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Note

Simultaneous determination of stevioside, rebaudioside A and C and dulcoside A in foods by high-performance liquid chromatography

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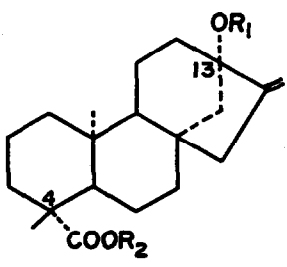
Some sweet-tasting compounds from the leaves of *Stevia rebaudiana* Bertoni, a plant native to Paraguay, have been reported[1,2]. These stevia sweeteners are similar in structure in that a steviol aglycone is connected at C-4 and C-13 to mono-, di- or trisaccharides consisting of glucose and/or rhamnose residues, as shown in Fig. 1. Stevioside has been shown to be the most effective sweetener, and in addition rebaudioside A and C and dulcoside A are also important. In Japan, food-grade stevia sweetener products have been used in a wide range of foods, so it is desirable to establish a simple, rapid and accurate method for the determination of these glycosides in various foods.

Various methods for the determination of stevioside and rebaudioside A after enzymatic or acidic hydrolysis of the stevia glycosides have been reported, including gas–liquid chromatography[3], thin-layer chromatography[4] and "thinchrography"[5], a combination of rod-type thin-layer chromatography and gas chromatography with flame ionization detection. However, these methods are non-specific for the determinaton of individual stevia sweeteners and are time consuming. Recently, several high-performance liquid chromatographic (HPLC) methods for the separation and determination of the stevia glycosides have been reported[6–12]. In particular, Ahmed and Dobberstein[9] and Makapugay *et al.*[11] developed excellent methods for the



	R_1	R_2
Stevioside	glc^{2}-glc	glc
Rebaudioside A	glc^{2,3}(glc, glc)	glc
Rebaudioside C	glc^{2}-rham, ^{3}-glc	glc
Dulcoside A	glc^{2}-rham	glc

glc ; β-glucopyranosyl
rham ; α-rhamnopyranosyl

Fig. 1. Structures of stevioside, rebaudioside A and C and dulcoside A.

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separation and determination of eight *Stevia rebaudiana* diterpene glycosides. These methods have been applied either to plant materials[6,9,11] or sweetener products[7,8]. Two HPLC methods have been applied to actual foodstuffs[10,12], but included only two typical stevia glycosides, namely stevioside and rebaudioside A.

The simultaneous determination of the four stevia sweeteners including rebaudioside C and dulcoside A in foodstuffs is reported here. A clean up treatment for stevioside, rebaudioside A and C and dulcoside A in beverages, soy sauce, candy and pickled radish using a reversed-phase cartridge and an HPLC method for their determination is described.

EXPERIMENTAL

Stevioside, rebaudioside A and C and dulcoside A of analytical-reagent grade were supplied by Maruzenkasei (Hiroshima, Japan). Amounts of 25 mg of these standard sweeteners were accurately dissolved in 25 ml of water and diluted with the HPLC mobile phase to obtain final concentrations of 5, 10, 20, 50, 70 and 100 μg/ml.

The mobile phase for HPLC was prepared by mixing acetonitrile (HPLC grade, Kanto Chemical, Tokyo, Japan) and deionized and distilled water (80:20, v/v).

A Shimadzu Model LC-6A HPLC system (Shimadzu Seisakusho, Kyoto, Japan) equipped with a column oven (Shimadzu CTO-6A) was used to deliver the mobile phase at a flow-rate of 0.8 ml/min. A normal-phase LiChrosorb NH_2 (5 μm) column (250 × 4 mm I.D.) (Merck, Darmstadt, F.R.G.), thermostated at 50°C, and a Shimadzu SPD-6AV detector operated at 210 nm were used.

The sample solutions were prepared as follows.

