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ORIGINAL PAPER

Improved HPLC method for the evaluation of the major steviol glycosides in leaves of *Stevia rebaudiana*

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Abstract A simple reversed-phase high-performance liquid chromatographic method has been developed for the determination of the major steviol glycosides, the diterpene sweeteners derived from *Stevia rebaudiana*. The method is based on a water extraction step and a solid-phase extraction (SPE) clean-up. Different SPE cartridges and two HPLC columns were tested in the separation of the main steviol glycosides stevioside and rebaudioside A. A good separation was performed on a Luna HILIC analytical column with a mobile phase consisting of acetonitrile/water (85:15 v/v). The calibration curves were linear from 10 to 800 μg/mL and the recoveries for stevioside and rebaudioside A were 99 ± 4.4 and 100 ± 5.0%, respectively. The applicability of this method was demonstrated in the analysis of stevioside and rebaudioside A from *Stevia* plants grown in two different areas in Germany. Stevioside and rebaudioside A contents showed statistically significant differences (*F*- and *t*-test) between the two harvests. Nevertheless, the total concentrations (>12%) and the ratio of stevioside to rebaudioside A (6:4) were similar to those found in the countries in which *Stevia rebaudiana* originates. Based on a comparison of yields from different harvests, we discussed whether *Stevia rebaudiana* can be economically grown in the temperate zones of the northern European hemisphere.

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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]. *Stevia* contains *ent*-kaurene-type diterpene glycosides, the most abundant of which are stevioside and rebaudioside A (Table 1). Minor components in this plant include rebaudioside C–F and dulcoside A (1–2% in total) [3–5]. Some evidence exists that steviolbioside and rebaudioside B are not genuine constituents of *S. rebaudiana* but rather are formed by partial hydrolysis during the extraction process [6, 7]. Since many years, dried *Stevia* leaves are used as natural sweeteners and their extracted steviol glycosides stevioside and rebaudioside A are approved for general food use in Australia, Argentina, Brazil, China, India, Israel, Japan, New Zealand, Paraguay, Russia, South Korea and a few other countries. In the USA, rebaudioside A and highly purified steviol glycosides received the GRAS status (generally recognised as safe) in 2008 and 2009, respectively, and are used as food additives [8–10]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established regulations for steviol glycosides demanding a purity level of at least 95% of the seven chemically defined steviol glycosides [11]. The JECFA also decided in 2008 to raise the ADI (acceptable daily intake) for steviol glycosides expressed as steviol from 2 to 4 mg/kg bodyweight [12]. Only in Europe, the use of the *Stevia* leaves and its extracted steviol glycosides is not authorised, with the only exception to this rule being France [13]. There, since August 2009, high purified

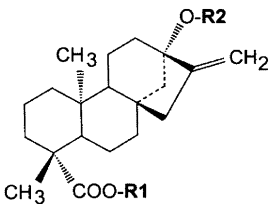
rebaudioside A has been allowed as a sweetener in specified foods as a preliminary authorisation for 2 years.

Steviol glycosides in *Stevia* leaves are usually obtained after hot water leaching that is sometimes followed by solid-phase extraction (SPE) [14–16]. Methods using chloroform and methanol for extraction [17] or even supercritical fluid extraction (SFE) have also been described [18–20].

For separation and quantification, HPLC analysis was applied using NH_2 [3, 14, 17, 18], C18 [15, 16, 21, 22] and carbohydrate columns [23]. NH_2 columns have a high selectivity for all steviol glycosides but poor reproducibility and long equilibration times, whereas reversed-phase columns are robust but show poor selectivity with regard to the separation of stevioside and rebaudioside A. This problem can be resolved by gradient elution [21] or by using two columns in series [18, 22].

Detection was achieved by UV [15, 17, 21–23], mass spectrometry [24–26], amperometric [27] or charged aerosol detection [28].

