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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · AUGUST 2011

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Characterization and Quantification of Hydroxycinnamate Derivatives in *Stevia rebaudiana* Leaves by LC-MS^{n}†

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Supporting Information

ABSTRACT: *Stevia rebaudiana* leaves are used as a zero-calorie natural sweetener in a variety of food products in Asian countries, especially in Japan. In this study, the hydroxycinnamate derivatives of *S. rebaudiana* have been investigated qualitatively and quantitatively by LC-MS^{n}. Twenty-four hydroxycinnamic acid derivatives of quinic and shikimic acid were detected, and 19 of them were successfully characterized to regioisomeric levels; 23 are reported for the first time from this source. These comprise three monocaffeoylquinic acids (M_r 354), seven dicaffeoylquinic acids (M_r 516), one *p*-coumaroylquinic acid (M_r 338), one feruloylquinic acid (M_r 368), two caffeoyl-feruloylquinic acids (M_r 530), three caffeoylshikimic acids (M_r 336), and two tricaffeoylquinic acids (M_r 678). Cis isomers of di- and tricaffeoylquinic acids were observed as well. Three tricaffeoylquinic acids identified in stevia leaves are reported for the first time in nature. These phenolic compounds identified in stevia might affect the organoleptic properties and add additional beneficial health effects to stevia-based products.

KEYWORDS: *Stevia rebaudiana*, chlorogenic acids, hydroxycinnamic acids, caffeoylquinic acids, caffeoylshikimic acids, tandem mass spectrometry

INTRODUCTION

Stevia rebaudiana is a plant belonging to the Asteraceae family of plants, which is native to Brazil and Paraguay. Due to the natural sweetness of its leaves, *S. rebaudiana* has caught attention in scientific and industrial fields to act as a natural zero-calorie sweetener in many applications in the food industry. The leaves contain ent-kaurene glycosides, comprising stevioside, rebaudiosides A, B, C, D, E, and F, and dulcoside A. All of these diterpene glycosides comprise a steviol backbone structure; they differ only in the glucose moiety at positions C13 and C19 (Figure 1). Stevioside is the main sweet-tasting glycoside in stevia and was reported to be 250−300 times sweeter than sucrose.[1] Rebaudioside A is the second most abundant ent-kaurene and sweetest compound in stevia; its sweetness is 400 times greater than that of sucrose, and it has more pleasant taste and is more water-soluble than stevioside.[2] The amounts of diterpene glycosides may vary depending on the growth conditions of stevia; however, stevioside accounts for 4−13% (w/w) and rebaudioside A accounts for 2−4% (w/w),[3] the other glycosides being present in lower concentrations.

The principal advantage of stevia metabolites is that they are natural, nonsynthetic products. Stevia leaves can be used in their natural state (fresh or dried form), due to their high sweetening intensity. Only small quantities are needed in comparison to white sugar to achieve comparable sweetness. The primary use of stevia is as a commercial sweetener; it is used in a wide range of products such as soft drinks, ice cream, chocolate, yogurt, and baked and cooked foods. Stevia products also have beneficial uses in various consumer care products such as toothpaste or mouthwashes.[4,5] Stevia may also be used for obesity, diabetics, dental caries, and therapeutic effects such as hypoglycemic activity.[6]

The majority of the annual stevia production of an estimated 4000 t is produced in China and South America. The stevia crop has been shown to be highly adaptable to cultivation in many other parts of the world. *S. rebaudiana* occurs naturally on acid soils of pH 4−5 but will also grow on soils with pH levels of 6.5−7.5, making it an interesting alternative to plants cultivated on poor soils such as tobacco.[7]

In addition to diterpene glycosides, a number of secondary plant metabolites have been identified from *S. rebaudina* including labdane-type diterpenes, triterpenoids and steroids, flavonoids, and oil components. From *S. rebaudiana*, 10 labdane-type diterpenoids were identified, including austroinulin, isoaustroinulin,[6] and sterebins (A−H).[8,9] A triterpenoid, lupeol 3-palmitate, was also separated from stevia.[10] As plant sterols, β-sitosterol, stigmasterol, and campesterol were identified from *S. rebaudiana*.[11]

Plant phenols are a large and diverse group of compounds including hydroxycinnamates, tannins, flavonoids, stilbenes, coumarins, lignans, and lignins.[12] Chlorogenic acids (CGAs) are the most common hydroxycinnamate derivatives observed in the plant kingdom. By definition, they are a large family of esters formed between quinic acid and one to four residues of certain *trans*-hydroxycinnamic acids, most commonly caffeic, *p*-coumaric, and ferulic; sinapic and dimethoxycinnamic acids also occur, and in some plant species various aliphatic acids may replace one or more of the *trans*-cinnamic acid residues.[13] CGAs are involved in biological functions in plants such as defense against pathogens and resistance to diseases. CGAs also participate in enzyme-catalyzed browning reactions that may adversely affect the color, flavor, and nutritional quality of dietary sources.[14]

