction was repeated two times each with 5 ml water. Supernatants were pooled and then centrifuged (10,000g, 5 min, 20°C). Supernatant was passed through $0.25 \mu\text{m}$ syringe filter prior to HPLC analysis.

The carbonated water (5 ml) was degassed by placing it on ultrasonic bath for 20 min at 30°C and then filtered through $0.25 \mu\text{m}$ syringe filter prior to HPLC analysis.

Instrumentation

An Agilent HPLC apparatus (LC 1260) equipped with UV detector, Open lab software and manual injector through sample loop of $20 \mu\text{L}$ was used for separation of SGs. Separation was carried out on Agilent ZORBAX C_{18} column (length: $4.6 \times 250 \text{ mm}$, particle size: $5 \mu\text{m}$) at 50°C . Column was flushed with methanol–water (80:20) before and after use. All samples were analyzed under isocratic conditions in accordance with the JECFA method (JECFA 2010). Mobile phase consisted of 32:68 mixtures of acetonitrile and 10 mM sodium phosphate buffer (pH 2.6) was used at a constant flow rate of 1 ml min^{-1} . Effluent was scanned at 210 nm.

Analysis of formalin-preserved flavoured milk

Formalin was added to flavoured milk samples containing combination of Reb A and Stev (100 mg kg^{-1} each) for achieving 0.4% (w/v) final concentration. The samples were mixed by inverting the sample bottles and stored at 30°C for 50 days. Samples were analysed at 0, 10, 20, 30, 40 and 50 days for Reb A and Stev by HPLC.

Method validation

Individual solutions of Reb A ($10\text{--}200 \mu\text{g ml}^{-1}$) and Stev ($3\text{--}100 \mu\text{g ml}^{-1}$) prepared in water-acetonitrile mixture (70:30) were used for (1) preparation of standard curve and (2) for determination of (a) retention time and (b) relationship between concentration and peak area. The standard curve was drawn by plotting concentration against peak area.

LOD and LOQ for Reb A and Stev were determined in acetonitrile:phosphate buffer (10 mM, pH 2.6) (32:68) in water and food samples spiked with SGs. LOD and LOQ in sample matrices were determined using the formula: $\text{LOD} = 3 \text{ (Standard Deviation of response)/slope}$ and

LOQ = 10 (Standard Deviation of response)/slope (International Conference on Harmonization 2005), respectively.

Accuracy is reported as percent recovery by the assay of known added amount of analyte in the sample. For the recovery studies, spiked samples were prepared by adding varying quantity of Reb A and Stev, both individually as well as in combination.

Recovery (%) of Reb A/Stev was calculated using the following formula:

$$\text{Percent recovery} = \frac{x}{y} \times 100$$

where, x, Peak area of SGs after extraction from product; y, Peak area of standard SGs.

The robustness of the method was evaluated by assaying test solutions in HPLC after slight but deliberately changing flow rate ($\pm 0.1 \text{ ml min}^{-1}$), the proportions of acetonitrile:buffer (30:70 and 34:66, v/v), pH of sodium phosphate buffer (± 0.1) and changing the column temperature ($\pm 2^\circ \text{C}$). Robustness study was done in flavoured milk samples spiked with Reb A and Stev in combination at 100 mg kg^{-1} each.

Application of neotame as internal standard

Appropriate volume of neotame standard solution prepared in water:acetonitrile mixture (70:30) was added to unspiked and SGs-spiked flavoured milk to achieve final concentration of 100 and 200 mg kg^{-1} . SGs and neotame were extracted and then analysed using HPLC as described above. A standard curve of neotame solubilized in water:acetonitrile (70:30) was also prepared.

Statistics

Precision of the measurement of the assay was done by calculation relative standard deviation (RSD). The standard deviation (SD), RSD and correlation coefficient (r^2) were calculated using Excel Software.

