GLYCOSIDES FROM *Stevia rebaudiana*

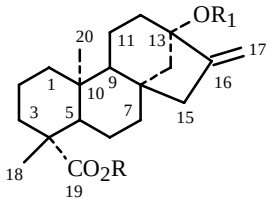
G. I. Kovylyaeva,[1] G. A. Bakaleinik,[1] I. Yu. Strobykina,[1]
V. I. Gubskaya,[1] R. R. Sharipova,[1] V. A. Al′fonsov,[1]
V. E. Kataev,[1] and A. G. Tolstikov[2]

UDC 547.913.6

A new laboratory method for isolating the glycosides stevioside and rebaudiosides A and C from leaves of Stevia rebaudiana *was proposed. According to HPLC, the glycoside contents in plants grown in Russia (Voronezh Oblast') and Ukraine (Crimea) were 5-6% (stevioside) and 0.3-1.3% (rebaudiosides A and C).*

Key words: glycosides, rebaudiosides, *Stevia rebaudiana* Bertoni, stevioside, steviol, isosteviol.

Leaves of *Stevia rebaudiana* Bertoni have been used for the last 20 years in countries of South America and Southeast Asia as a low-calorie sugar substitute [1]. Their sweetness is due to the glycosides stevioside (**1**), steviolbioside (**2**), and rebaudiosides A (**3**), B (**4**), C (**5**), D (**6**), E (**7**), F (**8**), and dulcoside A (**9**) [2]. Stevioside is 300 times sweeter than sugar but has a bitter aftertaste [1, 2]. The sweetness of rebaudiosides increases with increasing amount of sugar units bonded to the aglycon (steviol). However, their content in the plant material decreases at the same time [1-9].



1 - 9

1: R = β-Glc, R_1 = β-Glc^2-β-Glc
2: R = H, R_1 = β-Glc^2-β-Glc
3: R = β-Glc, R_1 = Glc^2-β-Glc
|3
β-Glc
4: R = H, R_1 = β-Glc^2-β-Glc
|3
β-Glc
5: R = β-Glc, R_1 = β-Glc^2-β-Rh
|3
β-Glc
6: R = β-Glc^2-β-Glc, R_1 = β-Glc^2-β-Glc
|3
β-Glc
7: R = β-Glc^2-β-Glc, R_1 = β-Glc^2-β-Glc
8: R = β-Glc, R_1 = β-Glc^2-β-Xyl
|3
β-Glc
9: R = β-Glc, R_1 = β-Glc^2-β-Rh

The quantitative glycoside composition of *S. rebaudiana* grown in Russia has not been reported. Therefore, the goal of the present work was to determine the contents of stevioside and rebaudiosides A and C in leaves of *S. rebaudiana* of Russian and Ukrainian origin.

1) A. E. Arbuzov Institute of Organic and Physical Chemistry, Kazan′ Scientific Center, Russian Academy of Sciences, 420088, Kazan′, ul. Arbuzova, 8, fax (843) 273 22 53, e-mail: kataev@iopc.knc.ru; 2) Institute of Chemical Engineering, Ural Division, Russian Academy of Sciences, Russia, 614990, Perm′, ul. Koroleva, 3. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 68-71, January-February, 2007. Original article submitted November 15, 2006.

0009-3130/07/4301-0081

TABLE 1. Quantitative Composition of Rebaudiosides in Various Samples of *S. Rebaudiana* Leaves

Collection site	Rebaudioside contents, % (per 100 g dry leaves)		
	Stevioside	Rebaudioside A	Rebaudioside C
Russia (Voronezh Oblast')	5.8	1.2	0.5
Ukraine (Crimea)	4.8	1.3	0.3
South Korea	5.5 [13, 14]	2.5 [13, 14]	1.4 [13, 14]
China	6.6 [13, 14]	3.7 [13, 14]	2.1 [13, 14]
Paraguay	4.6 [13, 14]	1.9 [13, 14]	0.9 [13, 14]
	9.1 [4]	3.8 [4]	0.6 [4]
Japan	7.7 [13, 14]	1.9 [13, 14]	0.9 [13, 14]
Canada	5.0 [12, 15]	0.3 [12, 15]	0.1 [12, 15]
Viet Nam	15.5 [12, 15]	3.8 [12, 15]	1.4 [12, 15]

