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Note

Improved high-performance liquid chromatographic separation of the *Stevia rebaudiana* sweet diterpene glycosides using linear gradient elution*,**

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Products obtained from the Paraguayan herb *Stevia rebaudiana* (Bertoni) Bertoni are currently commercially available for sweetening purposes in both Japan and Brazil[1,2]. In Paraguay, teas made from *S. rebaudiana* are prescribed by physicians for the treatment of diabetes, as a result of the reported hypoglycemic activity of this plant[3]. The cultivation of *S. rebaudiana* has been reported to occur not only in Japan, Brazil and Paraguay, but also in South Korea, Taiwan, Thailand, Indonesia and Laos[1,3–5]. Climatic conditions in California are also suitable for the growth of this species[6]. In addition, interest in *S. rebaudiana* has recently been expressed in the People's Republic of China[7].

Several investigators have devised high-performance liquid chromatographic (HPLC) analytical methods for the separation and quantitation of the *S. rebaudiana* sweet steviol (13-hydroxy-*ent*-kaurenoic acid) glycosides[8–17]. Such methods have been applied either to plant material[8–10,12,15,16] or to products[13,14,17] containing these sweet compounds. However, the separation of all eight of the known *S. rebaudiana* sweet diterpene glycos ides, namely stevioside, steviolbioside, rebaudiosides A–E and dulcoside A, has been achieved by only one group to date[15,16]. In the present communication, we report a more rapid, better resolved HPLC separation of the eight known *S. rebaudiana* sweet steviol glycosides, using an NH_2 phase-bonded column with linear gradient elution. The applicability of this method to the analysis of *S. rebaudiana* leaf samples of diverse geographic origin is demonstrated.

EXPERIMENTAL

Plant material

Five leaf samples of *S. rebaudiana* (Bertoni) Bertoni, of different geographic origins, were investigated. A sample grown in South Korea was provided by Oriental Pharma Company, Seoul, Korea, and samples cultivated in the People's Republic of

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China and Taiwan were supplied by ABIC International Consultants, Pine Brook, NJ, U.S.A. Two commercial samples of *S. rebaudiana* leaves of Paraguayan origin, manufactured by L. E. de Gasperi, Asunción, Paraguay, and Laboratories Miracle S.R.L., Asunción, Paraguay, respectively, were also investigated in this study. An additional quantity of *S. rebaudiana* leaves, cultivated in California and used for isolation work, was provided by Stevia, Arlington Heights, IL, U.S.A.

Chemicals

Quantities of stevioside, rebaudiosides A, C, D and E, and dulcoside A, were isolated from *S. rebaudiana* leaves, cultivated in California, as previously described[18,19]. Steviolbioside and rebaudioside B were obtained from stevioside and rebaudioside A, respectively, by alkaline hydrolysis[18]. All isolates exhibited data [m.p., $[\alpha]_D$, ultraviolet (UV) and infrared (IR) spectra, thin-layer chromatography (TLC)] consistent with literature values[19–23]. All test compounds were tested for purity by analytical TLC prior to use in the present study.

Apparatus

HPLC separations were carried out on a Beckman (Berkeley, CA, U.S.A.) Model 324 gradient liquid chromatograph, equipped with a 421 microprocessor system controller, Beckman Model 100A and 110A pumps with an added stop-flow valve, a dynamically stirred gradient mixing chamber, and a Model 210 sample injector valve, linked to a Perkin-Elmer (Norwalk, CT, U.S.A.) LC-85 variable-wavelength UV spectrophotometer detector with LC-75 Autocontrol, and an Altex C-R1A (Shimadzu Seisakusho, Kyoto, Japan) recorder.

Solvents

Solvents used in this study were of HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.), and were degassed through Ultipor NR 0.2-μm membrane filters (Rainin Instrument Co., Woburn, MA, U.S.A.), held in a microfiltering apparatus (Sibata, Tokyo, Japan).

