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The Leaves of *Stevia rebaudiana* (Bertoni), Their Constituents and the Analyses Thereof: A Review

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ABSTRACT: The plant *Stevia rebaudiana* is well-known due to the sweet-tasting *ent*-kaurene diterpenoid glycosides. Stevioside and rebaudioside A are the most abundant and best analyzed, but more than 30 additional steviol glycosides have been described in the scientific literature to date. Most of them were detected in the last two years. This paper reviews these new compounds and provides an overview about novel trends in their determination, separation, analysis, detection, and quantification. The detection and analysis of further constituents such as nonglycosidic diterpenes, flavonoids, chlorogenic acids, vitamins, nutrients, and miscellaneous minor compounds in the leaves of *Stevia rebaudiana* are reviewed as well. A critical review of the antioxidant capacity of *Stevia* leaves and its analysis is also included. These different aspects are discussed in consideration of the scientific literature of the last 10 years.

KEYWORDS: *Stevia rebaudiana, steviol glycosides, flavonoids, chlorogenic acids, vitamins, nonglycosidic diterpenes, steviamine, nutrient components, antioxidant capacity, ROS*

INTRODUCTION

Stevia rebaudiana Bertoni is a perennial herb of significant economic value due to its high content of natural, dietetically valuable sweeteners in its leaves.[1,2] Dried *Stevia* leaves have been used as natural sweeteners for many years, and their extracted steviol glycosides stevioside and rebaudioside A are approved as food additives in many countries throughout the world. In the USA, highly purified steviol glycosides received the GRAS (Generally Recognized As Safe) status in both 2008 and 2009.[3−5] The introduction of steviol glycosides on the European market as food additives with a purity of more than 95% is imminent after EFSA issued a positive opinion on their safety and raised the acceptable daily intake (ADI) for steviol glycosides, expressed as steviol equivalents, to 4 mg/kg BW/day in March 2010.[6] Final approval was given on 11 November 2011. The fate of *Stevia* leaves is less certain, as it is still being discussed in the European Union whether or not they are a "Novel Food".

More than 100 compounds have been identified in *Stevia rebaudiana*, the best known of which are the steviol glycosides, particularly stevioside and rebaudioside A, being the most abundant.[7] Many review articles on the composition of *Stevia rebaudiana* have been published. Within the last 10 years, the papers of Kinghorn, Kennelly, and Cerda-Garciá-Rojas [2,7,8] have constituted the most comprehensive reviews in English.

In our paper, the constituents of *Stevia* leaves are discussed by means of an extended review of the scientific literature in English over the last 10 years with a special focus on the analytical techniques.

STEVIOL GLYCOSIDES

New Steviol Glycosides. *Stevia rebaudiana* accumulates more than 30 steviol glycosides in varying concentrations. Amounts of total steviol glycosides up to 20% of the dry leaf weight are reported.[9] The best known steviol glycosides are stevioside and rebaudioside A, which have the highest content in the plant. Their concentrations vary widely depending on the genotype and cultivation conditions. For example, Kennelly[7] described the yield of stevioside from dried leaves varying from 5 to 22% and rebaudioside A contents from 25 to 54%. Ohta[12] described a yield of 9.2% stevioside and of 61.6% rebaudioside A, respectively, in the special species *S. rebaudiana* Morita, which was produced by selection and breeding of *S. rebaudiana* Bertoni.

Additionally, there are some minor diterpene glycosides, differing in the substitution on R^1, R^2, and/or R^3 of the *ent*-kaurene body (Table 1, Figure 1). The actual JECFA analytical method[10] lists nine different steviol glycosides (Table 1, Figure 1), but there were several more detected in the last two years. Ohta[12] found 10 new steviol glycosides (Table 1, Figure 1) in the special species *S. rebaudiana* Morita. Their structures could be confirmed using liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS^n) in negative ion mode, ^{1}H- and ^{13}C NMR spectroscopy, and several chemical studies. These steviol glycosides can be classified into four groups based on the structure of sugar chains linked at C13 (R^2): compounds containing the Glcβ1−2(Glcβ1−3)Glcβ1− chain (rebaudioside A family), the Rhaα1−2(Glcβ1−3)Glcβ1− chain (rebaudioside C family), the Xylβ1−2(Glcβ1−3)Glcβ1− chain (rebaudioside F family), and the Glcβ-1−3Glc− chain. Chaturvedula [13,14] isolated three isomers from commercially available *Stevia* leaf extracts belonging to the rebaudioside A and E family, respectively, one of which contained a fructose moiety. In addition, this research group detected two new steviol glycosides which are anomers of dulcoside A and rebaudioside C,[15] two with an α-glycosyl linkage,[16] one of which was reported previously,[12] and six diterpene glycosides with modifications in the kaurene body;[13,18] compound **4** was already

