Steviol Glycosides: Natural Non-Caloric Sweeteners

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Abstract

Steviol glycosides are a group of highly sweet diterpene glycosides isolated in only a few plant species of the Paraguayan shrub *Stevia rebaudiana*. Stevioside and rebaudioside A are the most abundant steviol glycosides which are

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responsible for its sweet taste and have commercial value all over the world as sugar substitute in foods, beverages, or medicines. More than 30 additional steviol glycosides have been described in the scientific literature to date implies a significant metabolic investment and poses questions regarding the benefits *S. rebaudiana* might gain from their accumulation. Due to the increase in demand for the major constituents in the leaves of *S. rebaudiana*, it is now grown commercially in a number of countries, particularly in China, Japan, Taiwan, Korea, Thailand, and Indonesia. It is a magical plant that offers sweetness with fewer calories and do not show any side effects after consumption on human health. They are thermostable even at higher temperatures making them suitable for use in cooked foods. Stevia cultivation and production would further help those who have to restrict carbohydrate intake in their diet, to enjoy the sweet taste with minimal or no-calories. During the past few decades, the nutritional and pharmacological benefits of these secondary metabolites have become increasingly apparent. While these properties are now widely recognized, many aspects related to their in vivo biochemistry and metabolism and their relationship to the overall plant physiology of *S. rebaudiana* are not yet understood.

Keywords
Stevia rebaudiana Bertoni • Diterpene Glycosides • History • Chemistry • Stability • Sensory • Metabolism • Analytical methods • Regulatory

1 Introduction

1.1 Stevia Leaf Extract and Steviol Glycosides

Stevia rebaudiana (Bertoni) Bertoni is a perennial shrub in the sunflower family of the Asteraceae (Compositae) found originally by the Guarani natives of Paraguay growing along the edges of the rainforest. It is often referred to as "The sweet herb of Paraguay" [1–3]. Dr. Moises Santiago Bertoni discovered this plant in 1888 and the plant was scientifically named as *Stevia rebaudiana* after a Paraguayan chemist Dr. Rebaudi in 1905. The Guarani and Mato-Grosso Indians believe *S. rebaudiana*, also known as stevia, was first used by their ancestors more than 1,500 years ago as a sweetener in foods and beverages and medicinal benefits. The leaves of stevia are about 30 times sweeter than sugar, whereas the compounds called steviol glycosides isolated from the leaves of stevia are the potently sweet diterpenoid glycosides stevioside and rebaudioside A [4, 5] are 200–300 times sweeter than sucrose; these compounds are also known as stevia sweeteners. Further, reports indicated that the extract of stevia has been used for centuries to sweeten food and beverages in other part of the world like Japan and China. Due to the increase in demand for the major constituents in the leaves of stevia which are the potently sweet diterpenoid glycosides, it is now grown commercially in a number of countries, particularly in China, Japan, Taiwan, Korea, Thailand, and Indonesia.

2 History/Progression of Steviol Glycoside Approval

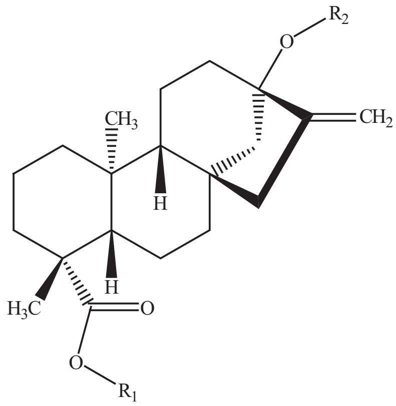
2.1 Molecules Covered by Current Regulation and Ninety Five Percent Purity

Crude extracts of stevia often sold as dietary supplements in some countries, but it is important to note that only purified stevia leaf extract has been evaluated and approved for use as an ingredient in foods and beverages by the leading regulatory agencies. Purified stevia leaf extract (also known as high-purity stevia) generally describes stevia that has 95 % or greater steviol glycoside content. This purity specification was set as part of a thorough safety review by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2008 and is supported by several regulatory authorities including the US Food and Drug Administration (FDA) and the European Commission.

JECFA has finalized specifications for steviol glycosides (95 % purity) including nine steviol glycosides, namely, stevioside; rebaudiosides A, B, C, D, and F; steviolbioside; rubusoside; and dulcoside A, on a dried basis (JECFA 2010) [6]. The European Food Safety Authority (EFSA) evaluated the safety of steviol glycosides as a food additive (sweetener) and expressed its opinion in 2010 which has been designated with E number. Steviol glycosides, E 960 is currently an authorized food additive in the European Union for use in several food categories and specifications; presently those specifications stipulate that steviol glycoside preparations contain not less than 95 % of the ten named steviol glycosides: stevioside; rebaudiosides A, B, C, D, E, and F; steviolbioside; rubusoside; and dulcoside A, on a dried basis. The specifications further define the preparations as comprising mainly (at least 75 %) of stevioside and/or rebaudioside A. The use of steviol glycosides as a food sweetener is regulated under the European Parliament and Council Regulation (EC) No 1333/2008 on food additives. Several other assessments were made for steviol glycosides as a sweetener by the Scientific Committee for Food (SCF in 1984–1999), the Joint FAO/WHO Expert Committee on Food Additives (JECFA in 2000–2010 time frame), and EFSA (2010–2015). JECFA and EFSA have established an Acceptable Daily Intake (ADI) for steviol glycosides as 4 mg/kg bw (body weight) per day, expressed as steviol equivalents. The difference between JECFA and EFSA is in the presence of an additional steviol glycoside rebaudioside E in EFSA. Further, High-Purity Rebaudioside M (minimum purity 95 %) has been approved GRAS status by FDA in 2014. Structures of various steviol glycosides covered by JECFA and EFSA along with their structures, molecular description, and approximate sweetness potency compared to sugar are given in Fig. 1.

3 Chemistry and Grouping of Steviol Glycosides

The two major steviol glycosides isolated from the leaves of *S. rebaudiana* are stevioside and rebaudioside A, and their structures were fully determined between 1955 and 1965 [7, 8]. Besides the major sweetening compounds, stevia also contains



Sweetener	R_1	R_2	Molecular Formula	Molecular Weight	Sweetness Potency
Rebaudioside A	Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	$C_{44}H_{70}O_{23}$	967.01	200
Rebaudioside B	H	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	$C_{38}H_{60}O_{18}$	804.88	150
Rebaudioside C	Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	$C_{44}H_{70}O_{22}$	951.01	30
Rebaudioside D	Glcβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	$C_{50}H_{80}O_{28}$	1129.15	221
Rebaudioside E	Glcβ(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-	$C_{44}H_{70}O_{23}$	967.01	174
Rebaudioside F	Glcβ1-	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	$C_{43}H_{68}O_{22}$	936.99	200
Stevioside	Glcβ1-	Glcβ(1-2)Glcβ1-	$C_{38}H_{60}O_{18}$	804.88	210
Steviolbioside	H	Glcβ(1-2)Glcβ1-	$C_{32}H_{50}O_{13}$	642.73	90
Rubusoside	Glcβ1-	Glcβ1-	$C_{32}H_{50}O_{13}$	642.73	114
Dulcoside A	Glcβ1-	Rhaα(1-2)Glcβ1-	$C_{38}H_{60}O_{17}$	788.87	30

glc = glucose rha = rhamnose xyl = xylose

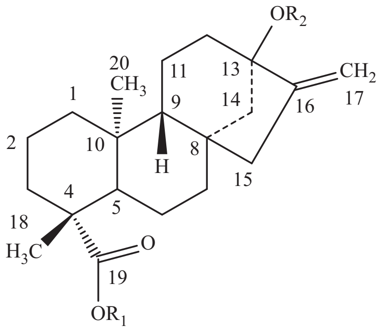
Fig. 1 Molecular formulae, molecular weights, structures and potencies of the *Stevia* sweeteners