High-performance liquid chromatography

The following operation conditions for HPLC were employed: column, Zorbax NH_2, 25 cm × 0.4 mm I.D. (DuPont, Wilmington, DE, U.S.A.); eluting solvent, 84–70% v/v acetonitrile–water (pH 5), changed over a period of 15 min; flow-rate, 2 ml/min; wavelength of UV detector, 210 nm; initial pressure, 1200 p.s.i.; sensitivity setting, 0.04 a.u.f.s.; recorder chart speed, 20 mm/min; temperature, ambient.

Beer's law curves were obtained from triplicate injections of different amounts of each standard diterpene glycoside, dissolved in methanol, as follows: stevioside, rebaudioside B, dulcoside A, 0.4, 0.8, 1.28, 1.6, 2.4, 3.2, 4.8 and 6.4 μg per injection; rebaudioside A and rebaudioside C, 0.8, 1.28, 1.6, 2.4, 3.2, 4.8 and 6.4 μg per injection; steviolbioside, 0.4, 0.8, 1.28, 1.6, 2.4, 3.2 and 4.8 μg per injection; rebaudioside E, 0.8, 1.28, 1.6, 2.4, 3.2 and 4.8 μg per injection, and rebaudioside D, 0.8, 1.28, 1.6, 2.4 and 3.2 μg per injection. Peak height measurements were made for each compound. In a similar manner, a Beer's law curve for dulcoside A was obtained at the 0.02 a.u.f.s. sensitivity setting. The weights of this compound applied per injection were 0.16, 0.48, 0.80, 1.2 and 1.6 μg.

Extraction of plant material

Powdered, dried *S. rebaudiana* leaf samples (1 g) were extracted in a Soxhlet apparatus sequentially with chloroform (200 ml) for 3 h and methanol (200 ml) for 5 h. The plant marc was then refluxed with distilled water (20 ml) for 1 h. Analytical TLC of the chloroform and aqueous extracts indicated that no detectable amounts of the sweet diterpene glycosides were present in either case, for all samples studied.

Methanol extracts were evaporated to dryness *in vacuo*, and dissolved in 50 ml portions of HPLC grade methanol. Aliquots (2 ml) of each solution were passed through prepared pre-columns (glass, 20 cm × 5 mm I.D.), packed with Permaphase ETH (DuPont). Portions (5 μl) of each leaf sample were subjected to HPLC in triplicate, with peak heights and retention times of each peak being recorded.

Recovery experiments

Standard stevioside, rebaudioside A and rebaudioside C (7.0, 4.0 and 3.0 mg, respectively) were added to 1.0 g of a marc obtained by the exhaustive methanol extraction of *S. rebaudiana* leaves. The spiked marc was taken through the entire extraction procedure, as described above, and triplicate 10-μl aliquots were chromatographed at a sensitivity setting of 0.04 a.u.f.s. A similar procedure was conducted on a further 1.0 g of methanol-exhausted *S. rebaudiana* marc that was spiked with 2.0 mg of dulcoside A, and chromatographed at the 0.02 a.u.f.s. sensitivity setting.

RESULTS AND DISCUSSION

In Fig. 1 the separation achieved by gradient elution HPLC of a mixture of the sweet diterpene glycoside constituents of *S. rebaudiana* leaves is shown. It was found not to be necessary to derivatize these compounds, since an adequate sensitivity was demonstrated by setting the UV detector to 210 nm during this analytical work. The retention time determined for each solute is listed in Table I, which also lists the slopes, *y*-axis (peak height) intercepts, and correlation coefficients of linear regression lines calculated for each standard from Beer's law curve determinations. For each standard, the detector response was observed as linear over the weight range expressed in the Experimental section. We have in the present study extended the use of an NH_2 phase-bonded HPLC column, as applied in certain previous HPLC studies on the *S. rebaudiana* steviol glycosides[8,13,14] to the separation of all eight of the sweet glycosides that are accumulated by the leaves of this species. The separation described herein is an improvement over an earlier attempt to resolve these eight compounds by HPLC[15,16] in offering a shorter analysis time and baseline separation between each component of the mixture chromatographed.