Several pharmacological activities of CGAs including antioxidant activity, the ability to increase hepatic glucose utilization,[15,16]

Received: June 1, 2011
Revised: July 15, 2011
Accepted: August 2, 2011
Published: August 02, 2011

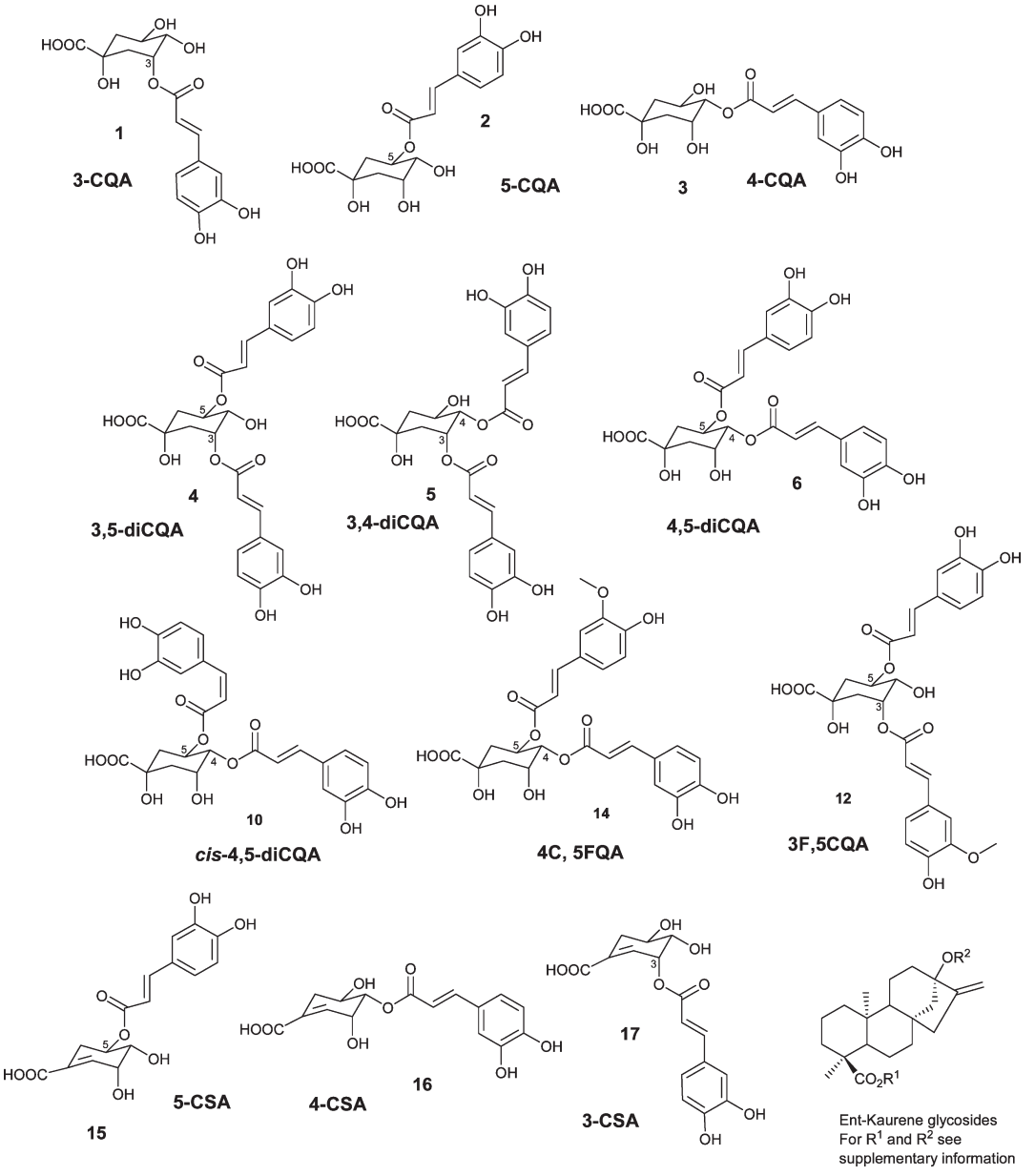

Figure 1. Structures and numberings of caffeoylquinic acids.

