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# Endocyclic double bond isomers and by-products from rebaudioside A and stevioside formed under acid conditions

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ABSTRACT

The hydrolysis reaction of rebaudioside A and stevioside was studied under mild and strong acidic conditions at different temperatures and times. Exposure of rebaudioside A to vigorous acid conditions yielded three different aglycone cores which were purified by silica gel chromatography: compound **1** (isosteviol), **2** (steviol) and **3** (endocyclic isomer of steviol). Four new glucoside degradation products with an *ent*-kaur-15-en-19-oic acid skeleton together with three known compounds were obtained after mild acid hydrolysis from rebaudioside A and stevioside. Compounds were identified based on extensive 1D and 2D NMR measurements and/or MS/MS fragmentation: compound **4** (*Endo*-steviolmonoside), compound **5**:(*Endo*-rebaudioside $G_1$), compound **6** (*Endo*-steviolbioside) and compound **7** (*Endo*-rubusoside) while compounds **8–10** were identified as *iso*-rebaudioside A (*Endo*-rebaudioside A) **(8)**, *iso*-rebaudioside B (*Endo*-rebaudioside B) **(9)** and *iso*-stevioside (*Endo*-stevioside) **(10)**. Crystal structure determination of compound **3** and **6** allowed confirmation of their structures by X-ray diffraction. Acidic beverages based on steviol glycosides as sugar substitutes should be properly storage for avoiding the loss of their organoleptic properties by formation of these or other hydrolysis products.

## 1. Introduction

Sucrose has historically played a leading role as a tabletop sweetener and in the commercial preparation of various foods and beverages. However, at present it has been determined that a significant number of chronic diseases are the consequence of the excessive consumption of sugar (Malik et al., 2010). Among these diseases, cardiovascular accidents, diabetes, and obesity are the cause of death of about 38 million people per year (WHO, 2013).

One way to prevent these diseases is by consuming non-caloric sweeteners to replace sucrose (Shankar et al., 2013; Hu and Malik, 2010). There are numerous natural compounds from plant species that perform this function (Kinghorn et al., 1986). Steviol glycosides, extracted from *Stevia rebaudiana* (Bertoni) Bertoni (Asteraceae), are among the most popular non-caloric sweeteners after recognition as safe molecules by the United States Food and Drug Administration (Andress, 2008; JECFA, 2010). Rebaudioside A and stevioside are the major tetracyclic diterpene glycosides of *Stevia* extracts with an *ent*-13-hydroxykaur-16-en-19-oic acid aglycone core. Both, rebaudioside A and stevioside are several times sweeter than sucrose but they impart undesirable sensory attributes such as bitterness, metallic and licorice tastes to foods and beverages sweetened with Stevia (Kroyer et al., 2010; Rocha and Bolini, 2015; Hellfritsch et al., 2012; Singla and Jaitak, 2016; Jaitak Vikas, 2015; Prakash et al., 2014a,b). However, rebaudioside A exhibits reduced bitterness compared to stevioside when used at low to medium sweetening levels (Prakash et al., 2008). Several soft drinks and juices are being prepared with steviol glycosides from *S. rebaudiana*, contributing to the health improvement of soft drink consumers. However, it's well known that soft drinks have low pH and steviol glycosides undergo double bond isomerization with sugar cleavage and Wagner-Meerwein rearrangement under acidic conditions (Lee, 2011; Avent et al., 1990; Prakash et al., 2012, 2014a,b; Chaturvedula et al., 2011a,b). Additionally, ingestion of steviol glycosides is non-caloric since the glycosides are not bioavailable, however their microbiome produced aglycone, steviol or its isomers, may well be adsorbed into the systemic circulation. Thus, preparation and characterization of those potential metabolites is warranted.

Herein, is described the isolation and structure elucidation of the

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acid hydrolysis reaction products of rebaudioside A and stevioside under vigorous and mild acidic conditions.

## 2. Materials and methods

### 2.1. Chemicals

$CH_3CN$, MeOH and $H_2O$ for HPLC and silica gel 60 F254 HPTLC plates were purchased from EMD Millipore (http://www.emdmillipore.com/). The bulk $CH_3CN$, MeOH, methyl *tert*-butyl ether (MTBE), AcOH, EtOAc were purchased from Reagents (http://www.reagents.com/). HCl, *n*-BuOH and $NaHCO_3$ were purchased from Fisher Scientific (https://www.fishersci.com/), THF from Alfa Aesar (https://www.alfa.com/). Flash silica was purchased from Sorbent Technologies (https://www.sorbtech.com/). NMR (perdeuterated) solvents were purchased from Cambridge Isotope Laboratories (https://www.isotope.com/).

### 2.2. Acid hydrolysis of rebaudioside A and stevioside

#### 2.2.1. Vigorous acid hydrolysis

20 g of rebaudioside A were dissolved in 100 mL of 1N HCl and heated at 80 °C over 3 h. The reaction mixture was submitted to liquid-liquid partition with EtOAc (5 × 200 mL) to yield 6.3 g of EtOAc extractable (*hydrolysis reaction 1*).

#### 2.2.2. Mild acid hydrolysis

Rebaudioside A (28 g and 30 g) and stevioside (21 g) were individually dissolved with heating in 140 mL of THF:$H_2O$ (6:1 v/v). Rebaudioside A and stevioside solutions were cooled to room temperature and 12 mL of HCl (37%) were added to form a solution of 1 N HCl. The reaction mixtures were initially monitored in milligram scale at 30 °C and 40 °C and after in gram scale at 40 °C for and 24 h and 66 h for rebaudioside A and 20 h for stevioside. Solutions were first neutralized with $NaHCO_3$ and then, the THF was removed in vacuo.

Rebaudioside A and stevioside solids were digested with EtOAc (3 × 100 mL) to afford 23.5 g (66 h), 27 g (24 h) and 15 g (20 h) of solids respectively which were dissolved in 10 mL of MeOH and 100 mL of $H_2O$. MeOH was evaporated and the aqueous solution was submitted to *n*-BuOH partitions (3 × 50 mL). Rebaudioside A *n*-BuOH layers furnished 9 g (Hydrolysis reaction 2) and 12 g (Hydrolysis reaction 3) while 7 g were obtained from stevioside *n*-BuOH layer (Hydrolysis reaction 4). All the solids showed a white color.