Table 1 Structures of steviol glycosides



Name	R1	R2
Steviol	H	H
Steviolbioside	H	Glu—1,2—Glu
Stevioside	Glu	Glu—1,2—Glu
Rebaudioside A	Glu	Glu—1,2—Glu | 1,3—Glu
Rebaudioside B	H	Glu—1,2—Glu | 1,3—Glu
Rebaudioside C	Glu	Glu—1,2—Rham | 1,3—Glu
Rebaudioside D	Glu—1,2—Glu	Glu—1,2—Glu | 1,3—Glu
Rebaudioside E	Glu—1,2—Glu	Glu—1,2—Glu
Rebaudioside F	Glu	Glu—1,2—Xyl | 1,3—Glu
Dulcoside A	Glu	Glu—1,2—Rham

Glu Glucose, *Rham* Rhamnose, *Xyl* Xylose

The aim of this work was to develop a simple HPLC method for the determination of the main steviol glycosides in *Stevia* leaves as well as in food that demands less organic solvents and minimises analysis time by the application of an effective SPE clean-up. Furthermore, the implementation of a HPLC column with a strong selectivity for all steviol glycosides, and good reproducibility and compatibility with MS detection were also aims of this study.

For this purpose, we tested three different solid-phase extraction cartridges and two different HPLC columns. The applicability of this new method for the determination of steviol glycosides in *Stevia* leaves was demonstrated using plant material from field trials established in different areas of Germany. Reliable quantitative analyses of *Stevia* sweeteners are necessary to determine if economic cultivation of this plant under European temperate zone conditions is possible.

Materials and methods

Chemicals and apparatus

HPLC-grade acetonitrile and methanol were obtained from VWR International (Leuven, Belgium). Solid-phase extraction (SPE) was carried out on a Baker 10 SPE system from Mallinckrodt Baker (Phillipsburg, USA) using Strata C18-E cartridges (100 mg, 1 mL, 55 µm, 70 Å) from Phenomenex (Aschaffenburg, Germany), Bakerbond C18 SPE cartridges (100 mg, 1 mL) from Mallinckrodt Baker (Phillipsburg, USA) and 3 M Empore C18-SD SPE cartridges (7 mm, 3 mL) from IVA (Meerbusch, Germany). RC membrane filters, 0.45 µm, Ø 25 mm, were purchased from IVA (Meerbusch, Germany).

Stevioside and rebaudioside A with minimum purity of 99.0% were obtained from WAKO Chemicals (Neuss, Germany).

A Grindomix GM 200 and a MM 2,000 mill from Retsch (Haan, Germany) were used for grinding the *Stevia* leaves, and a Hermle Z323 K centrifuge (Wehingen, Germany) for clarifying the extracts. The Nalgene centrifuge tubes, size 28 mL, were purchased from neoLab (Heidelberg, Germany), and the extractions were carried out in a Major Science reaction dry block from VWR International (Langenfeld, Germany).

HPLC conditions

Liquid chromatography under isocratic conditions was performed on a Varian system (Darmstadt, Germany) consisting of a ProStar 230 pump, a ProStar 410 autosampler, a 335 diode array detector set to a wavelength of 210 nm, a four-channel degasser, a Metatherm column

thermostat and the Galaxie CDS chromatography data system. To detect stevioside and rebaudioside A, two different analytical columns and mobile phases were tested. System A was equipped with a Luna HILIC (250 × 4.6 mm, particle size 5 µm, Phenomenex, Aschaffenburg, Germany) and the corresponding guard column (4 × 3.0 mm). The mobile phase consisted of acetonitrile/water (85:15 v/v) set to a flow rate of 1.0 mL/min kept at 36 °C. System B contained a Luna NH_2 100 A column (250 × 4.6 mm, particle size 5 µm, Phenomenex, Aschaffenburg, Germany) equipped with the corresponding guard column (4 × 3.0 mm) and kept at 36 °C. The mobile phase for this system was acetonitrile/water (75:25 v/v) used at a constant flow rate of 1 mL/min. The sample injection volume was 20 µL.

