# Determination of steviol glycosides in commercial extracts of *Stevia rebaudiana* and sweeteners by ultra-high performance liquid chromatography Orbitrap mass spectrometry

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**ABSTRACT**

*Stevia rebaudiana* extracts are used as sweeteners in several countries worldwide. Several extracts of diverse composition are available on the market, and their taste depends on the contents of the various steviol glycosides. This study presents an accurate method for the qualitative and quantitative determination of steviol glycosides in 40 *Stevia* extracts, 7 sweeteners and 3 *Stevia*-sweetened beverages by a UHPLC coupled to an Orbitrap mass spectrometer. The sub-2 µm amide column provided the separation of all the target analytes in a run time of 30 min with high resolution. The effect of different eluent compositions on the ionisation efficiency of the steviol glycosides was studied. The optimal ionisation conditions were achieved in negative mode using 0.05% formic acid. Under this condition, adducts were not found, $[M-H]^-$ were the main ions and the spontaneous loss of a glucose residue at C19 was reduced. The %RSD for intra- and inter-day precision for all eleven analytes varied from 2.1–4.2% and 3.0–5.1%, respectively. The recoveries from spiked *Stevia* extract samples were greater than 95% for all analytes. Rebaudioside A was the most abundant, ranging from 23–102%. Nine *Stevia* extracts and one drink were not compliant with the European Regulation. Isosteviol was under the LOD in all samples and steviol was found in four samples in quantities in the range 0.01–0.03%.

*Keywords:*

*Stevia rebaudiana* extract; Steviol glycosides; Sweetener; High-resolution mass spectrometry; Food.

## 1. Introduction

*Stevia rebaudiana* (*Stevia*) extract, a natural non-nutritive sweeteners, has drawn increasing attention because, in addition to being a non-cariogenic sweetener [1], it seems to have hypotensive, hypoglycaemic, antidiabetic, anticariogenic, antioxidant, hypotensive, antihypertensive, antimicrobial, anti-inflammatory and anti-tumour activities [2,3]. The sweetening effect of *Stevia* is due to glycosides of the aglycone steviol (ST), an *ent*-kaurene-type diterpene (Figure 1, table 1). To date, approximately 40 steviol glycosides have been identified in the leaves of *S. rebaudiana* [4]. Crude extracts of *Stevia* have a bitter or liquorice-like aftertaste, which can affect consumer liking, and stevioside (SV) seems to be the primary cause of the bitter aftertaste. Rebaudioside A ($R_A$) is a sweetener with a less pronounced aftertaste and, for this reason, nowadays, it is the principal component in *Stevia*'s commercial extracts. Overall, the sweetness increases with the number of glucose residues, and steviol glycosides bearing less β-glucosyl residues have higher bitter intensities [5]. Thus, to reduce the liquorice-like aftertaste, research efforts have focused on enriching the extract with $R_A$ [6] or enzymatically adding glucose residues to $R_A$ [7]. The enzymatic *trans*-glycosylation of the *Stevia* extract improves the sweet taste, but has led to the appearance of extracts containing steviol glycosides acceptable for use in Japan and the USA but not yet permitted by current European Union (EU) legislation.

Regarding legislation, in 2008, the Food and Drug Administration (FDA) stated that purified $R_A$ from *Stevia* could be considered GRAS (generally regarded as safe). In 2011, steviol glycosides were approved as food additives within EU, but *Stevia* extract should contain no less than 75% SV and/or $R_A$. Then, the 2016 regulation [8] stated that *Stevia* extract must contain not less than 95% of the approved 11 steviol glycosides, in any combination and ratio. The steviol glycosides actually permitted in the commercial *Stevia* extracts are SV, $R_A$, rebaudiosides B–F ($R_B$, $R_C$, $R_D$, $R_E$ and $R_F$,

respectively), rebaudioside M ($R_M$), dulcoside A (DuA), steviolbioside (Sb) and rubusoside (Ru) (Table 1).

Steviol glycosides degrade at temperature higher than 100 ˚C and acidic conditions enhanced the hydrolysis process [9]. Breakdown products like steviol and isosteviol must not be present in *Stevia* extract and food samples, as a general toxicological consensus is still missing. For this reason, baked goods sweetened with stevia extract are not permitted at the time in the EU. Thus, to comply with the regulatory agency's directives, a sensible and specific analytical method is mandatory to confirm the qualitative and quantitative determination of steviol glycosides in commercial *Stevia* extracts and foods sweetened with them.