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Table 1. New Steviol Glycosides (See Figure 1) (Glc = Glucose, Rha = Rhamnose, Xyl = Xylose)

name	R^1	R^2	structure	refs
steviolmonoside	H	Glcβ1−	1	JECFA[10]
rubusoside	Glcβ1−	Glcβ1−	1	JECFA[10]
steviolbioside	H	Glcβ1−2Glcβ1−	1	JECFA[10]
dulcoside A	Glcβ1−	Rhaα1−2Glcβ1−	1	JECFA[10]
(**1c**)	Glcβ1−	6-deoxyGlcβ1−2Glcβ1−	1	Chaturvedula[15]
stevioside	Glcβ1−	Glcβ1−2Glcβ1−	1	JECFA[10]
rebaudioside G	Glcβ1−	Glcβ1−3Glcβ1−	1	Ohta[12]
rebaudioside B	H	Glcβ1−2(Glcβ1−3)Glcβ1−	1	JECFA[10]
dulcoside B	H	Rhaα1−2(Glcβ1−3)Glcβ1−	1	Ohta[12]
rebaudioside A	Glcβ1−	Glcβ1−2(Glcβ1−3)Glcβ1−	1	JECFA[10]
(**1b**)	Glcβ1−	Glcβ1−6Glcβ1−2Glcβ1−	1	Chaturvedula[14]
(**2b**)	Glcβ1−	Glcβ1−2(Fruβ1−3)Glcβ1−	1	Chaturvedula[14]
rebaudioside C	Glcβ1−	Rhaα1−2(Glcβ1−3)Glcβ1−	1	JECFA[10]
(**2c**)	Glcβ1−	6-DeoxyGlcβ1−2(Glcβ1−3)Glcβ1−	1	Chaturvedula[15]
(**1f**)	Glcβ1−	Glcα1−3Glcβ1−2(Glcβ1−3)Glcβ1−	1	Chaturvedula[16]
(**2f**)	Glcβ1−	Glcα1−4Glcβ1−3(Glcβ1−2)Glcβ1−	1	Chaturvedula[16]
rebaudioside L	Glcβ1−	Glcβ1−6Glcβ1−2(Glcβ1−3)Glcβ1−	1	Ohta[12]
rebaudioside H	Glcβ1−	Glcβ1−3Rhaα1−2(Glcβ1−3)Glcβ1−	1	Ohta[12]
rebaudioside F	Glcβ1−	Xylβ1−2(Glcβ1−3)Glcβ1−	1	JECFA,[10] Starratt[11]
rebaudioside E	Glcβ1−2Glcβ1−	Glcβ1−2Glcβ1−	1	Ohta[12]
(**1a**)	Xylβ1−6Glcβ1−	Glcβ1−2Glcβ1−	1	Chaturvedula[13]
rebaudioside D	Glcβ1−2Glcβ1	Glcβ1−2(Glcβ1−3)Glcβ1−	1	
rebaudioside I	Glcβ1−3Glcβ1−	Glcβ1−2(Glcβ1−3)Glcβ1−	1	Ohta[12]
rebaudioside K	Glcβ1−2Glcβ1−	Rhaα1−2(Glcβ1−3)Glcβ1−	1	Ohta[12]
(**1d**)	Rhaα1−2Glcβ1−	Glcβ1−2(Glcβ1−3)Glcβ1−	1	Chaturvedula[17]
rebaudioside J	Rhaα1−2Glcβ1−	Glcβ1−2(Glcβ1−3)Glcβ1−	1	Ohta[12]
rebaudioside N	Rhaα1−2(Glcβ1−3)Glcβ1−	Glcβ1−2(Glcβ1−3)Glcβ1−	1	Ohta[12]
rebaudioside M	Glcβ1−2(Glcβ1−3)Glcβ1−	Glcβ1−2(Glcβ1−3)Glcβ1−	1	Ohta[12]
rebaudioside O	Glcβ1−3Rhaα1−2(Glcβ1−3)Glcβ1−	Glcβ1−2(Glcβ1−3)Glcβ1−	1	Ohta[12]

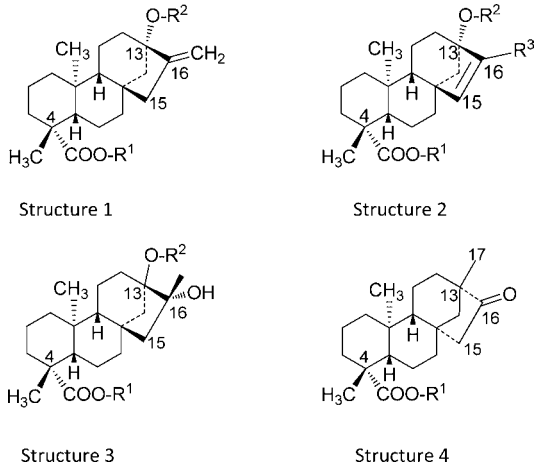

Figure 1. Four different kaurene body structures of steviol glycosides.

described[40] (Tables 1 and 2, Figure 1). 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, and also by means of chemical studies.