LC–MS conditions

An Agilent G1311A quaternary LC System was used consisting of a binary pump, a G1329A autosampler, a column oven set at 36 °C, a degasser and a G1314B VW detector set at 210 nm. MS detection was done with a Bruker HCTultra PTM Discovery Ion Trap System running in the negative electrospray ionisation (ESI) mode. The resulting MS parameters were lens 2, +71.5 V, capillary exit, −191.7 V, skimmer, −25.0 V, lens 1, +3.3 V, Oct 1 DC −8.41 V, Oct 2 DC −0.50 V, dry temperature, 350 °C, nebulizer, 58.63 psi, dry gas, 11.93 L/min, HV capillary 4,500 V, current capillary 9.766 nA. The mass traces of *m/z* 317, 479, 641, 803 and 965 were recorded, and identification of individual compounds was conducted by MS^n fragmentation. The LC–MS system was controlled by the Bruker Daltonik HyStar Software Version 3.2.44.0. The LC conditions for the separation of the steviol glycosides were the same as described earlier. HPLC injection volume was 10 µL.

For the preparation of stock solutions, the steviol glycosides were dried to a constant weight at 103 °C and then stored in an exsiccator until use. Twenty milligrams of dried steviol glycosides was dissolved in water to a final concentration of 1 mg/mL. Spiking solutions were prepared by diluting aliquots of the stock solutions with water and calibration solutions for HPLC analysis by diluting with acetonitrile/water (8:2 v/v) to obtain final concentrations of stevioside and rebaudioside A of 10–800 µg/mL.

Origin of the samples

For the recovery experiments, we used four different *Stevia* plants (Table 2) with stevioside concentrations from 5.5 to 10.6% and rebaudioside A concentrations from 3.1 to 7.3%.

The method was applied to *Stevia* leaf samples originated from cultivation experiments being established on two sites in Germany 200 km apart from each other and differing mainly in soil conditions. The first site had a fertile sandy loam (OVA) tending to upsilting after heavy rainfall, and the second site was a light loamy soil providing good drainage (BOR). At both sites, three different lots of land (replicates) were planted with 90 plants, respectively. Two plants of every replicate were sampled, and analyses of each plant were performed in duplicate.

Sample preparation

Stevia leaves were lyophilised at −25 °C. The samples were ground in a grinding mill (Grindomix GM 200) followed by a second step in a vibrating grinding mill (MM 2,000) to produce powder particles of 0.12 mm in size.

Extraction procedure

(A) Ground *Stevia* leaves (0.25 g) were weighed in a 10-mL centrifuge tube and extracted three times with water in a boiling water bath or a heating block set at 102 °C for 30 min. Each extract was cooled to room temperature and centrifuged (15 min, 8,500 U). The aqueous phases were transferred to a 25-mL volumetric flask that was filled to capacity after the last extraction step. This solution was used for SPE.

(B) The same procedure as described previously was carried out with acetonitrile/water (8:2 v/v) instead of water as the extraction solvent. This solution was filtered through a membrane filter (0.45 µm) and subjected to HPLC analysis.

Solid-phase extraction

Strata- and Bakerbond cartridges were conditioned with methanol (3 mL) and water (3 mL). Aliquots of the aqueous sample (0.1–0.4 mL) were added to the tubes, washed with water (3 mL) and acetonitrile/water (2:8 v/v) (5 mL) and then air-dried for 3 min. The steviol glycosides were eluted with a mixture of acetonitrile/water (8:2 v/v) (2 mL). The eluate was subjected to HPLC analysis.

The 3M discs were conditioned with methanol (0.25 mL) and water (0.5 mL). An aliquot of the aqueous sample (0.25 mL) was added to the tubes, washed with water (0.5 mL) and acetonitrile/water (2:8 v/v) (0.5 mL) and then air-dried for 1 min. For elution of the analytes, acetonitrile (0.5 mL) was added to the tubes and allowed to soak for 30 s. The steviol glycosides were eluted slowly without vacuum. The eluate was subjected to HPLC analysis.