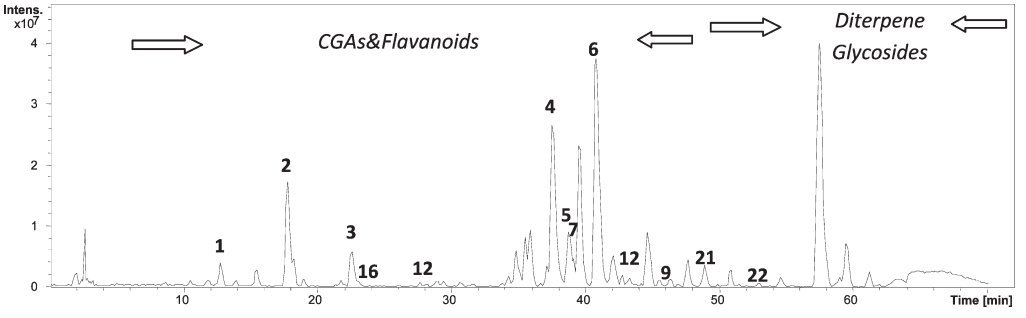

Figure 2. Base peak chromatogram of *Stevia rebaudiana* extract using ion trap MS in negative ion mode. For numbering, see Table 1.

inhibition of the HIV-1 integrase,[17,18] antispasmodic activity,[19] and inhibition of the mutagenicity of carcinogenic compounds[20] have been revealed by in vitro, in vivo, and human intervention studies so far. CGAs and their metabolites display additionally highly favorable pharmacokinetic properties.[21−23] Because the polyphenols in stevia might affect the organoleptic properties of

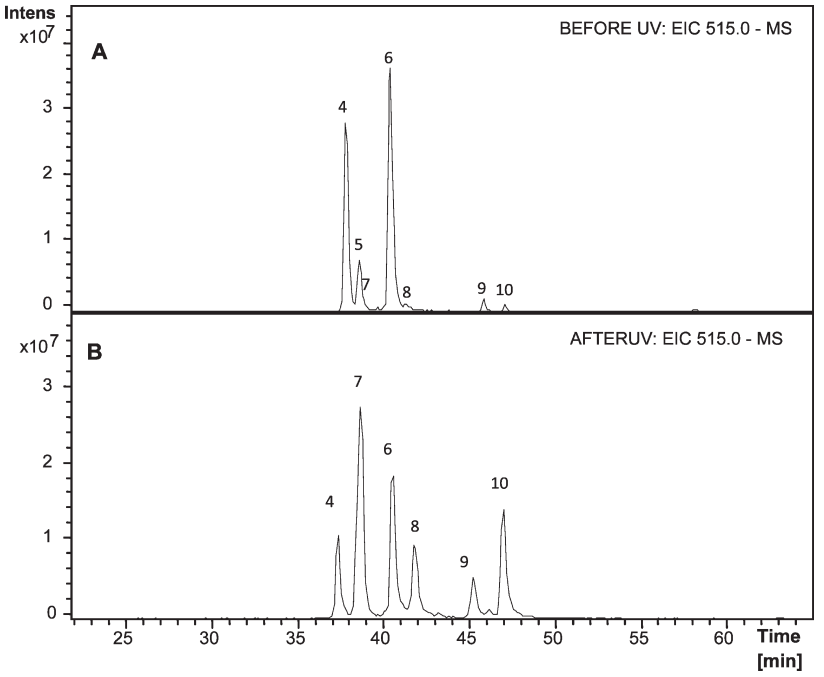

Figure 3. Extracted ion chromatograms (EIC) of m/z 515 in negative ion mode (A) before and (B) after UV irradiation.

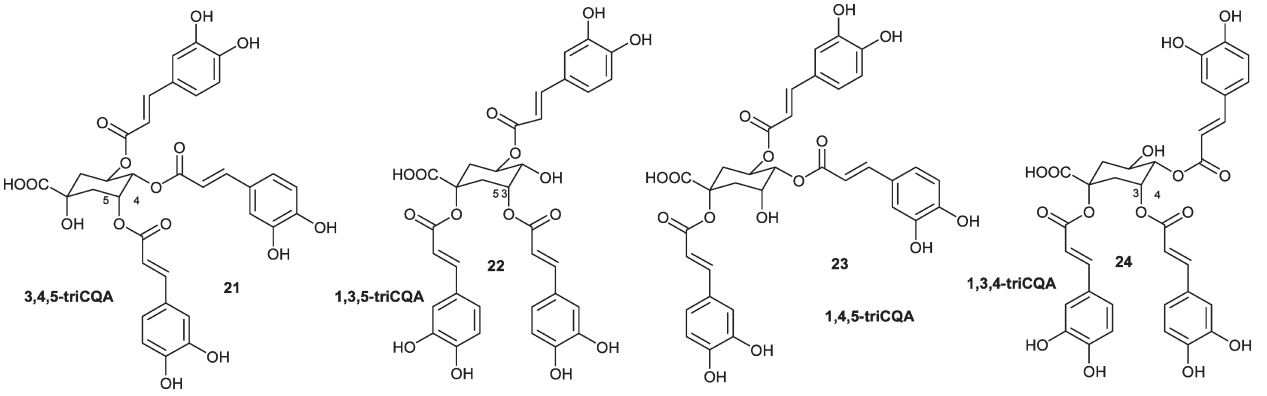

Figure 4. Structures and numbering of tricaffeoylquinic acids.

stevia-based product and could add additional health benefits to the product, the objective of the present study was to profile the phenolic content of *S. rebaudina* leaves with a particular emphasis on hydroxycinnamate derivatives.