Before the rebaudioside composition was analyzed quantitatively, we had to isolate the total glycosides and then the pure components from the plant material. The most complicated process in isolating rebaudiosides from the plant was the purification of the aqueous extracts from organic and inorganic impurities consisting of pigments (chlorophylls, xanthophylls, β-carotene, etc.), proteins, organic acids, resins, sesquiterpene lactones, etc. In addition to rather crude purification methods including only filtration of the aqueous extract through aluminum oxide (water eluent) [10], procedures combining several methods such as precipitation of impurities by hydroxides and purification over ion-exchange resins [11-13], precipitation by hydroxides and adsorption chromatography [14], electrolytic precipitation of impurities and purification over ion-exchange resins [15], precipitation of impurities by hydroxides and fractional extraction by solvents [16], and fractional extraction by solvents and adsorption chromatography [17] have also been reported.

Of the aforementioned combinations, we selected the most simple one, precipitation of impurities by hydroxides and fractional extraction of glycosides with butanol [16]. We tried $Ca(OH)_2$ and aqueous aluminum chloride followed by NaOH treatment to coagulate impurities from the evaporated aqueous solutions. The selection criterion was the clarification of the solution after neutralization. As it turned out, the method using aluminum chloride had several advantages over the reported $Ca(OH)_2$ method [11-14, 16] (solutions were clearer, precipitation occurred faster after neutralization, and the precipitate could be gravity filtered) and was chosen. After filtration, glycosides were extracted from the aqueous solution with butanol as before [16].

The butanol extracts were evaporated to dryness. The solid was a mixture of sweet glycosides (rebaudiosides) and highly colored impurities from *S. rebaudiana* leaves that redistributed from the aqueous solution into the butanol extract. The latter were removed by chromatography over a column of Al_2O_3. The optimal eluent was butanol:methanol:water. The solid obtained after distillation of the aqueous alcohol mixture was recrystallized from methanol to afford stevioside, the properties of which agreed with those in the literature (mp, IR spectrum, optical activity [3, 18], PMR spectrum [19]). The mother liquor remaining after stevioside crystallization was chromatographed over a column of silica gel impregnated with boric acid ($CHCl_3$:CH_3OH:H_2O eluent). This isolated rebaudiosides A and C, the properties of which agreed with those in the literature (mp, IR spectrum [4], optical activity, PMR spectrum [3, 20]).

In principle, the chromatographic separation over Al_2O_3 could be avoided and the aqueous extract could be immediately chromatographed (after evaporation of butanol) over silica gel impregnated with boric acid. However, in this instance several successive chromatographic separations would be needed. To the best of our knowledge, isolation of rebaudiosides A and C by such a method has not been reported.

Several methods are known for determining the quantitative content of glycosides in plant material (GC, capillary electrophoresis, IR spectroscopy [21-23]). However, the simplest and most reliable method is HPLC, which has been used to determine the composition of *S. rebaudiana* growing in various geographical areas [2, 7-9]. We used this method in our work. First we used reversed-phase chromatography (Partisil 5-ODS-3-C_{18}) with elution by CH_3OH:CH_3CN:H_2O (200:300:500) to identify glycosides (rebaudiosides) in *S. rebaudiana* extracts. However, these conditions were ineffective for separating stevioside from rebaudioside A, which were observed as a single peak. Thus, we used a column with Lorbaks NH_2 phase, which turned out to be exceedingly selective. Table 1 gives the quantitative data on the glycoside compositions of the studied specimens. Literature data for foreign samples from various geographical habitats are given for comparison.

Table 1 shows that the stevioside content was greatest for plants cultivated in Viet Nam [6, 9]. The highest content (3.7-3.8%) of rebaudioside A was observed in samples from Viet Nam [6, 9], Paraguay [2], and China [7, 8]. The content of rebaudioside C decreased as a function of cultivation region as follows: 2.1% (China) [7, 8], 1.4% (South Korea) [7, 8], 1.4% (Viet Nam) [6, 9], and 0.9% (Paraguay and Japan) [7, 8]. We note that the stevioside content in plants grown in Voronezh Oblast′ was about the same as that in samples from Ukraine, South Korea, and Canada whereas the rebaudioside A and C contents in the Russian and Ukrainian samples were about half that in the foreign samples.