Zimmermann[19] succeeded in confirming 5 of the 10 new steviol glycosides evaluated by Ohta[12] and one described by Chaturvedula[13] in leaves produced in Greece as well as in a commercially available *Stevia* extract from China, certified to contain 95% steviol glycosides. In addition, six new steviol glycosides which are isomers of dulcoside A, rebaudioside B, or stevioside and rebaudioside D, respectively, and two compounds which contain two hexose and one pentose moieties or four hexose and one deoxyhexose moieties were detected.[19] This classification was performed using only LC-ESI-MS^2 in negative ion mode; further confirmations have to be performed using NMR spectroscopy.

It should be discussed whether all of these new compounds, mainly those detected in purified leaf extracts, are genuine or artifacts due to purification procedures. Some evidence already exists that steviolbioside and rebaudioside B are not genuine constituents of *S. rebaudiana* but rather formed by partial hydrolysis during the extraction process.[7,20,21]

Isolation of Steviol Glycosides from Leaves. The sample cleanup of *Stevia* leaves is well established and has been reported for many years. Steviol glycosides from dried leaves are usually obtained after hot water leaching,[22,23] sometimes combined with a defatting step.[24] Pressurized hot water and microwave-assisted water extractions were observed to have higher or comparable efficiencies than heating under reflux.[25,26] 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.[27]

Jaitak[28] compared different extraction techniques. Conventional cold extraction was performed at 25 °C for 12 h, ultrasound extraction at 35 ± 5 °C for 30 min, and using microwave-assisted extraction, the extraction time could be reduced to one minute at 50 °C. Methods using chloroform and ethanol for extraction[29] or even supercritical fluid extraction (SFE) have also been published.[30−32] The extraction step is sometimes followed by solid phase extraction (SPE), mostly with C18 cartridges, to remove disturbing matrixes.[22,33] Quantitative extraction of steviol glycosides without further

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Table 2. New Steviol Glycosides with Changes in the *ent*-Kaurene Backbone (see Figure 1)

name	R^1	R^2	R^3	structure	refs
2	Glcβ–	Glcβ1−2-Glcβ1−	CH_2OH	2	Chaturvedula[13]
3	Glcβ1−	Glcβ1−2-Glcβ1−	CHO	2	Chaturvedula[13]
4	Glcβ1−	Glcβ1−2-Glcβ1−	CH_3	2	Chaturvedula,[13] Clos[40]
1	H	Glcβ1−2(Glcβ1−3)Glcβ1−	CH_3	2	Chaturvedula[18]
2	H	Glcβ1−2(Glcβ1−3)Glcβ1−		3	Chaturvedula[18]
3	Glcβ1−			4	Chaturvedula[18]

cleanup steps is also described,[22,34,35] but the high matrix load can disturb a satisfactory separation of all steviol glycosides.[22,36]

It is also possible to analyze *Stevia* leaves without any sample preparation[37] using desorption electrospray ionization mass spectrometry (DESI-MS). The semiquantitative experiments were performed directly on leaf fragments after an internal standard was spotted on the leaf and allowed to dry before analysis. The components of interest were inserted into the ion source by applying an aqueous spray solution.

Separation and Detection of Steviol Glycosides. High-performance liquid chromatography (HPLC) is the method of choice for the determination of steviol glycosides. Table 3 provides an overview of the various techniques. The initial chromatograms were produced on amino-based or reversed-phase columns (C18) in combination with UV-detection. All JECFA methods before 2010[38] 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 molecular polarity: the more glucose units are 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. This diterpene is poorly retained on these columns, and coelution occurs with some nonspecific matrix peaks.[36] In contrast to an amino-based column, the retention order is inverted on a C18 phase. Thus it is possible to detect steviol as well. Reversed-phase columns are robust but show poor selectivity with regard to the separation of stevioside and rebaudioside A. This problem could be solved by gradient elution[23,33] or by using two columns in series.[35]

Table 3. Separation and Detection of Steviol Glycosides Using HPLC

separation mode	columns	detection mode	refs
HPLC	amino	UV	22,38
HPLC	amino	PAD	45
HPLC	C18	UV	10,23,24
HPLC	C18 + C18	UV	35,44
HPLC	HILIC	UV	36
HPLC	Hydro-RP	UV	39
HPLC	Hydro-RP	CAD	40
HPLC	carbohydrate	MS	46
UHPLC	C18	MS	32,33
UHPLC	RP amide C16	UV + MS	26
UHPLC	HILIC	MS	19,42
UHPLC	PA + C18	UV	34
UHPLC	C18 + amino	TOF-MS	41
UHPLC	C18 + C 18	UV	43

In recent years, many new columns have come on the market, operating in the normal and reversed-phase mode as well. Hydrophilic liquid interaction chromatographic (HILIC) columns are, in general, normal-phase-based columns. Their retention order corresponds to that of amino columns, but they are more robust, have a short equilibration time, and are suitable for MS detection due to the absence of bleeding. Wölwer-Rieck[36] described an analytical method for the separation of nine steviol glycosides under isocratic conditions on a HILIC column using a water−acetonitrile mixture as mobile phase.