MATERIALS AND METHODS

The chlorogenic acids, 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid (chlorogenic acid), 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid, were purchased from PhytoLab (Vestenbergsgreuth, Germany). All other chemicals were purchased from Sigma-Aldrich (Bremen, Germany). Stevia leaves were purchased from a market in Bremen, Germany.

Sample Preparation. Two grams of *S. rebaudiana* leaves was immersed in liquid nitrogen, ground in a hammer mill, and extracted first with 150 mL of chloroform in a Soxhlet apparatus (Buchi B-811 extraction system) for 2 h and then with 150 mL of methanol for another 2 h. Solvents were removed from the methanolic extract in vacuo, and extracts were stored at −20 °C until required.

UV Irradiation. The prepared sample of stevia leaf extract (1 mL) was placed in a photoreactor (LuzchemLZC -4 V, Ottawa, Canada) under a shortwave UV lamp and irradiated at 245 nm for 40 min.

LC-MS^n. The LC equipment (Agilent 1100 series, Bremen, Germany) comprised a binary pump, an autosampler with a 100 μL loop, and a diode array detector with a light-pipe flow cell (recording at 320 and 254 nm and scanning from 200 to 600 nm). This was interfaced with an ion-trap mass spectrometer fitted with an ESI source (Bruker Daltonics HCT Ultra, Bremen, Germany) operating in Auto-MS^n mode to obtain fragment ions m/z. As necessary, MS^2, MS^3, and MS^4 fragment-targeted experiments were performed to focus only on compounds producing a parent ion at m/z 335.1, 337.1, 367.1, 529.2, or 677.3. Tandem mass spectra were acquired in Auto-MS^n mode (smart fragmentation) using a ramping of the collision energy. Maximum fragmentation amplitude was set to 1 V, starting at 30% and ending at 200%. MS operating conditions (negative mode) had been optimized using 5-caffeoylquinic acid[28] with a capillary temperature of 365 °C, a dry gas flow rate of 10 L/min, and a nebulizer pressure of 50 psi.

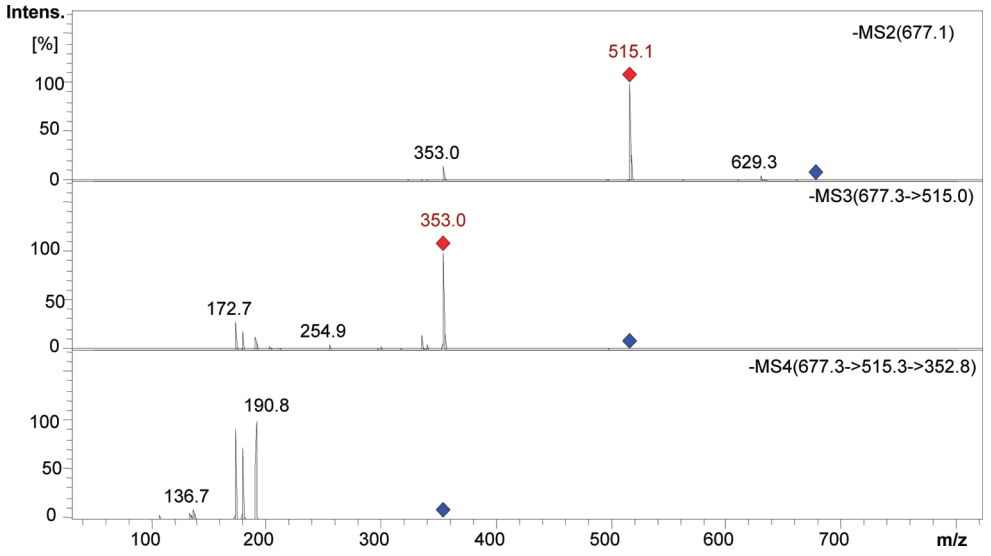

Figure 5. Tandem mass spectra of 1,3,5-triCQA in negative ion mode.

