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Analysis of Sweet Diterpene Glycosides from *Stevia rebaudiana*: Improved HPLC Method

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An improved analytical method was developed which may be applied to quality control of stevioside and rebaudioside A contents in dried leaves of *Stevia rebaudiana* before processing; in a selective sampling program searching for plants of higher yield in diterpene glycosides content; or when a large number of samples are sent to the laboratory for analysis. The procedure developed involves two steps: solvent extraction followed by an isocratic HPLC analysis. The sample, 1 g of dried leaves of *S. rebaudiana*, is ground and solvent-extracted with EtOH 70% (w/w) in Erlenmeyer flasks by shaking for 30 min in a 70 °C water bath. After the extract was cooled, it was filtered and analyzed by HPLC using an NH_2 column (250 × 4.6 mm) and a mixture of acetonitrile/water (80:20, v/v) as mobile phase, pH 5 adjusted with acetic acid. The detection was in the UV range at 210 nm (0.04 AUFS). Quantitation was performed by means of an external standard calibration curve for each analyte which had been obtained from standard solutions of pure stevioside and rebaudioside A. Working under these conditions there were no observed interference effects. The method saves time in sample preparation, and reduces sample handling and chromatographic analysis time, while having little loss of precision [coefficient of variation (CV%) between 1.8% and 3.0%] and recovery [between 98.5% and 100.5%]. The method was applied to 30 samples of *S. rebaudiana* from Misiones (Northeastern Argentina), and the stevioside content found ranged between 3.78 and 9.75% (weight) whereas Rebaudioside A content ranged between 1.62 and 7.27% (weight).

Keywords: *Sweetener, Stevia rebaudiana, stevioside, rebaudioside A, HPLC analysis*

INTRODUCTION

The Paraguayan herb *Stevia rebaudiana* (Bertoni), also known by natives as *Kaa-He-é* o "*hierba dulce*", has captured growing interest as a potential source of natural noncaloric sweeteners for use as a possible substitute for synthetic sweeteners. Its use has been approved in Brazil, Argentina, and Paraguay, as well as in China, Korea, and Japan (*1*).

Stevia rebaudiana contains six diterpene glycosides with an intense sweet taste, the most abundant and important being stevioside, with a sweetening power 300 times that of sucrose, and rebaudioside A, the second most abundant and 400 times sweeter than sucrose. The rest are of minor concern: rebaudioside C and dulcoside A, and at trace levels, rebaudioside E and D (*2*, *3*, *4*, *5*).

Many analytical methods have been applied for the separation and quantification of the sweet diterpene glycosides from the leaves of *S. rebaudiana*. Mizukami et al., (*6*) quantified only stevioside by a chemical method following an enzymatic hydrolysis, whereas Sakaguchi and Kan (*7*) quantified total glycoside content by means of gas chromatography after acid hydrolysis.

Thin-layer chromatography (*8*) has also been applied to identify the four more abundant glycosides: stevioside, rebaudioside A, sulcoside A, and rebaudioside C. References to high-performance liquid chromatography (HPLC) methods are found in the literature: some applied hydrophilic (OH) columns (*9*), other authors employed size exclusion chromatography (*10*, *11*), and the use of C18 columns after derivatization also has been reported (*12*).

Makapugay et al. (*13*) quantified not only the natural products content in the leaves of *S. rebaudiana*, but also the steviolbioside and the rebaudioside B, both hydrolysis products of atevioside and rebaudioside A, using a method consisting of Soxhlet extraction of the sample and HPLC separation. Dried and ground leaves of *S. rebaudiana* were extracted subsequently with chloroform and methanol, then the extract was evaporated to dryness, and the solid residue was dissolved in methanol (HPLC grade). After the sample was filtered and the volume was brought up to 50 mL, the sample was analyzed with a linear gradient chromatographic method using a mixture of acetonitrile/water with a NH_2 column and UV detection at 210 nm.

