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Research Article

STABILITY STUDY OF STEVIOL GLYCOSIDES IN MOCK BEVERAGES USING FLUORESCENT LIGHT EXPOSURE UNDER ICH GUIDELINES

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ABSTRACT

Photostability of a mixture of steviol glycosides was studied under fluorescent light exposure at 25 °C in mock beverages at pH 3.8 using International Conference on Harmonization (ICH) technical requirements covering the stability testing of new drug substances and products. Experimental results indicated that steviol glycoside mixture did not undergo any major decomposition with fluorescent light exposure for 2 weeks but yielded three minor compounds which were identified as 13-[(2-*O*-β-D-glucopyranosyl-*O*-β-D-glucopyranosyl)oxy]-17-hydroxy-*ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl ester, 13-[(2-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester, and 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-16-hydroxy-*ent*-kauran-19-oic acid β-D-glucopyranosyl ester, on the basis of ^{1}H & ^{13}C NMR, COSY, HSQC, HMBC, MS, MS/MS spectral data as well as hydrolysis studies. Also, the mass balance for the fluorescent exposed mixture of glycosides was calculated against their controls and was found as 98.3% supporting that any appreciable amount of undetected degradation products were formed under the conditions of the study.

Key words: Steviol glycoside mixture, Fluorescent light exposure, Stability, Degradation products.

INTRODUCTION

Recent interest of many soft drink manufacturers has driven their focus towards natural high-potency sweeteners in order to reduce calories by introducing non-caloric sweeteners into their systems. Extracts of *Stevia rebaudiana* (Bertoni) Bertoni, a perennial shrub of the Asteraceae (Compositae) family native to certain regions of South America (Paraguay and Brazil) often referred to as "the sweet herb of Paraguay" [1-2] resulted in the isolation of several potently sweet diterpenoid glycosides namely rebaudiosides A and D, stevioside, and dulcoside A which are known as stevia sweeteners [3]. These compounds are all glycosides of the diterpene *ent*-13-hydroxykaur-16-en-19-oic acid known as steviol [4]. Due to their continuing demand, there has been intense commercial interest on *S. rebaudiana* which is grown commercially in a number of countries, particularly in Japan, Taiwan, Korea, Thailand and Indonesia.

As a part of our continuing research to discover natural sweeteners, we have recently isolated several novel diterpene glycosides from the commercial extracts of the leaves of *S. rebaudiana* obtained from various suppliers around the world [5-9]. Apart from isolating novel compounds from *S. rebaudiana* and utilizing them as possible natural sweeteners or sweetness enhancers, we are also engaged in understanding the stability of the steviol glycosides in various systems of interest and their behavior profiles. In this article, we are describing the photostability of a mixture of steviol glycosides under fluorescent light exposure at 25° C in mock beverages at pH 3.8 using ICH technical requirements at 60% relative humidity. The composition of the steviol glycoside mixture studied in this experimentation is mainly the seven compounds namely stevioside (**1**), rebaudioside A (**2**), rebaudioside B (**3**), steviolbioside (**4**), rubusoside (**5**), dulcoside A (**6**), rebaudioside C (**7**), as well as the other eight compounds at relatively low abundance, rebaudioside D (**8**), 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosyl) ester (**9**), 13-[(2-*O*-(6-*O*-β-D-glucopyranosyl)-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid β-D-glucopyranosyl ester (**10**), 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl ester (**11**), rebaudioside F (**12**), 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-xylopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid β-D-glucopyranosyl ester (**13**), 13-[(2-*O*-β-D-xylopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid β-D-glucopyranosyl ester (**14**), 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-(4-*O*-α-D-glucopyranosyl)-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid β-D-glucopyranosyl ester (**15**). Further, the steviol glycosides studied belongs to two different classes of *ent*-kaurane diterpene glycosides: *ent*-13-hydroxykaur-16-en-19-oic acid (**1**-**10**, **12**-**15**) (Figure 1), and *ent*-13-hydroxykaur-15-en-19-oic acid (**11**) (Figure 2).

