Stability Studies of Stevioside and Rebaudioside A in Carbonated Beverages

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Stability of pure stevioside and rebaudioside A in carbonated phosphoric and citric acidified beverages during long-term storage was observed chemically, microbiologically, and organoleptically. Thin-layer chromatography and high-pressure liquid chromatography were used to follow the chemical degradation of these *Stevia* sweeteners. Some degradation of both sweeteners was observed after 2 months of storage at 37 °C; however, there were no significant changes at room temperature or below following 5 months of storage of stevioside, or 3 months of storage of rebaudioside A. Exposure to 1 week of sunlight did not affect stevioside but resulted in approximately 20% loss of rebaudioside A. Heating at 60 °C for 6 days resulted in 0–6% loss of the sweeteners.

Six sweet-tasting compounds have been reported in the leaves of *Stevia rebaudiana* Bertoni, a plant native to Paraguay: stevioside, rebaudiosides A, D, and E, and dulcosides A and B (Kohda et al., 1976; Sakamoto et al., 1977; Kobayashi et al., 1977; Crosby et al., 1979). Stevioside is a glycoside with a glucosyl and sophorosyl residue attached to the aglycon steviol; the latter has a cyclopentanoperhydrophenanthrene skeleton. The C4 and C13 of steviol are connected to the β-glucosyl and β-sophorosyl group, respectively. The structure of rebaudioside A is the same as that of stevioside except that the sophorosyl residue is replaced by a glucosyl-(1–3)-sophorosyl residue. The chemical structures of the six Stevia sweeteners and related compounds are shown in Figure 1. The Stevia sweeteners are similar in structure in that a steviol aglycon is connected at C4 and C13 to mono-, di-, or trisaccharides consisting of glucose and/or rhamnose residues.

Stevioside is the predominant sweetener, representing approximately 3–8% of dried leaves (Katayama et al., 1976). Due to its reported superior taste quality, rebaudioside A, the second most abundant sweetener in the *Stevia* plant (approximately 1% of dried leaves), has also received some attention (Kaneda et al., 1977). Dulcoside A constitutes approximately 0.2%, while the other three *Stevia* sweeteners represent 0.03–0.04% of the dried leaves. The sweetening powers of stevioside, rebaudioside A, and the dulcosides have been reported to be approximately 100 (Katayama et al., 1976), 130 (Tanaka et al., 1977), and 30 times (Kobayashi et al., 1977) that of sucrose, respectively.

So far, little detailed data have been available on the basic properties or the practical applications of the *Stevia* sweeteners in foods and beverages. Therefore, stability studies of the two most abundant *Stevia* sweeteners, stevioside and rebaudioside A, in two carbonated beverages are reported here.

MATERIALS AND METHODS

Chemicals. Reagent and chromatographic grades of chemicals were purchased from several well-known American suppliers. Steviolbioside (Wood et al., 1955) and rebaudioside B (Tanaka et al., 1977) were prepared from stevioside and rebaudioside A, respectively. Isosteviol was prepared according to the method of Mosettig et al. (1963). Crude, partially purified, and purified stevioside preparations, steviol, and a small sample of a purified rebaudioside A were obtained from confidential sources. Chromatographically (TLC and HPLC) pure rebaudioside A and a highly purified stevioside were used in the stability studies. Repeated crystallization of stevioside from dioxane–methanol (Wood et al., 1955) yielded a purified stevioside which still contained 8% steviolbioside (by HPLC). Rebaudioside A was obtained from crude stevioside by preparative column chromatography with subsequent solvent recrystallization.

Thin-Layer Chromatography (TLC). Precoated silica gel plates (F-254, E. Merck) were activated at 100 °C for 30 min and stored in a desiccator. Samples were developed to 10 cm from origins with $CHCl_3$–MeOH–H_2O (15:10:2) and detected by spraying with saturated $Ce(SO_4)_2$ in 65% H_2SO_4 and heating at 100 °C for 30 min. The plates were then kept in a desiccator. Since variations between TLC runs were noticed, a crude stevioside containing stevioside, rebaudiosides A and B, and steviolbioside was run along with the samples in order to relate the sample components with the known compounds. The R_f values were adjusted to correspond to the R_f of 0.49 for stevioside or the R_f of 0.38 for rebaudioside A.