### 2.3. Isolation procedure

6.3 g of the reaction mixture from vigorous acid hydrolysis of rebaudioside A (Hydrolysis reaction 1) were absorbed onto 60 g of celite and subjected to high efficiency normal-phase chromatography (7.5 i.d. × 50 cm, 10 μm) using two step gradient *wa*MTBE: *n*-heptane (20:80 v/v) and *wa*MTBE: *n*-heptane (40:60 v/v). 3 × 1 L and 1 × 100 mL forerun were collected followed with 12 × 120 mL fractions with *wa*MTBE: *n*-heptane (20:80) + 21 × 120 mL fractions with *wa*MTBE: *n*-heptane (40:60) (chromatography **1**). Fractions and forerun were analyzed by HPLC to afford six main pools following the pooling procedure previously described (Rodenburg et al., 2016). Compound **1** (4.2 g), **2** (46 mg) and **3** (438 mg) were purified from forerun 4- fraction 19; fractions 33–34 and fractions 37–50, respectively. Compound **3** was crystalized in MeOH (30 mg/mL) in form of needles and submitted to X-Ray analysis.

Solids (9 g, hydrolysis reaction 2) from rebaudioside A mild acid hydrolysis reaction mixture after 66 h were submitted to two runs in a high efficiency normal-phase chromatography (7.5 i.d. × 50 cm, 10 μm) spherical silica gel, (chromatography **2**). 6.2 g were dissolved in modified Reb C mobile phase (12 L Reb C + 10 L EtOAc), [Reb C mobile phase, 100:18:14:0.1 v/v/v/v of EtOAc:MeOH:$H_2O$:AcOH] and loaded into the column. Elution with modified mobile phase was begun and 2 × 1 L forerun were collected, followed by 50 × 100 mL fractions. All fractions were analyzed by HPLC and five pools were selected based on results from column analysis. **Pool 1.2**: forerun 2 (0.41 g); **pool 2.2**: fractions 1–3 (0.29 g); **pool 3.2**: fractions 7–9 (0.60 g); **pool 4.2**: fractions 11–14 (1.41 g) and **pool 5.2**: fractions 22–26 (1.35 g), pools designation is as follows**: pool x.y**, where **x**: pool number and **y**: chromatography number. In the second run 2.8 g were submitted to similar chromatographic conditions (chromatography **3**) to afford again five main pools based on results from column analysis. **Pool 1.3**: forerun 2 (0.16 g); **pool 2.3**: forerun 3 (0.73 g); **pool 3.3**: fractions 1–3 (0.14 g); **pool 4.3**: fractions 13–16 (0.27 g) and **pool 5.3**: fractions 17–19 (0.51 g). Compound **4** (410 mg) and **5** (160 mg) came from **pools 1.2** and **1.3** respectively. 200 mg from of the **pool 4.2** were submitted to a (2.5 × 40 cm, 10 μm) RP-C18 chromatography (Chromatography **4**) with $CH_3CN$:$H_2O$:AcOH (35:65:0.01 v/v/%). 3 × 120 mL and 1 × 60 mL forerun plus 25 fractions (1–4, 20 mL); (5–25, 10 mL) were collected and analyzed by HPLC. Two main pools were selected based on results from column analysis. **Pool 1.4**: fractions 1–14 (119.4 mg) and **pool 2.4**: fractions 15–19 (52.3 mg). **Pool 2.4** afforded compound **6** (52.3 mg). **Pools 5.2** and **5.3** were combined and digested with MeOH (200 mg/mL) to afford compound **8** (800 mg).

Solids (12 g, hydrolysis reaction 3) from rebaudioside A mild acid hydrolysis reaction mixture after 24 h were submitted to a high efficiency reversed-phase chromatography (7.5 i.d. × 50 cm, 10 μm). Sample (12 g) was dissolved in 200 mL of the mobile phase $CH_3CN$: $H_2O$: AcOH (30: 70: 0.01 v/v/%), loaded into column and elution begun. (Chromatography **5**) 3 × 1 L forerun were collected, followed by 43 × 100 mL fractions plus fractions 44 and 45 × 1 L each. All fractions were analyzed by HPLC and six pools were selected based on results from column analysis. Pools rich in rebaudioside A/*Endo*-rebaudioside A, **pool 3.5**: fractions 31–40 (700 mg) and **pool 4.5**: fractions 41–43 (1.45 g) were combined with 1.5 g of the same mixture previously obtained under similar procedure. 3.65 g was submitted to a high efficiency reversed-phase chromatography (7.5 i.d. × 50 cm, 10 μm). The sample was dissolved in 300 mL of the mobile phase $CH_3CN$: $H_2O$: AcOH (27: 73: 0.01 v/v/%) loaded into the column and elution begun. (Chromatography **6**). 9 × 1 L forerun were collected plus 45 fractions, 1–10 × 120 mL; 11–45 × 50 mL. All fractions were analyzed by HPLC and five main pools were selected. Compound **9** (110 mg) eluted in **pool 3.6**: fractions 25–27.

Solids (7 g, hydrolysis reaction 4) from stevioside mild hydrolysis reaction were dissolved in modified Reb C mobile phase (12 L Reb C + 10 L EtOAc) and loaded into a high efficiency normal-phase chromatographic column (7.5 i.d. × 50 cm, 10 μm spherical silica gel) and elution begun. Chromatography **7**, 1 × 1 L forerun plus 35 × 120 mL fractions were collected. All fractions were analyzed by HPLC and three main pools were selected based on results from column analysis. **Pool 1.7**: fractions 1–8 (1.6 g); **pool 2.7**: fractions 9–12 (3.2 g) and **pool 3.7**: fractions 13–19 (2.5 g). **Pool 3.7** was divided into portions; the first (1 g) was submitted to (2.5 × 40 cm, 10 μm) RP-C18 chromatography (Chromatography **8**) with $CH_3CN$: $H_2O$: AcOH (30: 70: 0.01 v/v/%). 4 × 120 mL forerun and 46 fractions, 1–5 × 20 mL; 6–46 × 10 mL were collected. Fractions were analyzed by HPLC and five main pools were selected, **pool 1.8**: fractions 1–5 (0.13 g); **pool 2.8**: fractions 6–16 (0.37 g); **pool 3.8**: fractions 17–21 (0.2 g); **pool 4.8**: fractions 22–24 (0.08 g) and **pool 5.8**: fractions 25–29 (0.06 g). The remaining 2.5 g of the pool 3.7 was again submitted to similar chromatographic conditions in a high efficiency reversed-phase chromatography (7.5 i.d. × 50 cm, 10 μm) (Chromatography **9**). 7 × 1 L + 1 × 200 mL forerun plus 29 fractions, 1–8 × 120 mL, 9–29 × 50 mL were collected. Three main pools were selected based on the HPLC chromatographic profile, **pool 1.9:** forerun 7–8 and fractions 1–10 (1 g); **pool 2.9**: fractions 11–14 (0.2 g) and **pool 3.9**: fractions 15–25 (0.3 g). Pools 3.8 and 3.9 were combined and afforded compound **10** (500 mg).

