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Note

Stevia rebaudiana

III. High-performance liquid chromatographic separation and quantitation of rebaudiosides B, D, and E, dulcoside A, and steviolbioside*

M. S. AHMED** and R. H. DOBBERSTEIN*
Stevia Company, Inc., 746 West Algonquin Road, Arlington Heights, IL 60005 (U.S.A.)
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The sweet diterpene glycosides of *Stevia rebaudiana* Bert. (Compositae) have attracted much attention in recent years as possible sugar substitutes. Stevioside, rebaudioside A and rebaudioside C are the major sweet constituents[1,2], while rebaudiosides D and E and dulcoside A are reported to be minor constituents[3,4]. Although Khoda *et al.*[5] reported the natural occurrence of rebaudioside B and steviolbioside in the plant, other workers believe that they are artefacts obtained by the hydrolysis of rebaudioside A and stevioside, respectively, during extraction and purification[6].

In previous communications, we reported the high-performance liquid chromatographic (HPLC) analysis of stevioside and rebaudioside A derivatives[7], as well as the separation and quantitation of underivatized stevioside, rebaudioside A, and rebaudioside C[8]. The latter publication also included a procedure for the efficient extraction of the diterpene glycosides from plant material. Other workers have also reported the HPLC separation and quantitation of underivatized stevioside and rebaudioside A[9,10]. This work reports the HPLC separation and quantitation of all eight known *Stevia rebaudiana* diterpene glycosides, which we believe have not been previously reported.

EXPERIMENTAL

Stevia rebaudiana glycosides

Stevioside, rebaudioside A, and rebaudioside C were isolated by the authors from the leaves of *S. rebaudiana* as previously described[7]. Steviolbioside and rebaudioside B were prepared by alkaline hydrolysis[7] of stevioside and rebaudioside A, respectively. Rebaudiosides D and E were kindly supplied by Professor O. Tanaka, School of Medicine, Hiroshima University, Hiroshima, Japan. Dulcoside A was obtained through the courtesy of Professor M. Kobayashi, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan.

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** Present address: Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, Cairo, Egypt.

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Apparatus

Liquid chromatographic separations were conducted with a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A liquid chromatograph equipped with a Rheodyne (Berkely, CA, U.S.A.) Model 7120 syringe-loading sample injector and a 100-μl sample loop, a Waters Assoc. Model 450 variable-wavelength spectrophotometer, and a Texas Instruments (Houston, TX, U.S.A.) Servo/Riter II portable recorder. Separations were carried out on two Waters Assoc. 30 × 0.78 cm I.D. Protein I-125 columns.

Solvents

Solvents for HPLC were reagent or certified grade and were redistilled in glass and degassed prior to use.

Stevia rebaudiana extraction for HPLC analysis

Dried, powdered (30 mesh) *S. rebaudiana* leaves (0.5 g) were extracted as previously described[8].

High-performance liquid chromatography

The operating conditions for HPLC were: ambient temperature, flow-rate of eluting solvent, 1-propanol, 1.0 ml/min; wavelength of UV detector, 210 nm; recorder chart speed, 3 in./h at 0.04 a.u.f.s. Standard solutions of rebaudiosides B, D, and E, dulcoside A, and steviolbioside were injected onto the column and their retention times determined.

Beer's law standard curves were obtained by injecting different quantities of the diterpene glycosides (dulcoside A, 0.5, 1, 5, 10, and 20 μg per 100 μl injection; steviolbioside and rebaudioside B, 5, 10, 20, 40, and 80 μg per 100 μl injection; and rebaudiosides D and E, 3.25, 7.5, 15, 30, and 60 μg per 100 μl injection) onto the column in triplicate and measuring the resulting peak heights.

RESULTS AND DISCUSSION

Our earlier publication[7] reported the HPLC analysis of stevioside and rebaudioside A following their hydrolysis to steviolbioside and rebaudioside B, respectively, and their subsequent esterification with *p*-bromophenacyl bromide. It was noted, however, that alkaline hydrolysis of rebaudiosides D and E also yielded rebaudioside B and steviolbioside, respectively. After many unsuccessful attempts to separate stevioside, rebaudioside A, and rebaudioside C and their derivatives, these underivatized glycosides were successfully separated on two Protein I-125 columns by 1-propanol as previously described[8]. When authentic samples of rebaudiosides B, D, and E, dulcoside A, and steviolbioside were obtained, it was determined that they were also resolved under the same chromatographic conditions. All of the glycosides gave linear detection responses when concentrations of 0.2–100 μg were employed. Beer's law slopes, *y*-axis (peak height) intercepts, and correlation coefficients were determined by linear regression calculations, and are shown in Table I, along with other HPLC characteristics. Typical separations of the known diterpene glycosides and an extract of *S. rebaudiana* are shown in Fig. 1.