High-Pressure Liquid Chromatography (HPLC). A Waters liquid chromatograph, Model ALC/GPC 201, equipped with a differential refractometer and a U6K injector and containing a μBondpak–carbohydrate analysis column was used during this work. The column was eluted with acetonitrile–water (80:20 v/v) at 1.0 mL/min, while maintaining the inlet pressure at 800 psi. Changes in refractive index were traced on a Varian A-25 dual-pen recorder.

For optimization of the quantitative analysis of stevioside and rebaudioside A in the beverage samples, it was found advantageous to concentrate the samples 5-fold by first flash evaporating them to dryness at room temperature followed by reconstituting in water. Since glucose was a possible degradation product of stevioside, and since HPLC peaks of glucose and stevioside were poorly resolved, stevioside in the beverages was extracted 4 times with equal volumes of water-saturated 1-butanol. The 1-butanol extracts were likewise flash-evaporated to dryness and 5-fold concentrates of the beverage samples were made. Injection size was usually 25 μL.

Preparation of Purified Rebaudioside A. Ten-gram portions of a crude stevioside preparation (containing 40% stevioside and 20% rebaudioside A by HPLC analysis) were placed on a silica gel column (Davison Chemical, Grade 62, mesh size 60–200, 5 cm i.d. × 23 cm) and eluted with $CHCl_3$–MeOH–H_2O (45:12:2 v/v/v).

The flow rate was maintained at 5 mL/min by gravity. Fractions of 100 mL each were collected and flash-evaporated at 40 °C to dryness. Elution was terminated after 38 fractions had been collected. Subsequent analysis of the HPLC fractions showed that almost all of the rebaudioside A had eluted. The yield of rebaudioside A with better than 90% purity (fraction no. 24–38) was 7.4%,

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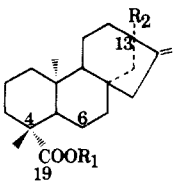

	R_1	R_2
Steviol	-H	-OH
Steviolbioside	-H	-OG(2,1)G
Stevioside	-G	-OG(2,1)G
Rebaudioside B	-H	-OG(2,1)G \(3,1)G
Rebaudioside A	-G	-OG(2,1)G \(3,1)G
Rebaudioside D	-G(2,1)G	-OG(2,1)G \(3,1)G
Rebaudioside E	-G(2,1)G	-OG(2,1)G
Dulcoside A	-G	-OG(2,1)Rh
Dulcoside B	-G	-OG(2,1)Rh \(3,1)G

G = β-glucopyranosyl

Rh = α-rhamnopyranosyl

Figure 1. Chemical structures of the *Stevia* sweeteners and related compounds.

representing roughly one-third recovery. Rebaudioside A in fractions 24–38 was then recrystallized twice from methanol to obtain chromatographically pure rebaudioside A.

Stability Studies of Pure Sweetener Solutions. *Heating in Neutral Solutions at 100 °C.* Two-milliliter portions of stevioside or rebaudioside A solution (6.5 mg/mL of H_2O) were sealed in separate 5-mL glass vials and heated for various time periods at 100 °C. TLC and HPLC were used to follow the progress of chemical degradation. For quantitative analysis, deviations from the peak heights at 0 h were used to calculate losses due to heat treatment.

Heating in Acidic Solutions. A citric acid system (0.22% w/v citric acid, 0.0348% w/v sodium citrate, pH 2.6) and a phosphoric acid system (0.04% w/v H_3PO_4, pH 2.4) were prepared into which either stevioside or rebaudioside A was dissolved at 6.5 mg/mL. The heating experiments were carried out at 60 °C for 0–137 and at 100 °C for 0–13 h, as described under Heating in Neutral Solutions at 100 °C.

Stability Studies in Carbonated Beverages. Long-term storage tests for each of the two sweeteners were carried out at 4 °C, room temperature (22 °C), and 37 °C. Carbonated citric acid and phosphoric acid containing beverages (citrus and cola types, respectively) were prepared with 0.1% of stevioside or rebaudioside A. No preservative was used. Unsweetened carbonated beverages were made and stored along with the stevioside or rebaudioside A sweetened carbonated beverages in order to determine possible effects due to nonsweetener components in the beverages. Samples of the sweetened and unsweetened carbonated beverages were kept in tightly closed glass bottles and stored at the specified temperatures. The beverages were chemically, microbiologically, and organoleptically monitored periodically for up to 5 months. HPLC and TLC were used to analyze sweetener losses and degradation products. Determinations of total microbial and yeast and mold counts were performed with plate count agar and orange serum agar, respectively. Taste tests were carried out by using expert tasters. The effect of short-term beverage exposure to elevated temperatures was evaluated by placing the beverages in an oven maintained at 60 °C for up to 137 h. The effect of sunlight was also studied by placing the products outdoors (at 10–25 °C) for approximately 1 week. During this time, the products were exposed to 3000 langleys of sunlight as monitored by an Eppley Black and White pyranometer for global and sky radiation.