More recently, Mauri et al. (*14*) have employed a capillary electrophoresis method to analyze *Stevia* glycosides, obtaining rebaudioside A and steviolbioside from a semipreparative HPLC.

The aim of this work is to develop an alternative method of analysis, requiring less time and solvent consumption (*15*), and using an extraction procedure that minimizes human and environmental exposure. This could be applied to the quality control of stevioside and rebaudioside A contents in dried leaves of *S. rebaudiana* before processing; in a selective sampling program; when searching for plants of higher yield in diterpene glycosides content; or when a large number of samples are sent to the laboratory for analysis.

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Table 1. HPLC Characteristics of Stevioside and Rebaudioside A

compound	retention time (min)[a]	slope[b]	*y*-axis intercept[b]	correlation coefficient[b]
stevioside	4.326	5.2367E−06	−0.0109	0.9998
rebaudioside A	6.097	6.4315E−06	−0.0299	0.9977

[a] For chromatographic conditions see Materials and Methods. [b] From peak area determinations.

MATERIALS AND METHODS

Samples. Method development was carried out on 30 samples of *S. rebaudiana* from Misiones (Northeastern Argentina). For analytical purposes, the samples were harvested at different places and at different times throughout the year, dried through exposure to sunlight, and kept in closed plastic bags in the laboratory until analysis.

Reagents and Apparatus. Analytical grade acetic acid, methanol, chloroform, and ethanol were obtained from Merck (Darmstadt, Germany). Methanol and acetonitrile/water (HPLC grade) from OmniSolv EM Science, Gibbstown, NJ, were used.

Three different HPLC systems were applied for this work. First, a Konik KNK 029-375 μP gradient-programmable liquid chromatograph with a NH_2 column LiChrospher 100, 5 μm, 250 × 4 mm (Merck, Darmstadt, Germany), helium solvent desgassing with linear UV−Vis 200 detector, and a Spectra Physics SP4290 integrator. The second system was a Shimadzu LC 6A with a NH_2 column Zorbax, 5 μm, 250 × 4.6 mm (Du Pont, Wilmington, DE) with UV detector SPD 6A, CR 3A recorder, and system controller SCL 6A. The third system included an HP series 1100 HPLC system (Hewlett-Packard, Avondale, PA), also with NH_2 column Zorbax, 5 μm, 150 × 4.6 mm (Du Pont, Wilmington, DE), autosampler G1313A, UV−DAD detection, and Chemstation integration software.

Extraction of Sweet Diterpene Glycosides from *S. rebaudiana*. The proposed method was compared with the Soxhlet extraction method of Makapugay et al. (*13*). Ten powdered (d_p<30 mesh ASTM), dried *S. rebaudiana* leaves replicate samples (1 g) were extracted in a Soxhlet apparatus sequentially with chloroform (200 mL) for 3 h and methanol (200 mL) for 5 h. Methanol extracts were evaporated in a rotovap to dryness in vacuo, and dissolved in 50-mL portions of HPLC-grade methanol. Aliquots (2 mL) of each solution were filtered (syringe filter, nylon, 0.22 μm, MSI, Westboro, MA) and passed through precolumn cleaning, then 5 μL was injected to the HPLC.

For the method under development, 10 replicate samples of dried and ground leaves of *S. rebaudiana* (1 g) were placed in 250-mL Erlenmeyer flasks and extracted by shaking for 30 min with 100 mL of EtOH 70% (w/w) in a 70 °C waterbath. After cooling, a 10-mL aliquot was filtered (filter paper, quantitative, routine ashless, Grade 42, Whatman, and syringe filter, nylon 0.22 μm, MSI Westboro), then 5 μL was analyzed by HPLC.

This procedure has many advantages when compared with other methods found in the literature in that it requires less time in sample preparation, and it also minimizes human risk and environmental impact by avoiding the use of MeOH and Cl_3CH.