The concentration levels of the sweet glycosides detected in five *S. rebaudiana* leaf samples were recorded in Table II. Fig. 1 shows a chromatogram of a sample extract of *S. rebaudiana* cultivated in the People's Republic of China, as analyzed by the present method. In order to obtain satisfactory analytical data for dulcoside A, a minor diterpene glycoside constituent of each of the five samples analyzed, it was necessary to evaluate each sample at two detector sensitivities. Thus, stevioside, rebaudioside A and rebaudioside C were analyzed in each sample at a detector setting of 0.04 a.u.f.s., and dulcoside A was determined in each case at 0.02 a.u.f.s. In ad-

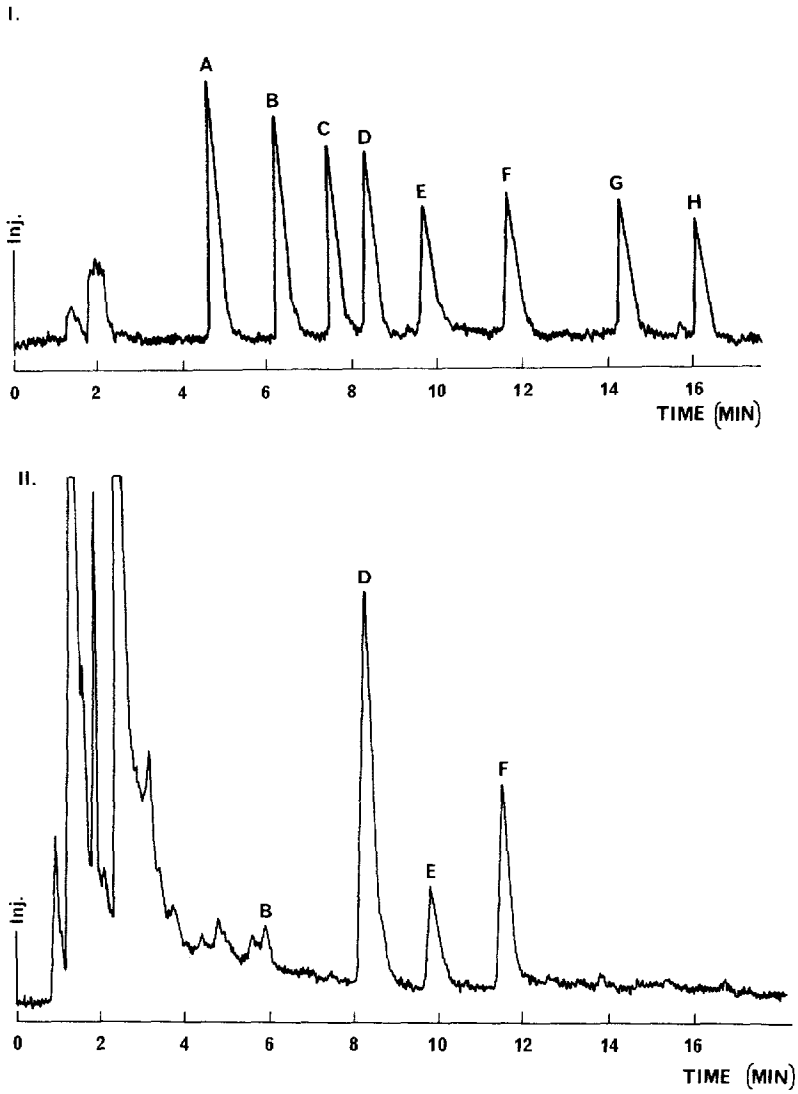

Fig. 1. Liquid chromatograms of sweet *ent*-kaurene glycosides. (I) Mixture of standard compounds. (II) Leaf extract of *Stevia rebaudiana* cultivated in the People's Republic of China. For operating conditions, see text. Peaks: A = steviolbioside; B = dulcoside A; C = rebaudioside B; D = stevioside; E = rebaudioside C; F = rebaudioside A; G = rebaudioside E; H = rebaudioside D.