Sweet-tasting steviol glycosides in plant material and food samples have been determined through different methods, including enzymatic hydrolysis [10], HPTLC [9], capillary electrophoresis [11], near-infrared spectroscopy [12], HPLC with UV detection [13, 14], UHPLC-evaporative light scattering [15], LC-fluorimeter [16], desorption electrospray ionisation MS [17], UHPLC-MS [18], LC-MS/MS [19–21] and LC in combination with hybrid quadrupole time-of-flight MS [22, 23]. Due to current legislation concerning the composition of the *Stevia* extract, MS is the analytical technique of choice for the identification and assay of targeted and untargeted species in complex mixtures, such as *Stevia* extract and *Stevia*-sweetened food. Thus, we aim to explore the capabilities of the UHPLC-high-resolution-Orbitrap MS (UHPLC-HR-MS), in full-scan acquisition mode and collision-induced dissociation, for the evaluation of isosteviol, steviol and steviol glycoside forms present in *Stevia* extracts, commercial sweeteners and *Stevia*-sweetened beverages, collected in the period 2015–2017. Moreover, both the mobile phase pH and the CID voltage effect on the ionization of the analytes were studied. In particular, the use of low and high collision energies as a tool for determining the sugar position on Steviol backbone was evaluated.

## 2. Materials and methods

### *2.1. Chemicals*

The compounds SV, $R_A$, $R_B$, $R_C$, $R_D$, $R_E$, $R_F$, $R_G$, $R_M$, DuA, Sb, Ru, ST and isosteviol (iST) were provided by Chromadex (Laguna Hills, CA, US); their purity was in the range 90-99%. Methanol, acetonitrile, ammonia and formic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). The dried *Stevia* extracts (DSE, S1–S40) were from European, North American and Chinese suppliers. Sweeteners (A1–A7) and beverages (B1–B3) were acquired from a local supermarket. Water was supplied by a Milli-Q apparatus (Millipore, Milford, MA).

### *2.2. Chromatographic columns tested*

A BEH Amide (150 × 2.1 mm, 1.7 µm, Waters, Milford, MA, USA), BEH Shield $C_{18}$ (150 × 2.1 mm, 1.7 µm, Waters), HSS $C_{18}$ (150 × 2.1 mm, 1.8 µm, Waters), Kinetex $C_{18}$ (150 × 2.1 mm, 1.7 µm, Phenomenex, Torrence, CA) and a Hypersil Gold $C_{18}$ (150 × 2.1 mm, 1.9 µm, Thermo Scientific, Mi, IT) column were evaluated. For the reverse-phase (RP) columns, the chromatographic conditions reported by Espinoza et al. [24] were applied.

### *2.3. Sample preparation and steviol glycoside determination by UHPLC-Orbitrap MS*

The DSE (50 mg) and dried sweeteners powder (1 g) were extracted with 30 mL of water:$CH_3CN$ (80:20, v/v), for 10 min under agitation. Then, water was added to adjust the volume of the clear solution to 50 mL. *Stevia*-sweetened beverages (1 mL) were diluted with 10 mL of water:$CH_3CN$ (80:20, v/v). The solutions were centrifuged at 1000 × *g* for 2 min, diluted with water:$CH_3CN$ (80:20, v/v) and 5 µL injected into the UHPLC system. Samples were extracted in triplicate and UHPLC-HR-MS analyses carried out in duplicate. The analysis was carried out on a UHPLC model Acquity (Waters) coupled with an HR Fourier transform Orbitrap mass

spectrometer (Exactive, Thermo Scientific, San Jose, CA), equipped with a HESI-II probe for ESI and a collision cell (HCD). The operative conditions were as follows: spray voltage -3.0 kV, sheath gas flow rate 45 (arbitrary units), auxiliary gas flow rate 10 (arbitrary units), capillary temperature 275°C, capillary voltage -95 V, tube lens -190 V, skimmer -46 V and heather temperature 60°C. The injection volume was 5 µL. The acquisition was made in the full-scan mode in the range $(m/z)^-$ 200–3000 u, using an isolation window of ±2 ppm. The automatic gain control (AGC) target, injection time, mass resolution, energy and gas in the collision cell were $1 \times 10^6$, 100 ms, 50 K, 20-40-60 V and $N_2$, respectively. The MS data were processed using Xcalibur software (Thermo Scientific). A BEH amide column (150 × 2.1 mm, 1.7 µm, Waters) was used for the separation. The column and samples were maintained at 35 and 20°C, respectively. The flow rate was 0.3 mL $min^{-1}$, and the eluents were 0.05% formic acid in water (A) and 0.05% formic acid in $CH_3CN$ (B). The UHPLC separation was accomplished by the following linear elution gradient: 13–17% A in 5 min, 17–20% A in 5 min, 20–35% A in 5 min and then, 35–50% B in 10 min. The calibration curves were constructed by dissolving 10 mg of each dried standard in 10 mL methanol. The working solutions were prepared in methanol in the range of 0.1–10 µg $mL^{-1}$ for SV, $R_A$–$R_G$, $R_M$, DuA, Sb and Ru, and 10–100 ng $mL^{-1}$ for ST and iST. Stock and working solutions were stored at -20 and -4°C, respectively.

