JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Photostability of Rebaudioside A and Stevioside in Beverages

JOHN F. CLOS, GRANT E. DUBOIS, AND INDRA PRAKASH*

The Coca-Cola Company, One Coca-Cola Plaza, Atlanta, Georgia 30313

The Coca-Cola Company and Cargill, Inc. have initiated the development and commercialization of the *Stevia rebaudiana* (Bertoni) derived sweetener rebaudioside A. Efforts were focused on high purity rebaudioside A (>97% by HPLC), commonly known as rebiana. In the course of the development program, extensive stability studies were carried out on rebiana, all supporting good stability for use in all food and beverage applications, including conditions where rebiana-sweetened beverages were exposed to light. Our findings on rebiana light stability refute those of an earlier study that suggested rebaudioside A to be unstable to sunlight exposure, while the structurally homologous stevioside is stable. We replicated the earlier study and found no significant photodegradation for either rebaudioside A or stevioside.

KEYWORDS: ***Stevia rebaudiana*** **(Bertoni); rebaudioside A; rebiana; stevioside; photostability**

INTRODUCTION

Extracts of the leaves of *Stevia rebaudiana* (Bertoni), a South American plant, have been popular for many years for their sweet taste. These so-called stevia sweeteners have become increasingly popular over the last several years because of consumer demand for all-natural foods and beverages, especially for ones low in calories. Stevia contains at least eight sweet-tasting compounds (see **Table 1**) (*1*). All are glycosides of the common aglycone steviol. They differ in the number (typically, three to five) and the type (typically, some combination of glucose, rhamnose, and xylose) of sugars attached. The two major compounds are rebaudioside A (**1**) and stevioside (**2**). They are reported to exhibit sweetness potencies, relative to a 10% sucrose reference, of 170 and 190, respectively (*2*). Rebaudioside A (purity >97% by HPLC) is also known as rebiana.

At the present time, many stevia products are on the market as sweeteners in Asian and Latin American countries. Currently, in the US and most other countries, neither the extract of these compounds from stevia nor any refined version of any of the sweet compounds can be used in foods or beverages or sold for any use other than as a dietary supplement (US). The Coca-Cola Company and Cargill, Inc. have formed a joint venture to commercially develop **1** as a sweetener for general use in food and beverages. During the development program of **1** (*3*), a full complement of stability studies was completed. Included among these stability studies on **1** were evaluations as dry powder, evaluations in model beverages and in buffers over a range of temperatures and pH values, and evaluations for photostability. In addition to our stability study work on **1**, we have also (1) developed methods for purification, (2) conducted sensory studies, (3) identified impurities present in both the raw stevia leaf extract as well as in the purified material, (4) determined degradation pathways, and (5) carried out multiple preclinical and clinical safety assessment studies (*4*).

Among the degradation studies necessary to assess sweetener viability, photostability is an important study, both for the bulk powder and in liquid formulations. Our testing protocol followed the International Conference on Harmonisation (ICH) guidelines for photostability testing of drug candidates (*5*). In addition to these studies, we felt it prudent to investigate a report by Chang and Cook (*6*) that significant degradation of **1** occurred after exposure to ca. 3000 langleys of sunlight (equivalent to approximately one week of sunlight during the summer), while no such loss occurred during the same light exposure of **2**. This is unexpected since such degradation was not seen in the ICH guideline studies on **1**, and since both **1** and **2** have the same steviol backbone, no difference in their photostability is expected.

Therefore, we repeated the Chang and Cook study on **1** and **2**. Here, we report our findings on the photostability of **1** and **2** in cola and lemon-lime beverages when exposed to sunlight.

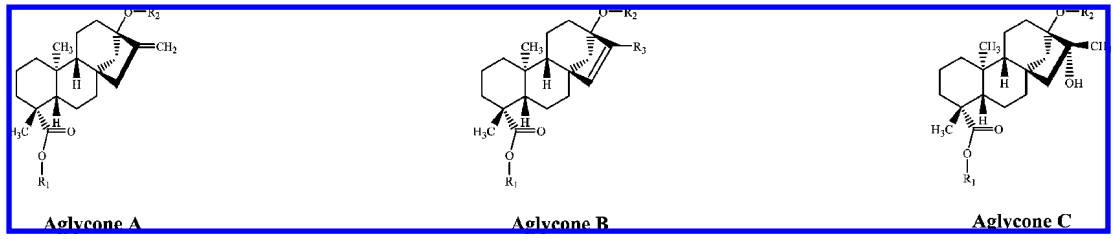
MATERIAL AND METHODS

Reagents and Chemicals. Quantitation of **1**, other steviol glycosides, and degradation products of **1** was enabled by the use of certified reference standards. All reference standards were isolated by AMRI (Bothell, WA) or prepared by The Coca-Cola Company and were certified by Chromadex (Irvine, CA). The compound numbers, common names, chemical structures, empirical formulas, and molecular weights of these reference standards are provided in **Table 1** as well as those of other compounds identified in the course of our studies. Glacial acetic acid (HOAc) was from EMD (Gibbstown, NJ), ammonium acetate (NH_4OAc) was from Fluka (a part of Sigma-Aldrich, Bellefonte, PA), 50% sodium hydroxide (NaOH) was from Mallinckrodt Baker (Phillipsburg, NJ), and 85% phosphoric acid (H_3PO_4) was from Fisher

* To whom correspondence should be addressed. Tel: 404-676-3007. Fax: 404-598-3007. E-mail: iprakash@na.ko.com.