In conclusion, we proposed an original laboratory method for isolating stevioside and rebaudiosides A and C from leaves of *S. rebaudiana* that consists of the following steps: aqueous extraction of glycosides, hydroxide coagulation of impurities using $AlCl_3 \cdot 6H_2O$ and NaOH, separation of the precipitate on a Schott funnel, evaporation of the aqueous solution, chromatography of the resulting solid over Al_2O_3, removal of the eluent and isolation of stevioside by recrystallization from methanol, chromatography of the mother liquor over silica gel impregnated with boric acid, removal of eluent and isolation of rebaudiosides A and C by recrystallization from methanol.

EXPERIMENTAL

TLC, column chromatography, and HPLC were used to separate the rebaudiosides. TLC was performed on Silufol plates with elution by ethylacetate:ethanol:water (13:2.7:2) and $CHCl_3$:CH_3OH:H_2O (15:6:1). Chromatograms were developed using α-naphthol (1%) in acetone with added phosphoric acid (10%).

HPLC was carried out on a Gilson liquid chromatograph equipped with a Rheodyne injector loop (20 μL) and a UV spectrophotometer with variable wavelength (200-900 nm) as a detector. We used an NH_2-phase and isocratic elution. Solvents were purified by the usual methods used for HPLC. The eluent was de-aerated by passing He through it. The chromatography conditions were Lorbaks NH_2 column (250 × 4.6 mm, Du Pont Chromatographia, USA), CH_3CN:H_2O (80:20 v/v) eluent, pH 5 (prepared eluent was acidified with conc. H_3PO_4). The eluent flow rate was 2 mL/min, λ = 211 nm, pressure 119-120 bar, room temperature, sensitivity 2 mV, recorder rate 10 mm/min. Quantitative analysis used the internal standard method. A calibration curve for stevioside was constructed in the concentration range 1.5-120 μg per 20 μL (it was linear). The calibration coefficient for stevioside was 0.99. The quantitative data were averaged over five parallel determinations.

Leaves of *S. rebaudiana* from the All-Russian SRI of Beet Sugar and Sugar (Ramon′, Voronezh Oblast′, Russia) and Pervomaisk collective farm (Tabachnoe, Crimea, Ukraine) were studied. Samples of leaf extracts for HPLC analyses were prepared as before [24]. Ground leaves (5 g) were soaked in aqueous methanol (50 mL, 49:1) and left for a day. Then the solution was decanted. The solid was washed with the same solution (20 mL) and again soaked in aqueous methanol (30 mL) for a day. The combined extract of glycosides was evaporated to dryness. The solid was dissolved in methanol (95%).

Matrix-activated laser desorption—ionization (MALDI) mass spectra were measured in a Dynamo MALDI TOF time-of-flight mass spectrometer (Thermo Bioanalysis Finnigan, USA). A UV-laser with wavelength 337 nm was used for the laser desorption. The matrix was dihydroxybenzoic acid. Samples were prepared by the dried-drop method. A mixture of the matrix in ethanol (1 mass %) and the analyte in methanol (0.1 mass %) was placed on a support and dried at 40°C. IR spectra were recorded on a Vector 22 spectrophotometer (Bruker) in the range 400-3600 cm^{-1} in KBr disks. PMR spectra were obtained on an Avance 600 instrument. Optical density was measured on a Perkin—Elmer 241 MC polarimeter in a 55-mm tube. Melting points were determined on a Boetius microstage.

Extraction of *S. rebaudiana* Leaves. Dry leaves (50 g) were placed in a 1-L round-bottomed flask with a reflux condenser. Distilled water (700 mL) was added and heated for 1 h. The extract was filtered. The combined filtrates from several extractions were concentrated in vacuo (40-60 mm Hg) to form a dark-brown glycerine-like liquid with the sour smell of rotten grass (syrup).

Purification of Pigments, Proteins, and Resins. Syrup (100 g, 83 mL, 50%) was diluted at room temperature to 500 mL with distilled water, stirred in a beaker to obtain a homogeneous mixture, treated with $AlCl_3 \cdot 6H_2O$ (41 g), and stirred until it dissolved. A loose precipitate formed. The mixture in the beaker was stirred and treated gradually with a solution of NaOH (19 g) in distilled water (25 mL). The dark-brown mixture with pH 5-6 of total volume 600 mL was left for 30 min until the precipitate was fully formed. The precipitate was filtered off in a Buchner funnel. Half of the evaporated filtrate was passed through a column (30 × 60 mm) packed with Al_2O_3. The column was eluted with distilled water to afford a light brown solution (250 mL).