other minor diterpene glycosides like rebaudiosides B, C, E, steviobioside, dulcoside A, isosteviol, and dihydroisosteviol. The most common composition of the wild variety of *S. reabaudiana* is often reported as follows: stevioside (5–10 % w/w), rebaudiosides A (2–5 %), and C (1 %), dulcoside A (0.5 %), rebaudiosides D, E, and F (0.2 %), rebaudioside M (0.05 %), and steviolbioside (0.1 %), on dry basis. Based on the genotype of the plant or due to the cultivation conditions, it was observed as a large difference in total steviol glycoside content as well as percentage steviol glycoside compositions. For example, the yield of stevioside from dried leaves varying from 5 % to 22 % whereas that of rebaudioside A content varies from 25 % to 54 %. A yield of 9.2 % stevioside and of 61.6 % rebaudioside A was described in the special species *S. rebaudiana* Morita, which was produced by selection and breeding of *S. rebaudiana* Bertoni [9]. Most of the glycosides isolated from this plant have the same diterpenoid backbone of steviol but differ in the content of saccharides. Many of these glycosides are natural sweeteners, which are not metabolized by humans and therefore do not provide energy in the diet, hence noncaloric. Though JECFA analytical method lists nine different steviol glycosides (Fig. 1), there were several more detected in recent years across the world present in trace quantities in dried leaf extracts originating from different cultivars. So far, over 40 steviol glycosides have been identified in *S. rebaudiana*, but the organoleptic properties of majority of these newly described steviol glycosides have not been reported due to lack of quantity for taste testing. Ohta et al. [9] found 10 new steviol glycosides in the special species *S. rebaudiana* Morita which was produced by selection and breeding of *S. rebaudiana* Bertoni and their structures were confirmed using liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) in negative ion mode, ^{1}H- and ^{13}C NMR spectroscopy, and several chemical studies. Chaturvedula and Prakash isolated a series of new steviol glycosides from commercially available stevia leaf extracts belonging to the rebaudioside A and E family, anomers of dulcoside A, and rebaudioside C, with an α-glycosyl linkage, and six diterpene glycosides with modifications in the *ent*-kaurene body [10–26]. Structural confirmations were performed using a quadrupole time-of-flight (Q-TOF) mass spectrometer equipped with an electrospray ionization (ESI) source operating in positive ionization mode, 1D and 2D NMR spectroscopic data as well as chemical studies. Ibrahim et al. [27] reported the presence of a new diterpene glycoside isolated from a commercial extract of the leaves of *S. rebaudiana*, namely, rebaudioside KA that was shown to be 13-[(*O*-β-D-glucopyranosyl)oxy]ent-kaur-16-en- 19-oic acid 2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl ester. Zimmermann succeeded in confirming 5 of the 10 new steviol glycosides evaluated in leaves produced in Greece as well as in a commercially available stevia extract from China, certified to contain 95 % steviol glycosides.

However, some evidence exists that rebaudioside B and steviolbioside are not native constituents of *S. rebaudiana* but are formed by partial hydrolysis during extraction [28], being thus artifacts of the extraction procedure. Hence, it is a debatable situation whether all of these new compounds, mainly those detected in purified leaf extracts, are genuine or artifacts due to purification procedures.

Majority of the steviol glycosides isolated so far are having mainly β-D-glucosyl moieties connected to the C-13 and C-19 position on steviol with varying attachments of sugars attached at individual positions. The structures of various steviol glycosides isolated along with their grouping, sugars attached, and corresponding references are provided below in Fig. 2:

In addition, three novel diterpene glycosides were isolated for the first time from the commercial extract of the leaves of *S. rebaudiana* and were identified as 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-Dglucopyranosyl) oxy] *ent*-kaur-15-en-19-oic acid, 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy]-16β-hydroxy-*ent*-kauran-19-oic acid, and 13-methyl-16-oxo-17-nor-*ent*-kauran-19-oic acid-β-D-glucopyranosyl ester on the basis of extensive 2D NMR and MS spectroscopic data as well as chemical studies [26].



S. No	Common Name	R1	R2	Reference
1) Steviol + Glucose				
1.1	Steviolmonoside	H	Glcβ1-	[9]
1.2	Steviol-19-O-β-D-glucoside	Glcβ1-	H	[29]
1.3	Rubusoside	Glcβ1-	Glcβ1-	[9]
1.4	Steviolbioside	H	Glcβ(1-2)Glcβ1-	[30]
1.5	Stevioside	Glcβ1-	Glcβ(1-2)Glcβ1-	[2]
1.6	Rebaudioside KA	Glcβ(1-2)Glcβ1-	Glcβ1-	[27]
1.7	Rebaudioside B	H	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[30]
1.8	Rebaudioside G	Glcβ1-	Glcβ(1-3)Glcβ1-	[9]
1.9		Glcβ(1-3)Glcβ1-	Glcβ1-	[31]
1.10	Rebaudioside E	Glcβ(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-	[23]
1.11	Rebaudioside A	Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[30]
1.12		Glcβ1-	Glcβ(1-6)Glcβ(1-2)Glcβ1-	[11]
1.13	Rebaudioside D	Glcβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[32]
1.14	Rebaudioside I	Glcβ(1-3)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[9]
1.15	Rebaudioside L	Glcβ1-	Glcβ(1-6)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[9]
1.16		Glcα(1-2)Glcα(1-4)Glcβ1-	Glcβ(1-2)Glcβ1-	[23]
1.17		Glcβ1-	Glcα(1-4)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[11]
1.18		Glcβ1-	Glcα(1-3)Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[12]
1.19		Glcβ1-	Glcα(1-4)Glcβ(1-3)[Glcβ(1-2)]Glcβ1-	[13]
1.20		Glcα(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[14]
1.21	Rebaudioside M	Glcβ(1-2)[Glcβ (1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[9, 25]
1.22		Glcβ(1-2)[Glcβ(1-6)]Glcβ1-	Glcβ(1-2)Glcβ1-	[31]
1.23		Glcβ(1-2)Glcβ1-	[Glcβ(1-3)Glcβ(1-6)]Glcβ1-	[24]
2) Steviol + Rhamnose + Glucose				
2.1	Dulcoside A	Glcβ1-	Rhaα(1-2)Glcβ1-	[34]
2.2	Dulcoside B	H	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	[9]
2.3	Rebaudioside C	Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	[24]
2.4		Rhaα(1-2)Glcβ1-	Glcβ(1-3)Glcβ1-	[35]
2.5	Rebaudioside H	Glcβ1-	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	[34]
2.6	Rebaudioside K	Glcβ(1-2)Glcβ1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	[9, 18]
2.7	Rebaudioside J	Rhaα(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[35]
2.8	Rebaudioside N	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[9, 20]
2.9	Rebaudioside O	Glcβ(1-3)Rhaα(1-2)[Glcβ(1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[21]
3) Steviol + Xylose + Glucose				
3.1		Glcβ1-	Xylβ(1-2)Glcβ1-	[15]
3.2	Rebaudioside F	Glcβ1-	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	[36]
3.3		Glcβ1-	Glcβ(1-2)[Xylβ(1-3)]Glcβ1-	[15]
3.4		Xylβ(1-6)Glcβ1-	Glcβ(1-2)Glcβ1-	[16]
3.5		Glcβ(1-2)[Glcβ (1-3)]Glcβ1-	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-	[22]
4) Steviol + Fructose + Glucose				
4.1		Glcβ1-	Glcβ(1-2)[Fruβ(1-3)]Glcβ1-	[10]
5) Steviol + deoxyGlucose + Glucose				
5.1		Glcβ1-	6-deoxyGlcβ(1-2)Glcβ1-	[16]
5.2		Glcβ1-	6-deoxyGlcβ(1-2)[Glcβ(1-3)]Glcβ1-	[17]
5.3		6-deoxyGlcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-	[19]

Fig. 2 Structures of various identified Steviol glycosides grouped on sugars attached to steviol backbone

4 Production

Stevia rebaudiana (Bertoni) Bertoni is a member of a New World genus of 150–300 species that belong to the tribe Eupatorieae of the family Asteraceace (sunflower family). The plant may reach a height of 80 cm when fully grown and is native to Paraguay in the Department of Amambay on the border of Paraguay with Brazil. Stevia plants require long daylight and water and can be grown all around the world, although most commercial stevia agriculture is done in mainland China. Steviol glycosides from dried leaves of *S. rebaudiana* are usually obtained after extraction with water or water-organic solvent mixture or organic solvent, precipitation of high molecular weight substances (sometimes combined with a defatting step), decolorization, purification, concentration, and drying. Pressurized hot water and microwave-assisted water extractions were observed to have higher or comparable efficiencies than heating under reflux. Ultrasonically assisted extraction is said to increase the yield by a factor of 1.5 at a lower temperature (68 °C) and to shorter extraction time (32 min) as compared to classical extraction. When compared different extraction techniques, it was found that conventional cold extraction was performed at 25 °C for 12 h, ultrasound extraction at 35 $\pm$ 5 °C for 30 min, and using microwave-assisted extraction, the extraction time could be reduced to 1 min at 50 °C. Methods using chloroform and ethanol for extraction or even supercritical fluid extraction (SFE) have also been published. The extraction step is sometimes followed by solid phase extraction (SPE), mostly with C18 cartridges, to remove disturbing matrixes. Stevioside and rebaudioside A are obtained from *S. rebaudiana* leaves in two stages. First step involved the extraction of steviol glycosides from the leaves with hot water or alcohol/water and the extract dried. In the second stage, further purification via alcohol/water crystallization yields very pure stevioside and/or rebaudioside A. In modern processes, hot-water extraction of stevia leaves gives a “primary extract” from which other plant components are then removed by flocculation. The cleared solution is passed through adsorption resins to concentrate the steviol glycosides, which are then eluted with alcohol. The dried eluate, comprising mixed steviol glycosides, may be stored and transported in this form before final purification. In this last step, the dried eluate is redissolved in a lower alcohol (pure or an aqueous solution) and recrystallized. Refluxing the dried solid in a methanol solution followed by cooling enables the isolation of stevioside. Rebaudioside A is obtained either from the filtrate after stevioside is removed or by directly crystallizing the solid with alcohol or aqueous alcohol. Traditionally, methanol has been used as the solvent, but ethanol has the advantage of selectively increasing the yield of rebaudioside A. Finally, the crystallized product is filtered and dried.