10.1021/jf801343e CCC: $40.75
Published on Web 08/22/2008

Table 1. Steviol Glycoside Sweeteners and Their Degradation Products



cpd no	common name	aglycone	Cpd type[a]	R_1	R_2	empirical formula	MW
1	rebaudioside A	A	SG	β-Glu[b]	2, 3-(β-Glu)$_2$-β-Glu-	$C_{44}H_{70}O_{23}$	967.01
2	stevioside	A	SG	β-Glu	2-β-Glu-β-Glu-	$C_{38}H_{60}O_{18}$	804.88
3	rebaudioside B	A	SG	H	2, 3-(β-Glu)$_2$-β-Glu-	$C_{38}H_{60}O_{18}$	804.88
4	steviolbioside	A	SG	H	2-β-Glu-β-Glu-	$C_{32}H_{50}O_{13}$	642.73
5	DAQ3	B ($R_3 = CH_3$)	DP/OI	β-Glu	2, 3-(β-Glu)$_2$-β-Glu-	$C_{44}H_{70}O_{23}$	967.01
6	DAQ9	B ($R_3 = CH_3$)	DP/OI	β-Glu	2-β-Glu-β-Glu-	$C_{38}H_{60}O_{18}$	804.88
7	DAQ1	C	DP/OH	β-Glu	2, 3-(β-Glu)$_2$-β-Glu-	$C_{44}H_{72}O_{24}$	985.03
8	DAQ8	C	DP/OH	β-Glu	2-β-Glu-β-Glu-	$C_{38}H_{62}O_{19}$	822.89
9	IMP-2	B ($R_3 = CH_2OH$)	DP/AO	β-Glu	2, 3-(β-Glu)$_2$-β-Glu-	$C_{44}H_{70}O_{24}$	983.01

[a] SG = steviol glycoside; /AR = degradation product/aglycone rearrangement; DP/OI = degradation product/olefin isomerization; DP/OH = degradation product/olefin hydration; DP/AO = degradation product/allylic oxidation. [b] β-Glu = β-D-glucosyl.

Scientific (Pittsburgh, PA), all of which were reagent grade. HPLC grade acetonitrile (MeCN) was purchased from Burdick & Jackson (Muskegon, MI). Water was purified using a Millipore system (Billerica, MA).

Although the analytical methods for steviol glycosides are known in the literature, we were not able to separate all rebaudioside A and stevioside degradation products using these methods (*7*). Therefore, we developed and validated the analytical methods reported herein.

Mobile Phase Preparation. All solvents were degassed for at least two minutes with helium before use. Method 1 is for the quantitation of all analytes except for steviol and isosteviol. Method 2 is for the quantitation of steviol and isosteviol.

Method 1 employed a three solvent mobile phase system. Solvent A (0.0284% NH_4OAc, 0.0116% HOAc) was prepared by dissolving 0.569 g of NH_4OAc and 0.231 mL of HOAc in 2 L of purified water and mixing thoroughly. Solvent B was 100% MeCN. Solvent C (0.040% HOAc) was prepared by adding 0.4 mL of HOAc to one liter of purified water and mixing thoroughly.

The mobile phase for Method 2 [45% 10 mM H_3PO_4 (pH 3.0) and 55% MeCN] was prepared by first diluting 1.15 g of 85% H_3PO_4 to 1 L with purified water and then adjusting the pH to 3.0 with 50% NaOH. Then, 450 mL of this solution was combined with 550 mL of MeCN, mixed thoroughly, and allowed to come to room temperature.

Standard Preparation. The diluent buffer was prepared by adjusting 1 L of water to pH 3.3 with glacial HOAc. The diluent solution was prepared by mixing 250 mL of MeCN with 750 mL of the diluent buffer. It was then allowed to come to room temperature.

Since they are at much higher concentrations, the standards for **1** and **2** were prepared separately, and the moisture content of the reference standards was measured by Karl Fischer titration each time the standards were prepared. This was necessary each time because of the hygroscopic nature of the compounds, as well as the fact that they easily gain or lose moisture with changes in humidity. The standards were prepared by weighing 21.0, 30.0, 39.0, 48.0, and 60.0 (each $\pm$ 0.5) mg in separate 100-mL volumetric flasks, diluting to volume with the diluent solution and stirring, if necessary, until dissolved. The concentrations were corrected for moisture and purity. They were injected once at the beginning and once at the end of the sequence, with additional sets of standards injected during longer runs. Standards are stable for 2 months when stored in a refrigerator set at 5 $\pm$ 3 °C.

Beverage Preparation and Sunlight Exposure. Model cola (pH 2.4) and lemon-lime (pH 2.6) beverages were prepared to simulate, as closely as possible, the beverage samples used in the study by Chang and Cook. The specifications for the beverages are provided in **Table 2**. Beverages of each matrix were prepared separately with **1** or **2**, as well as without sweetener added to monitor for the formation of interferences. When added, each of the sweeteners was at a concentration of 1000 mg/L. The two matrices with the two fortification types or without sweetener were stored in 10 oz clear glass bottles from All American Containers, Inc. (Forrest Park, GA) with crown closures from Taensa, S. A. (Guayaquil, Ecuador). Some of each type and fortification were wrapped in aluminum foil to protect from sunlight. The light-exposed and light-protected (control) samples were left in sunlight until the exposure reached a level of ca. 3000 langleys, which took approximately one week. A langley is "a unit of energy per unit area, equal to 1 gram-calorie/cm^2 commonly employed in radiation theory" (*8*). The outside temperature during exposure ranged from 18−23 °C for lows and from 30−34 °C for highs. Triplicate analyses of the samples were conducted by analyzing three bottles with each bottle being analyzed once. Samples without sweetener were not analyzed during the course of the study unless unknown peaks were detected in the fortified samples. Prior to analysis, the model beverage samples were decarbonated using sonication for approximately five minutes at room temperature and pressure. For analyses of **1** and **2** when they were added as the primary sweetener, the samples were diluted 2.5-fold with diluent solution. The samples were then transferred to autosampler vials and injected directly.