**Pool 2.7** (3.2 g) was submitted to a high efficiency normal-phase chromatography (Chromatography **10**) (7.5 i.d. × 50 cm, 10 μm) using

**Table 1**
$^{1}H$ NMR and $^{13}C$ NMR chemical shifts of compound **3–6**.

| Moiety | Position | 3[a] | | 4[a] | | 5[a] | | 6[a] | |
|---|---|---|---|---|---|---|---|---|---|
| | | $\delta_H$ (ppm) | $\delta_C$ (ppm) | $\delta_C$ (ppm) | $\delta_C$ (ppm) | $\delta_H$ (ppm) | $\delta_C$ (ppm) | $\delta_H$ (ppm) | $\delta_C$ (ppm) |
| Aglycone | 1 | 0.92; 1.92 | 41.8 | 0.89; 1.89 | 41.7 | 0.86; 1.83 | 41.7 | 0.83; 1.79 | 41.5 |
| | 2 | 2.32; 1.56 | 20.4 | 2.25; 2.25 | 20.3 | 2.24; 1.50 | 20.3 | 2.24; 1.48 | 20.1 |
| | 3 | 2.50; 1.11 | 39.2 | 2.47; 1.09 | 39.2 | 2.45; 1.07 | 39.2 | 2.46; 1.06 | 39.0 |
| | 4 | – | 44.4 | – | 44.4 | – | 44.4 | – | 44.2 |
| | 5 | 1.14 | 57.3 | 1.08 | 57.0 | 1.06 | 57.2 | 1.04 | 57.0 |
| | 6 | 2.23; 2.07 | 22.1 | 2.17; 2.01 | 22.0 | 2.16; 2.02 | 22.0 | 2.13; 1.95 | 21.8 |
| | 7 | 1.90; 1.79 | 33.4 | 1.69; 1.56 | 40.5 | 1.69; 1.54 | 40.5 | 1.60; 1.50 | 40.3 |
| | 8 | – | 48.7 | – | 48.8 | – | 48.7 | – | 49.1 |
| | 9 | 0.97 | 47.9 | 0.91 | 47.9 | 0.89 | 47.9 | 0.87 | 47.7 |
| | 10 | – | 40.6 | – | 40.6 | – | 40.6 | – | 40.3 |
| | 11 | 1.72; 1.72 | 21.9 | 1.58; 1.67 | 21.9 | 1.55; 1.66 | 21.7 | 1.60; 1.60 | 21.5 |
| | 12 | 1.75; 1.60 | 40.6 | 1.91; 1.91 | 31.2 | 1.89; 1.89 | 31.1 | 2.05; 1.98 | 30.0 |
| | 13 | – | 82.4 | – | 89.8 | – | 90.1 | – | 89.4 |
| | 14 | 2.47; 1.77 | 52.3 | 2.48; 2.14 | 48.5 | 2.48; 2.11 | 48.8 | 2.54; 2.03 | 48.5 |
| | 15 | 5.24 | 135.0 | 5.19 (s) | 136.5 | 5.21 (s) | 136.5 | 5.17 (s) | 135.4 |
| | 16 | – | 145.9 | – | 144.4 | – | 144.1 | – | 144.3 |
| | 17 | 1.89 | 12.7 | 2.01 | 13.4 | 1.98 | 13.4 | 2.03 | 13.0 |
| | 18 | 1.38 | 29.8 | 1.34 | 29.9 | 1.35 | 29.8 | 1.32 | 29.6 |
| | 19 | – | 180.6 | – | 180.6 | – | 180.5 | – | 180.4 |
| | 20 | 1.24 | 16.3 | 1.13 | 16.2 | 1.14 | 16.2 | 1.23 | 16.0 |
| Glc$\beta$-$C_{19}$ | 1′ | – | – | – | – | – | – | – | – |
| | 2′ | – | – | – | – | – | – | – | – |
| | 3′ | – | – | – | – | – | – | – | – |
| | 4′ | – | – | – | – | – | – | – | – |
| | 5′ | – | – | – | – | – | – | – | – |
| | 6′ | – | – | – | – | – | – | – | – |
| Glc$\beta$-$C_{13}$ | 1″ | – | – | 5.15 | 99.6 | 5.09 | 99.3 | 5.18 | 97.7 |
| | 2″ | – | – | 4.05 | 76.1 | 4.04 | 79.2 | 4.18 | 84.6 |
| | 3″ | – | – | 4.26 | 79.2 | 4.19 | 89.4 | 4.32 | 78.3 |
| | 4″ | – | – | 4.27 | 72.1 | 4.14 | 70.1 | 4.26 | 72.0 |
| | 5″ | – | – | 3.86 | 78.5 | 4.04 | 74.7 | 3.95 | 78.9 |
| | 6″ | – | – | 4.44; 4.33 | 63.3 | 4.54; 4.23 | 62.9 | 4.51; 4.44 | 63.2 |
| Glc$\beta$(1-X)[b] | 1‴ | – | – | – | – | 5.32 | 106.6 | 5.26 | 106.8 |
| | 2‴ | – | – | – | – | 4.05 | 76.1 | 4.12 | 77.5 |
| | 3‴ | – | – | – | – | 4.26 | 78.8 | 4.25 | 78.2 |
| | 4‴ | – | – | – | – | 4.20 | 72.1 | 4.26 | 71.6 |
| | 5‴ | – | – | – | – | 3.77 | 78.2 | 3.74 | 78.0 |
| | 6‴ | – | – | – | – | 4.54; 4.34 | 63.0 | 4.34; 4.27 | 62.7 |

*Endocyclic steviol* **(3)**; *Endo*-steviolmonoside **(4)**; *Endo*-rebaudioside $G_1$ **(5)**; *Endo*-steviolbioside **(6)**.
[a] NMR spectra were recorded in Pyr-$d_5$.
[b] X = 3 for compound **5**; X = 2 for compound **6**.

modified Reb C mobile phase (12 L Reb C + 10 L EtOAc). 1 forerun × 1 L plus 80 fractions, 1–4 × 100 mL; 5–80 × 50 mL. Three main pools were collected based on HPLC analysis of the fractions. **Pool 2.10**: fractions 31–32 (1.47 g) was again submitted to a high efficiency normal-phase chromatography (7.5 i.d. × 50 cm, 10 µm) (Chromatography **11**) using MTBE: MeOH: $H_2O$: AcOH (100: 15: 5: 0.01 v/v/v/%). 1 forerun × 1 L + 1 × 800 mL and 70 × 50 mL fractions were collected. Again, three main pools were selected, **pool 3.11**: fractions 34–40 (62 mg) showed a mixture of two compounds: rubusoside and compound **7**. Several efforts were made to purify compound **7** using silica and reversed-phase chromatographies but we were not successful in the purification of **7**.