Refining methods of stevioside into solvent partition extraction mainly methanol or water extraction and solvent partition extraction, incorporating mainly in situ precipitation with calcium hydroxide–carbon dioxide to remove impurities, similar to the purification process in the sugar industry. They also reported different methods of purification, such as adsorption, chromatography, ion-exchange, plasmid gel, or adsorption by activated carbon. JECFA also cited that the typical manufacture starts with extracting leaves with hot water, and the aqueous extract is then passed through an adsorption resin to trap and concentrate the component steviol glycosides. The resin is washed with methanol to release the glycosides, and the product is recrystallized with methanol. Ion exchange resins may be used in the purification process. The final product is commonly spray dried. There are several extraction patents for the isolation of steviol glycosides. Kinghorn et al. [5] has categorized the extraction patents into those based on solvent, solvent plus a decolorizing agent, adsorption and column chromatography, ion exchange resin, and selective precipitation of individual glycosides. In recent patents, methods such as ultrafiltration, metallic ions, supercritical fluid extraction with CO_2, and extract clarification with zeolite have been employed.

5 Stability of Steviol Glycosides

In order to be an effective sweetener in foods, beverages, and functional foods, stability of steviol glycosides in different matrices is one of the most important characters. Under controlled humidity conditions, dry powder of stevioside is stable for 2 years whereas rebaudioside A is stable for 3 years. Stevioside showed stability when incubated at a high temperature (120 °C) for one hour, although it degraded at temperatures above 140 °C, whereas rebaudioside A and D demonstrated higher stability at elevated temperatures to stevioside. At 200 °C, stevioside showed completed degradation, 63 % and 32 % rebaudioside A and rebaudioside D remain unchanged, respectively. Several investigations have reported in the literature about the stability of stevioside, rebaudiosides A, and D, and steviol glycoside mixture under room temperature, neutral pH, and sunlight conditions. These sweeteners can be used under pH conditions ranging from 2 to 10 and at temperatures of up to 120 °C.

The stability of a stevioside based sweetener in solutions formulated with different organic acids (citric, tartaric and phosphoric acid) and vitamins (thiamine, riboflavin, niacin and pyridoxine) under different storage conditions was evaluated and its application as a sweetener in coffee and tea was also assessed. An aqueous solution of the stevioside remained stable over a pH range of 2–10 at 80 °C. Stevioside content did not exhibit significant decomposition when incubated for up to 4 h with water-soluble vitamins at 80 °C. When subjected to extremely acid conditions, however, a significant decrease in stevioside concentration was observed, whereas ascorbic acid had a protective effect against the degradation of the stevioside. Moreover, both the major steviol glycosides of *S. rebaudiana* stevioside and rebaudioside A were slightly less stable in phosphoric acid compared to citric acid.

The major reaction pathways during degradation of steviol glycosides could be: 1. Isomerization of the C-16 olefin double bond to form the corresponding C-15 isomer (exocyclic to endo cyclic), 2. Hydration of the C-16 olefin to form an alcohol, and 3. Hydrolysis of the glycosidic ester at C-19 position to form a carboxylic acid (Fig. 3).

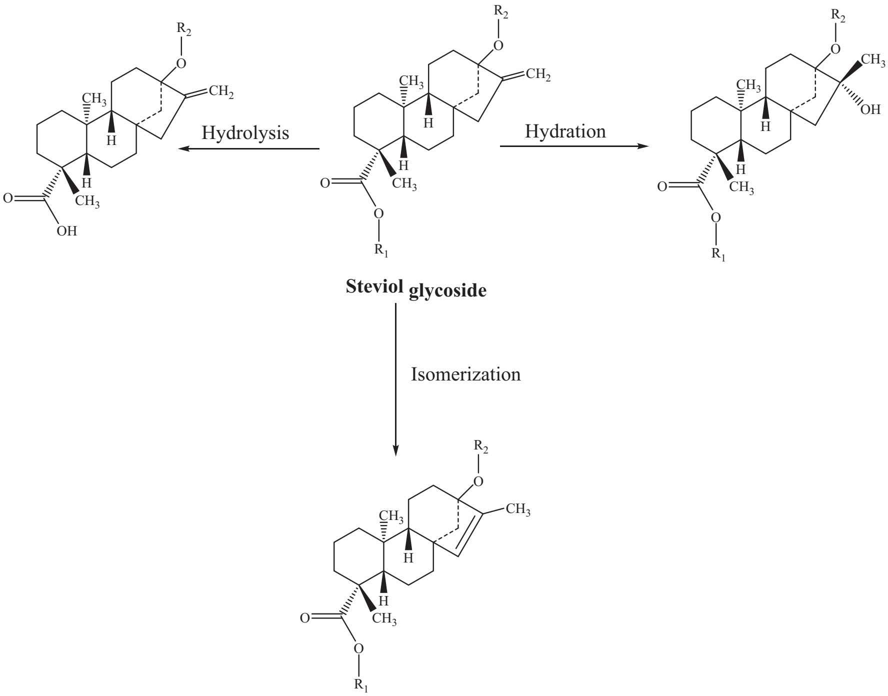


Fig. 3 Pathways of degradation of steviol glycosides

Photostability of a mixture of steviol glycosides was studied under fluorescent light exposure at 25 °C in mock beverages at pH 3.8 using International Conference on Harmonization (ICH) technical requirements for 2 weeks yielded three minor compounds which were identified as 13-[(2-*O*-β-D-glucopyranosyl*O*-β-D-glucopyranosyl)oxy]-17-hydroxy-*ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl ester (**a**), 13-[(2-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester (**b**), and 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-16-hydroxy-*ent*-kauran-19-oic acid β-D-glucopyranosyl ester (**c**) (Fig. 4). The mass balance was calculated against their controls and was found as 98.3 % suggested that there was not any appreciable amount of undetected degradation products were formed under the conditions of the study [36].

Similarly, the photostability of rebaudioside A was studied under fluorescent light exposure at 25 °C in mock beverages at pH 3.8 using ICH technical requirements indicated that rebaudioside A did not undergo any major decomposition with fluorescent light exposure for 2 weeks. Identification of the degradation products furnished two minor compounds which were identified as 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-Dglucopyranosyl) oxy]-17-hydroxy-*ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl ester (**d**) and 13-[(2-*O*-β-Dglucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-16β-hydroxy *ent*-kauran-19-oic acid β- D-

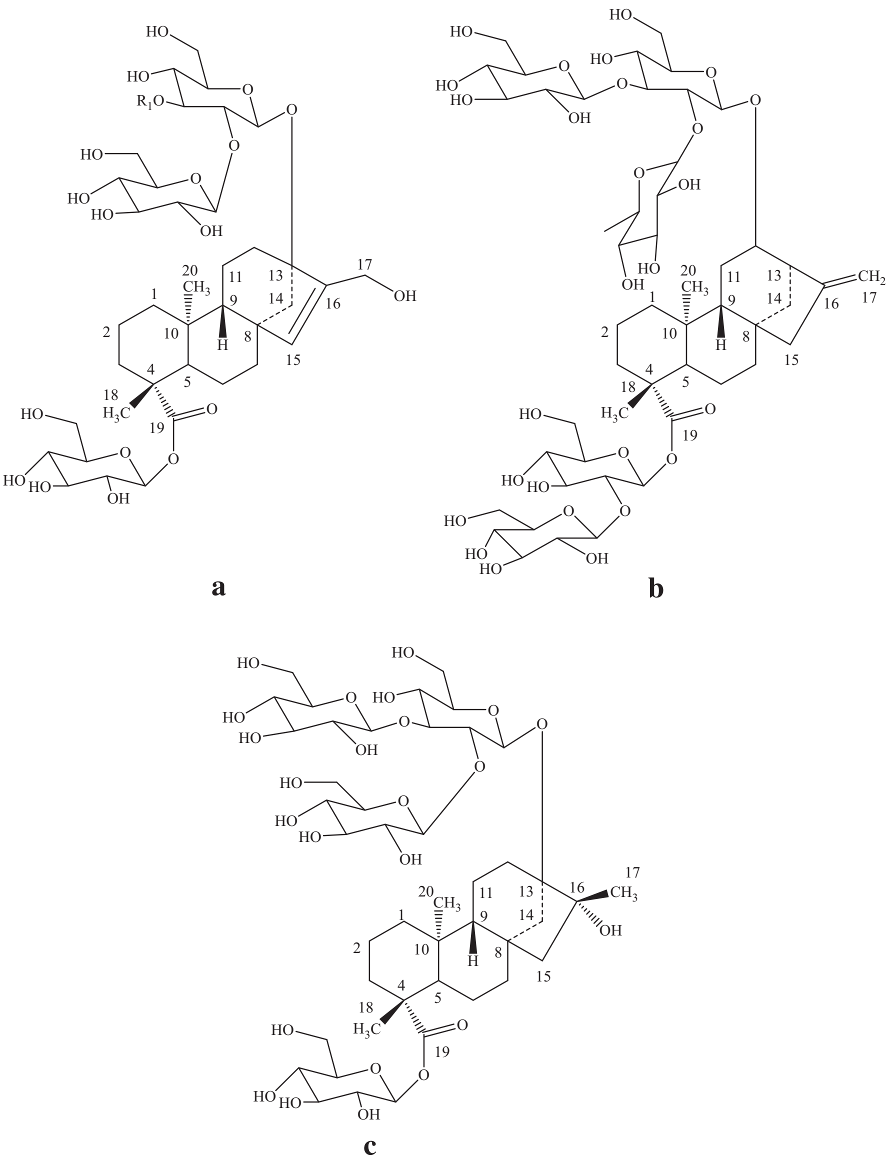
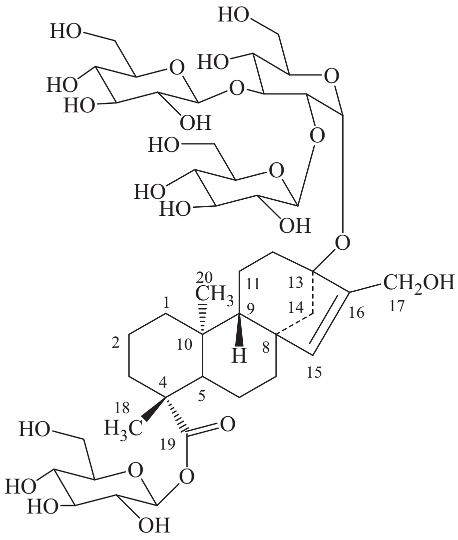