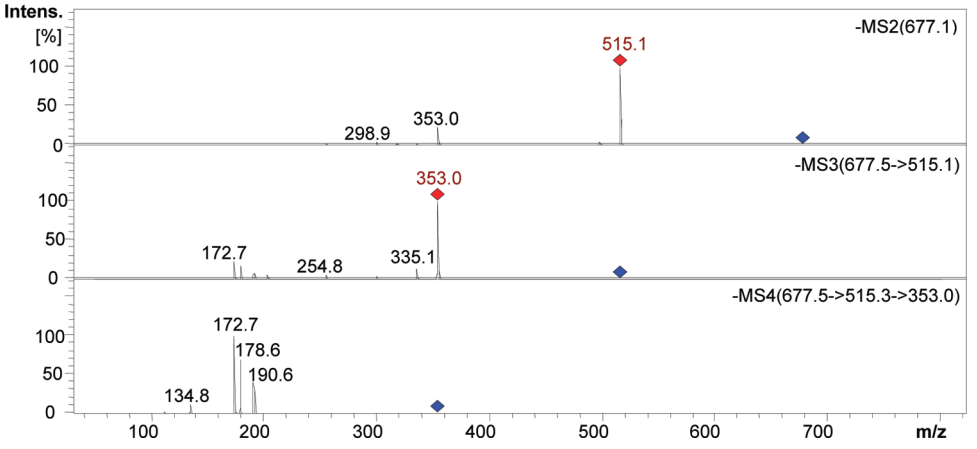

Figure 6. Tandem mass spectra of 3,4,5-triCQA in negative ion mode.

High-resolution LC-MS was carried out using the same HPLC equipped with a MicroTOF Focus mass spectrometer (Bruker Daltonics) fitted with an ESI source, and internal calibration was achieved with 10 mL of 0.1 mol/L sodium formate solution injected through a six-port valve prior to each chromatographic run. Calibration was carried out using the enhanced quadratic calibration mode.

HPLC. Separation was achieved on a 150 × 3 mm i.d. column containing diphenyl 5 μm with a 4 × 3 mm i.d. guard column of the same material (Varian, Darmstadt, Germany). Solvent A was water/formic acid (1000 + 0.05 v/v), and solvent B was methanol. Solvents were delivered at a total flow rate of 0.5 mL/min. The gradient profile was from 10 to 70% B linearly in 60 min followed by 10 min isocratic and a return to 10% B at 80 and 10 min isocratic to re-equilibrate.

Calibration Curve of Standard Compounds. Stock solutions of the standard compounds were prepared in methanol. A series of standard solutions was injected (5 μL) into the LC-MS system. The areas of the peaks of each standard from UV chromatograms were used to make the respective standard curves.

Synthesis of the Mixture of Regioisomers of Tricaffeoylquinic Acids. To a solution of quinic acid (96 mg, 0.5 mmol) and DMAP (16 mg, 0.12 mmol) in CH_2Cl_2 (10 mL) were added triethylamine (4 mL) and 3,4-diacetylcaffeic acid chloride (423 mg, 1.5 mmol) at room temperature. The reaction mixture was stirred for 6 h and acidified with 2 mol/L HCl (pH ≈1) and then stirred for an additional 3 h to remove the acetyl protecting groups. The layers were separated, and the aqueous phase was re-extracted with CH_2Cl_2 (1 × 20 mL) and EtOAc (2 × 20 mL). The combined organic layers were dried over Na_2SO_4 and filtered, and the solvents were removed in vacuo. The resulting esters were analyzed by HPLC-MS.

RESULTS AND DISCUSSION

Methanol extracts of stevia dry leaves were directly used for LC-MS analysis. Efficient separation and resolution were achieved with diphenyl packing and acetonitrile/water as solvent in the HPLC method. Negative ion mode was used for all MS measurements. The HPLC method used here constitutes a variation of methods employed previously,[24] with variations required to achieve sufficient separation of triacyl chlorogenic acids and ent-kaurene glycosides. In comparison to isolation of CGAs from green coffee beans, no removal of proteins/peptides by Carrez reagent was necessary.[13,24]

All data for chlorogenic acids and diterpene glycosides presented in this paper use the IUPAC numbering system,[32] and

Table 1. Tandem Mass Spectral Data of Hydroxycinnamates in *Stevia rebaudiana* Leaf Extract

