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Note

Potential sweetening agents of plant origin

I. Purification of *Stevia rebaudiana* sweet constituents by droplet counter-current chromatography*

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Stevia rebaudiana Bertoni (Compositae) is a sweet herb indigenous to elevated terrain on the borders of Brazil and Paraguay[1]. Eight sweet constituents have so far been isolated from this plant, namely, stevioside, steviolbioside, rebaudiosides A–E and dulcoside A[2–7]. In the last decade *S. rebaudiana* extracts and isolates have been used commercially in Japan to sweeten a variety of food products[8]. Currently, there is a great deal of interest in the potential use of stevioside and other *S. rebaudiana* constituents as non-nutritive sweetening agents for other markets around the world[9–13].

Our group recently became involved in a multi-disciplinary investigation of the physico-chemical and biological properties of certain plant-derived sweeteners. Prior to any biological testing, we have studied the applicability of a number of phytochemical isolation methods to purify individual sweet constituents of the plants under investigation. In this communication we wish to present details of a droplet counter-current chromatographic (DCCC) method for the rapid purification of stevioside, the most abundant sweet *ent*-kaurene glycoside from *S. rebaudiana*[2–4]. In addition, the suitability of this technique for the separation of other known *n*-butanol-soluble sweet principles of this plant (Fig. 1) will be discussed. DCCC has been used successfully for the separation of a variety of polar plant principles[14–17].

EXPERIMENTAL

Plant material

Stevia rebaudiana Bertoni (Compositae) was collected in its native habitat at

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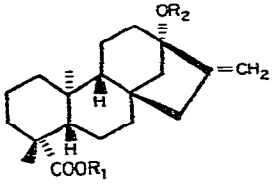

COMPOUND	R_1	R_2
Stevioside	-G	$-G^{2}\!-\!{}^{1}G$
Steviolbioside	-H	$-G^{2}\!-\!{}^{1}G$
Rebaudioside A	-G	$-G^{2}_{3}\!<\!{}^{1}G,\ {}^{1}G$
Rebaudioside B	-H	$-G^{2}_{3}\!<\!{}^{1}G,\ {}^{1}G$
Rebaudioside C (Dulcoside B)	-G	$-G^{2}_{3}\!<\!{}^{1}Rh,\ {}^{1}G$
Dulcoside A	-G	$-G^{2}\!-\!{}^{1}Rh$

G = glucose
Rh = rhamnose

Fig. 1. Structures of *n*-butanol-soluble sweet *ent*-kaurene glycosides from *Stevia rebaudiana*.

Cerro Kuatiá, near P. J. Caballero, Paraguay in April, 1981, by one of us (D.D.S.). Voucher specimens representing this collection have been deposited at the John G. Searle Herbarium of the Field Museum of Natural History, Chicago. An additional quantity of *S. rebaudiana* leaves, cultivated in California, was provided by Stevia Inc. (Arlington Heights, IL, U.S.A.).

Apparatus

DCCC separations were carried out on a Model-A instrument (Tokyo Rikakikai, Tokyo, Japan). Preliminary separations of certain *S. rebaudiana* sweet principles were achieved using preparative high-performance liquid chromatography (HPLC) on a Jobin-Yvon Chromatospac Prep 100 liquid chromatograph (Instruments SA, Metuchen, NJ, U.S.A.). Melting points were determined on a Kofler hot-stage instrument and are uncorrected, and optical rotations were obtained on a Perkin-Elmer 241 polarimeter. Ultraviolet (UV) and infrared (IR) spectra were recorded, respectively, using a Beckman Model DB-G grating spectrophotometer and a Beckman IR-18A spectrophotometer (polystyrene calibration at 1601 cm^{-1}).

Chemicals

Authentic samples of stevioside, rebaudioside A and rebaudioside C were generously provided by Professor O. Tanaka, Hiroshima University, Hiroshima, Japan. A reference sample of dulcoside A was donated by Professor H. Mitsuhashi, Hokkaido University, Sapporo, Japan.

Larger quantities of these *n*-butanol-soluble *S. rebaudiana ent*-kaurene glycosides were generated in the following manner. Dried *S. rebaudiana* leaves were macerated in methanol–water (4:1) and defatted with ethyl acetate. The diterpene glycosides listed in Fig. 1 were then partitioned into *n*-butanol from water, and a portion of the *n*-butanol fraction was subjected to preparative HPLC over silica gel 60 (Merck, Darmstadt, G.F.R.) eluted with mixtures of chloroform–methanol–water[4], using an elution pressure of 6 atm. Elution with chloroform–methanol–water (45:9:1) yielded separate fractions containing dulcoside A and stevioside, while elution with chloroform–methanol–water (45:12:1.5) yielded separate fractions containing rebaudioside A and rebaudioside C. Stevioside and rebaudioside A were isolated in a pure form without further chromatographic frationation by recrystallization from methanol. Additional small-scale gravity column chromatography over silica gel 60 of appropriate HPLC column cuts, using a similar elution profile to that listed above, was necessary to obtain dulcoside A and rebaudioside C in a form free from contamination by diterpene glycoside congeners. Dulcoside A was then obtained pure by recrystallization from methanol, while rebaudioside C was finally purified using the DCCC system described herein.