Fig. 4 Degradation compounds of steviol glycosides under fluorescent light exposure

glucopyranosyl ester (**e**) (Fig. 5). The mass balance for rebaudioside A was calculated against their control samples was found 99.1 %, suggesting any appreciable amount of undetected degradation products were formed under the conditions of the study [37].

This suggested that steviol glycoside mixture and purified rebaudioside A are considered relatively stable using the conditions of this study when exposed to fluorescent light.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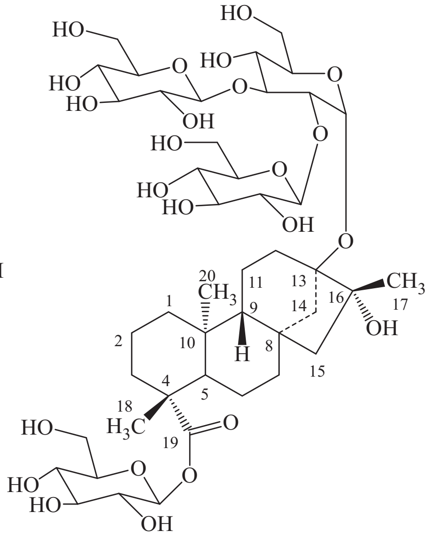
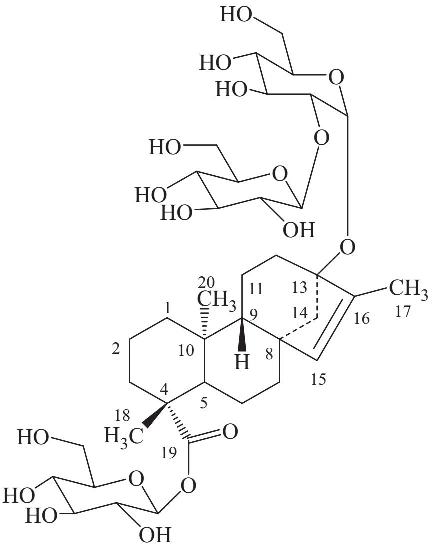

e

Fig. 5 Degradation compounds of Rebaudioside A under fluorescent light exposure

In another report, stability of a mixture of steviol glycosides was studied under a typical pH range and various temperatures that simulated both relevant and extreme beverage storage conditions. Thus, steviol glycosides were evaluated in mock beverage solutions by simulating formulations used in commercial cola soft drinks (pH 2.8 and pH 3.2), lemon-lime soft drinks (pH 3.8), and root beer soft drinks (pH 4.2) but lacking corresponding flavor components. Experimental results indicated that steviol glycoside mixture did not undergo any major decomposition but yielded two minor compounds which were identified as 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy] *ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl (**f**) and 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-16β-hydroxy-*ent*-kauran-19-oic acid β-D-glucopyranosyl ester (**g**) (Fig. 6). The stability of steviol glycosides in mock beverage solutions is pH, temperature, and time dependent; the rate and extent of degradation product formation is increased under acidic conditions (lower pH) and at higher temperatures with the majority of degradation product formation occurring after extended period of storage [38].

Likewise, stability of rebaudioside A was studied in mock beverage solutions by simulating formulations used in commercial cola soft drinks (pH 2.8 and pH 3.2), lemon–lime soft drinks (pH 3.8), and root beer soft drinks (pH 4.2) but lacking the flavor components. Samples were analyzed at scheduled intervals throughout the 26-week period for rebaudioside A, its known impurities, and degradation products. Experimental results indicated that rebaudioside A yielded six minor degradation compounds (**h-m**) (Fig. 7) whose structural characterization was performed on the basis of 1D (^{1}H, ^{13}C) and 2D (COSY, HSQC, HMBC) NMR, HRMS, MS/MS



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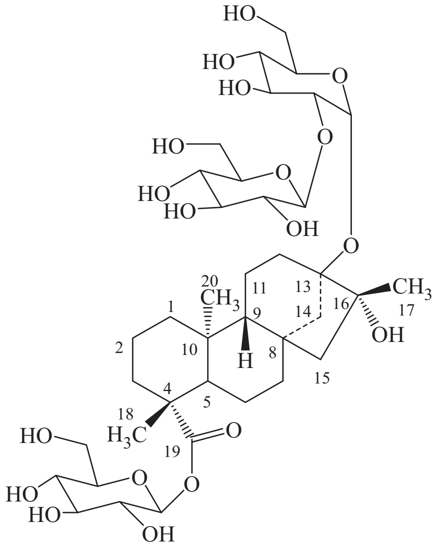

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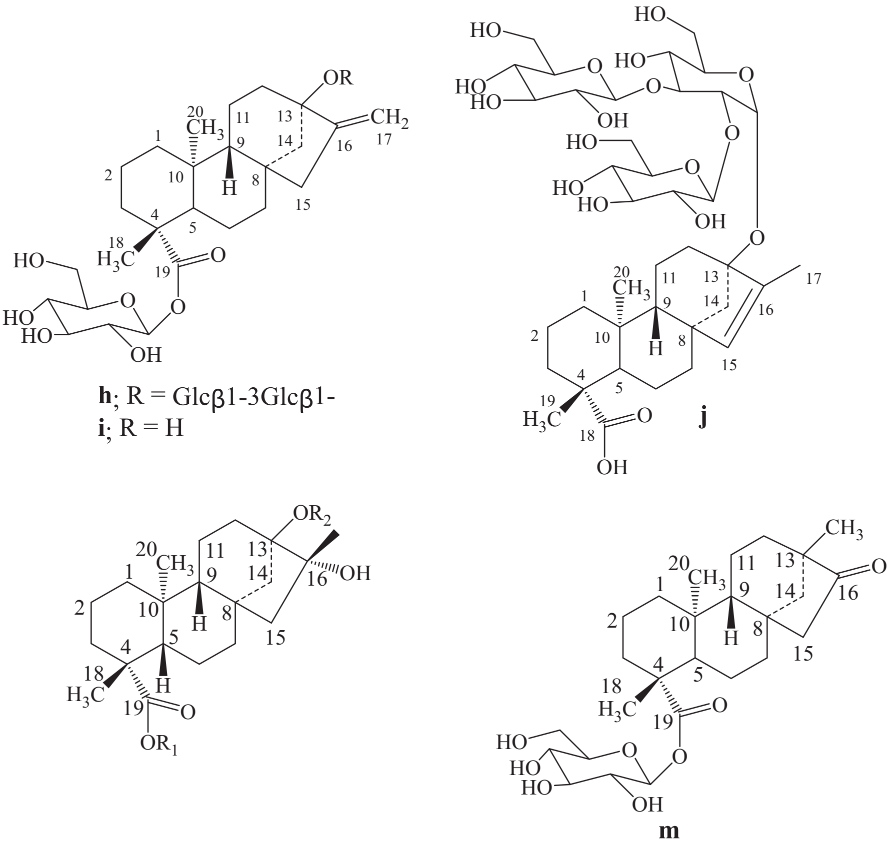
Fig. 6 Degradation compounds of steviol glycosides under acidic conditions

spectral data. Results indicated that the stability of rebaudioside A in mock beverage solutions is pH, temperature, and time dependent. The rate and extent of degradation product formation is increased under acidic conditions (lower pH) and at higher temperatures with the formation of degradation products mainly occurring after extended period of storage. But in each case, excellent mass balance was achieved at all conditions by the identification of known impurities and degradation products suggested that rebaudioside A is considered stable [39].