Beverage and soy sauce

The sample (5 g) was treated in a Sep-Pak C_{18} cartridge (Millipore), which was pre-wetted with 5 ml each of acetone, methanol and water. The cartridge was washed with 3 ml of water and 10 ml of acetonitrile–water (80:20) solution and eluted with 2 ml of the HPLC mobile phase. The eluate was diluted to 5 ml and a 30-μl portion of the solution was subjected to HPLC analysis.

Candy

A 50-g portion of sample was crushed and mixed in a mortar. After 5 g of the mixture had been weighed into a 50-ml glass beaker, 20 ml of water were added and the mixture was heated on a hot-plate to dissolve the solid. The solution was filtered through No. 2 filter-paper (Toyo Roshi, Tokyo, Japan) and the beaker and the filter-paper were washed with 5 ml of hot water. After cooling, the combined filtrate was placed on a Sep-Pak C_{18} cartridge and treated in the same manner as for beverage and soy sauce.

Pickled radish

A 50-g portion of sample was finely sliced and mixed. After 10 g of the mixture had been weighed into a 50-ml volumetric test-tube, 35 ml of water were added and homogenized with a Polytron (Model PT10-35; Kinematica, Littau, Switzerland). The homogenizer was washed with 5 ml of water. The washings were transferred into the tube and the combined solution was diluted to 50 ml with water. After the solution had

been mixed and centrifuged at 12 600 *g* for 10 min, the supernatant was filtered through No. 2 filter-paper. The first 5 ml of the eluate were discarded and the subsequent 25 ml aliquot was placed on a Sep-Pak C_{18} cartridge and treated in the same manner as for beverage and soy sauce.

RESULTS AND DISCUSSION

Mixed aqueous solutions of stevioside, rebaudioside A and C and dulcoside A were treated on a Sep-Pak C_{18} cartridge for sample preparation, rinsed with 3 ml of water and 10 ml of 20% acetonitrile to remove co-extracts as much as possible, and then eluted with 2 ml of acetonitrile–water (80:20). These stevia sweeteners were never eluted from the cartridge with less than 10 ml of 20% acetonitrile.

As shown in Fig. 2, the use of a LiChrosorb NH_2 normal-phase column with UV detection at 210 nm gave a sufficient baseline separation for the determination of these stevia sweeteners without interferences from other food components.

The method was applied to the analysis of beverage, soy sauce, candy and pickled radish. Typical chromatograms of the four kinds of food extracts are shown in Fig. 3. The peaks of stevioside in beverage, dulcoside A in soy sauce, stevioside and rebaudioside C in candy and rebaudioside A and C in pickled radish suffered slight interference with the appearance of unknown peaks close to the main peaks.

Plots of peak heights of the four stevia sweeteners showed a linear correlation over the concentrations range from 5 to 100 μg/ml. Table I shows the recoveries of the sweeteners fortified with standards at 20 and 100 ppm. The added standards were recovered in the range from 87.9 to 99.7% at the 20 ppm level, and from 93.2 to 97.8% at the 100 ppm level. The limit of detection for these stevia sweeteners in foods was 5 ppm.

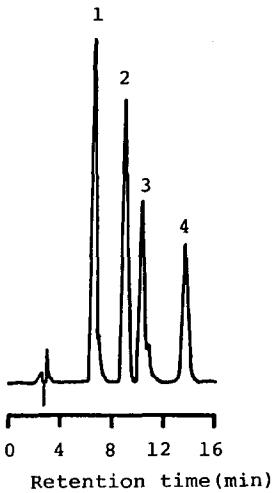

Fig. 2. Chromatogram of standard stevioside sweeteners (3 μg each). Peaks: 1 = dulcoside A; 2 = stevioside; 3 = rebaudioside C; 4 = rebaudioside A. Conditions: column, LiChrosorb NH_2 (5 μm) (250 × 4 mm I.D.); mobile phase, acetonitrile–water(80:20); flow-rate, 0.8 ml/min; detection, UV (210 nm); column temperature, 50°C.