Table 2 Recovery of stevioside and rebaudioside A after SPE with C18 cartridges

Compounds	Origin of spiked samples	Added (mg/g)	Detected (mg/g)	Recovery (%)	Mean recovery (%) ± RSD
Stevioside	Brasilia	0	55.3		99 ± 4.4
		25.7	78.6	97	
		26.3	81.3	99	
	Paraguay	0	70.9		
		13.9	78.0	92	
		13.9	89.9	106	
	Germany	0	106.0		
		13.8	114.3	96	
		34.6	147.2	105	
		34.6	135.7	97	
	Germany	0	81.6		
		8.4	90.3	100	
		8.4	88.2	98	
Rebaudioside A	Brasilia	0	72.5		100 ± 5.0
		29.9	110.6	108	
		30.5	102.7	99	
	Paraquaya	0	42.9		
		13.9	55.1	97	
		13.9	52.8	93	
	Germany	0	30.6		
		8.6	37.7	96	
		21.5	51.7	99	
		21.5	55.3	106	
	Germany	0	46.1		
		8.4	53.6	98	
		8.4	56.5	104	

Recovery of stevioside and rebaudioside after SPE

Strata and Bakerbond: Different types of ground and dried leaves of *S. rebaudiana* (0.25 g) were spiked with a standard solution (0.4–0.5 mL) containing 8.6–34.6 mg stevioside and rebaudioside A/g, extracted and cleaned up as described earlier.

Disc cartridge: *Stevia* leaf extract (1.0 mL) was spiked with 50, 100 and 200 µL of a standard solution (950.4 µg/mL) of stevioside or rebaudioside A, respectively. Using the same method, an unspiked *Stevia* leaf extract (1.0 mL) was diluted with 50, 100 and 200 µL of water. The spiked samples and the appropriate blanks were cleaned up via SPE and subjected to HPLC analysis as described earlier.

Method evaluation

Selectivity was determined by comparing the chromatograms obtained from the leaf samples with those of the standard solutions. Precision was calculated in terms of intra-day ($n = 10$) and inter-day repeatability ($n = 2$), with the 250 µg/mL standard solution of stevioside and rebaudioside A on the HILIC column and evaluated by calculating the relative standard deviation (RSD). Linearity was assessed by injecting standard solutions of stevioside and rebaudioside A from 10 to 800 µg/mL in triplicate. Qualitative determination was achieved by comparing the retention times of the standard solution with those of the samples. Quantification was possible by applying the calibration plot equations calculated by the least-squares method.

The accuracy of the method was determined by assessing the recovery and the appropriate relative standard deviation (RSD) in seven different leaf samples spiked with different amounts of the two steviol glycosides. The cultivation experiments were statistically evaluated by calculating the mean and the standard deviation of every land site. The resulting two experimental series ($n = 12$) were evaluated for outliers and the outlier-free series by *F*- and *t*-tests for significant differences based on a 95% confidence level.

Results and discussion

Quantitative extraction of the steviol glycosides with acetonitrile/water (8:2 v/v) and HPLC without sample clean-up is possible with a good appearance for the peaks of stevioside and rebaudioside A. Due to the high matrix load visible in the beginning of the chromatogram (Fig. 1b), the detection of steviol glycosides with shorter retention times than stevioside, such as steviolbioside and steviol, is not possible. Furthermore, the matrix load dramatically shortens the lifetime of the columns, and therefore the analytical method including SPE extraction (Fig. 1a) was applied.

SPE with Bakerbond and Strata cartridges

These two SPE cartridges tested were traditionally filled with loosely packed C18 particles.

The mean recovery of stevioside was 99 ± 4.4% ($n = 9$) and 100 ± 5.0% ($n = 9$) for rebaudioside A at the different spiking levels (Table 2). Having used *Stevia* leaves containing at least 10.6% stevioside and 7.3% rebaudioside A for the spiking experiments, stevioside concentrations up to 14% and rebaudioside A concentrations up to 10% can be analysed without further dilution or adaption.