Degradation of rebaudioside M, a minor sweet component of *S. rebaudiana* Bertoni, under simulated extreme pH and temperature conditions has been studied. Thus, rebaudioside M was treated with 0.1 M phosphoric acid solution (pH 2.0) and 80 °C temperature for 24 h, and experimental results indicated that rebaudioside M under low pH and higher temperature yielded three minor degradation compounds (**n**-**p**)(Fig. 8), whose structural characterization was performed on the basis of 1D (1H-, 13C-) & 2D (COSY, HSQC, HMBC) NMR, HRMS, MS/MS spectral data as well as enzymatic and acid hydrolysis studies [40].

Moreover, the stability of steviol glycosides in diverse food products semi skimmed milk, soy drinks, fermented milk drinks, ice cream, yogurt (made with both full cream and skimmed milk), dry biscuits, and jam was assessed. Storage conditions were those recommended for each type of food (−18 °C for ice cream, 6 °C for yogurt and 20 °C for skimmed and fermented milk). Stevioside recovery was between 96 % and 103 %, thus demonstrating that the steviol glycosides had not decomposed in any of the samples tested [41].



Compound	R_1	R_2
k	H	Glcβ1-2(Glcβ1-3)Glcβ1-
l	Glcβ1	Glcβ1-2(Glcβ1-3)Glcβ1-

Fig. 7 Degradation compounds of rebaudioside A under acidic conditions

6 Sensory Properties

6.1 Sweetness Potency

In the literature, stevioside, rebaudioside A, reabudioside D, and rebaudioside M are often reported as 150–250, 200–300, 200–350, and 200–450 times sweeter than sucrose, respectively. However, sweetness potency is strongly dependent on sucrose equivalency (SE) level for all high-potency sweeteners (HPS). Therefore, it is important to state the SE level at which sweetness potency has been determined.

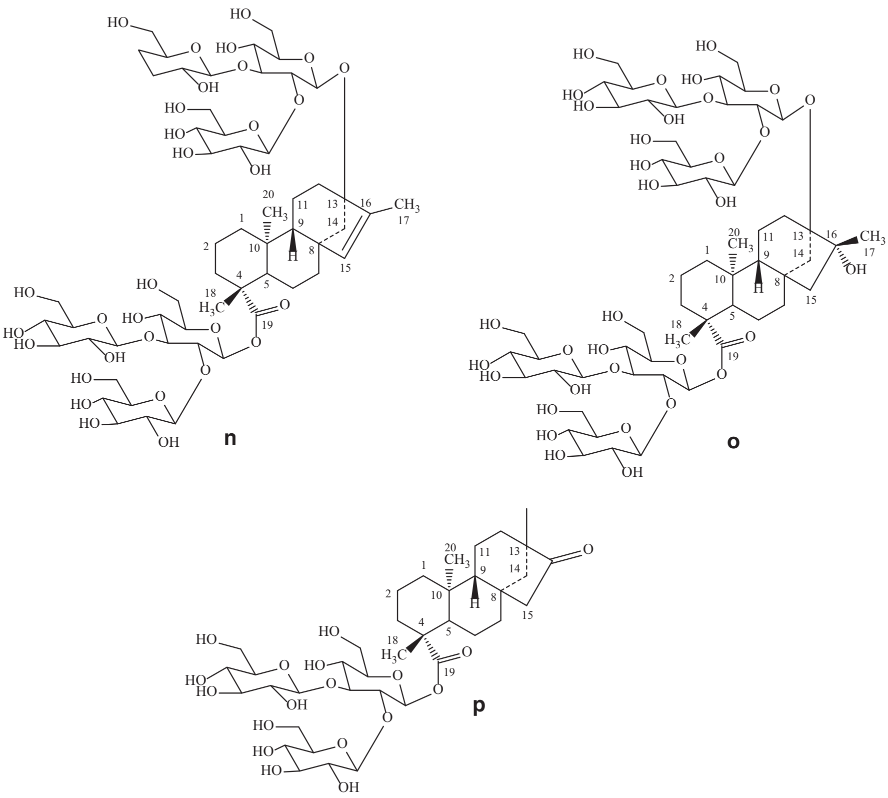


Fig. 8 Degradation compounds of rebaudioside M under acidic conditions

Sweetness potency is also system dependent and, therefore, it is important to also define the medium (e.g., water or phosphoric acid at pH 2.5). For comparison of different HPS, the most common medium is water (in the scientific literature where the medium is not specified, it is assumed to be water). While the choice of test medium is important, SE level is the major determinant of sweetness potency. There is, however, no industry-wide agreement on a common SE at which to report sweetness potency.

Typical use-levels of HPS in beverages are generally in the range of 4–10 % SE. Consequently, 6 % SE represents a reasonable average value at which to compare the sweetness potency of HPS in a plain water vehicle. At 6 % SE, the sweetness potency of rebaudioside A is 200 times [28], whereas rebaudioside M is 310 times [25] to that of sucrose. This is similar to the sweetness potency of aspartame, a HPS that is widely marketed and approved for addition to numerous foods and beverages in many countries, which is 180 times as potent at 6 % SE. Although the concentration-response functions for rebaudioside A ($R = 8.2C/(194 + C)$), rebaudioside M ($R = 14.2 \times C/(265 + C)$), and aspartame ($R = 25.5C/(1{,}160 + C)$) differ slightly in water, the three sweeteners are similar in sweetness over the range of SE levels. Rebaudioside M can be used as a single sweetener or blends to make zero calorie beverages.

6.2 Flavor Profile

As with most high-potency sweeteners, stevioside, rebaudioside A, rebaudioside D, and rebaudioside M exhibit clean sweetness at low SE levels but have other negative taste attributes (e.g., bitterness and black licorice) at higher SE levels. Stevioside exhibits much more bitterness than rebaudioside A, rebaudioside D, and rebaudioside M. Rebaudioside M also exhibits a clean sweet taste without any bitter or licorice aftertaste but is present at relatively low levels in currently available stevia plants [25, 28].

6.3 Sweetness Temporal Profile

Sweetness temporal profiles demonstrate changes in perception of sweetness over time. This property is key to the utility of a sweetener in foods and beverages and is complementary to its flavor profile. Every sweetener exhibits a characteristic Appearance Time (AT) and Extinction Time (ET). All high-potency sweeteners, in contrast to carbohydrate sweeteners, display prolonged ETs. This can be beneficial in some products such as chewing gum, where prolonged sweetness is desirable. Among all the steviol glycosides known today, rebaudioside M exhibits the quick onset (Fig. 9).

7 Food Applications

Based on the growing number of stevia-based products on the world market, such as drinks, table-top sweeteners, candy and other processed foods, personal hygiene products, and various delicacies, it is clear that the addition of steviol glycosides can increase the palatability and enjoyment of food by improving flavor and smell [42, 43]. The key factors affecting the stability of steviol glycosides are pH, humidity/moisture, and temperature, based on known results from aspartame and neotame. Products comprised carbonated and still nonalcoholic beverages, table-top sweetener formulations, chewing gum, yogurt, and cake were packed, stored (mostly at 25 °C and 60 % relative humidity), and evaluated at intervals using both chemical (HPLC) and sensory analyses. Sweetness was assessed using panels consisting of 35–50 persons. Samples were evaluated using a five-point scale of categories ranging from 5 (much too sweet) to 1 (not at all sweet). Samples were considered satisfactory if at least 80% of the panelists rated the sweetness in category 3 (just about right) or above. Key findings were by Prakash et al. [28] on soft drinks (100–600 ppm): rebaudioside A remained acceptably sweet throughout 26 weeks

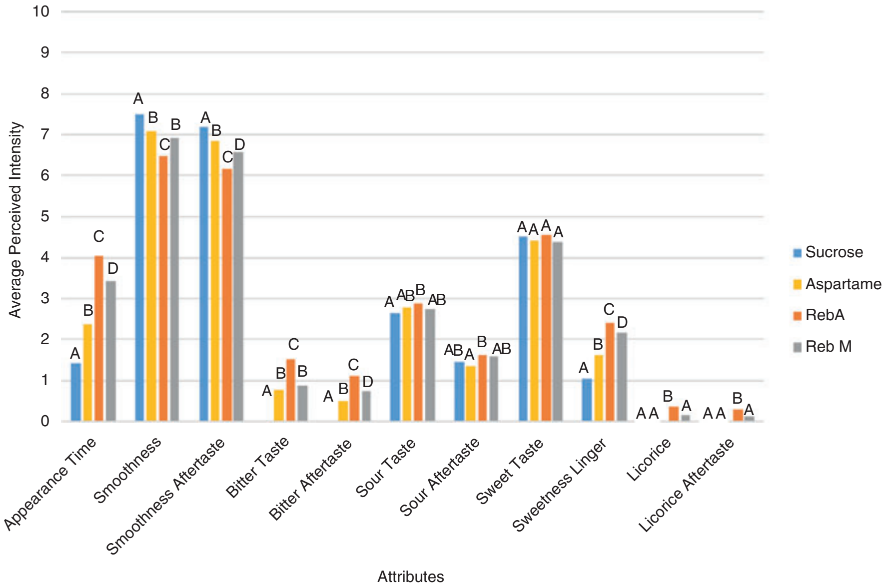


Fig. 9 Descriptive Analysis results of specific attributes for sucrose, aspartame, rebaudioside A, and rebaudioside M sweeteners at 400 ppm at room temperature in water

storage (cola and lemon-lime); for comparison, most soft drinks are consumed within 16 weeks of production.