Table I. Retention Times (t_R) and Capacity Factors (k') of *Stevia* Glycosides[a]

compound	t_R, min	k'
steviolbioside	8.1	0.76
stevioside	11.4	1.48
glucose	12.9	1.80
rebaudioside B	13.7	1.98
rebaudioside A	18.6	3.04
sucrose	20.7	3.50

[a] $k' = (t_R - t_0)/t_0$ where $t_0 = 4.6$ min (H_2O). Waters μBondapak-carbohydrate column; mobile phase, CH_3CN–H_2O (80:20 v/v); 1.0 mL/min.

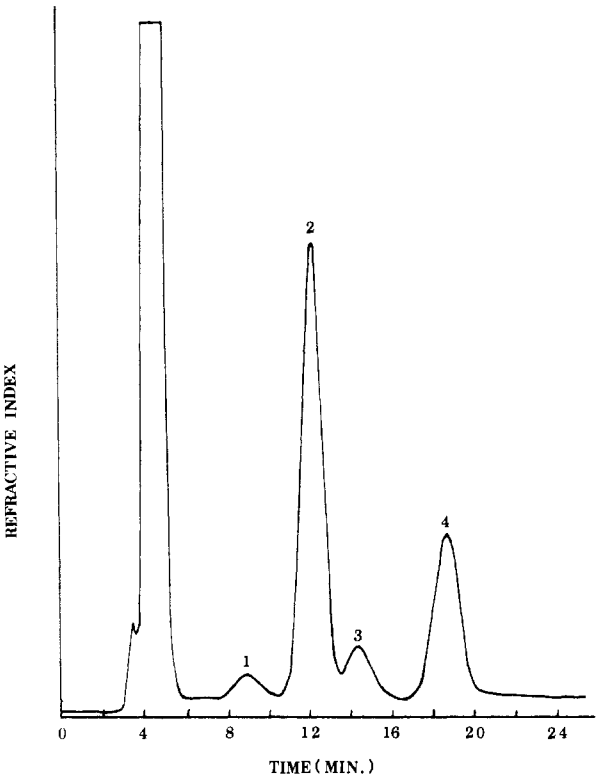

Figure 2. High-pressure liquid chromatography of *Stevia* glycosides in a crude stevioside (0.635 mg in H_2O) on a μBondpak–carbohydrate analysis column with acetonitrile–H_2O (80:20 v/v) as the eluant. 1 = steviolbioside; 2 = stevioside; 3 = rebaudioside B; 4 = rebaudioside A.

RESULTS AND DISCUSSION

HPLC of Reference Compounds. Typical retention times and capacity factors are shown in Table I. An HPLC pattern of a crude stevioside preparation is shown in Figure 2. The four peaks in Figure 2 were found to correspond to steviolbioside, stevioside, rebaudioside B, and rebaudioside A by TLC and column chromatography on silica gel according to the procedure of Kohda et al. (1976).

Table II. Retention of Pure Stevioside and Rebaudioside A Solutions (6.5 mg/mL) during Heating of 100 °C[a]

	Neutral Solutions %	
time, h	stevioside	rebaudioside A
0	100	100
12	83.7	—
22	79.4	88.6
48	66.0	68.5
65	38.2	—

	Acidic Solutions %			
	stevioside		rebaudioside A	
	I	II	I	II
0	100	100	100	100
4	46.2	54.0	56.8	59.8
10	21.9	29.9	19.3	32.2
13	13.7	32.2	13.2	24.1

[a] (—) Not determined. (I) Phosphoric acid system. (II) Citric acid system. HPLC peak heights were used for the above calculations.