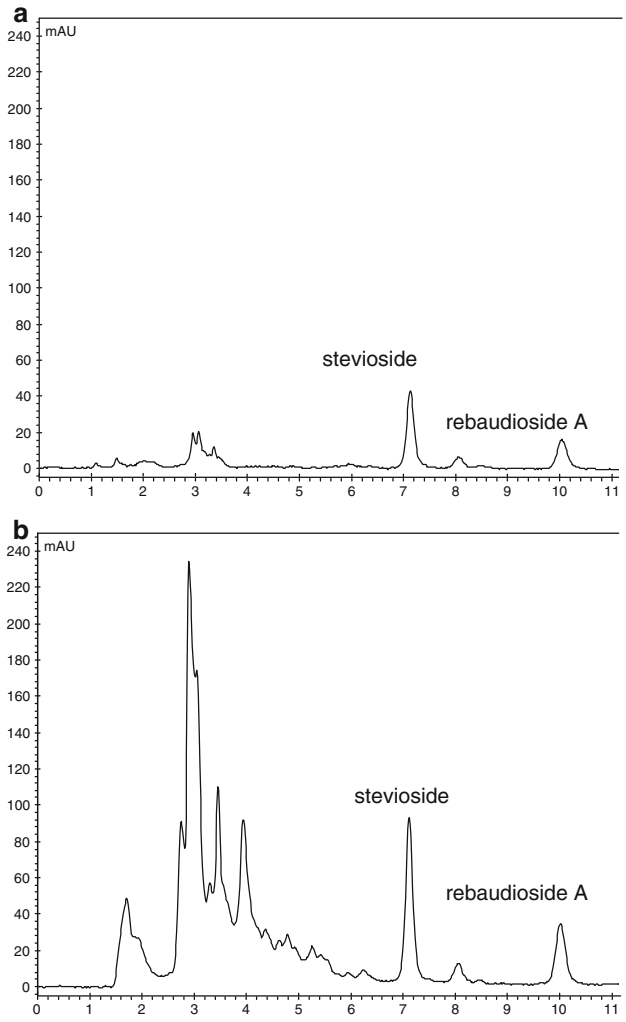


Fig. 1 Chromatograms of a *Stevia* leaf extract (**a**) after and (**b**) without SPE clean-up

SPE with disc cartridges

In these cartridges, the sorbent particles were trapped within an inert matrix of a PTFE disc resulting in thin membranes. The particle-loaded membranes were covered by polypropylene pre-filters of different densities. This construction was conducive to more efficient adsorption of particles with reduced solvent consumption and time required for the procedure (2.5 mL solvent for disc cartridges vs. 16.2 mL solvent required for Strata and Bakerbond cartridges). However, the recoveries obtained in this method showed poor reproducibility. They varied from 39–129% (data not shown) when spiking the water extract of *Stevia* leaves with 79.2, 39.6 or 19.8 µg/mL stevioside and rebaudioside A, respectively. Despite numerous procedural modifications (change in solvent, amount of solvent, drying time, duration of the elution), it was not possible to improve and stabilise the recovery.

Choice of HPLC column

The HPLC columns used in this study differed in their designs and modes of separation. The NH_2 column operates in the normal-phase mode, whereas the Phenomenex HILIC column retains a water-enriched layer of dihydroxypropane groups chemically bonded to silica to provide an alternative hydrophilic interaction liquid interface chromatography (HILIC) mode. This mode resembles the normal-phase mode. It has been assumed [29] that retention occurs by interaction of polar analytes with a stagnant layer of water, and the discussion is still ongoing whether retention in HILIC is a partition or an adsorption process. Obviously, HILIC is nevertheless suitable for analytes with poor retention in reversed-phase HPLC.

The NH_2 column showed the same retention pattern as the HILIC phase, as the retention of the analytes raised in order of increasing hydrophilicity. That result means that in the case of steviol glycosides, the retention times increase with the number of glucose units attached to the *ent*-kaurene skeleton.