Isolation of Stevioside by Butanol Extraction. The aqueous solution (250 mL) was extracted by commercial butanol in a separatory funnel (12 × 30 mL). The butanol was removed to afford light-green crystals (13 g).

Recrystallization of Stevioside. The butanol extract (13 g) was placed in a flask with a reflux condenser and diluted with methanol (200 mL). The solution was refluxed for 30 min and filtered through a Schott funnel. The crystals that formed on standing (2.5 g) were recrystallized twice from methanol, yield 2.5 g (5% per 100 g dry leaves), $C_{38}H_{60}O_{18}\cdot 3H_2O$, mp 201-203°C (MeOH) (lit. mp 198-202°C [18], 196-198°C [3]), $[\alpha]_D^{22}$ -33.7° (*c* 6.6, H_2O), $[\alpha]_D^{23}$ -23.8° (*c* 16.8, CH_2H_5OH) {lit. $[\alpha]_D^{20}$ -39.3° (*c* 5.7, H_2O), $[\alpha]_D^{20}$ -29.6° (*c* 3.56, C_2H_5OH) [3]}. IR spectrum (KBr, ν, cm^{-1}): 3200-3600 (OH), 1729 (COO), 1656 (C=CH_2), 1076, 1036 (C–OH). Mass spectrum (MALDI, *m*/*z*, I_{rel}, %): 827 (100) $[M + Na]^+$, 843 (35) $[M + K]^+$. The PMR spectrum agreed with that published [19].

Column Chromatography over Al_2O_3. The semicrystalline mass resulting from hydroxide purification from proteins and resins (see above) was treated with methanol (100 mL) and refluxed for 20 min. The methanol was decanted. The operation was repeated twice. After removal of methanol, the pasty residue was dissolved in BuOH:MeOH:H_2O and chromatographed over a column (30 × 90 mm) packed with Al_2O_3. Evaporation of the aqueous alcohol from the effluent formed a yellowish crystalline substance (0.5 g), recrystallization of which from methanol produced stevioside (0.3 g). Then the mother liquor was used to isolated rebaudiosides A and C.

Isolation of Rebaudiosides A and C. The mother liquor left after recrystallization of stevioside was chromatographed over a column packed with silica gel impregnated with boric acid [245 g silica gel treated with boric acid solution (400 mL, 0.1 N) and dried at 120°C] with elution by $CHCl_3$:CH_3OH:H_2O (30:10:1). Chromatography, removal of eluent, and recrystallization from methanol afforded rebaudiosides A and C.

Rebaudioside A. Yield 0.7 g (1.4% per 100 g dry leaves), mp 235°C (MeOH), $C_{44}H_{70}O_{23}\cdot 3H_2O$; $[\alpha]_D^{20}$ -17.5° (*c* 0.1588, MeOH) {lit. $[\alpha]_D^{20}$ -15.3° (*c* 0.4, MeOH) [3, 20]}. IR spectrum (KBr, ν, cm^{-1}): 3200-3600 (OH), 1728 (COO), 1646 (C=CH_2), 1076, 1035 (C–OH). Mass spectrum (MALDI, *m*/*z*, I_{rel}, %): 989 (100) $[M + Na]^+$, 1005 (46) $[M + K]^+$. The PMR spectrum agreed with that published [3, 20].

Rebaudioside C. Yield 0.1 g (0.2% per 100 g dry leaves), mp 186-188°C (MeOH) (lit. mp 193-195°C [4]), $C_{44}H_{70}O_{22}\cdot 3H_2O$, $[\alpha]_D^{20}$ -31° (*c* 0.1464, MeOH) {lit. $[\alpha]_D^{20}$ -28.7° (*c* 3.55, MeOH) [3]}. IR spectrum (KBr, ν, cm^{-1}): 3200-3600 (OH), 1731 (COO), 1644 (C=CH_2), 1077, 1034 (C–OH). Mass spectrum (MALDI, *m*/*z*, I_{rel}, %): 977 (100) $[M + Na]^+$, 989 (66) $[M + K]^+$. The PMR spectrum agreed with that published [3, 20].