EXPERIMENTAL

Major computer systems

The major computer systems used for this study are:

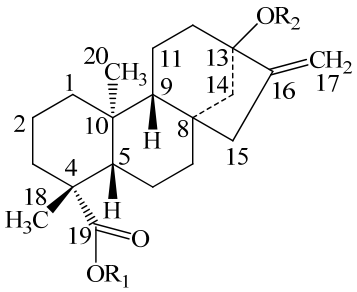
1. Metasys and/or REES (monitor and document facility storage and environmental conditions with constant temperature rooms (if applicable)
2. Waters Empower Chromatography Manager (data acquisition and result calculation system)
3. eNotes (official study communication)
4. Laboratory Information Management System (sample and assay tracking)
5. Nautilus 2001 R2 B2 (Thermo Lab Systems) (sample and assay tracking)

Reference standards and other compounds

All reference standards were isolated by AMRI (Bothell, WA) or prepared by The Coca-Cola Company and were certified by Chromadex (Irvine, CA). The steviol glycoside mixture used in this study mainly contained the seven compounds namely stevioside (**1**), rebaudiosides A & B (**2**-**3**), steviolbioside (**4**), rubusoside (**5**), dulcoside A (**6**), and rebaudioside C (**7**) which were quantitated using their corresponding standard compounds. The relatively low abundant glycosides rebaudiosides D (**8**) and F (**12**), 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl ester (**11**), and 13-[(2-*O*- β -D-glucopyranosyl-3-*O*-(4-*O*-α-D-glucopyranosyl)-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid β-D-glucopyranosyl ester (**15**), were identified by comparison of their retention times with known reference standards whereas the other compounds greater than or equal to 0.100%, were quantitated with stevioside (**1**). For compounds which reference standards or their standard retention times are not available (**9**-**10**, **13**-**14**), identification and

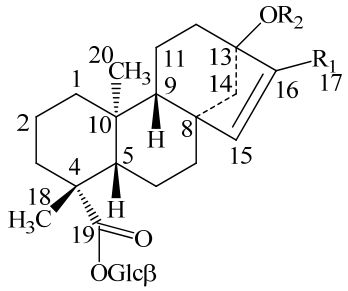
characterization was achieved on the basis of extensive spectroscopic data (^{1}H & ^{13}C NMR, COSY, HSQC, HMBC, MS, MS/MS) and in comparison with the data reported in the literature [5-10, 15]. The compound numbers, empirical formulae and molecular weights (adjusted to two decimal places) of the reference standards are provided in Table 1.

Figure 1



Compound	R_1	R_2
1	Glcβ1	Glcβ1-2Glcβ1-
2	Glcβ1	Glcβ1-2(Glcβ1-3)Glcβ1-
3	H	Glcβ1-2(Glcβ1-3)Glcβ1-
4	H	Glcβ1-2Glcβ1-
5	Glcβ1	Glcβ1-
6	Glcβ1	Rhaα1-2Glcβ1-
7	Glcβ1	Rhaα1-2(Glcβ1-3)Glcβ1-
8	Glcβ1-2Glcβ1-	Glcβ1-2(Glcβ1-3)Glcβ1-
9	Rhaα1-2Glcβ1-	Glcβ1-2(Glcβ1-3)Glcβ1-
10	Glcβ1-	Glcβ1-6Glcβ1-2Glcβ1-
12	Glcβ1	Xylβ1-2(Glcβ1-3)Glcβ1-
13	Glcβ1	Glcβ1-2(Xylβ1-3)Glcβ1-
14	Glcβ1	Xylβ1-2Glcβ1-
15	Glcβ1	Glcβ1-2Glcα1-4(Glcβ1-3)Glcβ1-

Figure 2



Compound	R_1	R_2
11	CH_3	Glcβ1-2Glcβ1-

Glcβ: β–D-glucopyranosyl; Glcα: α–D-glucopyranosyl;
Rhaα: α–L-rhamnopyranosyl; Xylβ: β–D-Xylopyranosyl

Table 1: Molecular weights and formulae of steviol glycosides

Compound	Molecular formula	Molecular weight
1	$C_{38}H_{60}O_{18}$	804.88
2	$C_{44}H_{70}O_{23}$	967.01
3	$C_{38}H_{60}O_{18}$	804.88
4	$C_{32}H_{50}O_{13}$	642.73
5	$C_{32}H_{50}O_{13}$	642.73
6	$C_{38}H_{60}O_{17}$	788.88
7	$C_{44}H_{70}O_{22}$	951.02
8	$C_{50}H_{80}O_{28}$	1129.15
9	$C_{50}H_{80}O_{27}$	1113.15
10	$C_{44}H_{70}O_{23}$	967.01
11	$C_{38}H_{60}O_{18}$	804.88
12	$C_{43}H_{68}O_{22}$	936.99
13	$C_{43}H_{68}O_{22}$	936.99
14	$C_{37}H_{58}O_{17}$	774.86
15	$C_{50}H_{80}O_{28}$	1129.15