Results and discussion

Reb A and Stev are the most abundant and representative compounds of SGs present in *S. rebaudiana* and the commercial preparations from *Stevia* (Gonzalez et al. 2014). In present study, a method has been standardized to quantify Reb A and Stev together in various food matrices. SGs were extracted from dairy food matrices by addition of Carrez solutions and acetonitrile, while water was employed as an extracting agent for extracting these glycosides from jam and chewing gum. HPLC analytical conditions as mentioned in materials and methods for assay

were optimized and were selected after set of experiments using different combinations. These combinations included using different mobile phases (methanol/water, methanol/phosphoric acid, acetonitrile/phosphoric acid, acetonitrile/water, acetonitrile/sodium phosphate buffer, etc.), varying the mobile flow rate (ranging from 0.1 to 0.4 ml min^{-1}), varying column temperatures (20 – 70°C) and changing the flow rate program from linear to gradient (results not shown). Representative chromatograms of individual Reb A, individual Stev and combination thereof on reverse phase column are shown in Fig. 1. Reb A and Stev gave sharp peaks at retention time of 4.79 and 4.93 min in HPLC, respectively. However, when sample containing both of these SGs, was attempted for separation, overlapping but identifiable two peaks corresponding individual SGs were obtained. With the application of Open Lab Software, it has been possible to calculate area under each of these two overlapping peaks. Difference in structure of these two SGs resulted in difference in retention time and amplitude of peak (Fig. 1). Standard curve for Reb A and Stev was linear in concentration range 10 – 200 and 3 – $100 \text{ } \mu\text{g ml}^{-1}$, respectively. Linear regression equation derived for Reb A and Stev were

$$\text{Reb A } y = 6733.45x - 17,489.2$$

$$\text{Stev } y = 10,690x + 10,238;$$

where x = concentration in $\mu\text{g ml}^{-1}$ and y = peak area in absorbance units.

The r^2 values of derived equations for estimation of Reb A and Stev were 0.999 and 0.998 , respectively.

Estimation of SGs in food matrices

The representative chromatogram of SGs extracted from various food matrices are shown in Figs. 2 and 3. It is clear from chromatogram that besides SGs, other components from food matrices are extracted. These components are also separated under the conditions optimized for separation of SGs. Major peaks appeared at retention time between 2.4 and 3.0 min in unspiked food samples. Also, additional major peaks with retention time between 3.5 and 4.5 min were observed with jam, carbonated water and chewing gum (Fig. 3). There is no major or minor peaks at or around retention time corresponding to Reb A and Stev in unspiked food samples. Resolution of Reb A and Stev in spiked food samples from other peaks of food origin suggested that extraction and separation protocols used in present investigation can be used for estimation of SGs in dairy and non-dairy foods.

Small deviation in retention time of Reb A and Stev has been noted when these SGs were extracted from matrices. Accuracy of method has been evaluated by calculating recovery of SGs from spiked foods. For ensuring reliability

Fig. 1 Chromatogram of 100 mg kg⁻¹ rebaudioside A (Reb A) and 100 mg kg⁻¹ stevioside hydrate (Stev) when injected individually (inset) or in combination thereof. Retention time of Reb A and Stev was 4.79 and 4.93 min, respectively

of accuracy across the foods, both dairy and non-dairy foods were spiked individually either with Reb A or Stev or combination of these two SGs in 50:50 ratio at different concentrations. Each experiment was repeated three times and each analysis was performed in triplicates. Recovery of both SGs was close to 100% (Table 1). Low RSD values ($\leq 2.58\%$) indicate that estimation method described for SGs in food matrices are within the acceptable limit. The extraction of SGs from complex food matrix such as milk and yoghurt has been challenge. Generally C18 SPE cartridges have been employed to exclude fat and protein from dairy matrix for the sample preparation (Jookan et al. 2012; Yamamoto et al. 2016). In the present study, proteins and fat have been removed from the dairy sample by use of combination of Carrez solution and 20% acetonitrile. Most of the low molecular weight compounds such as sugars are soluble in 20% acetonitrile (Sharma et al. 2009) and quantitative recovery of SGs in the present study indicate the suitability of such conditions. Method described here can reduce cost of estimation and extraction requires acetonitrile, potassium ferrocyanide and zinc acetate which are normally available in chemical laboratory. Recently Bartholomees et al. (2016) reported a method for estimation of total SGs as steviol equivalents wherein extracted SGs were converted to isosteviol before their separation and estimation on RP-HPLC using fluorometric detector. The method has good accuracy but requires complete

hydrolysis of SGs and also method cannot estimate individual SGs. The method described here has sufficient sensitivity and can measure two major SGs with accuracy.