Table 2. Beverage Specifications

parameter	cola	lemon-lime
carbonation (volumes)	3.6	3.8
density (kg/L)	0.99848	0.9978
acidity (%w/v)	0.1246	0.1000
pH	2.4	2.6

Table 3. Solvent Program for Method 1

time (min)	% A	% B	% C	comments
0.0	75	25	0	initial
8.5	75	25	0	hold
10.0	71	29	0	linear
16.5	70	30	0	linear
18.5	0	34	66	linear
24.5	0	34	66	hold
26.5	0	52	48	linear
29.0	0	52	48	hold
31.0	0	70	30	linear
37.0	0	70	30	hold
37.1	0	90	10	linear
40.0	0	90	10	hold
43.0	75	25	0	step

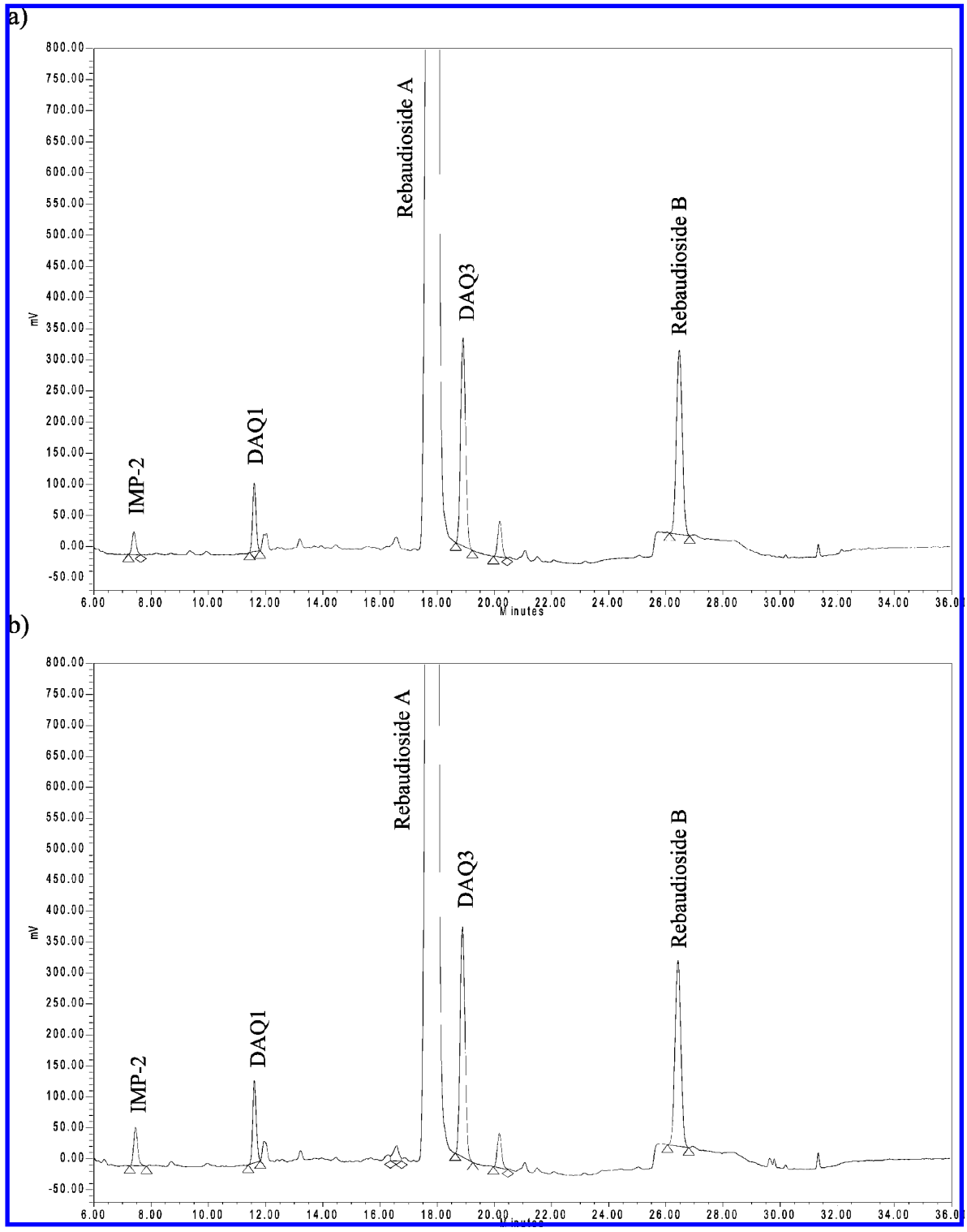


Figure 1. HPLC-CAD chromatograms for rebaudioside A (**1**) in (a) control and (b) light-exposed lemon-lime soft drinks.

Isolation and Characterization of Degradation Products. Degradation products of rebaudioside A and stevioside were isolated from a sunlight stability cola beverage (pH 2.4) samples through a series of liquid chromatographic steps by AMRI, Bothell, WA.