*2.4. Method validation*

External standards were used to quantify SV, $R_A$–$R_G$, $R_M$, DuA, Ru, Sb, ST and iST in *Stevia* extracts, sweeteners and beverages. Calibration curves were constructed for each standard at six concentration levels; four independent determinations were performed at each concentration, and regression analysis was employed to determine the linearity of the calibration graphs. The lower

limit of quantification (LLOQ) was defined by the lowest injected inter-day concentration, whose resultant relative standard deviation (RSD%) was < 20%. The limit of detection (LOD) was defined by the lowest concentration the assay can differentiate from background levels. The matrix effect of the procedure was determined by a recovery test, described elsewhere [18]. Briefly, three sweetener samples were spiked with different amounts of the analytes (20, 50, 100 μg) and each sample extracted in triplicate and analysed in duplicate, to evaluate recovery. The peak identity was confirmed by co-chromatography with an authentic standard, for comparison of the deprotonated ion and fragments. Quantitative analysis was performed following the deprotonated ion $[M-H]^-$. Precision was determined according to Gardana et al. [18]. The ruggedness of the proposed UHPLC-HR-MS method was estimated by two analysts, evaluating the amounts of ST glycosides in a sample of DSE. Each analyst performed 10 tests, and standard and extract solutions were injected in triplicate. Robustness was estimated by varying several chromatographic conditions, such as flow rate ±0.05 mL $min^{-1}$, column temperature ±5°C, formic acid in eluent 0.01–0.10%, pH of the eluent 2.3–9.0 (adjusted by $NH_4OH$), spray voltage ±0.2 kV and capillary voltage ±5 V. Data were analysed by Wilcoxon test, considering a significance level of $p>0.05$. The solution of the *Stevia* extract, sweetener, food and calibration standards were stored at 4°C. For analysis, the solutions were placed in the autosampler at 20°C and their stability evaluated overnight.

Statistical analyses were performed using Excel and Statistica software (Statsoft, Tusla, OK, USA). Results were reported as media ± expanded uncertainty.

## 3. Results and discussion

### *3.1. Chromatographic conditions*

The percentage mean recovery values of the extraction for steviol glycosides from spiked DSEs were in the range of 95–102%. The repeatability and inter-day precision ranges were 1.5–3.1% and 2.2–3.8%, respectively. The results were statistically compared to the effect of an external factor on the degree of reproducibility of the UHPLC-HR-MS method, and it was found there was no significant difference in the amount of the analytes ($p=0.550$). Regarding robustness, slight variations in flow rate and column temperature did not change the peak shape and resolution. Moderate variations in MS parameters, such as voltages and temperatures, did not significantly influence the quantitative analysis. Standard solutions and *Stevia* extracts in methanol proved stable in the autosampler at 20°C overnight (RSD < 1.1%). The negative ion mass spectra of $R_A$, obtained at different formic acid percentages in the eluent, are reported in Figure 2. At 0.01% (Figure 2A) the main ion had *m/z* 1079.6161, corresponding to $[M+2HCOOH+Na]^-$, while the intensity of the deprotonated $[M-H]^-$, deglucosylated $[M-Glc-H]^-$, dimeric $[2M-H]^-$ and $[2M+2HCOOH+Na-H]^-$ forms was low. On the contrary, no adducts were found with 0.05% formic acid, and the main ion was $[M-H]^-$, with the characteristic fragment $[M-H-Glc]^-$. Besides the deprotonated molecules, the dimer $[2M-H]^-$ was present in much lower quantities (Figure 2B). At more than 0.05% formic acid, the intensity of the deprotonated ion decreased with the consequent increase of the deglucosylated form (Figure 2C). By increasing pH values, the ion evaporation should have been preferentially favoured. Contrary to expectations, the proton transfer reaction at a low pH produced deprotonated molecules more efficiently than the ion evaporation mechanism at a higher pH. In particular, the abundance of the deprotonated $R_A$ decreased up to 40% when the buffer pH increased from 6 to 9 in the eluent. The decreased sensitivity was due to an increase in the deglucosylated form $[M-Glc]^-$ and

probably also to the suppression by ammonium formate or neutralisation of negative charges by ammonium ions. Thus, the best sensitivity and the greater structural information was obtained using 0.05% formic acid in the eluents. A good chromatographic separation was achieved for most of the steviol glycosides, using a sub-2 µm amide column. In contrast, SV and $R_A$, which represent the major components in many extracts, could not be separated at the baseline, using the RP columns. Regarding samples containing poly-glycosylated steviol, such as those obtained by *trans*-glycosylation, the RP columns were not able to separate all the glycosylated derivatives of ST.