Preparation of mock beverage samples

Bottles of mock beverages containing deionized water, potassium benzoate, tri-sodium citrate (dihydrate), citric acid (anhydrous), potassium chloride, sodium chloride, magnesium chloride, and calcium sulfate (anhydrous) were prepared at pH 3.8 by The National Food Laboratory, Livermore, CA (The NFL) per The NFL Project Number PR7784-B and were stored refrigerated by Covance in a desiccator at 5 ±3° C.

Reagents and chemicals

Glacial acetic acid (AcOH) 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 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).

Mobile phase preparation

All solvents were degassed for at least two minutes with helium before use. The HPLC method employed was a three solvent mobile phase system: Solvent A (0.0284% NH_4OAc, 0.0116% AcOH) which was prepared by dissolving 0.569 g of NH_4OAc and 0.231 ml of AcOH in two liters of purified water and mixing thoroughly; Solvent B was 100% MeCN; and Solvent C (0.040% AcOH) was prepared by adding 0.4 ml of AcOH to one liter of purified water by mixing thoroughly [10].

Dilution buffer and standard compounds preparation

The diluent buffer was prepared by adjusting one liter of water to pH 3.3 with glacial AcOH. 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 to at 5 $\pm$ 3 °C.

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 auto sampler 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 minutes 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. The RP-HPLC employed a Phenomenex (Torrance, CA) Synergi-Hydro column (250 mm x 4.6 mm, 4 µm) with a Phenomenex Security guard C_{18} cartridge and a tertiary solvent mobile phase (A: 0.040% NH_4OAc/AcOH buffer, B: MeCN and C: 0.040% AcOH). The column was at a temperature of 55°C and the flow rate was 1.0 ml/minute. The injection volume of each sample was 100 µl, which were kept at ambient temperature while in the auto sampler. 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 band width). CAD was used for the analysis of all steviol glycosides with a total run time of 43 min. NMR spectra were acquired on Bruker Avance DRX 500 MHz and Varian Unity Plus 600 MHz instruments using standard pulse sequences. MS data were generated with a Waters Premier Quadrupole Time-of-Flight (Q-TOF) mass spectrometer equipped with an electrospray ionization source operated in the positive-ion mode and a ThermoFisher Discovery OrbiTrap in the electrospray positive mode. Samples were diluted with water-acetonitrile (1:1) containing 0.1% formic acid and introduced via infusion using the onboard syringe pump.

Analysis procedure

For the RP-HPLC method, the column was flushed with 50 ml of 90% MeCN to waste before use and the samples were bracketed with standards by injecting them at the beginning and at the end of a run for accuracy of their retention times. The details of the solvents used for the RP-HPLC gradient method are given in Table 2.

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.

Identification and spectroscopic data for the degradation products

*13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-17-hydroxy-ent-kaur-15-en-19-oic acid β-D-glucopyranosyl ester (**16**)*: colorless oil; ^{1}H NMR (500 MHz, CD_3OD) δ 1.00 (s, 3H, C_{20}-CH_3), 1.21 (s, 3H,

C_{18}-CH_3), 4.11 (d, *J*=14.0 Hz, 1H, C_{17}-H), 4.29 (d, *J*=14.0 Hz, 1H, C_{17}-H), 4.58 (d, *J*=7.8 Hz, 1H), 4.66 (d, *J*=7.8 Hz, 1H), 5.37 (d, *J*=8.2 Hz, 1H), 5.36 (s, 1H, C_{15}-H); MS (ESI): $[M+H]^+$ 983.5; $[M-H]^-$) 981.4; +ESI TOFMS *m/z* 821.3817 (calcd. for $C_{38}H_{61}O_{19}$: 821.3807).

*13-[(2-O-α-L-rhamnopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oic acid-(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) ester (**17**)*: colorless oil; ^{1}H NMR (500 MHz, CD_3OD) δ 0.93 (s, 3H, C_{20}-CH_3), 1.24 (s, 3H, C_{18}-CH_3), 4.84 (s, 1H, C_{17}-H), 5.14 (s, 1H, C_{17}-H), 4.50 (d, *J*=7.8 Hz,1H), 4.65 (d, *J*=7.8 Hz,1H), 4.71 (d, *J*=8.2 Hz,1H), 5.42 (d, *J*=7.8 Hz,1H), 5.37 (d, *J*=8.2 Hz,1H); +ESI TOFMS *m/z* 1113.4999 (calcd for $C_{50}H_{81}O_{27}$: 1113.4965).