Isolated stevioside, rebaudioside A, rebaudioside C and dulcoside A exhibited closely comparable data [m.p., $[\alpha]_D$, IR, UV, thin-layer chromatography (TLC)] to authentic samples of these compounds.

Steviolbioside and rebaudioside B were obtained from stevioside and rebaudioside A, respectively, by alkaline hydrolysis according to published conditions[18]. These reaction products, when purified by recrystallization from methanol provided m.p., optical rotation and TLC data consistent with literature values[3,4,19].

Extraction and fractionation of S. rebaudiana leaves

Milled *S. rebaudiana* leaves (10 g), collected in Paraguay from a native habitat, were macerated in 4 × 200 ml methanol–water (4:1). Macerates were combined and solvent was removed under reduced pressure to yield 3.5 g of dried residue. This residue was dissolved in water (200 ml) and defatted with 7 × 100 ml ethyl acetate. The aqueous layer was then partitoned with *n*-butanol (6 × 50 ml), and the combined organic layer washed with water and evaporated to dryness to produce a residue. This extractive was dissolved in methanol (30 ml), stood at 5°C for 12 h, and crude stevioside (0.8 g) was precipitated, filtered, washed with methanol and dried in a vacuum oven. The remaining filtrate, constituting the *S. rebaudiana n*-butanol fraction minus the majority of the stevioside originally present, was evaporated to dryness to produce 1.3 g of a solid.

Droplet counter-current chromatography

The solvent system chloroform–methanol–isopropanol–water (11:9:4:8) was used for all DCCC separations reported in this communication. Ascending development was employed, with the upper layer of the partitioned solvent acting as the mobile phase. Solutes were dissolved in the stationary phase (10 ml) and injected into a 10-ml sample chamber. Fractions (5 ml) were collected at a rate of 10 ml/h in a Buchler Fractomette Alpha-200 (Buchler, Fort Lee, NJ, U.S.A.) automatic fraction collector, and were weighed on removal of solvent. All separations were carried out at ambient temperature.

RESULTS AND DISCUSSION

In Fig. 2 a DCCC elution chromatogram of crude stevioside (200 mg), obtained by methanol precipitation from an *n*-butanol fraction of *S. rebaudiana* collected in the field in Paraguay, is presented. Pure stevioside (120 mg; 4.8%, w/w; m.p. 202–204°C) (lit.[3] 198–202°C), uncontaminated by any other *S. rebaudiana* constituents, including rebaudioside C (see below), was collected as crystals in fractions 31–42 on removal of solvent. A total solvent consumption of only 750 ml was needed for the DCCC purification of stevioside and the isolation was achieved within 36 h after the commencement of the solvent partitioning of *S. rebaudiana* leaves. This process was highly reproducible and required very little technical manipulation during the DCCC operation.

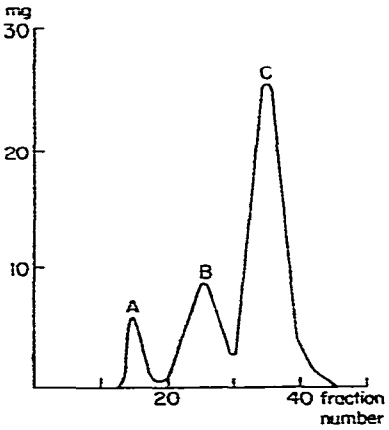

Fig. 2. DCCC elution chromatogram of crude stevioside (200 mg), crystallized by methanol from an *n*-butanol fraction of *S rebaudiana*. Solvent system, chloroform–methanol–isopropanol–water (11:9:4:8), upper layer used as mobile phase. A = Solvent front; B = rebaudioside A; C = stevioside.