*3.2. Identification of steviol glycosides*

Figure 3 reports the MS chromatogram of the samples S11, S40 and S15, acquired in the range 200–3000 u. Thirty-one compounds were separated, including $R_A$ (peak 10) and SV (peak 6), identified by co-chromatography (Sb, Ru, SV, $R_A$–$R_G$, $R_M$ and DuA), and on the basis of their molecular weight and fragmentation pattern evaluation (Table 2). Regarding steviol glycosides behaviour in the ESI source, it has been noted that those with glucose residues at C19 spontaneously lose one glucose moiety. Conversely, in the chromatograms of those with no residues at C19, such as $R_B$ and Sb, the deglucosylated form $[M\text{-}Glc\text{-}H]^-$ was not detected (Supplementary Fig. S1). The cleavage of the ester linkage at C19 obtained at low collision energy has also reported by Ohta et al. [25]. All steviol glycosides showed a consecutive loss of sugar moieties in the collision cell of the deprotonated ion $[M\text{-}H]^-$.

Sample S11 was a typical, low-purified *Stevia* extract, containing mainly SV, a low percentage of $R_A$ and relatively high amounts of $R_C$ and $R_B$. Sample S40, collected in 2015, must have $R_A$ and SV, and their declared content above 80%. The UHPLC-MS analysis showed that SV was not present and instead, $R_B$, an isomer of SV, was detected. According to the EU legislation under way

in 2015, sample S32 was not compliant. The chromatogram of sample S15 was rather different from most of the analysed samples, with several peaks eluted before and after the $R_A$. Notably, the presence of peaks with *m/z* equal or greater than 1111.4814 ($R_H$), 1127.4763 ($R_D$) and 1289.5295 ($R_M$) suggests that this sample was probably obtained by *trans*-glycosylation. Thus, this sample was also not compliant with the EU directive because it contains unapproved compounds, such as $R_G$, $R_H$, $R_I$ and their isomers. The chromatogram of the commercial beverage sample B1 was very similar to that of extract S15. Thus, for its production, a non-conforming extract was used.

The chromatograms of the samples B1, B2 and B3 also displayed the presence of peaks at 11.2 and 11.5 min, containing a set of ions with *m/z* from 341.1082 to 2736.9128 u (Supplementary Fig. S2). The detected ions differed by 342.1164 u, which corresponds to a sucrose residue. Thus, samples B1, B2 and B3 contain a series of oligomers of glucose (*n*=16), probably maltodextrin. It should be noted that the presence of maltodextrin or other starch derivatives was not reported on the label.

Compounds 18 and 19 had *m/z* 1273.5344 u and spontaneously produced fragments with *m/z* 1111.4814 and 1127.4763 u, indicating a corresponding loss of one hexose and one deoxyhexose moiety at C19. In the collision cell, peak 19 showed a consecutive loss of hexose moieties, while compound 18 also produced fragments with *m/z* 965.4232 u, due to the loss of a deoxyhexose residue. As reported previously [24, 25], and identified during the course of our study, the dissociation of the ester-linked at C19 occurred before that of the ether bound at C13. Thus, compounds 18 and 19 have one deoxyhexose, likely rhamnose, at C13 and C19, respectively, and therefore, 19 could be rebaudioside N [24], and 18, its isomer.

Peaks 12 and 13, with a deprotonated ion of 1111.4810 u, were isomers, but only compound 12 spontaneously produced fragments with *m/z* 949.4280, indicating the loss of one hexose moiety at

C19. Thus, peak 12 was identified as $R_H$. Compound 15, with *m/z* 1127.4760 u, was an isomer of $R_D$ and gave a fragmentation pattern similar to the latter. Thus, peak 15 has been identified as $R_I$. Peaks 20–23 had *m/z* of 1289.5290 u and in the collision cell, gave a consecutive loss of hexose residues. Thus, they were isomers with different numbers of glucose residues linked to the C19- or C13-glucosyl moiety of SV. Peaks 24–31were ST linked with up to 12 hexose residues at positions C19 and C13. These compounds were probably *trans*-glycosylation products of SV and $R_A$, obtained by the action of a glucosyl transferase [26].