Compound **7** *[DAQ 1, (13-[(2-O-β-D-Glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-16-hydroxy-kauran-18-oic Acid, β-D-Glucopyranosyl Ester].* MP: 177−181 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 0.86 (m, 1H, C_1-H), 0.90 (m, 1H, C_9-H), 0.98 (s, 3H, C_{20}-H), 1.07 (m, 1H, C_3-H), 1.10 (m, 1H, C_5-H), 1.20 (s, 3H, C_{19}-H), 1.25 (s, 3H, C_{17}-H), 1.36 (m, 1H, C_7-H), 1.42 (d, J = 13.7 Hz,1H, C_{15}-H), 1.43 (m, 1H, C_2-H), 1.58 (m, 1H, C_7-H), 1.58 (d, J = 13.7 Hz,1H, C_{15}-H), 1.64 (m, 1H, C_{11}-H), 1.74 (m, 1H, C_{12}-H), 1.79 (m, 1H, C_6-H), 1.80 (m, 1H, C_{11}-H),1.83 (m, 1H, C_1-H), 1.84 (m, 1H, C_{14}-H), 1.92 (m, 1H, C_2-H), 1.97 (m, 1H, C_6-H), 1.98 (m, 1H, C_{12}-H), 2.02 (d, J = 11.5 Hz,1H, C_{14}-H), 2.05 (d, J = 11.9 Hz,1H, C_3-H), 3.15 (m, 1H, C_{40}-H), 3.27 (m, 1H, C_{34}-H), 3.37 (m,1H, C_{22}-H), 3.65 (m, 1H, C_{28}-H), 3.73 (m, 1H, C_{29}-H), 4.67 (d, J = 7.8 Hz,1H, C_{33}-H), 4.70 (d, J = 8.2 Hz,1H, C_{27}-H), 4.88 (d, J = 7.8 Hz,1H, C_{39}-H), 5.37 (d, J = 8.2 Hz,1H, C_{21}-H); ^{13}C NMR (125 MHz, CD_3OD) δ 16.0, 19.8, 20.5, 22.1, 22.8, 28.6, 30.6, 38.7, 41.5, 42.3, 43.0, 44.7, 55.7, 56.1, 58.1, 75.1, 75.7, 78.7, 79.9, 87.5, 87.9, 95.4, 96.9, 103.6, 103.9, 178.3. MS (ESI) calculated for $C_{44}H_{72}O_{24}$: 985.03; found: ($[M]^+$)985.5, ($[M]^-$) 983.6.

Compound **3** *[DAQ 3, 13-[(2-O-β-D-Glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)- oxy]-kaur-15-en-18-oic Acid, β-D-*