Some papers[39,40] describe the use of a Hydro reversed-phase column. This column combines extreme hydrophobic selectivity with polar end-capping and is suitable for strong nonpolar and polar compounds, respectively. For the separation of polar compounds, it is possible to use, if desired, 100% aqueous mobile phase without a collapse of the stationary phase. Hoekstra[39] used a linear gradient consisting of 1% tetrahydrofurane in water and acetonitrile. He thus separated 12 steviol glycosides, including steviol and isosteviol. Clos[40] used a gradient of a three-solvent mobile phase system (ammonium acetate/acetic acid buffer; acetic acid; acetonitrile) and separated four steviol glycosides and five degradation products as well.

The introduction of ultrahigh-pressure liquid chromatography (UHPLC), generally coupled with MS detectors, allows for some methods using columns with smaller dimensions and particle sizes (under 4 μm). After testing a variety of sub 2 μm and traditional HPLC columns, Gardana[33] achieved the best performance on a 1.8 μm C18 column (150 mm × 2.1 mm). He was able to separate eight known and two unknown steviol glycosides but not steviol. Steviol was quantified on the same column with a mobile phase of different composition.

Pól[26] used an RP amide C16 column (150 mm × 2.1 mm) and a binary gradient with an acetonitrile−water mixture.

Zimmermann[42] successfully tested four different HILIC columns with particle sizes between 1.8 and 3 μm. Isocratic elution was sufficient for the separation of up to seven steviol glycosides within 8 min. The robustness of the separation depended mainly on the ion strength and the aqueous percentage of the mobile phase.

Separation problems can be solved by using two-dimensional systems. Cacciola[34] used a normal phase (polyamine, PA) in the first dimension and a C18 column with sub 2 μm particles in the second dimension, and he successfully separated 10 steviol glycosides. Pól[41] combined a small C18 column with an amino column, both with 3 μm particle size, and Cabooter[43] successfully separated 26 peaks using an automatic column coupling device; these peaks could not be fully identified at that time. Two C18 columns (2.1 mm × 100 mm) with particle size of 1.7 μm gave the best results.

UV detection at 210 or 200 nm after HPLC separation was often used for steviol glycoside determination,[10,22,23,28,38,43,44] though it is not very sensitive, because the carboxylic acid and

olefin moieties attached to the steviol backbone are weak UV absorbers. The sensitivity can be raised by a factor of 3−5 using a charged aerosol detector (CAD).[40] Furthermore, sensitive detection of stevioside is possible using a pulsed amperometric detector (PAD).[45] After HPLC separation, the effluent was adjusted to a pH ≥ 12 using sodium hydroxide, and after passing through a reaction coil, stevioside was quantified in the amperometric detector with a limit of detection of 0.3 mg/L.

Mass spectrometry is the most sensitive detection method 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. Choi[32] 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[19] or dichloromethane[33] for ionization enhancement. In ESI MS/MS fragmentation, the steviol glycosides were readily confirmed through subsequent glycosidic losses of fragments of 162 Da. 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[12,19,46] 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.

Quantification by ^{1}H NMR spectroscopy is possible for the major components stevioside and rebaudioside A−C.[47] The solvent mixture pyridine-d_5-DMSO-d_6 (6:1) enables satisfactory separation. Similar results were obtained after comparing the quantitative results with those obtained using the JECFA method.[10] 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.

Another method for rapid quantification of the major steviol glycosides is near-infrared spectroscopy (NIRS).[48,49] Reference analysis was done using HPLC. Yu[48] was able to analyze stevioside and rebaudioside A, Hearn[49] the three major steviol glycosides in total as sum parameter. The lack of significant differences between the values predicted by the model and the values analyzed by HPLC makes NIRS a powerful tool for rapid analysis of the main steviol glycosides.

NON-GLYCOSODIC DITERPENES

Labdane-type diterpenes belong to this group of constituents. McGarvey[50] isolated six new sterebins I−N (Figure 2), which were structurally similar to the sterebins A−H described previously.[51,52] Ibrahim[53] was able to confirm the presence of sterebin E, sterebin E acetate, austroinulin, and iso-austroinulin, which have already been described,[54] and sterebin A acetate, which was reported for the first time. Identification of the compounds was performed using ^{1}H- and ^{13}C NMR, UV and IR spectroscopy, and MS. Figure 2 shows the designed structures.

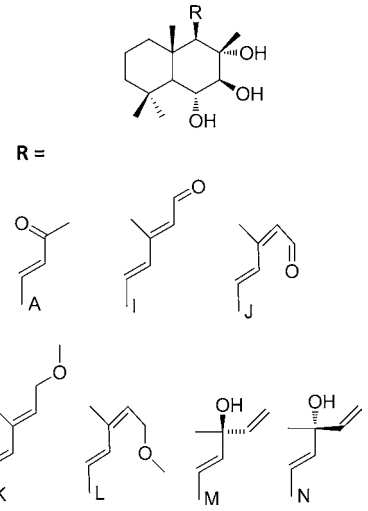

Figure 2. Structures of sterebins A, I−N.