### 2.4. Ultra-high-performance liquid chromatography-quadrupole time of flight-mass spectrometry analysis

The liquid chromatographic system was an Agilent Series 1290 comprised of the following modular components: binary pump, a vacuum solvent microdegasser, an autosampler with 100-well tray and a thermostatically controlled column compartment. Separation was achieved on an Agilent Zorbax C8 (2.1 × 50 mm, 1.8 µ) column. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at a flow rate of 0.2 mL/min. The separation was achieved as follows: 0 min, 70% A: 30% B, isocratic for next 20 min. One microliter of sample was injected. The column temperature was set at 35 °C. The mass spectrometric analysis was performed with a QToF-MS/MS (Model #G6530A, Agilent Technologies, Palo Alto, CA, USA) equipped with an ESI source with Jet Stream technology using the following parameters: drying gas ($N_2$) flow rate, 13.0 L/min; nebulizer, 35 psig, sheath gas temperature, 325 °C; sheath gas flow, 10 L/min; capillary, 3500 V; skimmer, 65 V; Oct RF V, 750 V; fragmentor voltage, 125 V. The sample collision energy was set at 10–70 eV. All the operations, acquisition and analysis of data were controlled by Agilent MassHunter Acquisition Software Ver. A.05.00 and processed with MassHunter Qualitative Analysis Software Ver. B.05.00. Each sample was analyzed in both positive mode in the range of $m/z$ = 100–1000. Accurate mass measurements were obtained by means of ion correction techniques using reference masses at $m/z$ 121.0509 (protonated purine) and 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine or HP-921] in positive ion mode. The compounds were confirmed in each spectrum.

### 2.5. General experiment procedure

Optical rotations were measured in MeOH at room temperature, using an Autopol IV instrument. Melting points were recorded using an OptiMelt automated Melting Point System from Standford Research Systems using open capillary. HRESIMS/MS for pure compounds were

acquired by direct infusion using an Agilent Model G6530A quadrupole time of flight mass detector equipped with an electrospray ionization interface and was controlled by Agilent software (Agilent MassHunter Work Station, A.05.00). All acquisitions were performed under negative ionization mode with a capillary voltage of 3500 V. $N_2$ was used as nebulizer gas (30 psig), as well as drying gas at 11 L/min at drying gas temperature of 325 °C. The voltage of PMT, fragmentor and skimmer was set at 750 V, 100 V and 65 V respectively. Full scan mass spectra were acquired from *m/z* 100–1700. Data acquisition and processing was done using the MassHunter Workstation software (Qualitative Analysis Version B.07.00).

1D and 2D spectra (COSY, gHSQC and gHMBC) were recorded on a Bruker Avance 500 NMR spectrometer at 298 K, operating at 500.13 MHz for $^1$H and 125.75 MHz for $^{13}$C using Pyridine-$d_5$. The chemical shifts are given in $\delta$ (ppm) and were calibrated using the residual solvent signals.

### 2.6. Physicochemical parameters of the new compounds

#### 2.6.1. 13-ent-kaur-15-en-19-oic acid (Endocyclic steviol isomer) (3)

Crystalline off-white solid; $[\alpha]_D^{25}$ −60.0 (*c* 0.1, MeOH), melting point 198–202 °C HRESIMS/MS *m/z* 317.2124 $[M-H]^-$ (calculated for $C_{20}H_{30}O_3$, 317.2122). $^1$H and $^{13}$C NMR spectroscopic data are summarized in Table 1.

#### 2.6.2. 13-[(β-D-glucopyranosyl)oxy]ent-kaur-15-en-19-oic acid (Endosteviolmonoside) (4)

Crystalline white solid; $[\alpha]_D^{25}$ −58.0 (*c* 0.1, MeOH), melting point 101–102 °C. HRESIMS/MS *m/z* 479.2639 $[M-H]^-$ (calculated for $C_{26}H_{40}O_8$, 479.2650), *m/z* 317.2027 (collision energy, 40 eV; intensity, 41%) (−162 Da), loss of one hexose. $^1$H and $^{13}$C NMR spectroscopic data are summarized in Table 1.

#### 2.6.3. 13-[(3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]ent-kaur-15-en-19-oic acid (Endo-rebaudioside $G_1$) (5)

Crystalline white solid; $[\alpha]_D^{25}$ −52.0 (*c* 0.1, MeOH), melting point 196–197 °C. HRESIMS/MS 641.3196 $[M-H]^-$ (calculated for $C_{32}H_{50}O_{13}$, 641.3179), *m/z* 479.2653 (94.8%) and *m/z* 317.2120 (14.9%) at collision energy, 40 eV; (−2 × 162 Da), loss of two hexoses. $^1$H and $^{13}$C NMR spectroscopic data are summarized in Table 1.

#### 2.6.4. 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]ent-kaur-15-en-19-oic acid (Endo-steviolbioside) (6)

Crystalline white solid; $[\alpha]_D^{25}$ −54.0 (*c* 0.1, MeOH), melting point 192–193 °C. HRESIMS/MS 641.3166 $[M-H]^-$ (calculated for $C_{32}H_{50}O_{13}$, 641.3179), *m/z* 479.2633 (58.7%) and *m/z* 317.2121 (10.5%) at collision energy, 40 eV; (−2 × 162 Da), loss of two hexoses. $^1$H and $^{13}$C NMR spectroscopic data are summarized in Table 1.

### 2.7. X-ray crystallography of compound (3) and (6)

The crystal structure of the endocyclic steviol isomer **3** was determined from a colorless crystal of the methanol solvate having dimensions 0.06 × 0.11 × 0.33 mm, using data collected at $T = 100$ K with MoKα radiation on a Bruker Kappa APEX-II DUO CCD diffractometer, equipped with an Oxford Cryostream cooler. The structure was solved using the program SHELXS-97 and refined anisotropically by full-matrix least squares on $F^2$ using SHELXL2014/7 (Sheldrick, 2008). H atoms were visible in difference maps, but were placed in idealized positions for the refinement, except for those of OH groups, which were refined. The absolute configuration was assigned from starting materials and is consistent with the Flack (1983) parameter based on resonant scattering of the light atoms. Crystal data: $C_{20}H_{30}O_3$ $CH_3OH$, $M_r = 350.48$, orthorhombic space group $P2_12_12_1$, $a = 7.4846(2)$, $b = 14.5254(4)$, $c = 17.4806(4)$ Å, $V = 1900.44(8)$ Å$^3$, $Z = 4$, $D_x = 1.225$ Mg m$^{-3}$, $\theta_{max} = 30.5°$, $R = 0.037$ for all 5813 unique data and 239 refined parameters. The Flack parameter is −0.6(3) and for 2028 Parsons, Flack and Wagner quotients (2013).