*13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-16β-hydroxy ent-kauran-19-oic acid β-D-glucopyranosyl ester (**18**)*: white powder; ^{1}H NMR (500 MHz, CD_3OD) δ 0.98 (s, 3H, C_{20}-CH_3), 1.20 (s, 3H, C_{18}-CH_3), 1.25 (s, 3H, C_{17}-CH_3), 4.67 (d, *J*=7.8 Hz,1H, C_{33}-H), 4.70 (d, *J*=8.2 Hz,1H), 4.88 (d, *J*=7.8 Hz,1H), 5.37 (d, *J*=8.2 Hz,1H); MS (ESI): $[M+H]^+$ 985.5; $[M-H]^-$) 983.6.

Table 2: RP-HPLC method for the separation of steviol glycosides

Time (min)	% of Mobile phase A	% of Mobile phase B	% of Mobile phase C
0.0	75	25	0
8.5	75	25	0
10.0	71	29	0
16.5	70	30	0
18.5	0	34	66
24.5	0	34	66
26.5	0	52	48
29.0	0	52	48
31.0	0	70	30
37.0	0	70	30
37.1	0	90	10
40.0	0	90	10
40.1	75	25	0
43.0	75	25	0

*General procedure for acid hydrolysis and determination of sugar configuration in **16-18:*** Each compound (500 µg) was hydrolyzed with 0.5 M HCl (0.5 mL) for 1.5 h. After cooling, the mixture was passed through an Amberlite IRA400 column and the eluate was lyophilized. The residue was dissolved in pyridine (0.25 mL) and heated with L-cysteine methyl ester HCl (2.5 mg) at 60ºC for 1.5 h, and then *O*-tolyl isothiocyanate (12.5 uL) was added to the mixture and heated at 60ºC for an additional 1.5 h. The reaction mixture was analyzed by HPLC: column Phenomenex Luna C18, 150 x 4.6 mm (5 u); 25% acetonitrile-0.2% TFA water, 1 mL/min; UV detection at 250 nm. The sugars were identified as D-glucose (*t*R, 12.21 and *t*R, 12.18 min) in compound **16** and **18**; and D-glucose (*t*R, 12.26 min) and L-rhamnose (*t*R, 21.32 min) for compound **17** [authentic samples, D-glucose (*t*R, 12.35) and L-glucose (*t*R, 11.12 min); D-rhamnose (*t*R, 11.73) and L- rhamnose (*t*R, 21.64 min)] [15][11].

*Enzymatic hydrolysis of **16-18**:* Each compound (250 µg) was dissolved in 2.5 ml of 0.1 M sodium acetate buffer, pH 4.5 and crude pectinase from *Aspergillus niger* (50 uL, Sigma-Aldrich, P2736) was added. The mixture was stirred at 50° C for 48 hr. The product precipitated out during the reaction and was filtered and then crystallized. The resulting products obtained from the hydrolysis of **16-18** were identified as *ent*-13, 17-dihydroxykaur-15-en-19-oic acid, steviol and *ent*-13, 16β-dihydroxykauran-19-oic acid [12] respectively by comparison of their ^{1}H NMR spectral data.

Table 3: Summary of original and light treated steviol glycosides at pH 3.8 (mg/l) [a]

Compound	Steviol Glycoside Mixture (Original)	Control Samples (Aluminum Foil Covered)	Fluorescent Light Treated
1	374	371	365
2	95.2	94.7	92.8
3	0.587	0.592	0.606
4	1.96	1.91	1.93
5	1.92	1.82	1.79
6	0.972	0.978	0.955
7	22.9	22.6	22.1
8	1.88	1.84	1.86
9	0.718	0.753	0.940
10	0.750	0.766	0.896
11	1.32	0.591	0.631
12	3.27	1.55	1.52
13	0.554	3.31	3.26
14	1.02	0.560	0.542
15	0.625	1.08	1.03
16	-	-	0.922
17	-	-	0.557
18	-	0.570	0.581
19	-	0.519	0.542
20	-	0.923	0.896
21	-	0.884	0.876
22	-	0.625	0.607

[a] Results are mean of three sample preparations

RESULTS AND DISCUSSION

The primary objective of this study was to assess the stability of a mixture of steviol glycosides under fluorescent light exposure at 25° C and relative humidity [RH] of 60% in mock beverage storage conditions. The pH used for this study was 3.8, which is similar to a lemon-lime beverage system. Also, we are herewith reporting the mass (mole) balances of the mixture of steviol glycosides and its major degradation products obtained during the course of study.