Reverse phase HPLC has been method of choice for the quantification of SGs either in the extracts of leaves of *S. rebaudiana* or in the food matrix. The two system which have been frequently reported in the literature include amino-based (Kolb et al. 2001; Ahmed and Smith 2002) and octadecyl silica (Jookan et al. 2012; Yamamoto et al. 2016) based stationary phases. Although, other detectors have been reported in the literature which include amperometric (Ahmed and Smith 2002), ELSD (Vanek et al. 2001) and MS (Pol et al. 2007), generally, the effluent is monitored at UV range close to 200 nm (Pol et al. 2007; Jookan et al. 2012). In the present study, C18 column has been used with UV detector and LOD, LOQ and recoveries for Stev and Reb A are comparable to those reported by others (Jookan et al. 2012; Bartholomees et al. 2016).

Method validation

The LOD and LOQ for Reb A and Stev were determined as per recommendation of International Conference on Harmonization, 2005 in acetonitrile:water mixture as well as in sample matrix. LOD and LOQ of Reb A (solubilized in acetonitrile:water mixture) were 0.25 and 0.75 $\mu\text{g ml}^{-1}$, respectively. Under similar conditions, LOD and LOQ of

Fig. 2 Chromatogram of extract from flavored milk and flavored yoghurt prepared with 100 mg kg⁻¹ rebaudioside A (Reb A) and 100 mg kg⁻¹ stevioside hydrate (Stev). **a** flavored milk without

steviol glycosides, **b** flavored milk with Reb A and Stev, **c** flavored yoghurt without steviol glycosides, **d** flavored yoghurt with Reb A and Stev

Stev were 0.10 and 0.375 µg ml⁻¹, respectively. The expected concentration of these analytes in the food sample is about 30 µg ml⁻¹ or above. Hence, SGs from selected food samples can be analyzed on RP-HPLC employing optimized analytical conditions. The LOD and LOQ range for Reb A in food samples were dependent on food and were between 1.057–1.834 and 3.525–6.114 µg ml⁻¹, respectively. Similarly, LOD and LOQ for Stev in food samples varied between 1.679–2.912 and 5.596–9.707 µg ml⁻¹, respectively.

The robustness of an analytical method is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness tests examine the effect that operational parameters have on the analysis results. For the determination of a method's robustness, method parameters like pH, flow rate, column temperature, etc., are varied within a realistic range, and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously

specified tolerance, the parameter is said to be within the method's robustness range. Recovery of Reb A and Stev did not change when measurements were made at (1) two different flow rate (0.9 and 1.1 ml min⁻¹), (2) two different ratio of acetonitrile and buffer (30:70 and 34:66), (3) two pH values of buffer (2.4 and 2.8) and (4) two different column temperature (48 and 52 °C) and was close to 100% (Table 2). The analytical method therefore remained unaffected by slight but deliberate changes in the analytical conditions and thus method is robust.

Use of internal standard

In this study, the suitability of salicin, aspartame and neotame were evaluated for their use as an internal standard. Salicin and aspartame eluted at retention time between 3 and 4 min and these peaks coincided with major peaks from food matrix (data not shown). Thus, salicin and aspartame cannot be used as internal standard for analysis of SGs from foods. Retention time of neotame was

Fig. 3 Chromatogram of extract from jam, carbonated water and chewing gum spiked with rebaudioside A (Reb A) and stevioside hydrate (Stev). **a** unspiked jam, **b** jam spiked with 180 mg kg⁻¹ Reb A and 180 mg kg⁻¹ Stev, **c** unspiked carbonated water, **d** carbonated

water spiked with 100 mg kg⁻¹ Reb A and 100 mg kg⁻¹ Stev, **e** unspiked chewing gum, **f** chewing gum spiked with 1750 mg kg⁻¹ Reb A and 1750 mg kg⁻¹ Stev