			MS^2							MS^3							MS^4						
				secondary peaks							secondary peaks							secondary peaks						
no.	compd	*m/z* (neg)	base peak	*m/z*	int	*m/z*	int	*m/z*	int	base peak	*m/z*	int	*m/z*	int	*m/z*	int	base peak	*m/z*	int	*m/z*	int	*m/z*	int
1	3-CQA	353.0	190.7	178.8	49	134.9	8			126.8	172.8	37	85.2	55	110.8	65	188.8	174.6	64	134.4	84		
2	5-CQA	353.0	190.7							126.8	172.7	49	85.1	61	110.8	21	108.8						
3	4-CQA	353.0	172.7	178.8	60	190.6	14	134.8	8	93.0	110.8	62	154.7	24									
4	3,5-diCQA	515.1	353.0	190.8	8					190.7	178.8	49	134.9	7			126.8	93.0	98	85.2	62	172.6	48
5	3,4-diCQA	515.1	353.0	335.0	12	172.8	17			172.8	178.8	67	190.8	57	134.8	10	93.0	110.8	41	83.2	6		
6	4,5-diCQA	515.1	353.0	299.0	3	254.9	7	172.8	17	172.8	178.6	52	190.8	28	135.0	7	93.0	110.8	89	83.0	18		
7	a *cis*-3,5-diCQA	515.1	353.0	190.8	10					190.7	178.8	50	172.8	11	134.9	10	85.0	126.8	85	93.0	53	172.7	36
8	a *cis*-4,5-diCQA	515.1	353.0	172.8	13					172.8	178.6	66	190.6	35	134.9	11	93.0	110.9	39	83.0	10		
9	*cis*-4,5-diCQA	515.1	353.0	172.7	7					172.7	178.8	66	190.8	59	134.9	12	93.0	110.9	23	83.0	19		
10	a *cis*-4,5-diCQA	515.1	353.0	172.8	12					172.7	178.6	76	190.6	70	134.8	18	93.0	110.8	39	83.0	6		
11	5-*p*-CoQA	337.1	190.7	162.8	6					126.8	172.7	42	108.8	44	92.8	32							
12	3F,5CQA	529.1	367.0	353.0	12	192.7	7	178.6	2	192.7	172.6	13	133.7	14			133.7	126.6	16				
13	C,FQA	529.1	367.1	349.0	7	178.7	10			178.7	160.8	73	134.8	85			134.7						
14	4C,5FQA	529.1	353.0	254.8	5	172.7	17			172.7	178.6	65	190.6	24	134.7	11	93.0	110.8	32	59.4	52		
15	5-CSA	335.1	178.7	172.8	11	134.8	20			134.7													
16	4-CSA	335.1	178.7	160.6	82	134.8	51			134.7													
17	3-CSA	335.1	178.7	160.8	4	134.8	42			134.8													
18	5-FQA	367.1	190.7	172.8	3					85.0	126.8	85	172.6	28									
19	FQA	367.1	178.7	190.8	33	160.8	12	134.8	72	134.7	106.8	5											
20	FQA	367.2	176.8							161.6	130.8	57											
21	3,4,5-triCQA	677.1	515.1	353.0	20					353.0	335.0	16	299.0	4	172.8	30	172.7	178.6	58	190.6	42	134.8	16
22	1,3,5-triCQA	677.1	515.1	353.0	16					353.0	335.0	15	254.9	5	172.7	28	190.8	178.6	72	172.6	90	136.7	10
23	triCQA	677.1	515.0	353.0	15					353.0	172.7	38	254.8	4			172.7	178.8	60	190.6	33	134.7	14
24	triCQA	677.1	515.1	353.0	20					353.0	172.7	22	254.8	4			172.8	178.6	87	190.8	90	134.7	15

structures are presented in Figure 1. Peak assignments of CGAs have been made on the basis of structure diagnostic hierarchical keys previously developed,[24−26] supported by means of their parent ion, UV spectra, and retention times relative to 5-CQA using validated methods in our laboratory.[24,27] More sensitive and more selective fragment-targeted MS^n experiments were used for quantitatively minor components. The base peak chromatogram of stevia extract is shown in Figure 2. Abbreviations and numbering are given in Figure 1. Stevia extract was analyzed by LC-MS^n in the negative ion mode using an ESI ion-trap mass spectrometer, allowing assignments of compounds to regioisomeric level, and also by high-resolution mass spectrometry using ESI-TOF in negative ion mode connected to LC. With the guidance of previous studies from Clifford,[24−29] three CQAs (**1**−**3**), seven di-CQAs (**4**−**10**), three FQAs (**18**−**20**), one *p*-CoQA (**11**), three CFQAs (**12**−**14**), three CSAs (**15**−**17**), and four tri-CQAs (**21**−**24**) were located in the chromatogram. For all compounds the high-resolution mass data were in good agreement with the theoretical molecular formulas, with a mass error of below 5 ppm, confirming the elemental compositions of all compounds investigated.

Reliable characterization of diterpene glycosides content in stevia is crucial. Because the structures of single glycosides are very similar, they have very similar retention times in LC and therefore result in overlapping of peaks in the chromatogram. In this paper, the general profile of diterpene glycosides in *S. rebaudiana* is given. Characterization of the compounds was achieved by ion-trap mass spectrometry with SIM, and confirmation of elemental composition was provided by ESI-TOF measurements (see the Supporting Information).