For quantitation purposes, pure stevioside and rebaudioside A were obtained from preparative column chromatography, and selected fractions were collected and purified. After successive recrystalization, the melting point reported in the literature was reached and the corresponding chromatographic analysis (HPLC) showed only one peak. Standard solutions of 0.3, 0.6, and 1.0 g L^{-1} of stevioside and rebaudioside A in MeOH were prepared. The parameters of external calibration curves were obtained by fitting experimental data through linear regression from replicate injections of standard solutions (see Table 1).

High-Performance Liquid Chromatography (HPLC). To optimize the separation of the sweet diterpene glycosides stevioside and rebaudioside A, the following HPLC operating conditions were used.

Gradient Elution. 84:16 to 70:30, v/v, acetonitrile/water (pH 5), linear gradient changed over a period of 15 min; flowrate, 2 mL min^{-1}; wavelength of UV detector, 210 nm, sensitivity adjusted to 0.04 AUFS; ambient temperature (*13*).

Isocratic Elution. 80:20, v/v, acetonitrile/water (pH 5), flowrate 2 mL min^{-1}; wavelength of UV detector, 210 nm, sensitivity adjusted to 0.04 AUFS; ambient temperature.

The compounds in each sample were identified by comparing their retention times with those of the standards referenced previously. Chromatographic characteristics of stevioside and rebaudioside A are shown in Table 1.

RESULTS AND DISCUSSION

The analytical method developed was compared with the Makapugay et al. (*13*) procedure, and many advantages were found. Figure 1 is a block diagram showing the simplicity of the developed analytical method.

Efficiency of Extraction. The wide range of stevioside and rebaudioside A content found in plant material requires a process of optimizing the extraction conditions. Several extracts were prepared using ethanol in the range from 100% to 40% (w/w), while the extraction

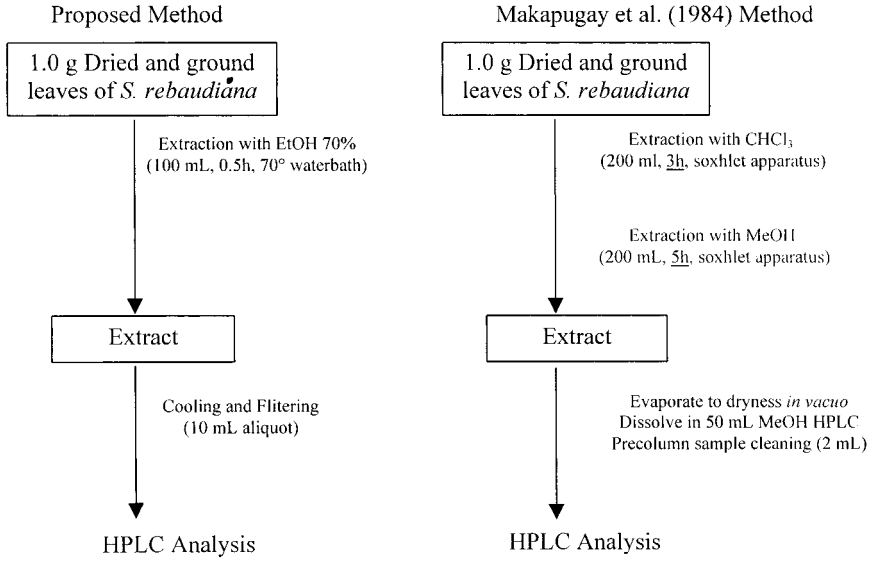

Figure 1. Comparison of analytical methodologies.