7.43 min (Fig. 4). Peak of neotame is well separated from the matrix peaks as well as SGs peaks. Further, there was linear relationship between response and concentration (20, 30, 50, 70, and 100 µg ml⁻¹) of neotame. The linear regression equation is $y = 609,561x - 146,651$ where x is

the concentration in µg ml⁻¹ and y is the peak area in absorbance units and r^2 is 0.987. Recovery of neotame both in flavoured milk and flavoured milk spiked with Reb A and Stev was close to 100%. Two derivatives, dihydrosteviol (Bartholomees et al. 2016) and steviol-19-β-

Table 1 Recovery (%) and relative standard deviation (RSD) (%) (n = 3) of rebaudioside A (Reb A) and stevioside hydrate (Stev) in various food samples

Sample	Steviol glycosides concentrations	% Recovery	% RSD
Flavored yoghurt	Reb A (200)	100.76	0.59
	Stev (200)	100.50	0.21
	Reb A ^a (100)	99.65	1.79
	Stev ^a (100)	99.72	1.26
Flavored milk	Reb A (200)	100.63	1.16
	Stev (200)	99.51	0.80
	Reb A ^a (100)	100.51	1.58
	Stev ^a (100)	99.69	2.58
Jam	Reb A (360)	102.00	1.02
	Stev (360)	101.30	1.02
	Reb A ^a (180)	101.41	0.91
	Stev ^a (180)	101.73	1.36
Carbonated water	Reb A (200)	100.40	0.53
	Stev (200)	101.59	0.54
	Reb A ^a (100)	100.84	0.78
	Stev ^a (100)	100.63	1.01
Chewing gum	Reb A (3500)	101.56	0.84
	Stev (3500)	101.59	0.94
	Reb A ^a (1750)	100.70	0.49
	Stev ^a (1750)	101.76	1.51

Figure in parenthesis indicates concentration of steviol glycosides in mg kg⁻¹ in spiked food samples

^aFood samples were spiked with both Reb A and Stev at equal concentrations

Table 2 Estimation of rebaudioside A (Reb A) and stevioside hydrate (Stev) in flavored milk spiked with glycosides each at 100 mg kg⁻¹ concentration under altered analytical conditions

Conditions	Recovery (%)		Retention time (min)		Relative standard deviation (%)	
	Reb A	Stev	Reb A	Stev	Reb A	Stev
Flow 0.9 ml min ⁻¹	101.83	100.50	4.83	4.97	0.17	0.21
Flow 1.1 ml min ⁻¹	101.91	100.04	4.64	4.81	0.25	0.29
Acetonitrile:buffer (30:70)	99.68	99.72	4.57	4.77	0.37	0.26
Acetonitrile:buffer (34:66)	99.91	99.65	4.88	5.01	0.48	0.79
Buffer pH 2.4	99.41	99.91	4.69	4.89	0.79	0.39
Buffer pH 2.8	99.21	98.71	4.81	4.96	0.68	0.28
Column temperature 48 °C	100.15	100.24	4.74	4.85	0.37	0.46
Column temperature 52 °C	100.63	100.44	4.81	4.95	0.35	0.63

Average of three observations (n = 3)

galactose-13-β-glucose (Amery et al. 2010) have been in use as internal standard but these are not commercially available.

Effect of addition of preservative to flavoured milk on estimation of SGs

As per the Food Safety and Standards Authority of India (FSSAI), formalin (INS No. 240) is used as a preservative to preserve dairy based samples for analytical purpose. Presence of formalin did not affect estimation of Reb A and Stev in flavored milk preserved with 0.4% formalin for at

least 50 days at 30 °C. Thus, formalin preserved flavoured milk samples can be used for estimation of SGs at later stage.

Conclusion

A simple extraction protocol for SGs from dairy or non-dairy foods has been described. The method of extraction and subsequent analysis on RP-HPLC can be employed in quality assurance laboratory as well as by regulatory agencies to ascertain the level of two major representative

Fig. 4 Chromatogram of extract of flavoured milk prepared with 200 mg kg^{-1} rebaudioside (Reb A), 200 mg kg^{-1} stevioside hydrate (Stev) and spiked with 100 mg kg^{-1} neotame as internal standard

of SGs (Reb A and Stev) in various food matrices. Quantitative recovery of SGs from foods after their extraction and analysis on reverse phase HPLC allow us to conclude that complete extraction of SGs has been achieved under experimental conditions. Extractant from food is compatible for analysis of SGs on reverse phase HPLC. Although, much sophisticated methods for SGs estimation have been reported in the literature, the simple protocol reported in this article will find practical applications. The method can also be used for formalin preserved samples.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