*3.3. Quantification of steviol glycosides*

The UHPLC-Orbitrap MS method was then applied to quantify the content of steviol glycosides in the DSE, sweeteners and beverages. The quantities, expressed as grams or milligrams (B1, B2 and B3) of the compound/100 g of dried material, are reported in Table 2. In all the *Stevia* extracts analysed, except samples S11 and S31, $R_A$ was the most abundant compound, with percentages in the range 23–99 g/100 g DSE. In particular, samples S2–S5, S22, S24, S33 and S34 contained almost exclusively $R_A$. Regarding samples produced in 2015, expiration 2017, none contained steviol glycosides not permitted, and their total percentage was higher than 92%. Despite this, samples S29 and S40 did not comply with EU legislation because the total amount of SV and $R_A$ was less than 75%. Sample S15, not compliant, contained approximately 18% of steviol glycosides of higher molecular weight not found in the other *Stevia* extracts. These compounds showed differences in the number and type of sugar moieties (glucose and/or rhamnose) at positions C13 and C19. Thus, these compounds could be *trans*-glycosylation products of steviol glycosides normally present in *Stevia* leaves, such as $R_A$, SV and $R_C$.

Samples A1–A2 (powder) and A5 and A7 (tablets) contained mainly $R_A$, in percentages higher than 95% of total steviol glycosides, suggesting that these commercial samples were produced with highly purified *Stevia* extracts. In contrast, samples A3, A4 and A6 contained more than 36, 49 and 21% SV, respectively, suggesting that partially purified *Stevia* extracts were used. Regarding beverages, samples B1, B2 and B3 contained approximately 7, 8 and 15 µg/mL $R_A$, respectively, and sample B1 also presented about 5 µg/mL of unauthorised steviol glycosides. $R_A$ represented more than 93% of the total steviol glycosides in sample B3 and not permitted compounds were not detected. Also, iST was under the LOD in all samples, and ST was found in four extracts (6, 12, 20 and 27) in quantities in the range 0.01–0.03% (Table 2). It should be noted that the latter extracts were not compliant with the current EU legislation.

## 4. Conclusion

An accurate analytical method using UHPLC-Orbitrap MS technique has been developed and evaluated to determine steviol glycosides in *Stevia* extracts, sweeteners and *Stevia*-sweetened beverages. Steviol-glycosides were analyzed by using negative ion ESI-HR-MS and CID. The negative ion spectra of steviol-glycosides exhibited important structure-related fragment under various CID voltages. Mass spectra at low-energy CID voltage exhibited a fragment ion produced by the selective cleavage of an ester linkage at C19, and those at high CID voltage a series of fragment ions providing information about the sizes and structures of the sugar chains at both C13 and C19. The sub-2 µm amide columns offer a superior efficiency than $C_{18}$ columns. The strength of the proposed method is the complete separation of all the peaks, the reproducible retention time and the specificity. Regarding weakness, the percentage of formic acid in the eluent affects the ionisation of the analytes and increases the spontaneous loss of a glucose residue. The latter could generate

misunderstanding in the untargeted analysis. Overall, due to its good performance, the UHPLC-HR-MS method could be used in quality control laboratories for the routine analysis of *Stevia* extracts and their commercial products.

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**Figure legends**

Figure 1. Steviol backbone ($R^1$=$R^2$=H) and isosteviol.

Figure 2. Negative ion mass spectra of rebaudioside A ($R_A$) obtained at A) 0.01%, B) 0.05% and C) 0.10% formic acid in the eluents.

Figure 3. Representative UHPLC-MS chromatograms obtained from the analysis of samples S7 (A), 32 (B) and S11 (C). See Table 1 for peak identification.

**Table 1.** High-resolution MS data of the steviol and steviol-glycosides found in *Stevia* extracts, sweeteners and beverages.