The low-polarity sterebins have no known pharmaceutical effects; their concentration should be minimized by developing new *Stevia* lines to enhance the levels of sweet-tasting diterpene glycosides.[50]

POLYPHENOLS

Quantitatively, polyphenols were mostly analyzed as a sum parameter using the Folin−Ciocalteu colorimetric method, and the total phenolic content obtained is expressed in gallic acid, tannic acid, or catechin equivalents/g or mg of extract or dried leaves. It should be mentioned that the Folin−Ciocalteu assay can be considered to be an additional method for the determination of the antioxidant capacity.[55] In this assay, a molybdatophosphoric heteropolyanion reduces phenols, yielding a colored product. This method is not very specific due to a lot of interfering substances, which include, in the case of *Stevia*, for example, sugars, organic acids, and metal ions.[55] In addition, the influence of the extraction agent is not negligible. For example, in an ethyl acetate extract of a crude 85% methanolic extract, 0.86 mg gallic acid equivalents/mg[56] and in 0.3 N HCL in methanolic extract, 25.18 μg gallic acid equivalents/mg,[57] were determined respectively, both calculated on the basis of dry weight. So it is not surprising that the results for polyphenols in *Stevia* differ significantly when using the Folin−Ciocalteu assay.[56−60]

Phenolic Compounds. Kim[58] analyzed phenolic compounds quantitatively by means of HPLC on a C18 column with gradient elution and diode array detection (DAD). The main phenolic compound was pyrogallol with 951.27 mg/100 g dry base water extract, followed by 4-methoxybenzoic acid (33.80 mg/100 g), *p*-coumaric acid (30.47 mg/100 g), 4-methylcatechol (25.61 mg/100 g), and sinapic and cinnamic acid (Figure 3).

Karaköse[61] detected 24 chlorogenic acids (CGAs) using LC-ESI-MS^n in negative ion mode and using LC-TOF with 23 compounds described for the first time in *Stevia rebaudiana* leaves (Table 4, Figure 3). Chlorogenic acids are esters of quinic or shikimic acid with cinnamic acids. Additionally, three mono- and three dicaffeoylquinic acids were quantified, respectively, after UV detection at 320 nm. Among the monocaffeoylquinic acids, 4-CGA was the most abundant compound (70.3 μg/g), and among all CQAs it was 3,5-diCQA

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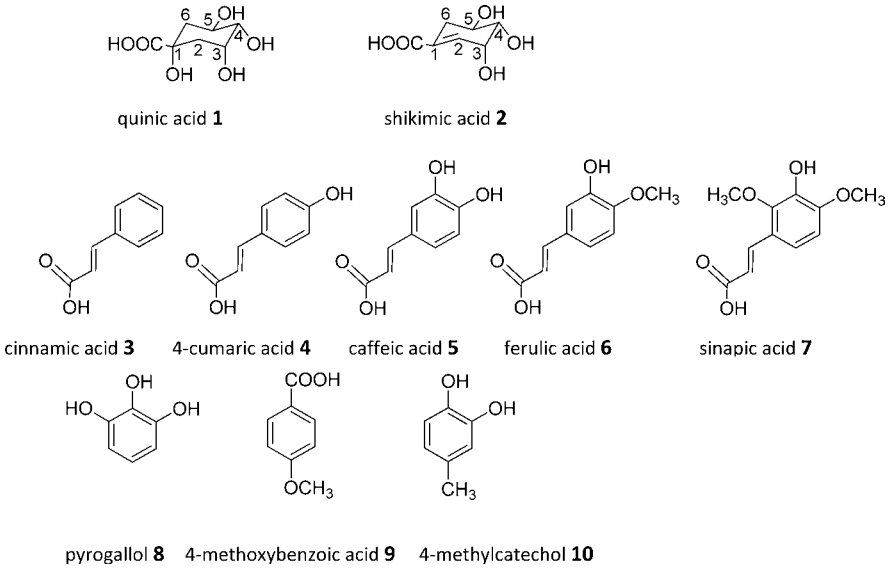

Figure 3. Substructures of chlorogenic acids (CGA).

Table 4. Chlorogenic Acids and Other Phenolic Compounds Detected in *Stevia rebaudiana* (See Figure 3)