1. Tabletop sweetener (200–2,000 ppm): rebaudioside A was tested in a number of formulations and sweetness remained stable for at least 52 weeks in all of them.
2. Chewing gum (300–10,000 ppm): rebaudioside A was considered stable and functional in chewing gum for 26 weeks.
3. Plain yogurt (100–1,000 ppm): No significant loss was measured during pasteurization (190 °F for 5 min) and fermentation. Rebaudioside A sweetness was stable throughout a 6-week storage period (40 °F).
4. White cake (200–1,000 ppm): No significant loss of sweetness was measured during the baking process (350 °F for 20–25 min) or during 5 days subsequent storage (25 °C and 60 % RH).
5. Other products (100–1,000 ppm): rebaudioside A has been successfully formulated into cereals and cereal-based foods, dietary supplements, pharmaceuticals, edible gels, and confectionery products.

Specifically, stevia extracts have been used to sweeten low-calorie soft drinks, soy sauce, dried seafood, candy, ice cream, chewing gum, and yogurt in several countries, particularly in Japan, Korea, and Brazil. There have been several investigations undertaken aimed at evaluating the potential of stevia as a sweetener of specific products. Reports concluded that low-calorie yogurts could be manufactured using commercial sweeteners including stevia without modifying standard technological procedures [44]. A similar study, but applied to cake, assessed the rheological and microstructural properties and the final quality of cakes made by replacing sugar with stevioside and liquid sorbitol. The results showed that the addition of stevioside did not change the amylographic viscosity of wheat flour batter during heating and cooling, unlike sucrose, which increases this property which concluded that it is possible to replace sugar with stevioside using this cake recipe while maintaining the rheological properties of the final product.

The stevia glycosides which are natural noncaloric and low-energy sweeteners can be used as functional ingredients in nutrition, by diabetic and phenylketonouric patients as well as by obese people due to the ability to reduce the craving for sweet and fatty foods. Stevioside is very stable at high temperatures up to 200 °C and in a wide range of pH values, is not fermented, and does not support formation of plaque on the teeth. Accordingly, stevioside has a high degree of stability and can find application as natural stabilizer in a variety of dairy products, beverages, confectionery, and other foods. According to reported literature, healthy humans who consumed stevioside did not differ from controls in blood pressure and blood biochemical parameters, while no gastrointestinal uptake was detected for stevioside or steviol. Also, no genetic toxicity or mutagenicity was found for steviol glycosides. Additionally, rebaudioside A is stable in a wide variety of foods and beverages such as flavored ice-tea, juices, flavored milk, and “live” yogurt. Since these products are usually consumed cold, Fry et al. [45] found that rebaudioside A is significantly sweeter under these conditions. According to Brusick [46], human consumption of rebaudioside A does not pose a risk for genetic damages.

8 Blending

Blending of stevia sweeteners with other high-potency sweeteners may reduce bitterness and improve temporal profile and often result sweetness synergy. Lingering sweetness of steviol glycosides is able to be masked by addition of certain substances with floral and sweet note. Further, combinations with other high-potency sweeteners result in reduction of the sweetener cost as well as improvement in stability. The bitter after taste and lingering properties of steviol glycosides can be complemented to flavors of coffee, chocolate, spicy sauces, and to the stringency and bitterness of tea. Overall blending of sweeteners result in suppression or addition or synergy.

At higher concentrations of steviol glycosides, mainly rebaudiosde A and stevioside (>6 % sugar equivalence) exhibit “off-taste” and this could be the reason these sweeteners are unlikely to be used as a sole sweetener. This limitation can be addressed by blending with other nutritive and nonnutritive sweeteners, especially for stevia sweetener rebaudioside A. Examples for nutritive sweeteners those go well with rebaudioside A include sorbitol and xylitol belongs to polyol family, and carbohydrates such as glycerol, fructose, glucose, sucrose, and HFCS. Similarly, the nonnutritive sweeteners suitable to be partnered with reabudioside A are Luo

Han Guo extract; monatin; brazzein; erythritol; sweet tasting amino acids such as glycine, alanine, and serine; and the artificial sweeteners like aspartame, acesulfame potassium, cyclamate, sucralose, and saccharin. These blending formulations may create a good tasting, improved temporal profile and reduced lingering.

9 Metabolism

Studies have been conducted in humans to document the metabolism of steviol glycosides. Steviol glycosides are not absorbed in the small intestine and once steviol glycosides reach the colon, gut bacteria hydrolyze steviol glycosides into steviol by snipping off their glucose units. Steviol is then absorbed via the portal vein and primarily metabolized by the liver forming steviol glucoronide and then excreted in the urine. Research has shown that there is no accumulation of stevia (or any by-product of stevia) in the body during metabolism. It is a result of this essentially poor absorption in the digestive tract which ultimately contributes to the fact that stevia has zero calories and does not raise blood glucose or insulin levels when digested.

Studies in rats [47] and other animal models, including chickens [3], hamsters [48], and pigs [49], indicated that stevioside is not readily absorbed from the GI tract. Available evidence from in vitro metabolism studies suggests that bacteria in the colon of rats and humans can transform various stevia glycosides into steviol [29]. Slow absorption of steviol was indicated by detection in the plasma of rats given oral stevioside [50]; however, did not detect plasma steviol following oral administration of steviosides to rats. In studies with human and rat liver extracts, Koyama et al. [47] demonstrated that steviol can be converted to various glucuronides. Excretion of metabolites of stevioside after oral doses has been shown in urine and feces in rats and hamsters [48]. Oral doses in pigs led to the detection of steviol glycoside metabolites in feces but not in urine [49]. Steviol was shown to be more readily transported with in vitro intestinal preparations than various steviosides [47, 49].

Renwick et al. [51] reviewed studies on microbial hydrolysis of steviol glycoside concluding that stevioside and reabudioside A are not absorbed directly and both are converted to steviol by gut microbiota in rats and in humans. This hydrolysis occurs more slowly for reabudioside A than for stevioside may be associated with the molecular structure. Studies have shown that steviol-16,17 epoxide is not a microbial metabolite. The authors concluded that there is a single hydrolysis product and that toxicological studies on stevioside are relevant to the safety assessment for reabudioside A. In a human study with 10 healthy subjects, Geuns et al. [52] measured blood, urine, and fecal metabolites in subjects that received 3 doses of 250 mg of purified stevioside (>97 %) three times a day for 3 days. Urine was collected for 24 h on day 3, and blood and fecal samples were also taken on day 3. Free steviol was detected in feces but not in blood or urine. Steviol glucuronide was detected in blood, urine, and feces. Approximately 76 % of the total steviol equivalents dosed were recovered in urine and feces. Based on these measurements, the authors concluded that there was complete conversion of stevioside in the colon to steviol, which was absorbed and rapidly converted to the glucuronide.

Metabolism of steviol glycosides in humans and rats is the same, but the pattern of metabolite excretion is different [53]. In humans, as in rats, both stevioside and rebaudioside A are metabolized by bacteria in the lower gut to steviol, which is absorbed into the portal blood system and transported to the liver where it is glucuronidated, the same process observed in the rat. However, in humans most steviol glucuronide appears in the plasma instead of the bile. Peak plasma concentrations of steviol glucuronide in humans occur approximately 8 and 12 h post-dosing for stevioside and rebaudioside A, respectively. Like the rat, peak plasma metabolite concentrations were lower after rebaudioside A ingestion than after stevioside ingestion. The half-life of steviol glucuronide in human plasma is approximately 14 h. Steviol glucuronide is the major excretion form of absorbed steviol in humans, and excretion occurs primarily via the urine rather than the feces. Only a small amount of steviol excretion occurs via the feces in humans. The metabolism of orally ingested steviol is interesting but not relevant for the understanding of steviol glycoside metabolism or safety. Humans do not ingest steviol, and orally ingested steviol kinetics are quite different compared to those of steviol glycosides.