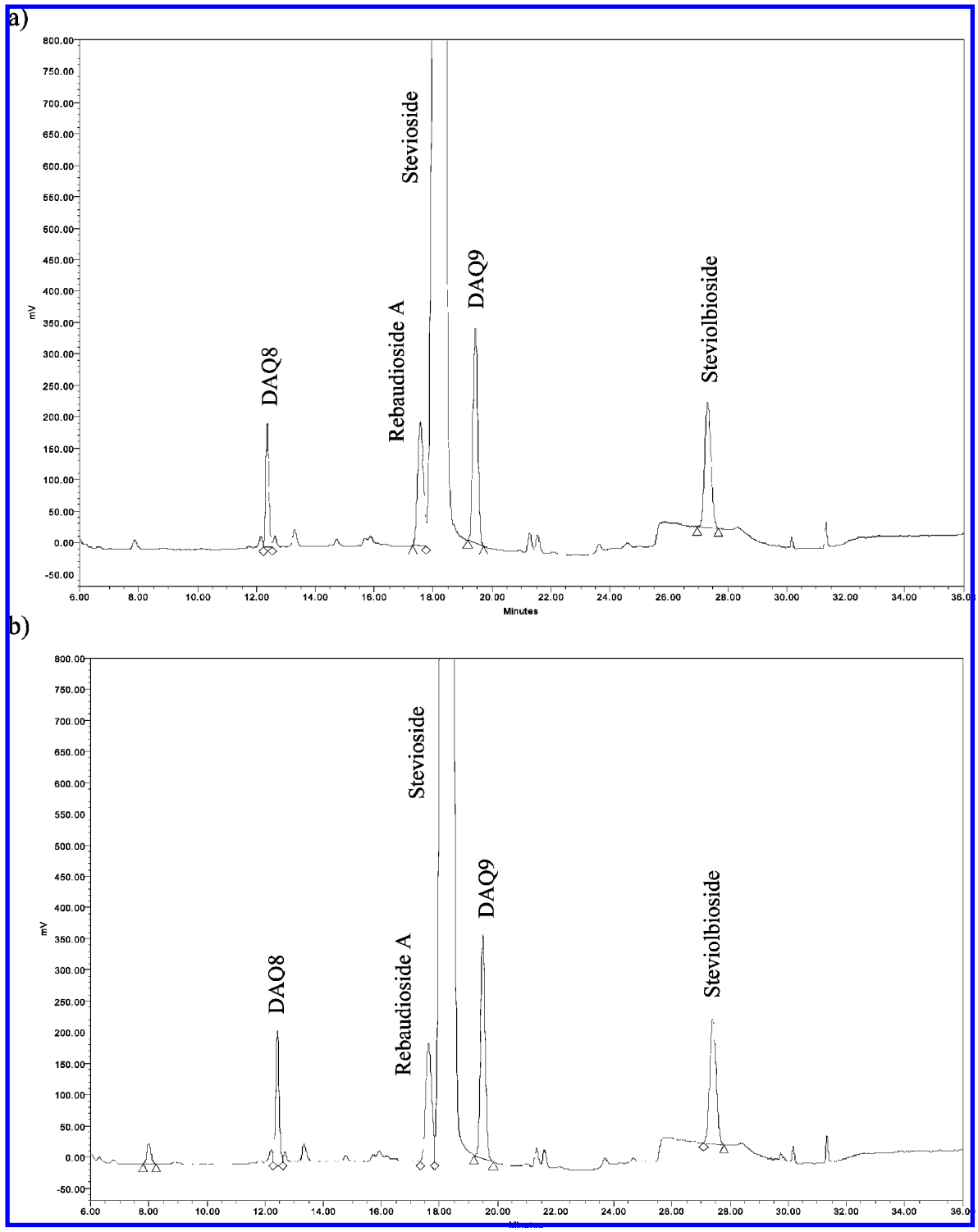


Figure 2. HPLC-CAD chromatograms for stevioside (**2**) in (a) control and (b) light-exposed lemon-lime soft drinks.

Glucopyranosyl Ester]. MP: 254−255 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 0.85 (m, 1H, C_1-H), 0.86(m, 1H, C_9-H), 0.97 (s, 3H, C_{20}-H), 1.05 (m, 1H, C_3-H), 1.11 (m,1H, C_5-H), 1.21 (s, 3H, C_{19}-H), 1.42 (m, 1H, C_2-H), 1.48 (m,1H, C_7-H), 1.50 (m, 2H, C_6-H, C_{11}-H), 1.6 (m, 1H, C_7-H), 1.62 (m,1H, C_{12}-H), 1.66 (m, 2H, C_{11}-H, C_{12}-H), 1.67 (m,1H, C_{14}-H), 1.71 (s, 3H, C_{17}-H), 1.83 (m, 1H, C_6-H), 1.84 (m, 1H, C_1-H), 1.96 (m, 1H, C_2-H), 2.13 (d, J = 12.2 Hz,1H, C_3-H), 2.22 (d, J = 9.6 Hz,1H, C_{14}-H), 3.25 (m, 1H, C_{40}-H), 3.27 (m, 1H, C_{34}-H), 3.35 (m, 1H, C_{22}-H), 3.38 (m, 2H, C_{30}-H, C_{41}-H), 3.46 (m, 1H, C_{23}-H), 3.61 (m, 1H, C_{28}-H), 3.73 (m, 1H, C_{29}-H), 4.64 (d, J = 8.5 Hz,1H, C_{27}-H), 4.66 (d, J = 7.8 Hz,1H, C_{33}-H), 4.80 (d, J = 8.2 Hz,1H, C_{39}-H), 5.12 (s, 1H, C_{15}-H), 5.39 (d, J = 8.9 Hz, 1H, C_{21}-H); ^{13}C NMR (125 MHz, CD_3OD) δ 12.1, 15.8, 19.9, 21.5, 28.7, 30.5, 38.8, 40.5, 41.6, 48.4, 48.9, 57.9, 75.2, 79.9, 87.2, 95.4, 96.9, 103.4, 104.0, 136.9. MS (ESI) calculated for $C_{44}H_{70}O_{23}$: 967.01; found: ($[M]^+$ 967.4, ($[M]^-$) 965.8.

Compound **8** *[DAQ 8, (13-[(2-O-β-D-Glucopyranosyl-β-D-glucopyranosyl)oxy]-16-hydroxy-kauran-18-oic Acid, β-D-Glucopyranosyl Ester].* MP: 225−227 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 0.85 (m, 1H, C_1-H), 0.90 (m, 1H, C_9-H), 0.99 (s, 3H, C_{20}-H), 1.05 (m, 1H, C_3-H), 1.10 (m, 1H, C_5-H), 1.20 (s, 3H, C_{19}-H), 1.26 (s, 3H, C_{17}-H), 1.36 (m, 1H, C_7-H), 1.42 (d, J = 13.7 Hz,1H, C_{15}-H), 1.41 (m, 1H, C_2-H), 1.36 (m, 1H, C_7-H), 1.58 (d, J = 14.4 Hz,1H, C_{15}-H), 1.63 (m, 1H, C_{11}-H), 1.74 (m, 1H, C_{12}-H), 1.83 (m, 1H, C_6-H), 1.63 (m, 1H, C_{11}-H),1.83 (m, 1H, C_1-H), 1.85 (m, 1H, C_{14}-H), 1.94 (m, 1H, C_2-H), 1.96 (m, 1H, C_{12}-H), 2.01 (m,1H, C_{14}-H), 2.16 (d, J = 11.9 Hz,1H, C_3-H), 3.36 (m,1H, C_{22}-H), 5.37 (d, J = 8.2 Hz,1H, C_{21}-H); ^{13}C NMR (125 MHz,

Table 4. Effect of Sunlight on the Stability of Rebaudioside A (**1**) and Stevioside (**2**) in Cola and Lemon-Lime Carbonated Soft Drinks

	Analytes (mg/L)[a]								
sample	1	2	3	4	5	6	7	8	9
Summary of Lemon-Lime pH 2.6 Sunlight Treated Samples (mg/L)									
rebaudioside A-control	949		12.1		13.7		3.55		2.54
rebaudioside A-sunlight exposed	914		12.5		15.4		4.61		4.84
stevioside-control	8.68	924		8.37		13.3		4.72	
stevioside-sunlight exposed	8.59	914		8.74		14.5		5.51	
Summary of Cola pH 2.4 Sunlight Treated Samples (mg/L)									
rebaudioside A-control	939		13.8		25.9		7.48		2.47
rebaudioside A-sunlight exposed	881		16.9		44.6		13.2		3.15
stevioside-control	8.39	922		9.30		24.0		8.26	
stevioside-sunlight exposed	8.08	885		11.9		42.2		14.1	

[a] Initial concentration of rebaudioside A (953 mg/L) and stevioside (939 mg/L) in cola and lemon-lime carbonated soft drinks.