A very small, lower-quality twinned crystal of a mixed water-methanol solvate of endo-steviolbioside **6** was used to confirm its connectivity and relative configurations. Data were collected at T = 100 K on a Bruker Kappa APEX-II DUO CCD diffractometer, equipped with a Cu microfocus X-ray source. Structure solution and refinement was carried out as for **3**, except that disordered solvent was removed using the SQUEEZE procedure, water H atoms could not be located, and OH hydrogen atoms were not refined. Crystal data: $C_{32}H_{50}O_{13}$. $H_2O$, $M_r = 705.27$, monoclinic space group $P2_1$, $a = 12.025(2)$, $b = 7.7676(13)$, $c = 18.882(13)$ Å, β = 90.597(12)°, $V = 1763.6(6)$ Å$^3$, $Z = 2$, $D_x = 1.328$ Mg m$^{-3}$, $\theta_{max} = 59.2°$, $R = 0.110$ for 4153 observed (of 6542 unique) data and 423 refined parameters.

Supplementary crystallographic data for 3 are contained in Cambridge Structural Database deposition CCDC-1539128, and for 6 CCDC-1539676. This data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336 033; or deposit@ccdc.cam.ac.uk.

## 3. Results and discussion

Vigorous acid hydrolysis of rebaudioside A yielded three main aglycones. Compound **1** showed a HRESIMS spectrum with a deprotonated molecular ion at *m/z* 317.2128 $[M-H]^-$ (calculated for $C_{20}H_{30}O_3$, 317.2122). This compound was isolated in gram quantities and identified as isosteviol (4.2 g, 21% of rebaudioside A starting weight). Compound **2** was purified as the minor compound of the aglycone mixture, showing a HRESIMS spectrum with a deprotonated molecular ion at *m/z* 317.2122 $[M-H]^-$ (calculated for $C_{20}H_{30}O_3$, 317.2122) and was identified as steviol (46 mg, 0.23% of rebaudioside A starting weight), the aglycone core of the tetracyclic diterpene glycosides of *S. rebaudiana*. Chemical shifts of both compounds were assigned based on NMR experiments and by comparison with carbon chemical shifts reported in literature for methyl ester derivatives. Compound **3** was isolated from the mixture of reaction products (438 mg, 2.19% of rebaudioside A starting weight) displaying a deprotonated molecular ion at *m/z* 317.2124 $[M-H]^-$ (calculated for $C_{20}H_{30}O_3$, 317.2122) and identified by 1D and 2D NMR experiments as the *endo* isomer of steviol (13-*ent*-kaur-15-en-19-oic acid), Table 1. Chemical structures of compounds **1-3** are shown in Fig. 1. We were fortunate to grow high quality crystals from compound **3** allowing X-Ray structural confirmation (Fig. 2).

Compound **3** was previously obtained after vigorous acid hydrolysis of stevioside using a mineral acid (Avent et al., 1990). However, products obtained by Avent et al. (1990) were isolated from the reaction mixture using chromatography (silica gel modified with 10% $AgNO_3$) after preparation of their methyl esters. Herein, we were able to separate the three isomers underivatized using a two steps gradient in a high efficiency normal-phase chromatography (McChesney and Rodenburg, 2014). Isosteviol is the main byproduct from vigorous acid hydrolysis of rebaudioside A followed by the endocyclic steviol isomer and in a very low yield steviol. Most probably, the same three aglycones in similar ratios should be found after hydrolysis of other steviol glycosides. Acid hydrolysis is not a convenient approach to produce steviol in quantities since it is the minor product of the reaction.

Additionally, acid hydrolysis of rebaudioside A (28 g) and stevioside (21 g) were both set at room temperature and at 40 °C (1 N HCl). No hint of degradation products under mild acidic conditions was observed at room temperature after one week. However, several degradation products were observed at 40 °C after 24 h and 66 h for rebaudioside A and 20 h for stevioside. RP-C18 HPLC chromatograms of rebaudioside A sampling after acid hydrolysis are shown in Fig. 3.

Mild acid hydrolysis of rebaudioside A at 40 °C at 24 h showed a mixture of rebaudioside A/*Endo*-rebaudioside A and rebaudioside B/