Fig. 3 shows the separation obtained when equal weights (30 mg) of the diterpene glycosides listed in Fig. 1 were submitted to DCCC. Compounds were eluted in a series representative of the number of sugar units attached to the *ent*-kaurene nucleus, with the more polar compounds being eluted before less polar compounds. While there are considerable differences in polarity in the six compounds shown in the DCCC chromatogram in Fig. 3, the efficiency of the method was such that almost complete resolution of every component in the mixture was obtained. Attempts to resolve rebaudioside C and stevioside better by varying the solvent composition were unsuccessful. Rebaudiosides D and E, two sweet *S. rebaudiana* constituents omitted from Fig. 1 because they are more soluble in water than in *n*-butanol, were not included in the present investigation.

Rebaudioside C and residual stevioside were also found to elute together when 160 mg of the Paraguayan *S. rebaudiana* *n*-butanol fraction, after crude stevioside removal, was submitted to DCCC (Fig. 4). Therefore, a preliminary separation procedure is necessary to resolve rebaudioside C from stevioside before DCCC, using this solvent system, may be applied to the purification of rebaudioside C from *S.*

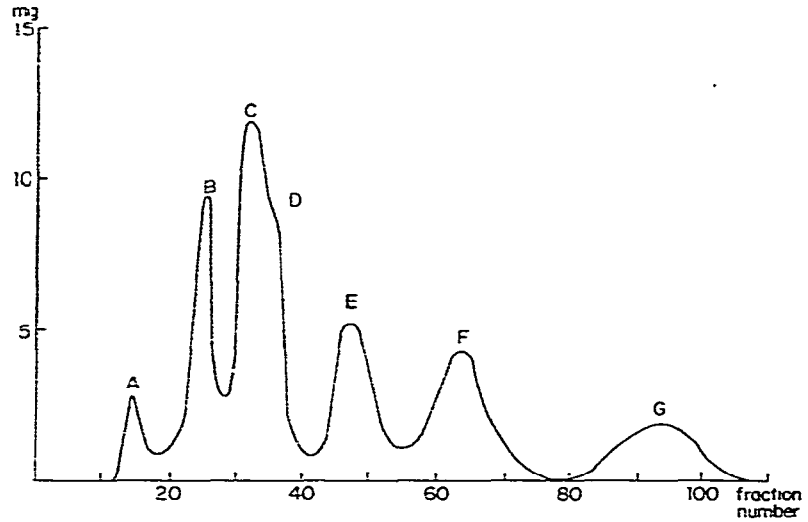

Fig. 3. DCCC elution chromatogram of a mixture (30 mg per compound) of *S. rebaudiana* sweet principles. A = Solvent front; B = rebaudioside A; C = rebaudioside C; D = stevioside; E = dulcoside A; F = rebaudioside B; G = steviolbioside.

rebaudiana. However, pure rebaudioside A (40 mg; m.p., 236–238°C) (lit.[4] 242–244°C) and pure dulcoside A (15 mg; 0.9%, w/w; m.p. 188–190°C) (lit.[7] 193–195°C) were obtained after recrystallization from methanol of the solutes in fractions 21–29 and 42–52, respectively (Fig. 4). The combined yield of rebaudioside A from this *S. rebaudiana* sample, as obtained in the DCCC runs represented in Figs. 2 and 4, was 4.25% (w/w) (total weight obtained pure 65 mg). Rebaudioside B and steviolbioside were not detected in this Paraguayan source of *S. rebaudiana* leaves, although these compounds have been found as natural products in *S. rebaudiana* cultivated in Japan[4].

Thus the DCCC methodology described in this paper constitutes a rapid, direct preparative procedure for the small-scale isolation of stevioside, rebaudioside A and

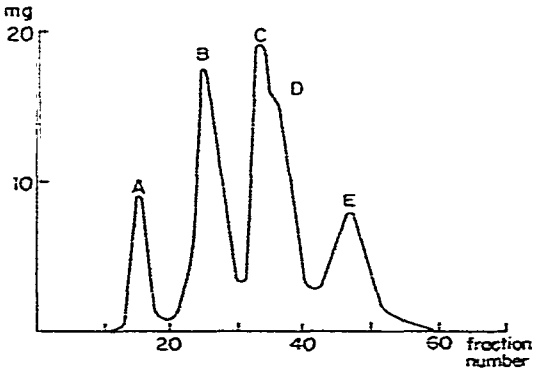

Fig. 4. DCCC elution chromatogram of an *n*-butanol fraction (160 mg), after methanol precipitation, of leaves of *S. rebaudiana* collected in Paraguay. A = Solvent front; B = rebaudioside A; C = rebaudioside C; D = stevioside; E = dulcoside A.

dulcoside A in high purity from a crude extract of *S. rebaudiana*. The method is also applicable to the final purification of the other main *n*-butanol-soluble *S. rebaudiana* sweet principle, rebaudioside C, after preliminary column chromatography has been performed to resolve this compound from stevioside.

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