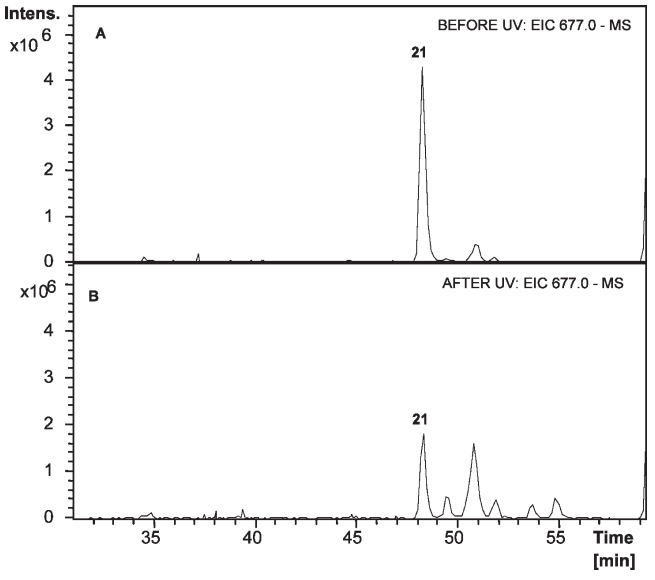

Figure 7. Extracted ion chromatograms (EIC) of *m/z* 677 in negative ion mode (A) before and (B) after UV irradiation.

Table 2. Quantities of Mono- and Di-CQAs in *S. rebaudiana* Leaves

compd	concn range	calibration curve	correl coeff	calcd amount (μg/g)
3-CQA	1 μg/mL−1 mg/mL	$Y = 4.457x - 460.04$	0.99	35.5
5-CQA	1 μg/mL−3 mg/mL	$Y = 17.719x - 2361.90$	0.99	44.3
4-CQA	0.07 μg/mL−1 mg/mL	$Y = 13.288x - 1223.00$	0.99	70.3
3,5-diCQA	0.09 μg/mL−1 mg/mL	$Y = 5.3176x - 529.76$	0.99	145.6
3,4-diCQA	0.07 μg/mL−1 mg/mL	$Y = 14.789x - 1401.40$	0.99	28.6
4,5-diCQA	0.03 μg/mL−04 mg/mL	$Y = 16.251x - 697.77$	0.99	37.2

Characterization of Caffeoylquinic Acids (M_r 354) and Dicaffeoylquinic acids (M_r 516). Three peaks were detected at m/z 353.1 and assigned using the hierarchial keys previously developed[24] as well-known 3-CQA, 5-CQA, and 4-CQA. Three dicaffeoylquinic acid isomers were identified by their parent ion m/z 515.2 and were assigned as 3,5-diCQA, 3,4-diCQA, and 4,5-diCQA using the hierarchial keys.[24,26] Three further peaks present as minor components showed fragmentation patterns similar to that of 4,5-diCQA. We have recently reported on cis isomers of chlorogenic acids present in plant tissue exposed to UV light, which have formed in a photochemical trans−cis isomerization reaction.[30] To confirm if the remaining three peaks correspond to cis isomers, the extract was irradiated with UV light at 245 nm for 40 min. After irradiation, a significant increase in the intensities of two peaks (9 and 10 in Figure 3) was observed, if compared to their corresponding trans isomers from the original plant extract. In addition, a significant increase was observed in the intensity of *cis*-3,5-diCQA (7 in Figure 3) peak accompanied by a decrease of the 3,4-diCQA (5 in Figure 3) peak. This finding suggests that under the chromatographic conditions employed the cis isomer is coeluting with 3,4-diCQA (Figure 3).

On the basis of increased intensity after UV irradiation and fragmentation pattern, three additional cis isomers were observed for 4,5-diCQA. One of these isomers was assigned as *cis*-4,5-diCQA (**9**), and two of them were assigned as cis−trans (a *cis*) isomer, but the distinction between 4-*cis*,5-*trans*-diCQA and 4-*trans*-5-*cis*-diCQA was not possible (**8** and **10**).

Characterization of Feruloylquinic Acid (M_r 368), *p*-Coumaroylquinic Acid (M_r 338), and Caffeoylferuloylquinic Acid (M_r 530). Only one peak was detected at m/z 337.1, which was identified as 5-*p*-CoQA according to its fragmentation pattern. Three peaks were detected at m/z 367, and one of them was identified as 5-FQA; the other two peaks could not be assigned due to their uncommon fragmentation pattern.

A targeted MS^3 experiment at m/z 529.2 ($[M - H^+]^-$) applied to the extract located three peaks, and two of them were identified as 3F,5CQA and 4C,5FQA on the basis of their characteristic fragmentations in MS^2 and MS^3 spectra. The assigments are achieved using the hierarchial keys previously developed, and mass spectra published previously are not presented here.[24,31]

Characterization of Caffeoylshikimic Acids (M_r 336). Caffeoylshikimic acids (CSA) have been reported in date palms, sweet basil, and carrot,[32−35] and they have been characterized to regioisomeric level in yerba maté leaves by tandem mass spectra previously.[36] This class of compounds is reported here for the first time from the Asteraceae family of plants. A targeted MS^3 experiment at m/z 335.1 ($[M - H^+]^-$) applied to the extract located three peaks, and they were identified by their fragmentation patterns as 5-CSA, 4-CSA, and 3-CSA (**15**−**17**).[36] All three regioisomers show m/z 178 (caffeic acid fragment) in their MS^2 spectra. 4-CQA shows an intense characteristic fragment ion at m/z 160, which is absent in the MS^2 spectra of 3-CSA and 5-CSA.