name	structure (see Figure 3)	refs
3-caffeoylquinic acid (3-CQA)	1 + 5	Karakköse[61]
5-caffeoylquinic acid (5-CQA)	1 + 5	Karakköse[61]
4-caffeoylquinic acid (4-CQA)	1 + 5	Karakköse[61]
3,5-dicaffeoylquinic acid (3,5-diCQA)	1 + 5 + 5	Karakköse[61]
3,4-dicaffeoylquinic acid (3,4-diCQA)	1 + 5 + 5	Karakköse[61]
4,5-dicaffeoylquinic acid (4,5-diCQA)	1 + 5 + 5	Karakköse[61]
a *cis*-3,5-dicaffeoylquinic acid (a *cis*-3,5-diCQA)	1 + 5 + 5	Karakköse[61]
a *cis*-4,5-dicaffeoylquinic acid (*cis*-4,5-diCQA)	1 + 5 + 5	Karakköse[61]
cis-4,5-dicaffeoylquinic acid (*cis*-4,5-diCQA)	1 + 5 + 5	Karakköse[61]
a *cis*-4,5-dicaffeoylquinic acid (a *cis*-4,5-diCQA)	1 + 5 + 5	Karakköse[61]
5-*p*-coumaroylquinic acid acid (5-*p*-CoQA)	1 + 4	Karakköse[61]
caffeoyl-feruloylquinic acid (CFQA)	1 + 5 + 6	Karakköse[61]
4-caffeoyl-5-feruloylquinic acid (4C,5FQA)	1 + 5 + 6	Karakköse[61]
5-caffeoylshikimic acid (5-CSA)	2 + 5	Karakköse[61]
4-caffeoylshikimic acid (4-CSA)	2 + 5	Karakköse[61]
3-caffeoylshikimic acid (3-CSA)	2 + 5	Karakköse[61]
5-feruloylquinic acid (5-FQA)	1 + 6	Karakköse[61]
feruloylquinic acid (FQA)	1 + 6	Karakköse[61]
feruloylquinic acid (FQA)	1 + 6	Karakköse[61]
3,4,5-tricaffeoylquinic acid (3,4,5-triCQA)	1 + 5 + 5 + 5	Karakköse[61]
1,3,5-tricaffeoylquinic acid (1,3,5-triCQA)	1 + 5 + 5 + 5	Karakköse[61]
tricaffeoylquinic acid (triCQA)	1 + 5 + 5 + 5	Karakköse[61]
3,4,5-tricaffeoylquinic acid (triCQA)	1 + 5 + 5 + 5	Karakköse[61]
pyrogallol	8	Kim[58]
4-methoxybenzoic acid	9	Kim[58]
4-coumaric acid	4	Kim[58]
4-methylcatechol	10	Kim[58]
sinapic acid	7	Kim[58]
cinnamic acid	3	Kim[58]

with 145.6 μg/g. Total chlorogenic acid was found to be 370 μg/g of dry leaf.

Flavonoids. This group of compounds is widely present in fruits, vegetables, grains, nuts, seeds, spices, etc. These compounds have antioxidant capacities.

The flavonoids detected in *Stevia* leaves belong to the subgroups of flavonols and flavones (Table 5, Figure 4). They were identified using two-dimensional UHPLC-DAD[34] and LC-MS/MS and spectroscopic methods (^{1}H and ^{13}C NMR, IR, and 2D NMR).[56,62] All flavonoids except quercetin-3-*O*-(4-*O*-*trans*-caffeoyl)-α-L-rhamno-pyranosyl-(1−6)-β-D-galactopyranoside[62] have already been described.[7]

Quantitatively, they were analyzed as total flavonoid content using an aluminum chloride colorimetric method[56] and the Folin−Ciocalteu assay.[56−58] The results differ considerably because of different extraction and calculation modes. For example, the total flavonoid content in the ethyl acetate extract of a crude 85% methanolic extract was 0.83 mg quercetin equivalents/mg[56] and 15.64 μg quercetin equivalent/mg in water extract,[58] respectively.

WATER-SOLUBLE VITAMINS

There is only one paper which deals with water-soluble vitamin content in *Stevia* leaves.[58] Folic acid was found to be the major compound (52.18 mg/100 g dry base of extract), followed by ascorbic acid (14.98 mg/100 g dry base of extract) and vitamin B2 (0.43 mg/100 g dry base of extract). The vitamins were analyzed using HPLC-DAD on a C18 column with gradient elution.

MISCELLANEOUS CONSTITUENTS

Phytosterols and Triterpenes. Two authors[53,63] confirmed the presence of the phytosterols β-sitosterol, stigmasterol, and lanosterol and the triterpene β-amyrin; all of these compounds have already been discussed.[7,8]

Volatile Constituents. Ibrahim[53] detected nine hydrocarbons and four aliphatic alcohols, namely cyclodecanol, hexadecanol, isoheptadecanol, and dotriacontanol as volatile constituents.

Forty different components were identified in the essential oil fraction of five different genotypes of *Stevia*, with the main constituents being spathulenol (13.4−40.9%), caryophyllene oxide (1.3−18.7%), β-caryophyllene (2.1−16.0%), and β-pinene (5.5−21.5%).[64]