A precise separation of stevioside and rebaudioside A and other steviol glycosides not quantified here could be achieved with the NH_2 just as well with the HILIC column. Figure 2 shows typical chromatograms of the two columns. Linearity between 10 and 800 µg/mL is excellent with $R^2 > 0.999$ (Table 3). The purified samples of the different

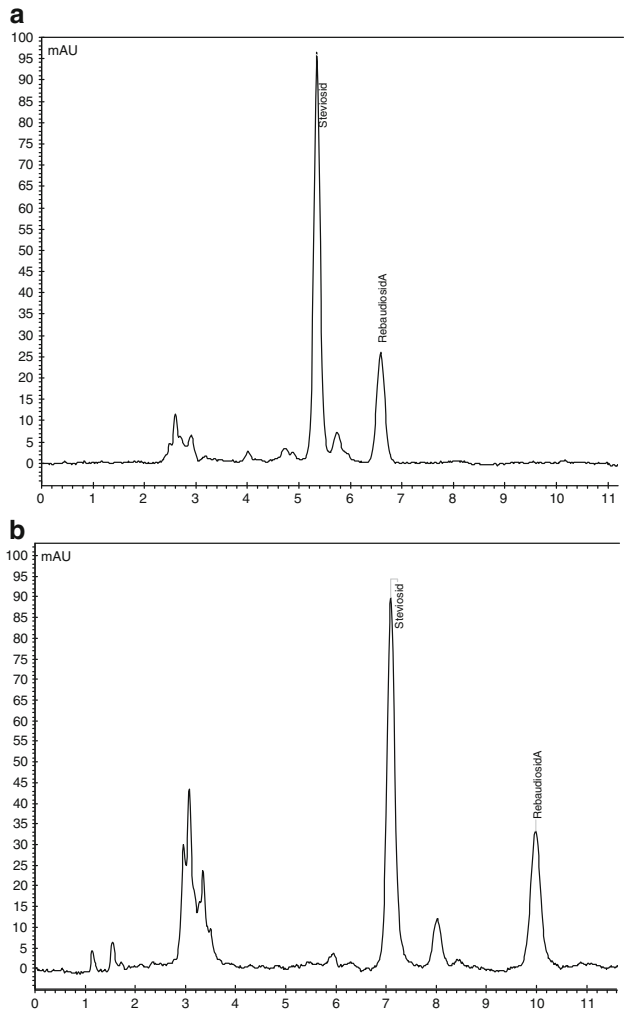


Fig. 2 Chromatograms of a *Stevia* leaf extract separated on the Luna NH_2 (**a**) and Luna HILIC column (**b**)

land sites were analysed on both columns. *F*- and *t*-tests of the corresponding data revealed no significant differences between the concentrations of stevioside and rebaudioside A. Nevertheless, we decided to use the HILIC column in further experiments despite slightly longer retention times for stevioside and rebaudioside A because we could obtain more robust results with shorter equilibration times. Furthermore, HILIC showed almost no bleeding and was more suitable for the use in LC–MS detection applied in further investigations. We evaluated the precision of the HILIC column to have an intra-day precision of 0.43–0.59% for stevioside and 0.16–0.25% for rebaudioside A and inter-day precision values of 0.52% for stevioside and 0.21% for rebaudioside A, respectively.

The identity of stevioside and rebaudioside A was additionally confirmed by LC–MS detection with electrospray ionisation (ESI) in the negative ion mode. The molecular ions of stevioside (*m/z* 803) and rebaudioside A (*m/z* 965) as well as their typical fragment ions resulting from the successive elimination of glucose were clearly observed and matched with the signals identified by UV detection. These results correspond to similar investigations by LC–MS [24].

Application to real samples

The method developed here was used to verify the stevioside and rebaudioside A concentrations in cultivation experiments. These experiments were carried out to determine whether *Stevia* can be successfully and economically cultivated in Europe. The natural habitat of the short day plant *S. rebaudiana* is located in the subtropics with an annual mean temperature of 21 °C and an annual precipitation between 1,500 and 1,600 mm [30], whereas in the temperate zones of the northern European hemisphere, long day conditions prevail during the growing season and the annual mean temperature is about 9 °C with annual precipitation of about 600 mm. Despite these adverse conditions, 5 years of field trials with *Stevia rebaudiana* showed that this plant is worthy for cultivation in temperate latitudes [31–33].