ACKNOWLEDGMENT

The work was supported financially by the RFBR (grants No. 04-03-32133 and 04-03-32063).

REFERENCES

1. G. K. Podporinova, *Pishch. Promst.*, **12**, 61 (2004).
2. J. E. Brandle, A. N. Starratt, and M. Gijzen, *Can. J. Plant Sci.*, **78**, 527 (1998).
3. M. Kobayashi, S. Horikawa, I. H. Degrandi, J. Ueno, and H. Mitsuhashi, *Phytochemistry*, **16**, 1405 (1977).
4. Y. Sakamoto, K. Yamasaki, and O. Tanako, *Chem. Pharm. Bull.*, **25**, 844, 3437 (1977).
5. A. D. Kinghorn and D. D. Soejarto, *Economic and Medicinal Plant Research*, Academic Press, London (1985).
6. A. G. Lyakhovkin, A. P. Nikolaev, and V. B. Uchitel′, *Stevia, Sweetgrass: A Domestic Medicinal and Food Plant* [in Russian], ZAO Ves′ (1999).
7. Y. Hashimoto, M. Morijasu, S. Nakamura, S. Ichiguro, and M. Komuro, *J. Chromatogr.*, **161**, 403 (1978).
8. H. C. Marapugay, N. P. D. Nanaayakkara, and A. D. Kinghorn, *J. Chromatogr.*, **283**, 390 (1984).
9. J. E. Brandle and N. Rosa, *Can. J. Plant Sci.*, **72**, 1263 (1992).
10. V. A. Zubtsov, E. I. Milorodova, E. Yu. Yurova, S. S. Ryasenskii, and L. L. Osipova, RF Pat. No. 2,111,969 (1998); *Byull. Izobret.*, No. 20 (1998); A. N. Starratt, C. W. Kirby, R. Poks, and J. E. Brandle, *Phytochemistry*, **59**, 367 (2002).
11. H. Okane and L. Kamata, *Jpn. Kokai Tokkyo Koho*, Jpn. Pat. No. 52057198, May 11, 1977; *Chem. Abstr.*, **87**, 99049d (1977).

12. Research Foundation for the Development of Industries, *Jpn. Kokai Tokkyo Koho*, Jpn. Pat. No. 57005663, Jan. 12, 1982; *Chem. Abstr.*, **96**, 141440n (1982).
13. H. Ichizone, *Jpn. Kokai Tokkyo Koho*, Jpn. Pat. No. 54012400, Jan. 30, 1979; *Chem. Abstr.*, **90**, 148704n (1979).
14. S. Li, *Zhongguo Tiaoweipin*, **9**, 10 (1984); *Chem. Abstr.*, **102**, 147684j (1985).
15. K. Miwa, *Jpn. Kokai Tokkya Koho*, Jpn. Pat. No. 54103900, Aug. 15, 1979; *Chem. Abstr.*, **92**, 82420y (1980).
16. K. Takamura, M. Kawaguchi, and C. Isono, *Jpn. Kokai Tokkyo Koho*, Jpn. Pat. No. 52047961, Apr. 16, 1977; *Chem. Abstr.*, **87**, 132568x (1977).
17. I. C. C. Mantovaneli, E. C. Ferretti, M. R. Simoes, and C. F. da Silva, *Braz. J. Chem. Eng.*, **21**, 449 (2004).
18. H. B. Wood, R. Allerton, H. W. Diel, and R. G. Fletcher, *J. Org. Chem.*, **20**, 875 (1955).
19. G. Colombo, S. Riva, and B. Danieli, *Tetrahedron*, **60**, 741 (2004).
20. H. Kohda, R. Kasai, K. Yamasaki, K. Murakami, and O. Tanaka, *Phytochemistry*, **15**, 981 (1976).
21. B. Nikolova-Damyanova, V. Bankova, and S. Popov, *Phytochem. Anal.*, **5**, 81 (1994).
22. Y. Kitada, M. Sasaki, and Y. Yamazoe, *J. Chromatogr.*, **474**, 447 (1989).
23. M. S. Ahmed, R. H. Dobberstein, and N. R. Farnsworth, *J. Chromatogr.*, **192**, 387 (1980).
24. Z. Yagliardi, A. Amato, A. Basili, and Y. Cavazzuttu, *Ann. Chim. (Rome)*, **76**, 39 (1986).