- Ahmed MJ, Smith RM (2002) Determination of stevioside by high-performance liquid chromatography with pulsed amperometric detection. *J Sep Sci* 25:170–172
- Amery R, Jookan E, Duquenne B, Geuns J, Meesschaert B (2010) Determination of steviol glycosides in various dairy matrices and soy drink. In: Genus MC (ed). *Stevia: science, no fiction. The Proceedings of the 4th EUSTAS Stevia symposium*, vol 4, pp 69–82
- Aranda-Gonzalez I, Moguel-Ordóñez Y, Betancur-Ancona D (2015) Determination of rebaudioside A and stevioside in leaves of *S. rebaudiana* Bertoni grown in Mexico by a validated HPLC method. *Am J Anal Chem* 6:878–885
- Bartholomees U, Struyf T, Lauwers O, Ceunen S, Geuns JM (2016) Validation of an HPLC method for direct measurement of steviol equivalents in foods. *Food Chem* 190:270–275
- Benford DJ, Hill F, Schlatter J, DiNovi M (2009) Steviol glycosides (addendum). In: Joint FAO/WHO Expert Committee on Food Additives (JECFA) Safety evaluation of certain food additives. Sixty-ninth meeting: food additives, pp 183–219. http://apps.who.int/iris/bitstream/10665/44063/1/9789241660600_eng.pdf. Accessed 8 June 2017
- Cardello HMAB, Da Silva MAPA, Damasio MH (1999) Measurement of the relative sweetness of stevia extract, aspartame and cyclamate/saccharin blend as compared to sucrose at different concentrations. *Plant Foods Hum Nutr* 54:119–129
- Chatsudhipong V, Muanprasat C (2009) Stevioside and related compounds: therapeutic benefits beyond sweetness. *Pharmacol Ther* 121:41–54
- Chester K, Tamboli ET, Singh M, Ahmad S (2012) Simultaneous quantification of stevioside and rebaudioside A in different stevia samples collected from the Indian subcontinent. *J Pharm Bioallied Sci* 4:276–281
- Choi YH, Kim I, Yoon KD, Lee SJ, Kim CY, Yoo KP, Kim J (2002) Supercritical fluid extraction and liquid chromatographic-electrospray mass spectrometric analysis of stevioside from *Stevia rebaudiana* leaves. *Chromatographia* 55:617–620
- De S (1991) Special milks. *Outlines of Dairy Technology*. Oxford University Press, pp 90–116
- EFSA (2010) Scientific opinion on the safety of steviol glycosides for the proposed uses as a food additive. EFSA Panel on Food Additives and Nutrient Sources added to Food (2010). *EFSA J* 8:1537
- EFSA (2015) Scientific opinion on the safety of the proposed amendment of the specifications for steviol glycosides (E 960) as a food additive. EFSA Panel on Food Additives and Nutrient Sources added to Food (2015). *EFSA J* 13:4316
- FSSAI (2012) Minutes of the tenth meeting of food authority (2012), Food Safety Standards Authority of India (FSSAI), New Delhi. http://old.fssai.gov.in/Portals/0/Pdf/minutes_of_authority_meeting%2820-11-2012%29.pdf. Accessed 8 June 2017
- Geuns JMC (2003) Stevioside. *Phytochemistry* 64:913–921
- Geuns J, Struyf T, Bartholomees U, Ceunen S (2009) EUSTAS protocol and round-robin testing of steviol glycosides by an internal standard method. In: *Proceedings of the 3rd–5th EUSTAS Stevia symposium*, vol 9, pp 1–26
- Gonzalez C, Tapia M, Perez E, Pallet D, Dornier M (2014) Main properties of steviol glycosides and their potential in the food industry: a review. *Fruits* 69:127–141
- Hearn LK, Subedi PP (2009) Determination levels of steviol glycosides in the leaves of *Stevia rebaudiana* by near infrared reflectance spectroscopy. *J Food Compos Anal* 22:165–168