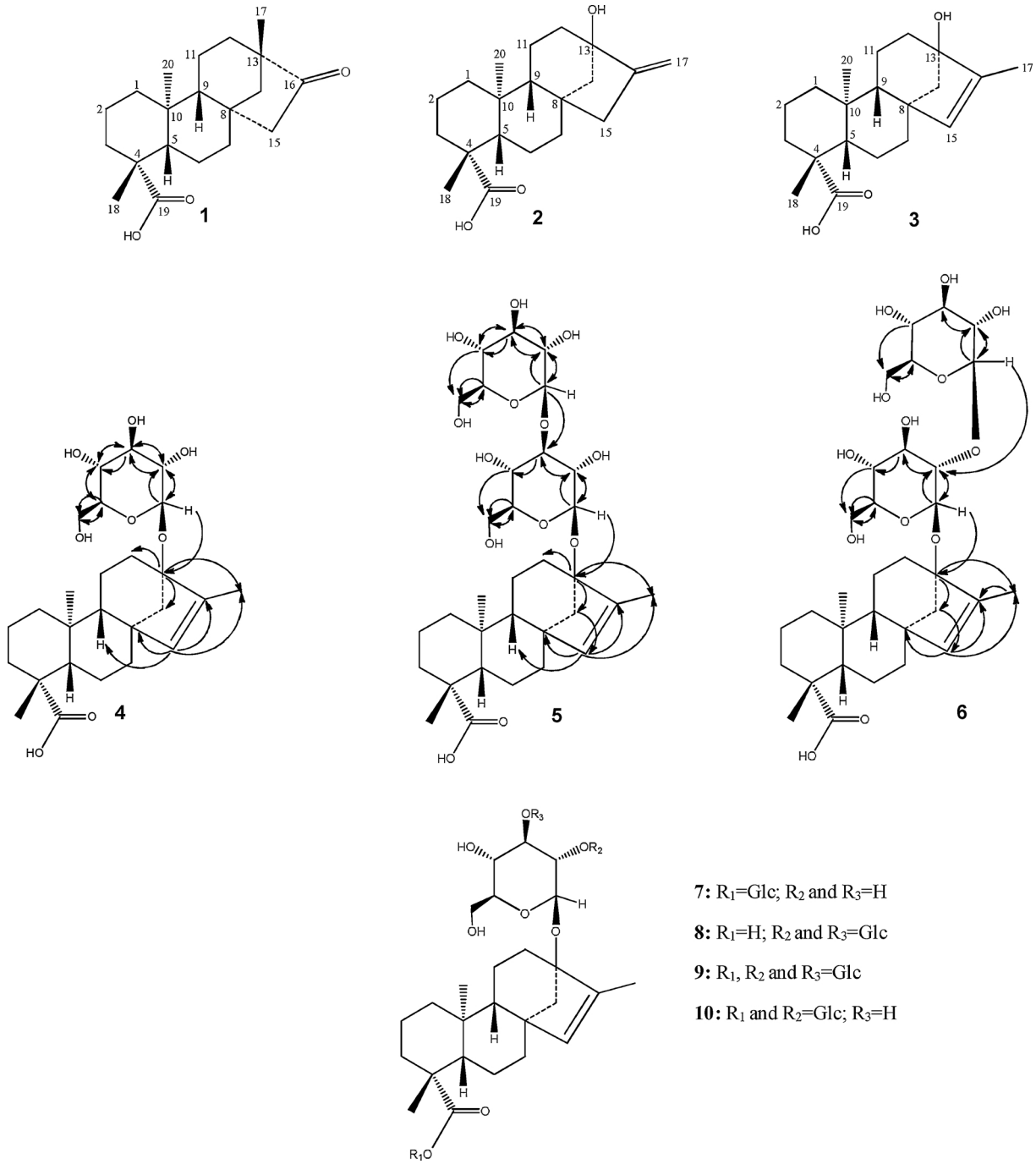


**Fig. 1.** Chemical structures of compounds Iso-steviol (**1**), Steviol (**2**), Endocyclic steviol (**3**), Endo-steviolmonoside (**4**), Endo-rebaudioside $G_1$ (**5**); Endo-steviolbioside (**6**), Endo-rubusoside (**7**), Iso-rebaudioside B (**8**), Iso-rebaudioside A (**9**) and Iso-stevioside (**10**) and crucial HMBC and COSY correlations for assignments of new compounds.

*Endo*-rebaudioside B as major compounds of the mixture (Fig. 3**A**). This result indicates that the first byproducts formed are due to the cleavage of the glucose from C-19 position and isomerization of the exocyclic double bond to the endocyclic isomer. On the other hand, longer incubation times of rebaudioside A in mild acidic conditions yielded mainly a mixture of endocyclic diterpene glycoside isomers after cleaving one or more monosaccharide from the C-13 moiety (Fig. 3**B**). Similar results were observed for stevioside in 20 h of mild acidic incubation. Rebaudioside A, a water soluble steviol glycoside, has been used as a non-caloric sweetener in several soft drinks and juices. As we observed, an increase in temperature under acidic conditions trigger a degradation of rebaudioside A into several shorter endocyclic steviol glycosides and aglycones. Most of these byproducts have limited water solubility e.g.: *endo*-rebaudioside B, *endo*-steviolbioside and *endo*-steviolmonoside, thus an inappropriate storage of beverages containing steviol glycosides as sugar substitute should change its organoleptic properties. The same attention should be given to other steviol glycosides (rebaudiosides M and D) used as sugar substitutes in acidic beverages, and further studies should be carried out directly in acidic beverages.

Endocyclic degradation products were purified from the reaction mixture to make them available in quantities for further studies their organoleptic and physicochemical properties.

Hydrolysis products from rebaudioside A and stevioside, after work

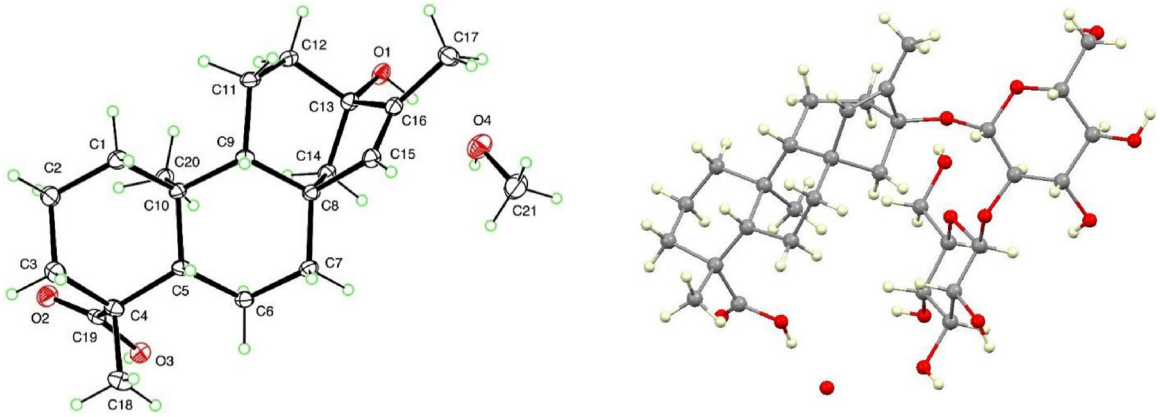


**Fig. 2.** X-ray ellipsoid drawings of compound 3 and 6 were generated at the 50% level (ORTEP) and using the software Mercury, respectively.

up, were submitted to chromatographic separation. $^{1}H$ and $^{13}C$ NMR data of **pool 1.2** showed a mixture of the same three aglycones previously reported herein after vigorous acid hydrolysis (**Supporting information** pages 1 and 2).

Compound **4** (410 mg, 1.5% of Rebaudioside A) was purified as a crystalline white solid exhibiting a negative specific rotation $[\alpha]_D^{25}$ −58.0 (*c* 0.1, MeOH) and a melting point of 101–102 °C. HRESIMS/MS data in negative mode showed a deprotonated molecular ion at *m/z* 479.2639 $[M-H]^-$, (calculated *m/z* 479.2650 $[M-H]^-$) suggesting a molecular formula $C_{26}H_{40}O_8$. One product ion at *m/z* 317.2027 was observed at collision energy of 40 eV, characteristic of the core of steviol tetracyclic diterpenes due to the loss of one hexose (−162 Da) from the C-13 portion as previously described for diterpene glycosides from *S. rebaudiana* (Perera et al., 2017a,b; Ibrahim et al., 2016).