| Peak | Acronym | $R_1$ | $R_2$ | $[M-H]^-$ |
|---|---|---|---|---|
| | ST | -H | -H | 317.2122 |
| 1 | Sb | -H | -Glc-Glc(2→1) | 641.3179 |
| 2 | Ru | -Glc | -Glc | 641.3179 |
| 3 | DuA | -Glc | -Glc-α-Rha(2→1) | 787.3758 |
| 4 | $R_B$ | –H | -Glc-Glc(2→1)-Glc(3→1) | 803.3716 |
| 5 | $R_G$ | -Glc | -Glc-Glc(3→1) | 803.3715 |
| 6 | SV | -Glc | -Glc-Glc(2→1) | 803.3716 |
| 7 | $R_C$ | -Glc | -Glc-Glc(3→1)-Rha-(2→1) | 949.4280 |
| 8 | $R_F$ | -Glc | -Glc-Glc(2→1)-Xyl(3→1) | 935.4129 |
| 9 | $R_C$ isomer | -Glc | -Glc-Glc-Rha | 949.4278 |
| 10 | $R_A$ | -Glc | -Glc-Glc(2→1)-Glc(3→1) | 965.4242 |
| 11 | $R_E$ | -Glc-Glc(2→1) | -Glc-Glc(2→1) | 965.4241 |
| 12 | $R_H$ | -Glc | -Glc-Glc-Rha-Glc | 1111.4814 |
| 13 | $R_H$ isomer | -H | -Glc-Glc-Rha-Glc-Glc | 1111.4814 |
| 14 | $R_D$ isomer | | | 1127.4771 |
| 15 | $R_I$ | -Glc-Glc | -Glc-Glc-Glc | 1127.4770 |
| 16 | $R_M$ | -Glc-Glc-Glc | -Glc-Glc-Glc | 1289.5274 |
| 17 | $R_D$ | -Glc-Glc(2→1) | Glc-Glc(2→1)-Glc(3→1) | 1127.4763 |
| 18 | $R_N$ isomer | -Glc-Glc-Glc | -Glc-Glc-Rha | 1273.5344 |
| 19 | $R_N$ | -Glc-Glc-Rha | -Glc-Glc-Glc | 1273.5345 |
| 20 | ST-$Glc_6$ | | | 1289.5274 |
| 21 | ST-$Glc_6$ | | | 1289.5274 |
| 22 | ST-$Glc_6$ | | | 1289.5274 |
| 23 | ST-$Glc_6$ | | | 1289.5274 |
| 24 | ST-$Glc_7$ | | | 1451.5800 |
| 25 | ST-$Glc_7$ | | | 1451.5802 |
| 26 | ST-$Glc_7$ | | | 1451.5800 |
| 27 | ST-$Glc_8$ | | | 1613.6340 |

| | | |
|---|---|---|
| 28 | ST-$Glc_9$ | 1775.6860 |
| 29 | ST-$Glc_{10}$ | 1937.2422 |
| 30 | ST-$Glc_{11}$ | 2099.7951 |
| 31 | ST-$Glc_{12}$ | 2261.8500 |

ST, Steviol; SV, Stevioside; $R_A$, Rebaudioside A; $R_B$-$R_I$, $R_M$, $R_N$, Rebaudiosides; DuA, Dulcoside A; Sb, Steviolbioside; Ru, Rubusoside; glc, Glucose; Rha, Rhamnose; Xyl, Xylose.

**Table 2.** Amount of steviol, isosteviol and steviol-glycosides in *Stevia* extracts (S1–S40, g $100g^{-1}$), sweeteners (A1–A7, g $100g^{-1}$) and beverages (B1–B3, mg $100mL^{-1}$). Data are reported as media ± expanded uncertainty (x±U).