CD_3OD) δ 16.0, 19.8, 20.6, 22.2, 23.1, 28.6, 31.2, 38.9, 41.7, 42.2, 43.1, 55.7, 56.1, 58.1, 76.0, 78.0, 83.2, 95.5, 97.3, 105.5. MS (ESI) calculated for $C_{38}H_{62}O_{19}$: 822.39; found: ($[M]^+$)823.4, ($[M]^-$) 821.4.

Compound **6** *[DAQ 9, 13-[(2-O-β-D-Glucopyranosyl-β-D-glucopyranosyl)-oxy]-kaur-15-en-18-oic Acid, β-D-Glucopyranosyl Ester].* ^{1}H NMR (500 MHz, CD_3OD) δ 0.85 (m, 1H, C_1-H), 0.86(m, 1H, C_9-H), 0.99 (s, 3H, C_{20}-H), 1.05 (m, 1H, C_3-H), 1.10 (m,1H, C_5-H), 1.20 (s, 3H, C_{19}-H), 1.40 (m, 1H, C_2-H), 1.48 (m,1H, C_7-H), 1.52 (m, 1H, C_{11}-H), 1.62 (m, 1H, C_7-H), 1.63 (m,1H, C_{12}-H), 1.69 (m, 1H, C_{12}-H), 1.67 (m,1H, C_{14}-H), 1.71 (s, 3H, C_{17}-H), 1.81 (s, 3H, C_6-H), 1.84 (m, 1H, C_1-H), 1.95 (m, 1H, C_2-H), 1.98 (m, 1H, C_6-H), 2.13 (d, J = 13.3 Hz,1H, C_3-H), 2.24 (d, J = 10.0 Hz,1H, C_{14}-H), 3.29 (m, 1H, C_{40}-H), 3.35 (m, 1H, C_{22}-H), 3.43 (m, 1H, C_{28}-H), 4.63 (d, J = 8.5 Hz,1H, C_{27}-H), 4.55 (d, J = 7.8 Hz,1H, C_{39}-H), 5.10 (s, 1H, C_{15}-H), 5.37 (d, J = 8.2 Hz, 1H, C_{21}-H); ^{13}C NMR (125 MHz, CD_3OD) δ 12.3, 15.9, 19.9, 21.6, 21.8, 28.6, 30.0, 38.9, 40.5, 41.8, 48.2, 48.6, 58.1, 82.4, 95.5, 96.9, 105.0, 136.6. MS (ESI) calculated for $C_{38}H_{60}O_{18}$: 804.38; found: ($[M]^+$ 805.3.4, ($[M]^-$) 803.3.

Compound **9** *[Imp-2, 13-[(2-O-β-D-Glucopyranosyl-3-O-β-D-glucopyranosyl]-β-D-glucopyranosyl)-oxy]-17-hydroxy- kaur-15-en-18-oic Acid, β-D-Glucopyranosyl Ester].* ^{1}H NMR (500 MHz, CD_3OD) δ 0.87 (m, 1H, C_1-H), 0.91(d, J = 8.5 Hz,1H, C_9-H), 0.99 (s, 3H, C_{20}-H), 1.06 (m, 1H, C_3-H), 1.12 (m,1H, C_5-H), 1.21 (s, 3H, C_{19}-H), 1.41 (m, 1H, C_2-H), 1.54 (m,1H, C_7-H), 1.55 (m, 2H, C_{11}-H), 1.65 (m, 1H, C_7-H), 1.68 (m,1H, C_{12}-H), 1.74 (m,1H, C_{14}-H), 1.79 (m, 1H, C_{12}-H), 1.83 (m, 1H, C_6-H), 1.86 (m, 1H, C_1-H), 1.97 (m, 1H, C_2-H), 2.14 (d, J = 12.6 Hz,1H, C_3-H), 2.30 (d, J = 10.4 Hz,1H, C_{14}-H), 3.21 (m, 1H, C_{40}-H), 3.27 (m, 1H, C_{34}-H), 3.37 (m, 3H, C_{22}-H, C_{30}-H, C_{41}-H), 3.62 (m, 1H, C_{28}-H), 3.74 (m, 1H, C_{29}-H), 4.11 (d, J = 14.0 Hz, 1H, C_{17}-H), 4.29 (d, J = 14.0 Hz, 1H, C_{17}-H), 4.69 (d, J = 7.8 Hz,1H, C_{27}-H), 4.66 (d, J = 7.8 Hz,1H, C_{33}-H), 4.84 (d, J = 7.8 Hz,1H, C_{39}-H), 5.37 (d, J = 8.2 Hz, 1H, C_{21}-H), 5.38 (s, 1H, C_{15}-H); ^{13}C NMR (125 MHz, CD_3OD) δ 15.8, 19.8, 21.4, 21.6, 28.6, 30.7, 38.8, 40.3, 41.7, 47.7, 49.6, 58.0, 59.2, 75.2, 75.5, 79.8, 87.6, 95.5, 96.8, 103.5, 104.1, 137.0. MS (ESI) calculated for $C_{44}H_{70}O_{24}$: 982.43; found: ($[M]^+$ 983.5, ($[M]^-$) 981.4.