2D-gHSQC of compound **4** showed the presence of three methyl singlets at $\delta_H$ 1.13; 1.34 and 2.01 ppm, eight methylene and two methine protons at $\delta_H$ 0.91 and 1.08 ppm, and an olefinic proton at $\delta_H$ 5.19 ppm, suggesting the presence of an *ent*-13-hydroxykaur-15-en-19-oic acid skeleton core (Avent et al., 1990). The endocyclic compound was identified as a mono β-glucosyl diterpene, evidenced by the signal observed at $\delta_H$ 5.15 ppm ($J$ = 7.7 Hz) thus supporting the MS information previously described. $^{1}H$ and $^{13}C$ chemical shifts are shown in Table 1.

The relative position of the sugar attachment was established by assignment of $^{3}J$ HMBC cross peak between anomeric proton H-1″ (5.15 ppm) and C-13 (89.8 ppm) confirming the attachment of the glucose unit at position C-13. The position of the protons and carbons 2–6 of the glucose were assigned by $^{3}J$ HMBC and COSY correlations (Fig. 1). Compound **4** was thus named 13-[(β-D-glucopyranosyl)oxy]*ent*-kaur-15-en-19-oic acid (*Endo*-steviolmonoside) and assigned structurally as shown in Fig. 1.

Compound **5** (160 mg, 1.6% of Rebaudioside A) was purified as a crystalline white solid exhibiting a negative specific rotation $[\alpha]_D^{25}$ −52.0 (*c* 0.1, MeOH) with a melting point of 196–197 °C. HRESI-MS/MS experiment showed a molecular ion at *m/z* 641.3196 $[M-H]^-$, (calculated *m/z* 641.3179 $[M-H]^-$) suggesting a molecular formula $C_{32}H_{50}O_{13}$. Two main product ions at *m/z* 479.2653 (94.8%) and *m/z* 317.2120 (14.9%) were observed at CE = 40 eV characteristic of the sequential loss of two hexoses (−2 × 162 Da) from the C-13 portion (Perera et al., 2017a,b).

2D-gHSQC of compound **5** showed the presence of three methyl singlets at $\delta_H$ 1.14; 1.35 and 1.98 ppm, eight methylene and two methine protons at $\delta_H$ 0.89 and 1.06 ppm, and an olefinic proton at $\delta_H$ 5.21 ppm, suggesting the presence of an *ent*-13-hydroxykaur-15-en-19-oic acid skeleton core (Avent et al., 1990). The endocyclic compound was a di-β-glucosyl diterpene, evidenced by the signals observed at $\delta_H$ 5.09 ppm ($J$ = 7.7 Hz) and 5.32 ppm ($J$ = 7.9 Hz) supporting the MS information previously described. $^{1}H$ and $^{13}C$ chemical shifts are shown in Table 1.

The relative position of each sugar attachment was established by assignment of gHMBC spectra. $^{3}J$ HMBC correlations between anomeric proton H-1″ (5.09 ppm) and C-13 (90.1 ppm) confirmed the attachment of the first glucose unit at position C-13. The $^{3}J$ HMBC correlation between H-1‴ (5.47 ppm) and C-3″ (89.4 ppm) was used to establish the attachment of the second glucose to the position C-3′ of the first glucose. The position of the protons and carbons 2–6 of the glucoses were assigned by $^{3}J$ HMBC and COSY correlations (Fig. 1). Compound **5** was thus named 13-[(3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]*ent*-kaur-15-en-19-oic acid (*Endo*-rebaudioside $G_1$) and assigned structurally as shown in Fig. 1.

Compound **6** (52 mg, 0.2% of Rebaudioside A) was purified as a crystalline white solid exhibiting a negative specific rotation $[\alpha]_D^{25}$ −54.0 (*c* 0.1, MeOH) with a melting point of 192–193 °C. Again, HRESI-MS/MS experiment showed a molecular ion at *m/z* 641.3166 $[M-H]^-$, (calculated *m/z* 641.3179 $[M-H]^-$) suggesting that compound is an isomer of compound **5**. Two main product ions at *m/z* 479.2633 (58.7%) and *m/z* 317.2121 (10.5%) at CE = 40 eV were observed, characteristic of the sequential loss of two hexoses (−2 × 162 Da) from the C-13 portion (Perera et al., 2017a,b). As previously reported, steviol glycosides having a Glc(1-3)Glc- arrangement at C-13 elute in RP-C18 earlier than those containing the same steviol-C13-disaccharides arranged at Glc(1-2)Glc- (Perera et al., 2017a,b,c). Compound **6** eluted later than **5** suggesting a C-13 moiety arranged as follows: Glc(1-2)Glc-.

2D-gHSQC of compound **6** also showed the presence as well of three methyl singlets at $\delta_H$ 1.23; 1.32 and 2.08 ppm, eight methylene and two methine protons at $\delta_H$ 0.87 and 1.04 ppm, and an olefinic proton at $\delta_H$ 5.17 ppm, suggesting the presence of an *ent*-13-hydroxykaur-15-en-19-oic acid skeleton core (Avent et al., 1990). The endocyclic compound was a di-β-glucosyl diterpene isomer of compound **5** evidenced by the signal observed at $\delta_H$ 5.18 ppm ($J$ = 7.9 Hz) and 5.26 ppm ($J$ = 7.8 Hz) supporting the MS information previously described. Arrangement of the C-13 moiety was corroborated by assignment of gHMBC spectra. $^{3}J$ HMBC correlations between anomeric proton H-1″ (5.18 ppm) and C-13 (89.4 ppm) confirmed the attachment of the first glucose unit at position C-13. The $^{3}J$ HMBC correlation between H-1‴ (5.26 ppm) and C-2″ (89.4 ppm) was used to establish the attachment of the second glucose to the position C-2″ of the first glucose. The position of H-2″ was confirmed through the COSY correlation between H-2″ (4.18 ppm) and H-1″ (5.18 ppm). The position of the other protons and carbons of the glucoses were assigned by $^{3}J$ HMBC and COSY correlations (Fig. 1). $^{1}H$ and $^{13}C$ chemical shifts of compound **6** named as 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]*ent*-kaur-15-en-19-oic acid (*Endo*-steviolbioside) are shown in Table 1.