The data demonstrate that the eight known diterpene glycosides of *S. re-*

baudiana can be rapidly and accurately analyzed by HPLC on a double Protein I-125 column using the procedures described in this communication.

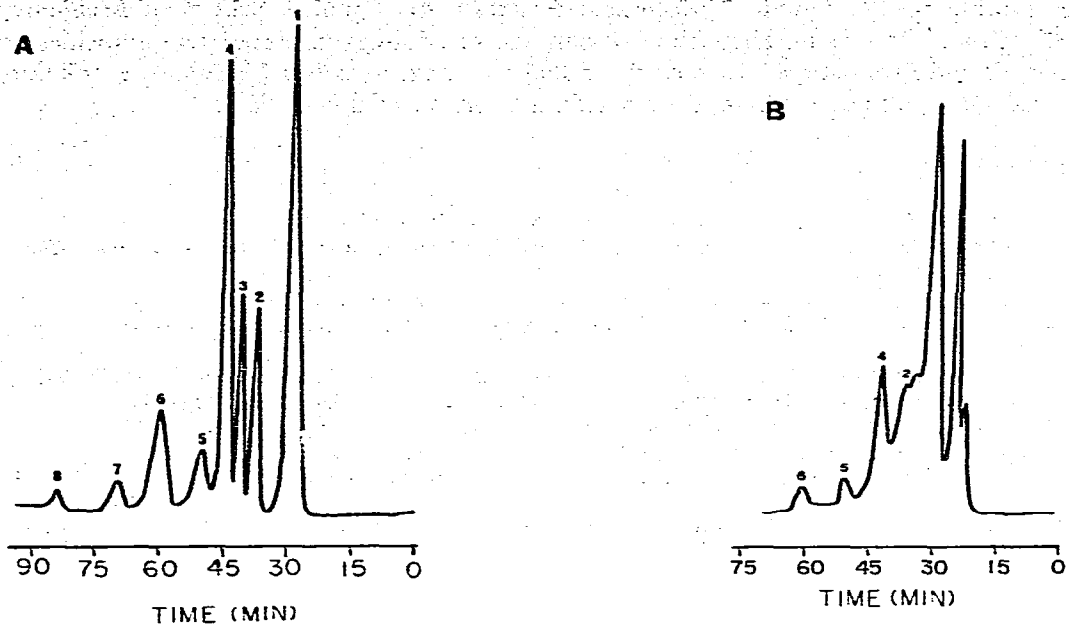

Fig. 1. Separation of *Stevia rebaudiana* diterpene glycosides. A, Mixture of standards; B, *Stevia rebaudiana* methanol extract. Operating conditions: two Protein I-125 columns in series; mobile phase, 1-propanol; flow-rate, 1 ml/min; detector, UV spectrophotometer (210 nm). Peaks: 1 = dulcoside A; 2 = steviolbioside; 3 = rebaudioside B; 4 = stevioside; 5 = rebaudioside C; 6 = rebaudioside A; 7 = rebaudioside E; 8 = rebaudioside D.

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF *STEVIA REBAUDIANA* DITERPENE GLYCOSIDES

Diterpene glycoside	*Retention time (min)**	*Minimum detectable amount (μg)*	*Slope*	*y-axis intercept*	*Correlation coefficient*
Dulcoside A	27.5	0.2	6.54	+ 3.65	0.998
Steviolbioside	37.7	0.5	2.33	+ 8.69	0.999
Rebaudioside B	40.9	0.5	1.55	+20.52	0.995
Rebaudioside E	65.0	2.0	1.39	+ 1.14	0.999
Rebaudioside D	84.0	2.0	1.29	− 3.18	0.998
Stevioside	43.2	1.0	1.68	− 1.16	0.999
Rebaudioside C	49.5	2.0	1.22	+ 0.52	0.999
Rebaudioside A	59.0	2.0	0.91	+ 0.35	0.999

* Chromatographic conditions as described under Experimental. The coefficient of variation for retention times varied from 0.11% to 2.30%. The mean coefficient of variation was 1.05%.

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