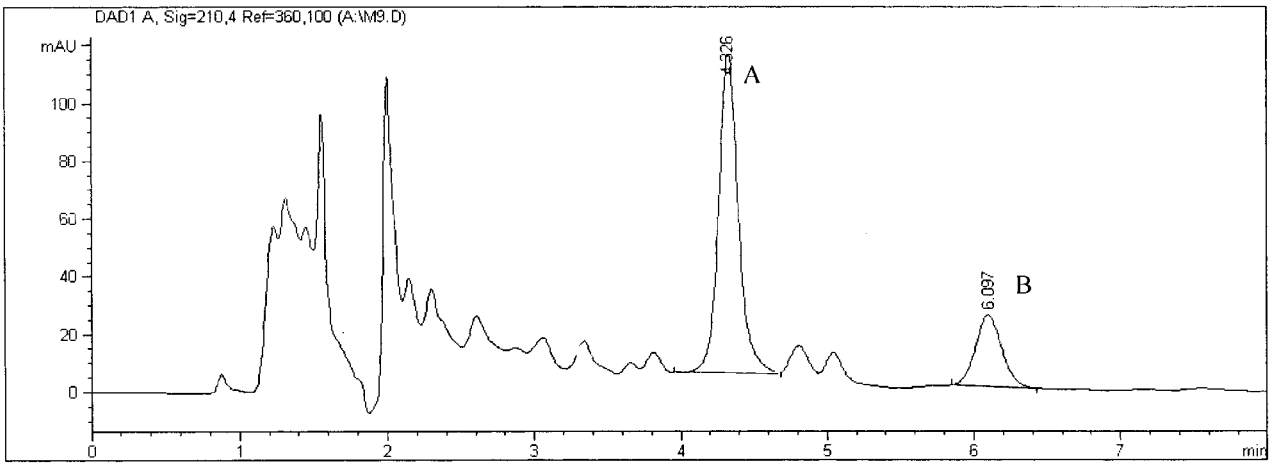

Figure 2. Typical HPLC chromatogram from *S. rebaudiana* extract. Isocratic elution mode recorded at 210 nm (0.04 AUFS): (A) stevioside, (B) rebaudioside A.

temperature was varied from ambient to 78 °C for EtOH 100% and 88 °C for EtOH 40%.

As it was required to reach an effectiveness close to that of the Soxhlet procedure, the extraction time was also studied. A series of experiments was performed with the solvent mixture, EtOH 70% (w/w), and temperature, 70 °C, obtained from the step before, while the extraction time was changed from 10 min to 120 min. It was found that concentration reached a maximum value and remained constant after 30 min of shaking in the water bath.

The above procedure led to an optimal solvent extraction mixture of EtOH 70% (w/w), an extraction temperature of 70 °C, and an extraction time of 30 min.

Chromatographic Analysis. As was expected, the chromatographic analysis time was reduced significantly. In the gradient elution mode the HPLC analysis takes longer than 15 min, taking into account the time to achieve the separation, and the equilibration time (5 min) back to initial conditions.

Working under an isocratic elution mode, only 7 min was required to separate the components of interest, without affecting resolution (Figure 2). No interference with other analytes or degradation products was observed to date.

All HPLC columns used for the experiments have shown a very good performance for more than 1000 analyses without regeneration.

Repeatability. The precision was established from experiments with one sample. Each method was applied on 10 replicates and the precision was evaluated by measuring the coefficient of variation (CV) of stevioside and rebaudioside A contents in the sample. In the method of Makapugay et al., the precision found was 2.15% for stevioside and 2.96% for rebaudioside A, and in our method the precisions were 2.25% and 3.03% for stevioside and rebaudioside A, respectively (Table 2). Both methods fall under the same order of magnitude of precision.

Recovery of Added Stevioside and Rebaudioside A. The accuracy of the method (Table 3) was determined by adding amounts of pure crystals of stevioside (20, 40, 60, and 80 mg/g) and rebaudioside A (10, 20, 30, and 40 mg/g) to three samples with different contents of these analytes, and analyzing each according to the experimental method. It was found that the recovery of added stevioside ranged from 96 to 101%, and the recovery of added rebaudioside A ranged from 99 to 103%.