The stability of a steviol glycoside mixture was evaluated in mock beverage solutions by simulating formulations used in commercial lemon-lime soft drinks (pH 3.8), but lacking the flavour components. Three sets of mock beverages at pH 3.8 were prepared as described above were taken and the mixture steviol glycosides at a concentration of about 500 mg/l was added to glass bottles which were covered with plastic wrap. An additional three sets of rebaudioside-A mixture in mock beverage bottles at pH 3.8 at similar concentration as above were covered with plastic wrap as well as an aluminium foil and were studied as control samples. Bottles were placed side-by-side with a validated chemical actinometrical system to ensure that the specified light exposure is obtained and exposed to a minimum of 1.2 million lux hours and not less than 200 watt hours/m^2 exposure to near ultra-violet light at 25° C as defined in the ICH guidelines [13] for a 2 week period. Samples were analysed using the HPLC method as stated above for steviol glycosides, their known impurities and degradation products, as well as unidentified compounds that are greater than or equal to 0.100% from the starting concentration of steviol glycosides. All samples were treated identically during analysis to minimize assay bias. From the results shown in Table 3, it was found that there was almost no change in the concentration of the seven major steviol glycosides **1**-**7** in both the control and fluorescent light treated steviol glycoside mixtures. Further it was found that the control steviol glycoside mixture covered with plastic wrap and aluminium foil showed five additional compounds **18**-**22** and the fluorescent light exposed steviol glycoside mixture resulted in the identification of seven compounds **16**-**22**. The results indicated that the fluorescent treated steviol glycoside mixture resulted in the identification of two additional compounds **16** and **17** against their controls samples.

The structures of the three additional identified compounds **16**-**18** from light treated samples and control samples were characterized on the basis of extensive spectroscopic data (^{1}H & ^{13}C NMR, COSY, HSQC, HMBC, MS, MS/MS) and the details are given below.

Compound **16** was obtained as colourless oil, and its molecular formula was assigned as $C_{38}H_{60}O_{19}$ from the HRESI mass spectrum. The ^{1}H NMR spectrum of **16** showed the presence of two methyl singlets at δ 1.00 and 1.21, eight methylene and two methine protons between δ 0.86-2.32, an oxymethylene group as a doublet of doublets at δ 4.11 (*J* = 14.0 Hz) and 4.29 (*J* = 14.0 Hz) and a trisubstituted olefinic proton at δ 5.36 (s). Its ^{1}H NMR spectrum also showed three anomeric protons as doublets at δ 4.58 (*J*=7.8, 1H), 4.66 (*J*=7.8, 1H), and 5.37 (*J*=8.2, 1H). The NMR data for **16** were very similar to stevioside (**1**), except for the absence of an exocyclic double bond and a methylene group. A close comparison of the ^{1}H and ^{13}C NMR values of **16** with stevioside (**1**) suggested the migration of the exocyclic double bond from C-16/C-17 to C-15/C-16 with an additional hydroxyl group present at the C-17 position. This was supported by the ^{13}C NMR values for the oxymethylene group at C-17 which appeared at δ 59.2 and a trisubstituted double bond between C-15 and C-16 which were observed at δ 136.6 and 146.9, respectively. Acid hydrolysis of **16** afforded D-glucose that was identified by preparing its corresponding thiocarbamoyl-thiazolidine carboxylate derivative with L-cysteine methyl ester and *O*-tolyl isothiocyanate, and in comparison of its retention time with the standard sugars as described in the literature comparison [11]. Enzymatic hydrolysis of **16** furnished an aglycone which was identified as *ent*-13, 17-dihydroxykaur-15-en-19-oic acid on the basis of its ^{1}H NMR spectral data [12]. The large coupling constants observed for the three glucose anomeric protons suggested their β-orientation similar to stevioside (**1**) and rebaudioside A (**2**). COSY, HSQC, HMBC and 1D-TOCSY correlations suggested that **16** has a 2-subustituted β-D-glucobiosyl unit at C-13 and β-D-glucosyl substituent unit as an ester at C-19 on *ent*-13, 17-dihydroxykaur-15-en-19-oic acid, the aglycone moiety. On the basis of above spectral and chemical studies as well as by comparing the spectral data reported in the literature [9], the structure of **16** was established as 13-[(2-*O*-β-D-glucopyranosyl-β-D glucopyranosyl)oxy]-17-hydroxy-*ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl ester as shown in Figure 3.