| Sample | Year | Sb | Ru | DuA | $R_B$ | SV | $R_C$ | $R_F$ | $R_A$ | $R_E$ | $R_D$ | $R_M$ | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S1 | 2017 | nf | nf | nf | 0.4±0.02 | 0.5±0.02 | 1.1±0.04 | nf | 96.3±3.1 | nf | nf | nf | 98.3±2.9 |
| S2 | 2017 | nf | nf | nf | nf | 0.1±0.004 | nf | nf | 98.6±3.2 | nf | nf | nf | 98.7±3.2 |
| S3 | 2017 | nf | nf | nf | 0.01±0.0003 | 0.04±0.002 | nf | nf | 98.9±3.3 | nf | nf | nf | 99.0±2.8 |
| S4 | 2017 | nf | nf | nf | nf | 0.07±0.003 | 0.01±0.0003 | nf | 99.4±3.2 | nf | nf | nf | 99.5±3.4 |
| S5 | 2017 | nf | nf | nf | nf | 0.02±0.001 | nf | nf | 99.0±3.2 | nf | nf | nf | 99.0±2.6 |
| S6 | 2017 | 0.3±0.01 | 1.1±0.04 | 0.9±0.03 | 11.1±0.4 | 0.3±0.01 | 4.3±0.1 | nf | 75.4±2.5 | nf | nf | 0.003±0.0001 | 93.4±2.9 |
| S7 | 2017 | 0.01±0.003 | 0.2±0.01 | 0.1±0.003 | 1.6±0.05 | 30.3±1.2 | 3.4±0.1 | 0.02±0.0006 | 56.2±1.9 | 0.05±0.002 | 0.02±0.0006 | 0.001±0.0003 | 91.8±2.9 |
| S8 | 2017 | 0.2±0.06 | 0.9±0.03 | 0.4±0.01 | 1.1±0.04 | 8.5±0.3 | 2.1±0.07 | 0.01±0.0003 | 75.4±2.3 | 0.03±0.001 | 0.04±0.001 | nf | 88.7±2.5 |
| S9 | 2017 | 0.2±0.06 | 0.4±0.01 | 0.2±0.006 | nf | 5.9±0.2 | 4.4±0.1 | 0.02±0.0006 | 84.4±2.7 | 0.04±0.001 | 0.06±0.002 | 0.001±0.0003 | 95.6±3.2 |
| S10 | 2017 | 0.06±0.002 | 0.2±0.01 | 0.4±0.01 | nf | 37.3±1.3 | 0.07±0.002 | 0.01±0.0003 | 57.9±1.9 | 0.02±0.0006 | 0.01±0.0003 | nf | 96.0±3.3 |
| S11 | 2017 | 1.1±0.04 | 0.7±0.02 | 1.8±0.06 | 5.3±0.2 | 45.5±1.6 | 5.5±0.2 | 0.9±0.03 | 29.4±1.1 | 0.05±0.002 | 0.7±0.02 | 0.002±0.0006 | 90.9±2.6 |
| S12 | 2017 | 0.1±0.003 | 0.2±0.01 | 0.3±0.01 | nf | 1.4±0.05 | 0.1±0.003 | nf | 61.6±2.0 | nf | nf | nf | 64.4±1.3 |
| S13 | 2017 | nf | 0.1±0.001 | 0.3±0.01 | nf | 9.5±0.4 | 3.8±0.1 | 0.95±0.03 | 80.1±2.6 | 0.01±0.003 | 0.02±0.0006 | nf | 94.7±2.5 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S14 | 2017 | 0.4±0.013 | 0.1±0.001 | nf | nf | 9.5±0.4 | 3.7±0.1 | 0.7±0.02 | 83.1±2.7 | nf | nf | nf | 97.5±3.1 |
| S15 | 2017 | 0.3±0.01 | 0.6±0.02 | 2.4±0.08 | 5.2±02 | 12.6±0.5 | 15.4±0.5 | 1.84±0.06 | 23.3±0.9 | 13.7±0.4 | 2.1±0.07 | 1.7±0.05 | 97.2±2.8 |
| S16 | 2017 | 0.4±0.013 | 0.6±0.02 | nf | nf | 7.8±0.3 | 4.3±0.1 | 0.3±0.01 | 80.3±2.6 | nf | nf | nf | 93.7±3.1 |
| S17 | 2017 | nf | nf | 0.2±0.006 | 2.3±0.07 | 7.8±0.3 | 0.9±0.03 | nf | 87.2±2.8 | nf | nf | nf | 98.4±3.2 |
| S18 | 2016 | 0.07±0.002 | 0.9±0.03 | 1.7±0.05 | nf | 7.1±0.2 | 2.1±0.06 | nf | 84.7±2.7 | nf | nf | nf | 96.6±2.8 |
| S19 | 2016 | 1.1±0.04 | 2.3±0.06 | 2.1±0.07 | 1.1±0.04 | 21.3±0.8 | 4.3±0.11 | 0.4±0.01 | 62.6±2.0 | 0.01±0.0003 | 0.04±0.001 | 0.001±0.0003 | 95.2±3.3 |
| S20 | 2016 | 0.04±0.001 | 0.1±0.003 | 0.2±0.06 | nf | 1.8±0.08 | 0.4±0.01 | nf | 59.7±2.1 | nf | nf | nf | 62.3±2.0 |
| S21 | 2016 | 0.02±0.001 | 0.06±0.002 | 0.2±0.006 | nf | 5.3±0.2 | 2.1±0.06 | 0.75±0.02 | 90.8±2.9 | 0.01±0.003 | 0.02±0.0006 | nf | 99.3±3.1 |