Instrumentation and Conditions. An Agilent (Wilmington, DE) 1200 HPLC, including a quaternary pump, a temperature controlled column compartment with additional 6-port switching valve, an autosampler, and a UV absorbance detector, was used for the analysis. A charged aerosol detector (CAD), ESA, Inc. (Chelmsford, MA), was also used for the analysis. The scale on the CAD was 100 pA, and the filter was set to medium. The switching valve diverted the first 5.5 min of each injection away from the CAD detector to prevent fouling of the detector. The system was controlled using Waters (Milford, MA) Empower software. For Karl Fischer moisture analysis, titration was performed using a Metrohm 784 KFP Titrino titrator.

Method 1 employed a Phenomenex (Torrance, CA) Synergi-Hydro column (250 mm × 4.6 mm, 4 μm) with a Phenomenex Security Guard C_{18} cartridge and a tertiary solvent mobile phase (A, 0.040% NH_4OAc/HOAc buffer; B, MeCN and C: 0.040% HOAc). These solvents were used according to the gradient defined in **Table 3**. The column was at a temperature of 55 °C, and the flow rate was 1.0 mL/minute. The injection volume was 100 μL for both samples and standards, which were kept at ambient temperature while in the autosampler. Ultraviolet (UV) detection at 215 nm was used for analysis of both **1** and **2**, and 210 nm was used for **2** when analyzing for it as an impurity. In all cases for UV detection, a 4 nm bandwidth was used with a reference wavelength of 260 nm (100 nm bandwidth). The CAD was used for the analysis of all other impurities and degradation products. The run time was 43 min.

Method 2 was an isocratic separation that used a Phenomenex Prodigy ODS (3) column (150 × 2 mm, 5 μm). The mobile phase was 45% 10 mM H_3PO_4 (pH 3.0) and 55% MeCN. The column was at a temperature of 40 °C, and the flow rate was 0.3 mL/min. The injection volume was 100 μL for both samples and standards, which were kept at ambient temperature while in the autosampler. Ultraviolet detection at 210 nm (4 nm bandwidth) and a reference wavelength of 260 nm (100 nm bandwidth) was used for the analysis. The run time was 12 min.

Analysis Procedure. For Method 1, the column was flushed with 50 mL of 90% MeCN to waste before use. In both Methods 1 and 2, the samples were bracketed with standards by injecting them at the beginning and at the end of a run. Additional sets of standards were inserted into longer runs.

Quantitation of Analytes. Each analyte was identified by retention time matching with reference standards. The area response of each analyte was determined for the samples and standards. Full fit 1/x weighted linear regression standard curves for the UV detector data were prepared by plotting analyte concentrations in mg/L. In a similar manner, the CAD detector data were fitted to a 1/x weighted quadratic standard curve line. The Empower data acquisition software was used to prepare the calibration curves and to calculate concentrations of analytes.

RESULTS AND DISCUSSION

Chromatographic Separation. Typically, stevia sweeteners are separated on an amino (NH_2) column since the main differences between them are the number and type of glycoside moieties attached to steviol (**Table 1**). This is acceptable when analyzing stevia extracts or purified materials dissolved in an organic or principally organic solvent (e.g., 80/20 MeCN/water). However, for analyses of principally aqueous samples such as beverages, only small volumes (<10 μL) can be injected, which leads to poor detection limits. Injection of larger volumes leads to loss of chromatographic resolution since water is a strong solvent for NH_2 columns. Because of this issue, a reversed phase (i.e., C_{18}) column is generally preferred when analyzing beverages. Reversed phase columns are also preferred because they have better reproducibility than NH_2 columns and are more durable. However, on a standard C_{18} column, **1** and **2** coelute as do rebaudioside B (**3**) and steviolbioside (**4**). Therefore, for beverage analysis, a column was needed that could handle

large injections of aqueous beverages and, at the same time, provide increased interactions with the analyte glycosidic moieties.

Recently, staionary phases have been developed that address the limitations of the older bonded polar and bonded nonpolar phase columns. One such stationary phase is incorporated in the Synergi-Hydro column from Phenomenex. These columns are manufactured with both 2 and 4 μm stationary phase particle sizes. Unfortunately, the 2 μm columns are limited to a length of 50 mm, which does not provide enough resolution to separate all of the analytes. Therefore, a 4 μm stationary phase particle size/250 mm column was used with the drawback of having longer run times. This column was chosen as it provided the resolution of all analytes in the study.

Detection sensitivity was also a key factor considered in the development of Method 1. The steviol aglycone contains both carboxylic acid and olefin moieties. Although these are weak UV absorbers, they give an adequate signal at 210 nm to meet the required quantitation limit (LOQ) of 0.5 mg/L. However, for degradation products lacking olefinic unsaturation (i.e., **7** and **8**), the LOQ is much higher. Therefore, analytes **7** and **8** required an alternative method of detection. Charged aerosol detection (CAD) is a relatively recent type of detection, which has been commercialized by ESA, Inc. The CAD is similar to evaporative light scattering detectors (ELSD) in that it evaporates the mobile phase (and therefore requires volatile mobile phases), and it is a universal detector for most nonvolatile analytes. However, CAD detection is different from ELSD in that the CAD transfers a charge to the analytes, and this charge is measured, whereas ELSD measures the light scattered by analytes.

We found that the CAD provides about 3−5 times better sensitivity for analytes of this study. One drawback of CAD detection is that it broadens peaks slightly, thus degrading the resolution between **1** and **2**. Therefore, since **1**−**6** and **9** have olefinic unsaturation as chromophores, they have an adequate LOQ using UV detection and were quantitated by this detection method. We found LOQs for **7** and **8** to be significantly affected by the eluant program. For this reason, a somewhat complex eluant program was employed such that a flat baseline is achieved at the time of analyte elution.