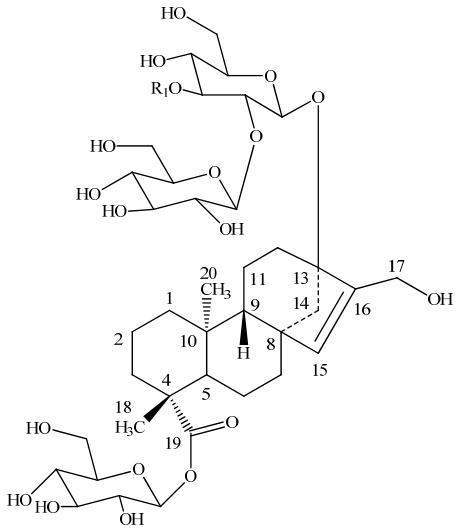


Figure 3: Structure of compound **16**

Compound **17** was also obtained as a colorless film, and its molecular formula was assigned as $C_{50}H_{80}O_{27}$ from the positive ESI TOF mass spectrum at 1113.4999. The ^{1}H NMR spectrum of **17** showed the presence of two methyl singlets at δ 0.93 and 1.24, nine methylene and two methine protons between δ 0.85-2.23, two protons as singlets at δ 4.84 and 5.14 of an exocyclic double bond; similar to **1**. The presence of five sugar units in its structure was supported by the ^{1}H NMR spectrum of **17** which showed the

presence of anomeric protons at δ 4.50, 4.65, 4.71, 5.37 and 5.42 which were inferred as four hexose moieties and a deoxyhexose unit from the ESI MS/MS spectrum of **17** that showed the fragment ions at *m/z* 951, 789, 627 and 465 as well as further fragmentation of *m/z* 951 to *m/z* 805. Acid hydrolysis of **17** afforded sugar units which were identified as D-glucose and L-rhamnose by preparing their corresponding thiocarbamoyl-thiazolidine carboxylate derivatives as described in **16**. Based on the results from NMR and MS/MS spectral data as well as hydrolysis experiments of **17**, it was confirmed that this compound is having steviol backbone with four β-D-glucosyl substituents and an α-L-rhamnosyl moiety in its structure. Enzymatic hydrolysis of **17** furnished an aglycone which was identified as steviol [12]. On the basis of COSY, HSQC, HMBC and 1D-TOCSY correlations it was suggested that **17** has a 2-*O*-α-L-rhamnosyl-3-*O*-β-D-glucosyl-β-D-glucosyl unit at C-13 and a 2-subustituted β-D-glucobiosyl unit as an ester at C-19 on steviol. The anomeric proton of the L-rhamnosyl unit was appeared at δ 5.42 had a coupling constant of 1.2 Hz indicating its α-configuration similar to dulcosides A and B [14]. The large coupling constants observed for the four anomeric protons of the D-glucosyl moieties at δ 4.50 (d, *J* = 7.8 Hz), 4.65 (d, *J* = 7.8 Hz), 4.71 (d, *J* = 8.2 Hz), 5.37 (d, *J* = 8.2 Hz), and 5.42 (d, *J*=7.8 Hz), suggested their β-orientation similar to **1** and **16**. Comparison of the spectral data with rebaudioside K isolated from *S. rebaudiana* Morita [15] which was obtained by hybridization breeding of *S. rebaudiana* Bertoni, suggested the structure of **17** as 13-[(2-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyra-nosyl)oxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-β-D -glucopyranosyl-β-D-gluco-pyranosyl) ester (Figure 4).