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Table 5. Flavonoids Detected in Leaves of *Stevia rebaudiana* (See Figure 4)

name	R^1	R^2	R^3	R^4	R^5	refs
Flavonols						
quercetin	OH	OH	H	OH	OH	Li[62]
quercetin-3-*O*-β-D-arabinoside	OH	OH	H	*O*-arabinoside	OH	Ghanta,[56] Li[62]
quercetin-3-*O*-β-D-rhamnoside	OH	OH	H	*O*-rhamnoside	OH	Ghanta,[56] Li[62] Cacciola[34]
quercetin-3-*O*-glucoside	OH	OH	H	*O*-glucoside	OH	Cacciola[34]
quercetin-3-*O*-rutinoside	OH	OH	H	*O*-rutinoside	OH	Cacciola[34]
quercetin-3-*O*-(4-*O*-*trans*-caffeoyl)-α-L-rhamno-pyranosyl-(1−6)-β-D-galactopyranoside	OH	OH	H	[4-*O*-*trans*-caffeoyl-α-L-rhamnopyranosyl-(1−6)-β-D-galactopyranoside]	OH	Li[62]
kaempferol-3-*O*-rhamnoside	H	OH	H	*O*-rhamnoside	OH	Ghanta[56]
Flavones						
apigenin	H	OH	H	H	OH	Ghanta[56]
apigenin-4′-*O*-β-D-glycoside	H	*O*-glycoside	H	H	OH	Ghanta[56]
apigenin-7-*O*-β-D-glycoside	H	OH	H	H	*O*-glycoside	Cacciola,[34] Li[62]
luteolin	OH	OH	H	H	OH	Ghanta,[56] Li[62]
luteolin-7-*O*-β-D-glycoside	OH	OH	H	OH	*O*-glycoside	Li,[62] Cacciola[34]

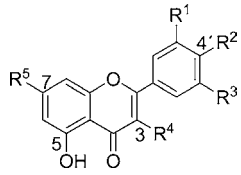

Figure 4. Backbone structure of flavonoids.

Polyhydroxy Indozilidine Alkaloid. For the first time, an indozilidine iminosugar alkaloid named steviamine (Figure 5)

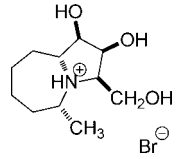

Figure 5. Structure of steviamine.

was isolated from *Stevia* leaves.[65] This alkaloid family is known to occur in *Hyacinthaceae*, but it has never been reported in *Asteraceae*. Different pharmacological and ecological activities have been contributed to iminosugars, the inhibitory effect against glucosidase being the most studied.[66]

NUTRIENT COMPOSITION

Table 6 provides an overview of the nutrient composition of *Stevia* leaves analyzed by several authors. The results varied considerably because the plants originated in different countries, such as Egypt,[67] Spain,[72] Paraguay,[72] India, and Pakistan.[60,68,69] The methods used for the determination of moisture, ash, protein, fat, and crude fiber were standard procedures.[70] Minerals were analyzed using atomic absorption spectroscopy.

For the determination of total carbohydrates, several non-standardized methods were used or their content was calculated by difference;[67] the monosaccharides fructose and glucose were analyzed enzymatically.[71]

Stevia leaves have a low fat and a considerable protein content. Essential amino acids content is 7.7 g/100 g. Potassium is the mineral with the highest content, followed by calcium. Interestingly, the water extract of *Stevia* leaves contains monosaccharides such as glucose and fructose but their contents are low. Oxalic acid with a high yield of 2.3 g/100 g[68] can be considered as an adverse compound. For example, spinach which is called to be high in oxalic acid contains 0.12−1.3 g/100 g.

The lipid fraction is rich in unsaturated fatty acids such as linoleic and linolenic acid (Table 7).[63,69]

ANTIOXIDANT CAPACITY

In traditional antioxidant theory, it was postulated[73] that free radicals and reactive oxygen species (ROS) are related to aging, cancer, atherosclerosis, and further degenerative diseases. Antioxidants were said to remove these reactive species and to thus promote health. Antioxidants are widely present in fruits and plants, and they belong to several chemical classes. There are four general sources of antioxidants: enzymes such as superoxide dismutase and catalase, large molecules such as albumin and ferritin, small molecules such as ascorbic acid, gluthathione, uric acid, tocopherols, carotenoids, (poly)phenols, and others.[55] To assess the free radical scavenging activity of natural products, there are many tests available which differ in measurement principles, reactants, and reaction mechanism. This variety exists because there is no uniform definition for

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Table 6. Nutrient Composition of Dried *Stevia* Leaves (g/100 g or mg/100 g)

	refs						
	69	68	72		60		67
			Paraguay	Spain	summer	monsoon	
moisture (%)		7	7.2	8.8	7.7	8.6	5.37
ash (%)	13.12	11	7.7	8.1	8.4	9.5	7.41
protein (%)	20.42	10	15.5	12.1	12.0	12.9	11.41
total carbo-hydrates (%)	35.20	52**					61.93
fructose (%)			1.4	1.2			
glucose (%)			0.95	0.63			
fat (%)	4.34	3	5.0	3.6	2.7	3.7	3.73
crude fiber (%)		18	12.1	9.7			15.52
calcium (mg/100 g)	1550	464.4			722	808	17.7
total essential amino acids (g/100 g)							7.7
total nonessential amino acids (g/100 g)							3.7
oxalic acid (mg/100 g)		2295					
phosphorus (mg/100 g)	350	11.4					
iron (mg/100 g)	36.3	55.3			31.1	31.3	5.89
sodium (mg/100 g)	160	190			32.7	63.4	14.93
potassium (mg/100 g)	2510	1800			839	730.3	21.15
magnesium (mg/100 g)	500						3.26
manganese (mg/100 g)	9.8						2.89
molybdenum (mg/100 g)	0.1						
selenium (mg/100 g)	0.06						
zinc (mg/100 g)	6.4						1.26
copper (mg/100 g)	1.0						0.73
cobalt (mg/100 g)	0.03						