Characterization of Tricaffeoylquinic Acid (M_r 678). Four triacyl CQA isomers (Figure 4) were detected in the stevia extract at 677 for tricaffeoyls in neg. mode and confirmed as tricaffeoyl derivatives by targeted MS^4 experiments. Assignment of regiochemistry was assisted by an independent synthesis of a mixture of all four possible regioisomers of tricaffeoylquinic acids. The chromatogram of the mixture of all theoretically possible four regioisomers of tricaffeoylquinic acid obtained through synthesis showed two well-resolved peaks with retention times and MS data identical to those present in the stevia extract along with an intense broad peak in a retention time range where the two remaining isomers in the stevia extract were observed (see the Supporting Information). Detailed studies of the tandem mass spectra at various retention times within the broad peak suggest that this broad peak must correspond to two distinct unresolved regioisomers of tricaffeoylquinic acid. Comparison of the chromatogram of the synthetic mixture with the extract allowed unambiguous assignment of the two regioisomers in the extract by identity of the fragmentation pattern compared to the synthetic mixture. Identification of 1,3,5-triCQA in the extract was followed automatically due to the absence of an MS^4 base peak at m/z 173 corresponding to a dehydrated MS^2 base peak of the quinic moiety characteristic of 4-acylated isomers. The MS^4 base peak at m/z ~191 and a secondary peak at m/z 178 (72% of base peak) suggest the 3,5-disubstitution pattern (Figure 5). 3,4,5-triCQA was identified by comparison to material described previously.[36,37] (Figure 6)

The two remaining peaks might be cis isomers of 3,4,5-triCQA and 1,3,5-triCQA, or they can correspond to either 1,4,5-tri-CQA and 1,3,4-tri-CQA or any of their cis isomers (see Table 1). However, current information does not allow us to discriminate unambiguously between these regioisomers at the moment. To probe whether cis isomers were present, the extract was again irradiated with UV light, and after chromatographic analysis, a significant increase in the intensity of the peaks of 4-acylated isomers was observed (Figure 7). Otherwise, the experiment was inconclusive. It is worth noting that in theory for each tricaffeoyl derivative eight stereoisomers with various trans−cis stereochemistries are possible, thus increasing the total number of isomeric tricaffeoylquinic acids to 32. Given the identity of MS data and the absence of characteristic shoulders in the UV spectra characteristic for *cis*-caffeoyl derivatives, we tentatively assign the two remaining isomers as 1,4,5-tri-CQA and 1,3,4-tri-CQA. Only 3,4,5-tri-CQA has been previously reported in nature, whereas the remaining isomers are reported here for the first time.

Quantification of Caffeoylquinic Acids. Following the qualitative profiling of chlorogenic acids in *S. rebaudiana*, we decided

to quantify the levels of selected compounds. Chlorogenic acid standard solutions were analyzed by LC-MS using the same chromatographic method as used for stevia leaf extracts. For six selected monoacyl- and diacylquinic acids, calibration curves were obtained using six-point calibration from the UV chromatogram recorded at 320 nm. The individual amounts calculated for mono- and dicaffeoylquinic acids are listed in Table 2, which also lists the correlation coefficient of linear regression for each standard sample and the concentration range.

Among the monocaffeoylquinic acids, 4-CQA was found to be the most abundant compound, and among all CQAs 3,5-diCQA was found to be the most abundant compound. The total chlorogenic acid amount determined here is around 370 μg/g of dry leaf.

In this study we profiled the chlorogenic acids in *S. rebaudiana* employing LC-MS^n and LC-TOF techniques. A total of 24 chlorogenic acids were detected in *S. rebaudiana* leaves, with 23 compounds described for the first time from this source. Tri-CQAs were reported for the first time from *S. rebaudiana* with three regioisomers found for the first time in nature. CSAs were characterized for the first time from a plant belonging to the Astareceae family by using tandem mass spectrometry. Quantification of selected mono- and di-CQAs was achieved by using the UV chromatogram with total chlorogenic acid levels found to be 370 μg/g of dry leaf.

■ ASSOCIATED CONTENT

Supporting Information. Additional EIC of triacyl CGAs, MS^2 + MS^3 data of all compounds mentioned in the text, table of high-resolution MS-TOF data for compounds identified, and structures of ent-kaurene terpenes. This material is free of charge via the Internet at http://pubs.acs.org

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Funding Sources

Financial support from the European Union (project DIVAS) is gratefully acknowledged.

■ ACKNOWLEDGMENT

We acknowledge the technical assistance of Anja Müller.

■ DEDICATION

†This paper is dedicated to Prof. M. N. Clifford on the occasion of his 65th birthday.

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