For comparative purposes to determine whether toxicological studies conducted previously with stevioside would be applicable to the structurally related glycoside, rebaudioside A, toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol were examined in rats [54]. Orally administered single doses of the radiolabeled compounds were extensively and rapidly absorbed with plasma concentration-time profiles following similar patterns for stevioside and rebaudioside A. Radioactivity from orally administered steviol glycosides slowly increased in plasma over a period of hours and was excreted primarily via the feces within 48 h of oral dosing. While the half-life of plasma radioactivity was 5 h in male rats and 10 h in female rats, other kinetic parameters were similar in males and females. Both steviol and steviol glucuronide were identified in plasma. Peak plasma levels of radioactivity were slightly lower for rebaudioside A compared to stevioside. As indicated above, this is not unexpected given the time required to remove an additional glucose moiety present in rebaudioside A. Less than 2 % of the radioactivity was found in the urine and virtually no residual radioactivity was observed in any organ 96 h after dosing. The predominant compound observed in bile from cannulated rats was steviol glucuronide, while steviol was the predominant compound found in rat feces. Radioactivity in the feces accounted for 97–98 % of the administered dose of both stevioside and rebaudioside A demonstrating that excretion of steviol glucuronide via bile is the major excretory route of steviol in the rat. The authors concluded that the overall data on toxicokinetics and metabolism indicate that rebaudioside A and stevioside are handled in an almost identical manner in the rat after oral dosing. In a randomized, double blind, cross-over study in healthy male subjects, Wheeler et al. [53] assessed the comparative pharmacokinetics of steviol and steviol glucuronide following single oral doses of rebaudioside A and stevioside. Steviol glucuronide was eliminated from the plasma, with similar t1/2 values of approximately 14 h for both compounds. Administration of rebaudioside A resulted in a significantly (approximately 22 %) lower steviol glucuronide geometric mean Cmax value (1,472 ng/mL) than administration of stevioside (1,886 ng/mL). Steviol glucuronide was excreted primarily in the urine of the subjects during the 72-h collection period, accounting for 59 % and 62 % of the rebaudioside A and stevioside doses, respectively.

Pharmacokinetic analysis indicated that both rebaudioside A and stevioside were hydrolyzed to steviol in the gastrointestinal tract prior to absorption. The majority of circulatory steviol was in the form of steviol glucuronide indicating rapid first-pass conjugation prior to urinary excretion. Only a small amount of steviol was detected in urine (rebaudioside A: 0.04 %; stevioside: 0.02 %). The investigators concluded that rebaudioside A and stevioside underwent similar metabolic and elimination pathways in humans with steviol glucuronide excreted primarily in the urine and steviol in the feces. No safety concerns were noted as determined by reporting of adverse events, laboratory assessments of safety, or vital signs [53]. Another pharmacokinetic investigation was done as a toxicokinetic (TK) phase of a dietary study to determine the potential of rebaudioside A toxicity in rats at levels up to 2,000 mg/kg bw/day. Rebaudioside A and total steviol were detected in peripheral blood of rats during daily administration of 2,000 mg/kg bw/day of rebaudioside A at extremely low levels, with mean plasma concentrations of approximately 0.6 and 12 ug/mL, respectively. Estimates of absorbed dose for rebaudioside A and total steviol were approximately 0.02 % and 0.06 %, respectively, based on the amounts measured in urine collected over 24 h in comparison to daily administered dietary dose to rats. Mean fecal rebaudioside A and measured hydrolysis products expressed as total rebaudioside A equivalents compared to daily administered dose results in an estimate of percent of dose recovered is 84 %.

10 Analytical Methods

Early analysis of sweet-tasting steviol glycosides involved Thin Layer Chromatography (TLC) coupled with densitometry, while other protocols like colorimetric detection has been used for the specific detection of isosteviol as its methyl ester. Several methods are known for determining the quantitative content of glycosides in plant material like gas chromatography or infrared spectroscopy; however, the simplest and most reliable method is HPLC, which has been used to determine the composition of *S. rebaudiana* growing in various geographical areas due to the easiness of sample preparation and the more satisfactory separation of stevioside, rebaudioside A, and other minor steviol glycosides, compared to methodologies based on TLC.

The initial HPLC analysis results were produced on amino-based or reversed-phase columns (C18) in combination with UV-detection. All JECFA methods before 2010 proposed the amino column as well. Amino-based stationary phases have a high selectivity for all steviol glycosides and provide good separation of the most abundant isomer pair rebaudioside B/stevioside and rebaudioside A/rebaudioside E. The separation order predominately depends on the glucose units attached to the *ent*-kaurene backbone, the higher is their retention time on the column. Accordingly, stevioside (3 glucose moieties) elutes before rebaudioside A (4 glucose moieties); both are well separated. Unfortunately, amino-based columns suffer from poor reproducibility and long equilibration times, and they cannot be used in combination with MS detection due to their strong bleeding. Moreover, they are not suitable for the determination of the aglycon steviol, which is poorly retained on these columns, and co-elution occurs with some nonspecific matrix peaks. An improved method has been developed by changing the HPLC conditions and including the use of an octadecylsilyl column instead of an amino-bonded column to enable the rapid and reliable determination of the nine steviol glycosides reported in JECFA by an isocratic HPLC-UV method. With the developed method, the nine steviol glycosides can be separately determined and identified using individual reference chemicals as standards, unlike the previous identification method, which was based on the relative retention times. In addition, the single stevioside quantification standard was replaced with both reference standards of stevioside and rebaudioside A. Importantly, the validation of the developed method was successful with the limits of quantification for the nine steviol glycosides were between 0.2 % and 0.6 %. The developed assay method for the nine steviol glycosides was proposed to JECFA and adopted as the revised assay method for the steviol glycosides specifications at its 73rd meeting in 2010 [6].

Eventually, highly specific HPLC-based analytical methods have evolved for the separation and quantitation of the different steviol glycosides with ever higher resolution and sensitivity, using a variety of different HPLC techniques like RP-HPLC, 110 2D-HPLC, 111 ultra-HPLC, 112 and 2D-ultra-HPLC48, using a wide variety of columns, namely, reversed-phase, amino, and HILIC with mobile phases AcCN and MeOH using either gradient or isocratic elution, under various detection systems (e.g., UV, DAD, and amperometry) [55]. As regulatory constraints require sensitive methods to analyze the sweet-tasting steviol glycosides in foods and beverages, a HILIC-MS/MS method was developed enabling the accurate and reliable quantitation of the major steviol glycosides stevioside, rebaudiosides A–F, steviolbioside, rubusoside, and dulcoside A by using the corresponding deuterated 16,17-dihydrosteviol glycosides as suitable internal standards. This quantitation not only enables the analysis of the individual steviol glycosides in foods and beverages but also can support the optimization of breeding and postharvest downstream processing of Stevia plants to produce preferentially sweet and least bitter tasting Stevia extracts. The determination of stevioside, rebaudioside A, and steviol was carefully pursued through different methods as indicated in the scientific literature, including enzymatic hydrolysis and chemical detection, high-performance TLC, over-pressured layer chromatography, capillary electrophoresis, high-speed counter-current chromatography, 2D-GC, quantitative NMR, near-infrared reflectance spectroscopy, and square-wave polarography. Recently, ambient ionization MS techniques such as desorption electrospray ionization (DESI) have been applied successfully for the direct analysis of steviol glycosides in *S. rebaudiana* leaves with minimal sample preparation [56]. HPLC technology and a near infrared (NIR) spectroscopy model was established to directly measure the stevioside glycosides (rebaudioside A and stevioside) content in the leaves of *S. rebaudiana* Bertoni. This model can be applied directly to measure the content of rebaudioside A and stevioside and resolved the problem of high cost and complex operation in using the current chemical laboratory methods. Though most HPLC-based methodologies use an external standard as reference for quantitation like rebaudioside A or stevioside, the major steviol glycosides from the plant with >99 % purity; recently, an internal standard has been developed for steviol glycoside analysis, namely, the 19-*O*-β-D-galactopyranosyl ester of steviolmonoside. Use of an internal standard allows for the correction of losses due to sample cleanup and is independent of errors in injection volume or detector sensitivity. A qualitative LC-TOF method was also proposed to evaluate steviol glycosides together with a validated HPTLC procedure with densitometric detection and a NIR procedure for the quantification of steviol glycosides. Recently, a semi-quantitative determination of steviol glycosides was also performed by desorption electrospray ionization mass spectrometry. As for steviol quantification, a validated an RP-LC method with fluorometric detection after derivatization by a coumarin byproduct has been reported. Recently, a study has concluded that ultra-HPLC methods with electrospray ionization mass spectrometry (UHPLC-MS) can be used for the routine evaluations of steviol glycosides in crude extracts [57].