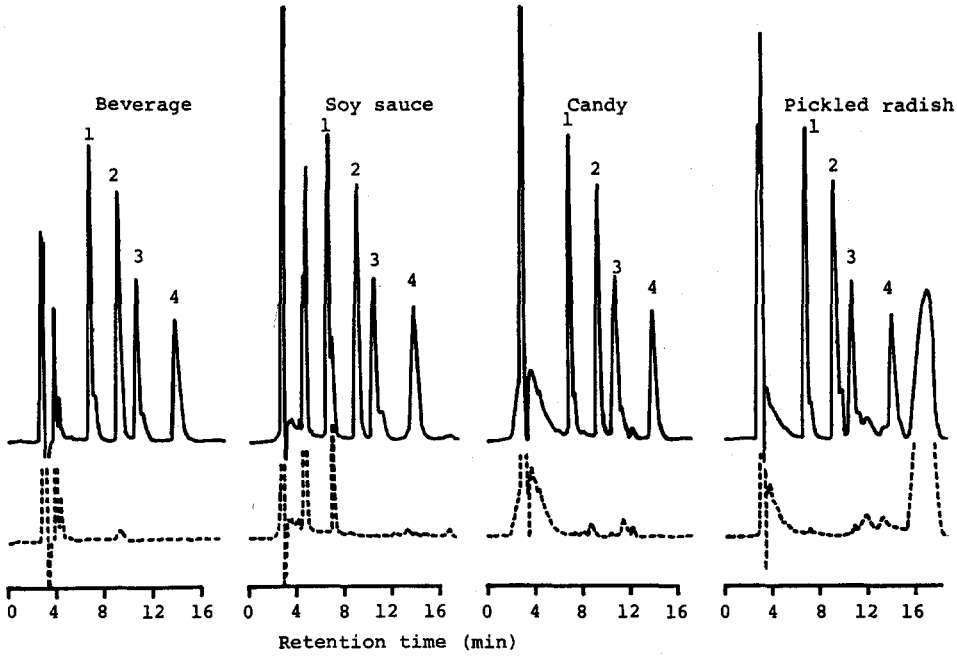

Fig. 3. Chromatograms of food samples without stevia sweeteners (broken curves), and the same samples after addition of 100 μg/g of stevia sweeteners (solid curves). Peak numbers as in Fig. 2.

In conclusion, the simultaneous determination of stevioside, rebaudioside A and C and dulcoside A in foods by HPLC was satisfactorily achieved. These stevia sweeteners were effectively separated, identified and quantitated on LiChrosorb NH_2 with detection at 210 nm. The proposed method allows the simple, rapid and accurate determination of the four stevia glycosides in foods, and is suitable for routine analysis.

TABLE I

RECOVERIES OF STEVIA SWEETENERS ADDED TO BEVERAGE, SOY SAUCE, CANDY AND PICKLED RADISH

Sample	*Added (μg/g)*	*Recovery[a] (%)*			
		Dulcoside A	*Stevioside*	*Rebaudioside C*	*Rebaudioside A*
Beverage	20	92.5 ± 1.1	100.2 ± 3.1	99.4 ± 3.3	100.0 ± 1.4
	100	94.1 ± 0.8	97.5 ± 0.9	95.6 ± 2.1	96.3 ± 1.1
Soy sauce	20	91.1 ± 1.9	91.5 ± 2.8	87.9 ± 1.8	90.6 ± 1.4
	100	95.9 ± 0.7	96.7 ± 0.8	96.8 ± 1.9	97.3 ± 1.3
Candy	20	90.8 ± 2.2	99.2 ± 3.6	98.6 ± 1.5	96.8 ± 2.7
	100	94.2 ± 1.3	95.9 ± 0.8	95.6 ± 1.5	96.6 ± 1.0
Pickled radish	20	94.3 ± 2.4	94.5 ± 2.3	91.1 ± 2.2	94.1 ± 3.1
	100	94.6 ± 1.8	93.5 ± 1.4	97.8 ± 2.6	93.2 ± 1.5

[a] Average ± standard deviation of four determinations.

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