Table 2. Study of Precision of the Determination of Stevioside and Rebaudioside A in Leaves of *S. rebaudiana* Using Soxhlet Extraction and Gradient HPLC Method (Method 1), and the Fast Extraction and Isocratic HPLC Method (Method 2)

	method 1		method 2	
	stevioside	rebaudioside A	stevioside	rebaudioside A
n	10	10	10	10
mean[a]	8.67	3.16	8.43	3.18
SD	0.18	0.09	0.19	0.09
% CV	2.15%	2.96%	2.25%	3.03%

[a] Reported as percent content in weight (dry basis).

Table 3. Study of the Recovery of the Determination of Stevioside and Rebaudioside A in Leaves of *S. rebaudiana* Using the Fast Extraction and Isocratic HPLC Method

added (mg/g) stevioside − rebaudioside A	recovery% stevioside	recovery% rebaudioside A
Sample 1		
20−10	98.7%	97.1%
40−20	99.3%	99.8%
60−30	102.1%	98.1%
80−40	98.4%	99.2%
mean	**99.6%**	**98.5%**
SD	0.0168	0.0119
% CV	1.69%	1.21%
Sample 2		
20−10	98.8%	97.9%
40−20	96.4%	100.2%
60−30	99.3%	100.6%
80−40	99.5%	103.1%
mean	**98.5%**	**100.5%**
SD	0.0142	0.0211
% CV	1.44%	2.10%
Sample 3		
20−10	98.2%	94.9%
40−20	98.4%	100.2%
60−30	101.0%	102.6%
80−40	103.1%	99.3%
mean	**100.2%**	**99.2%**
SD	0.0233	0.0321
% CV	2.33%	3.24%

Contents of Stevioside and Rebaudioside A in the Leaves of *Stevia rebaudiana* Analyzed. Thirty

Table 4. Stevioside and Rebaudioside A Contents of Several Samples of *S. rebaudiana* Using the Fast Extraction and Isocratic HPLC Method

sample	stevioside[a]	rebaudioside A[a]	total content[a]
1	3.78	2.60	6.38
2	3.80	3.46	7.26
3	4.76	2.10	6.86
4	4.79	3.18	7.97
5	5.24	1.62	6.86
6	5.29	2.04	7.33
7	5.78	3.28	9.06
8	5.79	5.08	10.87
9	5.83	7.01	12.84
10	5.93	4.85	10.78
11	6.04	2.13	8.17
12	6.20	5.47	11.67
13	6.23	2.86	9.09
14	6.42	2.42	8.84
15	6.72	4.86	11.58
16	6.94	2.37	9.31
17	7.05	3.23	10.28
18	7.20	3.92	11.12
19	7.42	4.57	11.99
20	7.52	6.17	13.69
21	7.63	2.20	9.83
22	8.07	5.48	13.55
23	8.11	4.89	13.0
24	8.18	6.19	14.37
25	8.57	3.18	11.75
26	8.89	4.28	13.17
27	8.92	7.27	16.19
28	9.06	3.92	12.98
29	9.12	6.88	16.0
30	9.84	3.08	12.92

[a] Reported as percent content in weight (dry basis).

samples of *S. rebaudiana* obtained from different places in Misiones (Northeastern Argentina) were analyzed. The amounts of stevioside and rebaudioside A found in each sample are shown in Table 4. It can be seen that there is a wide range of contents for both analytes and total sweet diterpene glycosides content. This may be related to the time of harvesting through the year as well as to the stage of development of the plant.

Also, a wide variability was found in the ratio rebaudioside A/stevioside which ranges from 0.28 to 1.2 in *S. rebaudiana*. This ratio should be as high as possible considering rebaudioside A has better sweetening properties than stevioside.

Research studies of a number of plant specimens having a good ratio of rebaudioside A/stevioside and total glycosides content are necessary to develop a scale-up procedure for extraction and purification of this natural sweetener.

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