- International Conference on Harmonization (ICH), Guideline (2005) Validation of analytical procedures: text and methodology. Q2 (R1), vol 1. Geneva
- JECFA—Joint FAO, WHO Expert Committee on Food Additives (2010) Steviol glycosides. Compendium of Food Additive Specifications, Monograph, 10
- Jookan E, Amery R, Struyf T, Duquenne B, Geuns J, Meesschaert B (2012) Stability of steviol glycosides in several food matrices. *J Agric Food Chem* 60:10606–10612
- Kochhar A, Dhindsa S, Sachdeva R (2008) Effect of stevia leaf (*Stevia rebaudiana*) powder supplementation and nutrition counselling on anthropometric parameters and gain in knowledge of the subjects. *Ethno-Medicine* 2:107–113
- Kolb N, Herrera JL, Ferreyra DJ, Uliana RF (2001) Analysis of sweet diterpene glycosides from *Stevia rebaudiana*: improved HPLC method. *J Agric Food Chem* 49:4538–4541
- Lemus-Mondaca R, Vega-Galvez A, Zura-Bravo L, Ah-Hen K (2012) *Stevia rebaudiana* Bertoni, source of a high-potency natural sweetener: a comprehensive review on the biochemical, nutritional and functional aspects. *Food Chem* 132:1121–1132
- McQuate RS (2011) Ensuring the safety of sweeteners from *Stevia*. *Food Technol* 65:42–49
- Minne VJ, Compennolle F, Toppet S, Geuns JM (2004) Steviol quantification at the picomole level by high-performance liquid chromatography. *J Agric Food Chem* 52:2445–2449
- Moussa MM, Zeitoun MM, Zeiton MA, Massoud MI (2003) Physico-chemical properties of stevia sweeteners as natural low caloric sweetener. *Alex J Agric Res* 48:61–75
- Pavlíček V, Tůma P (2017) The use of capillary electrophoresis with contactless conductivity detection for sensitive determination of stevioside and rebaudioside A in foods and beverages. *Food Chem* 219:193–198
- Perez E, Gonzalez C, Vaillant F, Lares M (2016) *Stevia* derivative and its potential uses in diabetic-directed foods. Review. *J Nutr* 3:1–20
- Pol J, Ostra EV, Karasek P, Roth M, Benesova K, Kotlarikova P, Caslavsky J (2007) Comparison of two different solvents employed for pressurised fluid extraction of stevioside from *Stevia rebaudiana*: methanol versus water. *Anal Bioanal Chem* 388:1847–1857
- Prakash I, Clos JF, Chaturvedula VSP (2012) Stability of rebaudioside A under acidic conditions and its degradation products. *Food Res Int* 48:65–75
- Sharma R, Rajput YS, Dogra G, Tomar SK (2009) Estimation of sugars in milk by HPLC and its application in detection of adulteration of milk with soymilk. *Int J Dairy Technol* 62:514–519
- USFDA (2016) GRAS Notice (GRN) No. 662. United States Food and Drug Administration. USFDA, Silver Spring. <https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm519307.pdf>. Accessed 8 June 2017
- Vanek T, Nepovim A, Valicek P (2001) Determination of stevioside in plant material and fruit teas. *J Food Compos Anal* 14:383–388
- Woelwer-Rieck U, Lankes C, Wawrzun A, Wust M (2010) Improved HPLC method for the evaluation of the major steviol glycosides in leaves of *Stevia rebaudiana*. *Eur Food Res Technol* 231:581–588
- Yadav SK, Guleria P (2012) Steviol glycosides from *Stevia*: biosynthesis pathway review and their application in foods and medicine. *Crit Rev Food Sci Nutr* 52:988–998
- Yamamoto S, Tahara S, Sugiki M, Miyakawa H, Uematsu Y, Yamajima Y, Monma K (2016) A rapid dialysis method for analysis of stevioside and rebaudioside A in foods. *J Food Hyg Soc Jpn* 57:155–159
- Yu C, Xu K, Shi Y (2011) The spectrum model established for measuring the contents of Rebaudioside A and Stevioside quickly in the leaves of *Stevia rebaudiana* Bertoni. *Energy Proc* 5:855–861