Several chromatographic attempts were made to purify compound **7**, however, we were not successful, thus, compound **7** was identified in

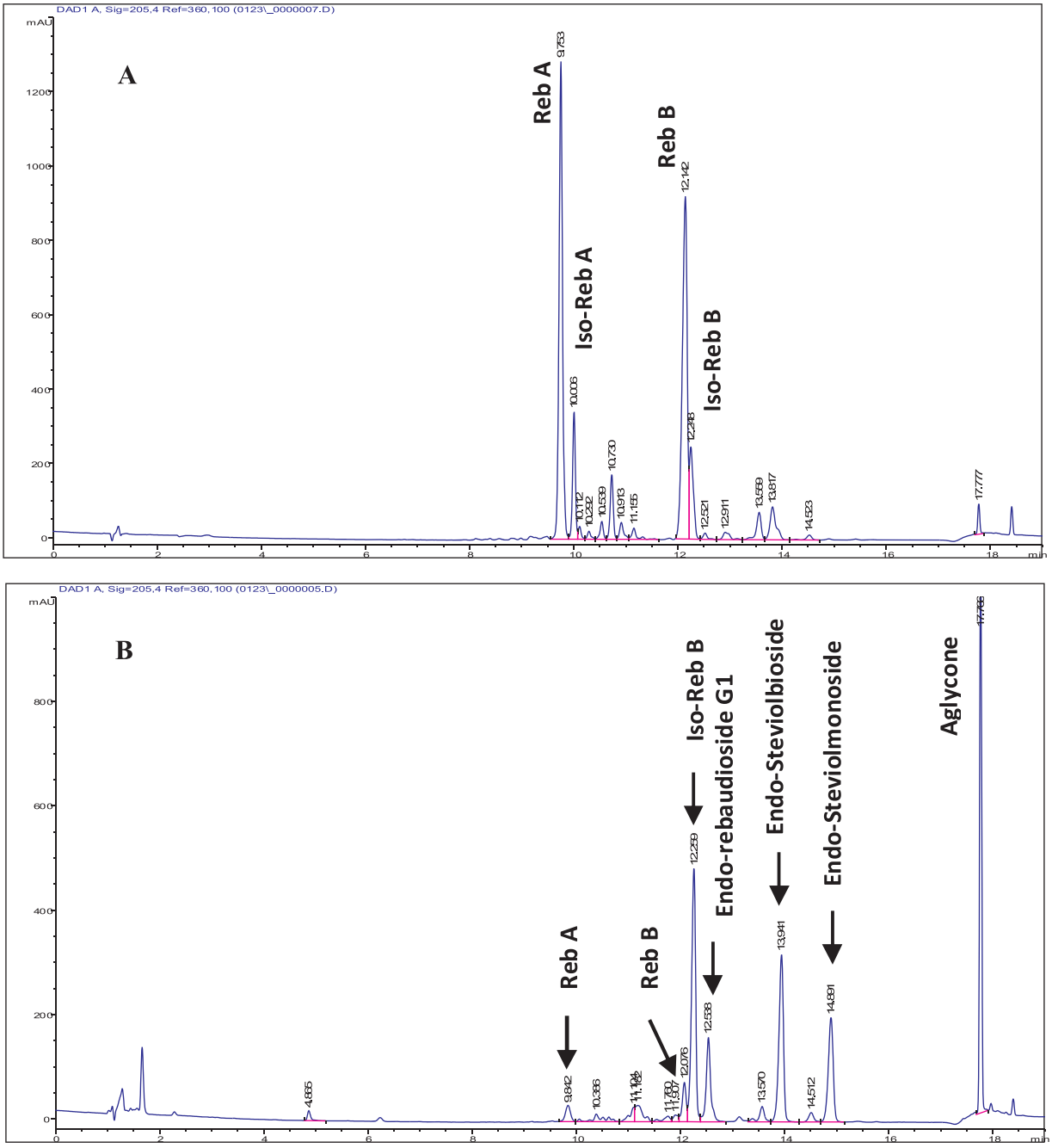


**Fig. 3.** HPLC chromatograms of the rebaudioside A hydrolysis products at 40 °C after 24 h **(A)** and 66 h **(B)**.

a mixture by LC–MS/MS. This mixture showed two peaks at 17.5 min and 18.2 min in RP-C18 column. The compound with the earlier elution time was identified as rubusoside by comparison of its retention time with that of standard and by MS/MS. The MS spectrum showed a deprotonated molecular ion at *m/z* 641.2610 and product ions at *m/z* 479.2610 and 317.2110 (Perera et al., 2017a). Compound **7** showed a deprotonated molecular ion at *m/z* 641.3152 $[M-H]^-$ (calculated *m/z* 641.3179 $[M-H]^-$) suggesting that compound **7** is an isomer of compound **5** and **6.** Compound **7** was easily differentiated from its isomers by ranging collision energies. A deprotonated ion at *m/z* 641.3152 together with a product ion at *m/z* 479.2609 at low collision energy (10 eV), characteristic of the loss of one glucose (−162 Da) from C-19 moiety was observed. Further loss of one glucose (–162 Da) was observed at highest collision energy (50 eV) to finally afford the aglycone at *m/z* 317.2141 (Perera et al., 2017a).

Compounds **8** (800 mg, 2.9% of Rebaudioside A), **9** (110 mg, 0.4% of Rebaudioside A) and **10** (500 mg, 2.4% of Stevioside) were also isolated as white crystalline solids and were identified by comparison of their HRESIMS/MS spectra obtained by arraying collision energies as well as by comparison of the proton, and carbon chemical shifts with those previously reported in literature (Chaturvedula et al., 2011a,b; Perera et al., 2017a,b,c; Thomas, 2011).

Vigorous hydrolysis of steviol glycosides studied using mineral acids yields mainly a mixture of aglycones (Avent et al., 1990) while vigorous acid hydrolysis controlling pH and time of reaction give different glycosides (Prakash et al., 2014a,b). In contracts, mild acidic conditions of some steviol glycosides studied produce mainly endocyclic steviol glycosides although hydrolysis products vary depending of the type of acid, pH, temperature and time of incubation (Prakash et al., 2012).

## 4. Conclusions

Vigorous and mild acid hydrolysis reactions of the major steviol glycosides from *Stevia rebaudiana* have been studied. Vigorous acid hydrolysis of rebaudioside A yielded a mixture of three different aglycones. A two-step gradient on silica gel was described to purify the intact aglycones in different yields: isosteviol > > > endocyclic steviol isomer > > steviol. Acid hydrolysis is not a convenient approach to produce steviol in quantities.

Mild acid hydrolysis of rebaudioside A and stevioside yielded several degradation products due to the formation of the endocyclic compounds and the cleavage of one or more glucose from C-19 and/or C-13 moieties. Most of degradation products were purified and structures were elucidated for the first time. Higher temperature storage of acidic beverages containing steviol glycosides as sugar substitutes should be given full attention since solubility of these degradation products in water is limited, hence, the organoleptic properties of the beverage may change dramatically.

## Conflict of interest

None.

## Acknowledgement

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.phytol.2018.04.007.

HRESIMS and $^{1}H$ and $^{13}C$ NMR spectroscopic data of purified compounds are available as Supporting information.

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