However, because the *Stevia* plant is not hardy enough to survive in winter, production had to rely on annual cultivation, where, in more southern latitudes, plants can be maintained up to 5 or 6 years [1]. Compared to this prolonged cultivation, annual replacement of the plants demands extra investment that must be compensated for by appropriate yields. From earlier studies, it is well known

Table 3 Linear regression curves of stevioside and rebaudioside A

Compound	Retention time (min)	Slope	*Y*-axis intercept	Determination coefficient, R^2
Stevioside[a]	7.0	4.4945	−19.763	0.9999
Rebaudioside A[a]	9.7	3.6650	−14.254	0.9999
Stevioside[b]	5.4	4.3080	−0.300	0.9998
Rebaudioside A[b]	6.6	3.2024	−5.903	0.9997

[a] For chromatographic conditions, see HPLC conditions for system A

[b] For chromatographic conditions, see HPLC conditions for system B

Table 4 Stevioside and rebaudioside A concentrations in the *Stevia* leaves harvested on two different sites

Land sites	Stevioside (g/100 g)	Rebaudioside A (g/100 g)	Stevioside (g/100 g)	Mean rebaudioside A (g/100 g)
OVA	7.84	4.85	7.90 ± 0.29	4.93 ± 0.44
	8.32	5.44		
	8.22	5.08		
	7.68	5.44		
	7.44	5.05		
	7.85	5.28		
	8.07	4.61		
	8.03	5.27		
	7.62	4.19		
		4.28		
		4.71		
BOR	6.97	4.47	7.78 ± 0.61	4.28 ± 0.29
	7.08	4.01		
	7.48	4.06		
	7.91	4.22		
	8.75	4.02		
	7.79	4.66		
	8.71	3.98		
	7.40	4.23		
	8.13	4.76		
	7.59	4.40		
Significant difference by *F*-test	Yes	No		
Significant difference by *t*-test	/	Yes		

that long day conditions promote any parameter of vegetative growth and even increase sweetener content in *S. rebaudiana* [34]. In the 5 years of field trials mentioned previously, the herbage yield (dry matter) varied from 3 t ha^{-1} in 2005 to 12 t ha^{-1} in 2004 [31]. This result corresponded to and even exceeded the yields reported by others (e.g. for Brasil: 3 t ha^{-1} [35], California: 3.6 t ha^{-1} [36], Canada: 3 t ha^{-1} [37], Japan: 4.5 t ha^{-1}, Korea: 4.4 t ha^{-1}, Paraguay: 3.8 t ha^{-1}, Spain: 5.4 t ha^{-1} [30] and India: 3.5 to 6.3 t ha^{-1} [38]).

In 2006, when *S. rebaudiana* was cultivated on two different land sites, herbage yield was significantly higher on the loamy sand soil: 11.3 t ha^{-1} for BOR when compared to 9.6 t ha^{-1} for OVA. To interpret this difference, it was important to understand how the main sweeteners, stevioside and rebaudioside A, were affected by these land sites. Table 4 shows the results. Comparing the outlier-free data series of the two land sites by *F*- and *t*-tests, significant differences for the stevioside and rebaudioside A concentrations could be estimated. However, concentrations were only slightly higher on the OVA site so that the difference in yields was not compensated.

As the sweetener concentrations were similar to those found in the countries in which *S. rebaudiana* originates, the higher yields under the long day conditions of the temperate zones are promising for an economic cultivation of the *Stevia* plant in Europe.

Conclusion

Steviol glycosides can be easily analysed after water leaching and SPE clean-up with C18 cartridges. HPLC can be performed on a HILIC column with recoveries of nearly 100%. Application of this method for the quantification of stevioside and rebaudioside A concentrations in *Stevia* plant leaves from different areas in Germany revealed that economical production of *Stevia rebaudiana* could be feasible in the temperate zones of Europe.

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