Mass spectrometry is one of the most sensitive detection methods for steviol glycosides. Frequently, these detectors operate in the electrospray ionization (ESI) negative ion mode 19, 24, 32, 33, 46 and are linked to HPLC. It has been stated that negative ion mode is 10 times more sensitive than positive ion mode. Using MS detection, poor resolution for some critical pairs of steviol glycosides can be acceptable because of the high selectivity of the MS detector. Mobile phases contain acetonitrile–water mixtures and additives such as ammonium formate or dichloromethane for ionization enhancement. In ESI MS/MS fragmentation, the steviol glycosides were readily confirmed through subsequent glycosidic losses of fragments of 162 Daltons. This makes it rather difficult to distinguish between isomers such as rubusoside/steviolbioside or stevioside/rebaudioside B, especially when LC resolution is not sufficient. Some authors confirmed that a distinction is possible when applying low, intermediate, and high -collision energies (20, 40, and 60 V, respectively) in MS detection. They were able to prove that the ester bond between the glucose moiety and the carboxyl group at C4 of the kaurene backbone fragments quite easily, even at low collision energies. The corresponding steviol glycosides (e.g., stevioside, rubusoside) could only be confirmed by their fragment ions. The bonds with and between the sugar chains at C19 are more stable, and the resulting steviol glycosides (i.e., steviolbioside, steviol monoside, rebaudioside B) have stable $[M - H]+$ ions even at higher voltage settings. Desorption electrospray ionization (DESI) mass spectrometry was found as a rapid, qualitative, and semi-quantitative method that does not require sample preparation for steviol glycosides estimation in stevia leaves [58].

Quantification by ^{1}H NMR spectroscopy is possible for the major components stevioside and rebaudioside A – C [47]. The solvent mixture pyridine-d5-DMSO-d6 (6:1) enables satisfactory separation of various steviol glycosides. Similar results were obtained after comparing the quantitative results with those obtained using the JECFA method. The advantage of this method is that NMR analysis does not require reference compounds and it is significantly faster than HPLC analysis. For quantification, the internal standard anthracene was used.

As regulatory constraints require sensitive methods to analyze the sweet-tasting steviol glycosides in foods and beverages, a HILIC-MS/MS method was developed enabling the accurate and reliable quantitation of the major steviol glycosides stevioside, rebaudiosides A – F, steviolbioside, rubusoside, and dulcoside A by using the corresponding deuterated 16,17-dihydrosteviol glycosides as suitable internal standards. This quantitation not only enables the analysis of the individual steviol glycosides in foods and beverages but also can support the optimization of breeding and postharvest downstream processing of stevia plants to produce preferentially sweet and least bitter tasting Stevia extracts.

11 Regulatory Status

Since 1995, the extract of the leaves of the plant *S. rebaudiana* with a mixture of steviol glycosides have been used as a dietary supplement in the US [3]. Based on the available information, no New Dietary Ingredient Notification for dietary supplement use of purified rebaudioside A has been made to the US-FDA. Since 1989 and prior to 2008, at least two GRAS petitions seeking authorization for the addition of stevioside or steviol glycosides to foods had been submitted to FDA. However, no authorizations had been issued by FDA in response to these filings, and subsequently these petitions were withdrawn. It appears that the previously available safety data including purity considerations for stevia, stevioside, or steviol glycosides were inadequate.

In December 2008, in response to Generally Recognized As Safe (GRAS) notifications submitted to the US Food and Drug Administration (FDA), the FDA stated it has no questions regarding the conclusion of expert panels that rebaudioside A is GRAS for use as a general purpose sweetener in foods and beverages, excluding meat and poultry. Rebaudioside A is a stevia sweetener isolated and purified from the leaves of the stevia plant. In June 2009, FDA stated it has no questions regarding the conclusion of an expert panel on the GRAS status of steviol glycoside extract with high rebaudioside A content for use as a tabletop sweetener. In 2010, the European Food Safety Authority (EFSA) assessed the safety of steviol glycosides from stevia and established an Acceptable Daily Intake (ADI) for their safe use. In November 2011, the European Commission authorized the use of steviol glycosides as a sweetener in foods and beverages [59]; it is also approved as a dietary supplement in the EU. Stevia and steviol glycosides have a long history of use in several countries, including Japan and Paraguay. In South America and in several countries in Asia, including China, Japan, and Korea, stevia derived-sweeteners are permitted as a food additive. The Food Standards Australia New Zealand (FSANZ) has completed its evaluation of an application for use of steviol glycosides in foods in 2008. FSANZ recommended that the Australia and New Zealand Food Regulation

Ministerial Council (Ministerial Council) amend the Australia New Zealand Food Standards Code to allow the use of steviol glycosides in food (FSANZ, 2008). In 2008, Switzerland's Federal Office for Public Health approved the use of stevia as a sweetener citing the favorable actions of JECFA. Subsequently, France published its approval for the food uses of rebaudioside A with a purity of 97 % (AFSSA, 2009). Based on a review of the international regulation of *Stevia rebaudiana* and the clinical evidence for safety and efficacy, the Natural Health Products Directorate, Health Canada (2009), has adopted the following guidelines for the use of stevia and steviol glycosides in Natural Health Products (NHPs) on September 18, 2009. Stevia sweeteners are approved for use in many other countries, including Korea, Mexico, Taiwan, China, Russia, Australia, Argentina, New Zealand, Colombia, Peru, Uruguay, Brazil, Malaysia, and Switzerland. Since 2008, the US FDA has issued "no questions" letters in response to the multiple GRAS notifications filed on Reb A and steviol glycosides.

In 2008 and 2009, the Food and Agriculture Organization/World Health Organization's Joint Expert Committee on Food Additives (JECFA), a global panel of food ingredient safety experts, and the United States FDA stated the use of high-purity steviol glycosides ($\geq$95 %) is safe for human consumption, with an ADI expressed in steviol equivalents of up to 4 mg per kilogram of body weight per day. The European Food Safety Authority (EFSA) in 2010 assessed the safety of steviol glycosides from Stevia and established an ADI for their safe use. Daily Intake (ADI) of 4 mg/kg body weight is expressed as steviol equivalents [60]. The ADI is listed in units of mg per kg of body weight. The European Commission on 11th November 2011 allowed the usage of steviol glycosides as a food additive which will probably lead to wide-scale use in Europe. In 2011, the European Commission authorized the use of high-purity steviol glycosides ($\geq$95 %) in foods and beverages across the European Union. Globally, scientists have concluded that Stevia sweeteners are safe for people of all ages. The Dietary Supplement Health and Education Act (DSHEA) passed in the United States in 1994 were also approved steviol glycosides to be used as a functional ingredient in dietary supplements [61]. Recently the Food Safety and Standards Authority of India (FSSAI) has allowed the use of natural sweetener stevia (steviol glycoside) in selected products, including soft drinks, dairy-based beverages, and desserts.

Though not exhaustive, below are links to key multiple global regulatory agencies that have approved the use of high-purity stevia extracts:

- Agence Française de Sécurité Sanitaire des Aliments (AFSSA, or French Agency for Food Safety)
- Codex Alimentarius Commission (CAC)
- European Food Safety Authority (EFSA)
- European Commission
- Food Standards Australia New Zealand (FSANZ)
- Health Canada
- Joint FAO/WHO Expert Committee on Food Additives (JECFA)
- US Food and Drug Administration (FDA)

12 Conclusion

The sweet herb *S. rebaudiana* (Bertoni) has a valuable future and is extensively used in various areas of the world. Stevia, steviol glycosides, and their metabolites have commercial value in number of countries as sugar substitutes in foods, beverages, and medicines. Judging by the published literature, the use of steviol glycosides as sweeteners is an area that requires much further research, due to both their high appeal and commercial potential. This is very important, not only for manufacturers but also in general for various food applications. In this context, much research is still needed, not only to develop and optimize steviol glycoside extraction but also to improve the taste of products sweetened with these compounds. Thus, the continued evaluation of these ingredients as regards aspects such as the intensity, persistence of sweet taste, and absence of other residual flavors is necessary in order to meet the demands of today's consumers and ensure their acceptance, preference, and choice by the general public. Furthermore, and given that there is currently a sizeable and growing market for the commercialization of Stevia-containing products, optimization of production and processing should be undertaken concurrently in order to avoid limitations in the supply of steviol glycosides, an aspect that could restrict their extensive use in the demanding future that lies ahead. Finally, the patterns of use of stevia and its approval by the major international regulatory organizations, who have confirmed both the safety of this product for human consumption and its stability over time, point towards its development as an ubiquitous sugar alternative. It is expected that steviol glycosides will be used mainly in the manufacture of beverages, along with other traditional foods (such as dairy products, bread and cakes, confectionery, etc.), table-top sweeteners, functional food and beverages, and nutritional supplements, in addition to their use in personal care products (such as toothpaste) and as an active pharmaceutical ingredient or excipient. Steviol glycosides, with rebaudioside A in particular, are widely used commercially as a healthy, noncariogenic, zerocalorie alternative for sucrose. In addition, many studies with both animal models and human volunteers have shown clearly the beneficial pharmacological effects of stevioside (37) against type-II diabetes, hypertension, metabolic syndrome, and atherosclerosis. Besides steviol glycosides, the leaves of S. rebaudiana are also known for their important natural antioxidants such as flavonoids and various phenolics, tannins, essential oils, and other compounds. These substances have antioxidant, antimicrobial, immunostimulatory, and sweetening activities. According to recent research, stevia can be used as a novel functional ingredient in the food, feed, and medical industry, although further research is needed to be continued.

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