Table 7. Fatty Acid Profile (g/100 g) of *Stevia* Leaf Oil

	refs	
	Tadhani[69a]	Korobko[63a]
palmitic acid (C 16:0)	27.51	2.11
palmitoleic acid (C 16:1)	1.27	
stearic acid (C 18:0)	1.18	2.03
oleic acid (C 18:1)	4.36	
linoleic acid (C 18:2)	12.40	9.32
linolenic acid (C 18:3)	21.59	24.95

[a]all values are calculated on a zero moisture basis per 100 g.

the antioxidant activity and because ROS can be generated by different kinds of radicals such as hydrogen peroxide, lipid peroxides, singlet oxygen, superoxide anion, hydroxyl radical, peroxy radical, peroxynitrite, etc.[74] Furthermore, a differentiation must be made between the mechanisms by which antioxidants can deactivate radicals. There are the hydrogen atom transfer (HAT) mechanism and the single electron transfer (SET) mechanism. HAT reactions are solvent- and pH-independent and rather quick, whereas SET reactions are pH-dependent and usually slow.[55] Most of the tests are absorbance tests in which the absorbance of the antioxidant was measured compared to a defined reference compound.

In *Stevia*, the antioxidant capacity was mostly determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.[56,58−60] The addition of antioxidants decreases the absorbance of a DPPH solution measured at 517 nm to a stable value. The antioxidant capacity was expressed as IC_{50}, which is the concentration of an antioxidant needed to trap 50% of DPPH.[75] Consequently, a low IC_{50} value indicates a high antioxidant capacity.

A similar test is the $ABTS^+$ or TEAC assay, which uses 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) as a coloring agent. Again, IC_{50} was calculated and compared to the IC_{50} of a quercetin standard.[56] Normally, TROLOX, a synthetic vitamin E derivative, is used as reference compound in the TEAC test.[76] The $ABTS^+$ or TEAC assays are suitable for compounds such as phenols, which have a redox potential lower than that of $ABTS^+$. Only then can a reduction of $ABTS^+$ occur.[55] Furthermore in *Stevia*, the ferric reducing/antioxidant power (FRAP) test,[57] a modified hydroxyl radical scavenging test using terephthalic acid (TPA),[77,78] the Nitroblue tetrazolium (NBT) test for superoxide anion radical scavenging activity,[58,59,78] the deoxyribose test for hydroxyl radical scavenging activity,[58,59] and the Greiss test for nitric oxide radical scavenging activity[59] were applied. All of these tests show remarkable antioxidant capacities of *Stevia* leaf extracts, sometimes with a high correlation to the total phenolic and flavonoid contents.[58] The results reveal significant differences and are not comparable as the based chemical reactions, and the parameters being determined vary considerably. Furthermore, different solvent media (water or organic) and standards used as references influence the results. These results evidently show that no single assay will accurately reflect all antioxidants and that too many analytical methods cause inconsistent results. The demand to standardize the methods for determining antioxidant capacity is comprehensible.[55]

In conclusion, steviol glycosides in *Stevia rebaudiana* are being intensively investigated because of their economic importance, and the search for new compounds is still ongoing. Along with the sweet-tasting diterpenes, the presence of nonglycosidic labdane diterpenes, flavonoids, phenolic compounds, vitamins, nutrients, phytosterols, triterpenes, essential oil components, and additional minor compounds has been described in the literature for many years. In the last 10 years, the focus was on the detection of new steviol glycosides, with more than 30 new ones being detected and

characterized. Additionally, new flavonoids, chlorogenic acids, hydrocarbons, and one iminosugar were also described. Apart from the steviol glycosides, *Stevia* contains substantial amounts of beneficial compounds as polyphenols, essential amino acids, minerals, and crude fiber, while the high oxalic acid content has an adverse effect. The iminosugar steviamine should be reproducibly detected, and its safety has to be monitored intensively.

Stevia leaves have a long history of use in many countries throughout the world without any negative effects.

For commercial use in food, *Stevia* leaves will not have a major role to play, much more important are their high-purified steviol glycosides authorized as food additives in many food categories, for example in Europe, with a purity of more than 95%. *Stevia* leaves will be used as sweetening compounds in tea mixtures or as water-based extracts in desserts and milk products or directly by the end-user in jams, stewed fruits, etc.[79] To guarantee the safety of the leaves, quality control procedures must be employed to ensure that standardization and safety requirements are met, as set by regulatory agencies. This should include the control of the most important constituents as well as the microbiological quality, the heavy metal content combined with a residue analysis of possible pesticides and herbicides.[79]

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