Stability Studies of Pure Sweetener Solutions. *Heating in Neutral Solutions.* HPLC and TLC showed that prolonged heating of stevioside solution (6.5 mg/mL) at 100 °C resulted in a decrease in the stevioside concentration with the appearance of the two degradation products steviolbioside and glucose. This is attributable to the rupture of the C19 ester bond in stevioside. Steviolbioside appeared in the TLC chromatogram after heating for 4 h and glucose was detected following 8 h of heating. Similar results were obtained for rebaudioside A, yielding rebaudioside B and glucose as the degradation products instead of steviolbioside and glucose. Thus, the C19 ester linking appeared to be the most heat-sensitive bond both in stevioside and in rebaudioside A. The results obtained by HPLC analyses showing the extent of degradation of stevioside and rebaudioside A at 100 °C are reproduced at the top of Table II.

Heating in Acidic Solutions. (*a*) *TLC Analyses.* Heating at 60 °C for up to 137 did not cause any appreciable degradation of stevioside or rebaudioside A. This was true both in the citric and the phosphoric acid systems. However, heating both the acid systems at 100 °C resulted in drastic changes. TLC showed that when stevioside was heated at 100 °C in citric acid steviolbioside, glucose and two unknown components (A, B) were detected after 4 h. After being heated for 10 and 13 h, an additional component (C) appeared along with traces of two components (D, E). It was noted that isosteviol had the same R_f value as component E and that the R_f value for steviol did not correspond to any of the unknowns. Stevioside degraded even more in phosphoric acid. Components A, B, and C, steviolbioside, and glucose appeared when stevioside was heated at 100 °C for 4 h. Furthermore, significant amounts of components D and E were seen following 13 h of heating. Fewer degradation products were observed for rebaudioside A under similar conditions. Thus, in the citric acid system, rebaudioside B, glucose, one unknown (N), and a trace of another unknown (M) were detected after heating for 4 h at 100 °C. The concentration of the degradation products increased with time during 13 h of study, but no additional degradation product appeared. Similar results were obtained in the phosphoric acid system except that degradation reactions were somewhat more pronounced as indicated by higher concentration of component M. The R_f values of the degradation products of stevioside and rebaudioside A are shown in Table III.

Table III. TLC Analyses of the Degradation Products of Stevioside and Rebaudioside A in Acidic Solutions Heated at 100 °C

	R_f
stevioside	0.49
unknown B	0.19
glucose	0.33
unknown A	0.42
stevioside	0.49
unknown C	0.66
steviolbioside	0.72
unknown D	0.95
E, isosteviol	1.00
rebaudioside A	0.38
unknown N	0.13
glucose	0.33
unknown M	0.48
rebaudioside B	0.53

Table IV. HPLC Analyses of the Degradation Products of Stevioside and Rebaudioside A in Acidic Solutions Heated at 100 °C[a]

	t_R
stevioside	1.00
steviolbioside	0.71
unknown 1	0.88
glucose	1.13
unknown 2	1.39
unknown 3	1.70
unknown 4	1.88
unknown 5	2.27
rebaudioside A	1.00
unknown i	0.56
glucose; rebaudioside B	0.67
unknown ii	0.87
unknown iii	1.37

[a] Note: The relative retention times (t_R) are based on the t_R of either stevioside or rebaudioside A as 1.

(*b*) *HPLC Analysis.* Parallel results were obtained, as shown in Table IV. In the citric acid system, one (unknown 2), three (unknowns 1,2, and 5), and four (unknowns 1, 2, 3, and 5) unknown peaks were detected when stevioside was exposed to 4, 10, and 13 of heating at 100 °C, respectively. Steviolbioside and glucose were the known degradation products. When stevioside was heated in phosphoric acid for up to 13 h at 100 °C, two unknown degradation products (unknowns 2 and 5) were detected at 4 h. Two more unknowns (1, 4) appeared at 10 and again at 13 h. Steviolbioside and glucose were also known degradation products in each case. HPLC analysis of rebaudioside A when heated at 100 °C in citric acid showed one unknown (iii) degradation product at 4 h and two (ii, iii) at 10 and 14 h. In the phosphoric acid system, another unknown (i) was found during 4–10 h of heating but not at 13 h. Rebaudioside B and glucose were detected during heating in both acidic systems.