dition to the satisfactory calibration data obtained for this compound at 0.04 a.u.f.s. (Table I), the detector response to dulcoside A at 0.02 a.u.f.s. was also found to be linear. A baseline correction mode was used in analyzing dulcoside A at 0.02 a.u.f.s. in the sample extracts. Recoveries of stevioside, rebaudioside A, rebaudioside C and dulcoside A in controlled experiments to determine the effectiveness of the extraction procedure for the plant material, were calculated as 96%, 89%, 90% and 92%, respectively. Steviobioside, rebaudioside B, rebaudioside D and rebaudioside E were

TABLE I

HPLC CHARACTERISTICS OF THE EIGHT KNOWN SWEET DITERPENE GLYCOSIDES OF *STEVIA REBAUDIANA*

Compound	*Retention time (min)**	*Slope***	*y-Axis intercept***	*Correlation coefficient***	*Minimum detectable*** amount (μg)*
Steviolbioside	4.5	2.36	−0.43	0.989	0.4
Dulcoside A	6.5	1.67	−0.11	0.992	0.4§
Rebaudioside B	7.4	1.56	−0.17	0.985	0.4
Stevioside	8.8	0.11	+1.40	0.999	0.4
Rebaudioside C	10.1	1.18	−0.11	0.991	0.8
Rebaudioside A	11.9	1.12	+0.13	0.989	0.8
Rebaudioside E	14.2	0.93	+0.19	0.992	0.8
Rebaudioside D	16.5	0.73	+0.23	0.976	0.8

* For conditions of chromatography, see Experimental.
** From peak height determinations.
*** UV detector 0.04 a.u.f.s.
§ Maximum detectable amount 0.15 μg with UV detector 0.02 a.u.f.s.

not detected in any of the extracts examined, because of their lack of abundance in these samples. It was established in separate experiments that all four of these compounds were extracted into the solvents used in this analytical method.

The sweet diterpene glycoside constituents of *S. rebaudiana* leaves have not only been quantitated by other groups using HPLC[8–10,22,15,16], but also by other methods comprising TLC–colorimetry[24], TLC–densitometry[25], gas–liquid chromatography[26,27], droplet counter-current chromatography[18], and an enzymatic procedure[28]. In providing a simultaneous assay of four underivatized *S. rebaudiana* sweet constituents, with a rapid analysis time and high specificity, the present method would appear to be highly advantageous when compared with these alternative approaches.

TABLE II

SWEET DITERPENE GLYCOSIDE LEVELS IN *STEVIA REBAUDIANA* LEAF SAMPLES OF DIVERSE GEOGRAPHIC ORIGIN DETERMINED BY HPLC

For conditions of chromatography, see Experimental.

Sample	*Source*	*Percentage (w/w) of dried leaf*			
		*Stevioside**	*Rebaudioside A**	*Rebaudioside C**	*Dulcoside A***
1	South Korea	5.5	2.5	1.4	0.66
2	People's Republic of China	6.6	3.7	2.1	0.53
3	Taiwan	8.1	3.5	1.4	0.53
4	Paraguay***	4.6	1.9	0.85	0.41
5	Paraguay§	5.5	3.4	1.5	0.54

* Determined with UV detector 0.4 a.u.f.s.
** Determined with UV detector 0.2 a.u.f.s.
*** Sample manufactured by L. E. de Gasperi, Asunción, Paraguay.
§ Sample manufactured by Laboratorios Miracle S.R.L., Asunción, Paraguay.

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