All of the compounds listed in **Table 1** are adequately separated using this method for accurate quantitation. No interferences were detected in the beverages. **Figures 1** and **2** show the chromatograms for the control and light-exposed samples of **1** and **2**, respectively.

Photostability of Rebaudioside A (1) and Stevioside (2). According to Chang and Cook, in cola and lemon-lime beverages, 22% and 18%, respectively, of **1** was lost after exposure to 3000 langleys of sunlight. It was curious, however, that under the same conditions, **2** was unaffected. Therefore, we undertook this study to determine if the Chang and Cook results could be replicated. Their paper did not give details of the beverages except that they were cola and citrus. We assumed the "citrus beverage" to be a lemon-lime beverage system. Other information in the Chang and Cook paper described model H_3PO_4 (pH 2.4) and citric acid (pH 2.6) systems. Therefore, we prepared cola beverage prototypes at pH 2.4 and lemon-lime beverage prototypes at pH 2.6.

Our results for the sunlight-promoted degradation of **1** and **2** are provided in **Table 4**. Overall, the results reported by Chang and Cook are not consistent with our findings. For **1**, in all cases, the light-exposed samples were within 3−7% of the light-protected control samples and, in the case of **2**, the light-exposed samples were within 1−7% of the controls. The minor degradation products of **2** were identified as **6** and **8** (**Table 1**), which are clearly analogous to **5** and **7**, respectively, derived from **1**. The fact that these degradation products are formed in similar amount in both control and sun-light exposed samples suggests that they are acid-promoted rather than sunlight-promoted degradation products.

In summary, a very carefully controlled study was performed using validated analytical methods where all analytes present at significant levels (>0.5%) were known or identified and where all analytes greater than 0.1% were quantified with primary reference standards. With this study, we have demonstrated that **1**, as well as **2**, is stable to sunlight exposure. We also found good mass balance agreement in the exposed samples relative to the controls (>95%). Also, contrary to the work of Chang and Cook, we found no significant difference in the photostability between **1** and **2**, which is to be expected on the basis of their similar structures.

Differences between our findings and those reported by Chang and Cook reported 25 years ago are likely due to differences between the analytical methods (sample preparation and chromatographic techniques). First, they injected 25 μL of sample. In the case of **1**, the beverage was taken to dryness and then reconstituted in water, which for an NH_2 column, is a poor choice as the sample solvent. In the case of **2**, they extracted with water-saturated 1-butanol and then reconstituted but did not define the solvent. This can lead to distortion of the peaks and accelerated deterioration of the column, both of which can lead to split peaks. The second issue is that their sample preparation methods are different for the beverages made with **1** from those made with **2**. This could lead to the differences in the chromatography between the two analyses. Another issue with the sample preparation procedure is that it concentrates the acids in the case of **1** when the beverage was taken to dryness and could have caused degradation. Acid-catalyzed addition of water across double bonds is known in the literature (*9*). This was not a problem for **2** because it was extracted into water-saturated butanol first.

ACKNOWLEDGMENT

We thank Corunda Pruitt, Valerie Mercier and Tyler Simpson for the preparation of model beverages.

LITERATURE CITED

(1) Kinghorn, A. D., Kim, N.-C. and Kim, D. H. L. Terpenoid Glycoside Sweeteners. In *Naturally Occurring Glycosides*; Ikan, R., Eds.; John Wiley & Sons: New York, 1999; pp 399−429.

(2) DuBois, G. E.; Dietrich, P. S.; Lee, J. F.; McGarraugh, G. V.; Stephenson, R. A. Diterpenoid sweeteners. Synthesis and sensory evaluation of stevioside analogues nondegradable to steviol. *J. Med. Chem.* **1981**, *24*, 1269–1271.

(3) Prakash, I.; DuBois, G. E.; Clos, J. F.; Wilkens, K. L.; Fosdick, L. E. Development of rebiana, a natural, non-calorie sweetener, *Food Chem. Toxicol.* **2008**, *46*, S75−S82.

(4) Rebaudioside A: An Assessment of Safety. *Food Chem. Toxicol.*, **2008**, *46*, S1−S92.

(5) International Conference on Harmonisation. *ICH Harmonised Tripartite Guideline, Stability Testing: Photostability Testing of New Drug Substances and Products, Q1B*, Current Step 4 Version; November, 1996.

(6) Chang, S. S.; Cook, J. M. Stability studies of stevioside and rebaudioside A in carbonated beverages. *J. Agric. Food Chem.* **1983**, *31*, 409–412.

(7) Kolb, N.; Herrera, J. L.; Ferreyra, D. J.; Uliana, R. F. Analysis of sweet diterpene glycosides from *Stevia rebaudiana*: Improved

HPLC method. *J. Agric. Food Chem.* **2001**, *8*, 105–111. (a) Ahmed, M. S.; Dobberstein, R. H. *Stevia rebaudiana*. III. High-performance liquid chromatographic separation and quantitation of rebaudiosides B, D, and E, dulcoside A, and steviolbioside. *J. Chromatogr.* **1982**, *245*, 373–376.

(8) *CRC Handbook of Chemistry and Physics*, 64th ed.; Weast, R. C., Ed; CRC Press, Inc: Boca Raton, FL, 1983; p, F-92.

(9) March, J. *Advanced Organic Chemistry*, 4th ed.; Wiley: New York, 1992; p 759.

Received for review April 30, 2008. Revised manuscript received June 27, 2008. Accepted July 30, 2008.

JF801343E