Quantitative HPLC analysis data for stevioside and rebaudioside A obtained during heating in acidic systems at 100 °C are shown at the bottom of Table II. These data confirmed TLC results which showed that both stevioside and rebaudioside A degraded much faster in acidic solutions than in neutral solutions. Furthermore, both sweeteners were slightly less stable in phosphoric acid than in citric acid, possibly partly due to the slightly lower pH.

Stability Studies in Carbonated Beverages. *Long-Term Storage Tests.* Stevioside exhibited no significant changes in HPLC and TLC analyses at 4 °C or at room temperature for at least 5 months when formulated in carbonated beverage containing 0.1% stevioside as the sole

Table V. Degradation of Stevioside and Rebaudioside A during Long-Term Storage in Carbonated Beverages (by HPLC Analysis)[a]

time, month	% degradation at storage temp of 4 °C I	4 °C II	22 °C I	22 °C II	37 °C I	37 °C II
			Stevioside			
0	N	N	N	N	N	N
0.6	N	—	N	—	N	—
1	—	N	—	N	—	N
2.0	—	N	—	N	—	6
2.3	N	—	N	—	17	—
3	—	N	—	N	—	10
4	N	N	N	N	36	17
5	N	—	N	—	40	—
			Rebaudioside A			
0	N	N	N	N	N	N
1	N	N	N	N	N	N
2	N	N	N	N	12	8
3	N	N	N	N	19	12
4	N	N	11	6	25	13

[a] (I) Phosphoric acid beverage. (II) Citric acid beverage. (N) Not significantly different from corresponding unsweetened beverage with 0.1% sweetener freshly added. (—) Not determined.

sweetener with phosphoric or citric acid as the acidulant. Degradation of stevioside was observed when both of the above samples were stored at 37 °C. A 36% loss in stevioside concentration was observed after 4 months at 37 °C in phosphoric acid beverage. The degradation of stevioside in the citric acid beverage at 37 °C was found to be at a slower rate, with a 17% loss after 4 months of storage. Detailed quantitative results from HPLC analyses are shown in Table V. Both HPLC and TLC showed that when stevioside degraded, steviolbioside, glucose, and an unknown with an HPLC relative retention time of 1.37 (stevioside = 1.00) were the only degradation products detected. Rebaudioside A showed no significant changes during 4 months of storage at 4 °C, 3 months at room temperature, or 1 month at 37 °C in either citric or phosphoric acid beverages. This sweetener again exhibited greater stability in the citric acid system than in the phosphoric acid system. Although a reduction in rebaudioside A concentration was observed by HPLC, no degradation products were observed. TLC chromatograms also did not reveal any spot other than that attributable to rebaudioside A.

Microbiological stability of the products during storage was excellent. Initial total counts were generally under 100 counts per mL, with zero total yeast and mold. Microorganisms were not detected from the experimental samples stored at 4–37 °C after 17 days. Also, no counts were obtained at any subsequent times. Organoleptically, no significant differences other than sweetness were found between the aged sweetened products and the aged unsweetened products with 0.1% sweetener added to the latter after storage. Tasters generally could not easily detect a loss in sweetness resulting from a 10% or less decrease in either the stevioside or rebaudioside A concentration.

Effect of Exposure to Hot Environment. When stevioside or rebaudioside A sweetened carbonated beverages were stored at 60 °C for 137 h, little or no reduction in sweetener concentration could be observed. Stevioside concentration did not decrease in citric acid beverages; however, a 4% decrease was noted in the phosphoric acid beverages. The corresponding figures for rebaudioside A were 3% in citric and 6% in phosphoric acidified beverages. Thus, both sweeteners appeared to be quite stable when the beverages were heated at 60 °C for a few days.

Effect of Sunlight Exposure. No significant changes in stevioside concentration were observed in either the phosphoric or citric acid beverages when exposed to 3000 langleys of sunlight. Surprisingly, rebaudioside A underwent considerable degradation under similar conditions with 22 and 18% losses in the phosphoric and the citric acid beverages, respectively. HPLC analysis showed a single peak trailing rebaudioside A. This same phenomenon was also observed by TLC. No other degradation products were observed and no off-tastes were found following sunlight exposures.

Registry No. Stevioside, 57817-89-7; rebaudioside A, 58543-16-1; steviolbioside, 41093-60-1; rebaudioside B, 58543-17-2; isosteviol, 27975-19-5; glucose, 50-99-7; H_3PO_4, 7664-38-2; citric acid, 77-92-9.

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