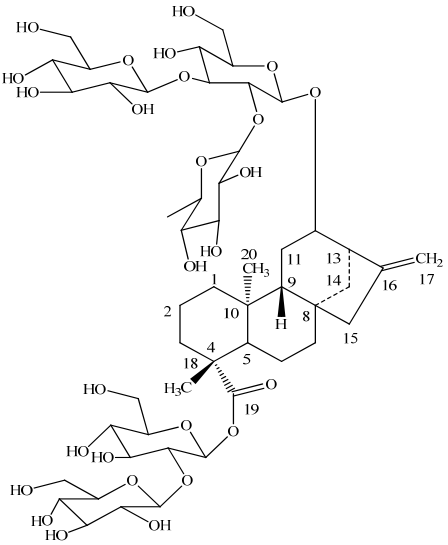


Figure 4: Structure of compound **17**

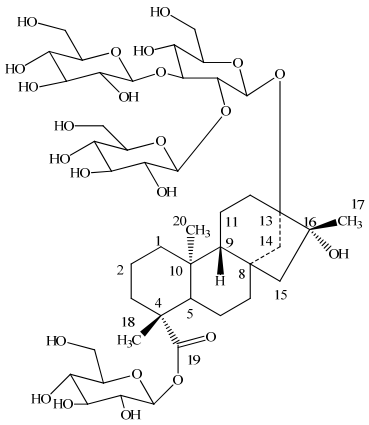


Figure 5: Structure of compound **18**

Compound **18** was obtained as white powder, and its molecular formula was assigned as $C_{44}H_{72}O_{24}$ from the ESI mass spectrum which showed $(M+H)^+$ ion at *m/z* 985.5 in its positive mode and $(M-H)^+$ ion at *m/z* 985.6 in its negative mode. The ^1H NMR spectrum of **18** showed the presence of three methyl singlets at δ 0.98, 1.20, and 1.25; four anomeric protons that were observed as doublets at δ 4.67 (*J*=7.8 Hz, 1H), 4.70 (*J*=8.2 Hz, 1H), 4.88 (*J*=7.8 Hz, 1H), and 5.37 (*J*=8.2 Hz, 1H). Acid hydrolysis of **18** afforded D-glucose as

mentioned for compounds **16** and **17**. Enzymatic hydrolysis of **18** furnished an aglycone that was identified as *ent*-13, 16β-dihydroxykauran-19-oic acid on the basis of NMR spectral data comparisons with the literature values [12]. Based on the COSY, HSQC, HMBC and 1D-TOCSY correlations, it was suggested that **18** has a 2,3-branched β-D-glucotriosyl moiety unit at C-13 and a β-D-glucosyl substituent unit as an ester at C-19 on the aglycone moiety, *ent*-13, 16β-dihydroxykauran-19-oic acid. Based on the above spectral and hydrolysis studies the structure of **18** was deduced as 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-16β-hydroxy *ent*-kauran-19-oic acid β-D-glucopyranosyl ester (Figure 5) consistent with literature reported values [10]. The structures of the other four compounds **19**-**22** were not characterized for the want of more material, but were considered to be having the similar molecular composition of stevioside for mass balance calculation. The retention times (t_R) for the four unknowns **19**-**22** are 13.6, 14.5, 23.1 and 23.8 respectively. The mole equivalents calculated from the analytical values of each compound on the basis of its molecular weight re in Table 4, which indicated that the steviol glycoside mixture that was treated with fluorescent light exposure for 2-weeks had a mass balance of 98.3 mole percent.

Table 4: Mass balance of light treated steviol glycosides at pH 3.8 (µmol/l)

Compound	Control samples (Aluminum foil covered)	Fluorescent light treated
1	461	453
2	97.9	96.0
3	0.736	0.753
4	2.97	3.00
5	2.83	2.78
6	1.24	1.21
7	23.8	23.2
8	1.69	1.69
9	0.676	0.844
10	0.792	0.927
11	0.523	0.559
12	1.93	1.89
13	3.53	3.48
14	0.598	0.578
15	1.39	1.33
16	-	1.12
17	-	0.500
18	0.579	0.590
19[a]	0.645	-
20[a]	1.15	1.11
21[a]	1.10	1.09
22[a]	0.777	0.754
Total	606	596
Fluorescent light treated vs Control samples		98.3%

[a] All the unknowns were assumed to have the molecular weight of stevioside (**1**) for quantitation

CONCLUSION

From the HPLC analysis of the fluorescent light exposed steviol glycoside mixture, it was found that there was minimal degradation at 25° C in mock beverages at pH 3.8 under 60% RH. Further, the high mass balance values found for the molar recoveries for both mixture of steviol glycosides in the fluorescent light exposure suggested that there was not any appreciable amount of undetected degradation products were formed under the conditions of the study. This suggested that steviol glycoside mixtures are considered relatively stable using the conditions of this study when exposed to fluorescent light.

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