| S22 | 2016 | nf | nf | nf | nf | 0.1±0.004 | 0.02±0.0006 | nf | 98±3.1 | nf | nf | nf | 98.1±2.9 |
| S23 | 2016 | nf | nf | nf | nf | 0.03±0.001 | 0.01±0.0003 | nf | 97.4±3.1 | nf | nf | nf | 97.4±2.7 |
| S24 | 2016 | nf | nf | nf | nf | 0.02±0.001 | 0.01±0.0003 | nf | 97.9±3.2 | nf | nf | nf | 97.9±3.3 |
| S25 | 2016 | nf | 0.1±0.003 | 0.8±0.03 | 0.1±0.003 | 2.4±0.09 | 0.5±0.02 | nf | 91.2±2.9 | nf | nf | nf | 95.1±2.8 |
| S26 | 2016 | nf | 0.1±0.003 | 0.5±0.02 | nf | 10.2±0.4 | 1.2±0.04 | nf | 83.2±2.7 | nf | nf | nf | 95.2±2.6 |
| S27 | 2016 | 0.2±0.006 | 0.6±0.02 | 1.4±0.04 | 5.6±0.2 | 0.4±0.02 | 1.2±0.04 | nf | 62.6±2.0 | 0.02±0.0006 | 0.03±0.001 | nf | 72.4±1.9 |
| S28 | 2015 | 0.1±0.003 | 0.3±0.01 | 0.2±0.006 | 0.8±0.03 | 8.4±0.3 | 4.4±0.1 | 0.8±0.02 | 77.1±2.5 | 0.03±0.001 | 0.02±0.0006 | nf | 92.1±2.7 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S29 | 2015 | 1.0±0.03 | 1.9±0.06 | 2.4±0.08 | 1.5±0.05 | 26.2±0.9 | 10.1±0.3 | 2.5±0.08 | 46.9±1.6 | 0.01±0.0003 | 0.01±0.0003 | nf | 92.5±3.0 |
| S30 | 2015 | 1.5±0.05 | 1.2±0.04 | 1.5±0.05 | 1.2±0.04 | 33.6±1.2 | 6.7±0.2 | nf | 48.5±1.6 | 0.01±0.0003 | 0.02±0.0006 | nf | 94.2±2.6 |
| S31 | 2015 | 2.1±0.07 | 0.9±0.03 | 1.1±0.04 | 1.1±0.04 | 42.1±1.4 | 4.4±0.1 | 1.6±0.05 | 40.9±1.4 | nf | nf | nf | 94.2±2.0 |
| S32 | 2015 | nf | 1.2±0.04 | 1.5±0.05 | 2.2±0.07 | 38.6±1.4 | 3.9±0.1 | 2.2±0.07 | 43.1±1.4 | nf | nf | nf | 92.7±2.7 |
| S33 | 2015 | nf | nf | 0.1±0.03 | nf | 0.3±0.001 | nf | nf | 99.1±3.2 | nf | nf | nf | 99.5±3.1 |
| S34 | 2015 | nf | nf | 0.1±0.03 | nf | 0.4±0.01 | 0.1±0.003 | nf | 97.3±3.1 | nf | nf | nf | 97.9±3.2 |
| S35 | 2015 | nf | 0.3±0.01 | 1.8±0.06 | 1.3±0.04 | 28.2±1.1 | 10.2±0.3 | nf | 53.4±1.7 | 0.2±0.006 | 0.04±0.001 | nf | 95.4±3.2 |
| S36 | 2015 | 0.3±0.01 | 1.3±0.04 | 2.3±0.07 | 1.1±0.03 | 48.1±1.7 | 8.4±0.3 | 0.5±0.02 | 32.4±1.1 | nf | nf | nf | 94.4±3.3 |
| S37 | 2015 | 1.2±0.04 | 1.8±0.06 | 1.5±0.05 | 2.5±0.08 | 43.8±1.5 | 4.8±0.2 | 2.0±0.07 | 44.7±1.4 | nf | nf | nf | 102.3±3.4 |
| S38 | 2015 | 0.8±0.03 | 1.1±0.04 | 1.8±0.06 | 1.0±0.03 | 41.1±1.5 | 11±0.3 | 2.6±0.08 | 40.8±1.3 | nf | nf | nf | 100.1±3.5 |
| S39 | 2015 | 1.0±0.03 | 0.8±0.03 | 1.5±0.05 | 1.2±0.04 | 40.8±1.6 | 10.4±0.3 | 2.6±0.08 | 42.0±1.3 | nf | nf | nf | 100.5±3.2 |
| S40 | 2015 | nf | 0.03±0.001 | 0.03±0.001 | 25.6±0.8 | 0.02±0.007 | 0.04±0.002 | 0.2±0.007 | 68.7±2.2 | 0.11±0.004 | 0.12±0.004 | 0.1±0.003 | 95.0±3.0 |
| A1 | 2016 | nf | $5x10^{-4}±2x10^{-5}$ | $2x10^{-4}±6x10^{-6}$ | nf | $8.9x10^{-3}±3x10^{-4}$ | $4x10^{-3}±1x10^{-4}$ | nf | 0.8±0.03 | nf | nf | nf | 0.8±0.03 |
| A2 | 2017 | nf | nf | nf | nf | $1x10^{-3}±3x10^{-5}$ | nf | nf | 1.0±0.03 | nf | nf | nf | 1.0±0.04 |
| A3 | 2017 | 0.3±0.01 | 0.7±0.02 | 0.2±0.007 | 0.03±0.001 | $4x10^{-3}±2x10^{-4}$ | $1x10^{-3}±1x10^{-4}$ | nf | 4.4±0.2 | 0.01±0.0004 | 0.01±0.0004 | nf | 1.2±0.05 |

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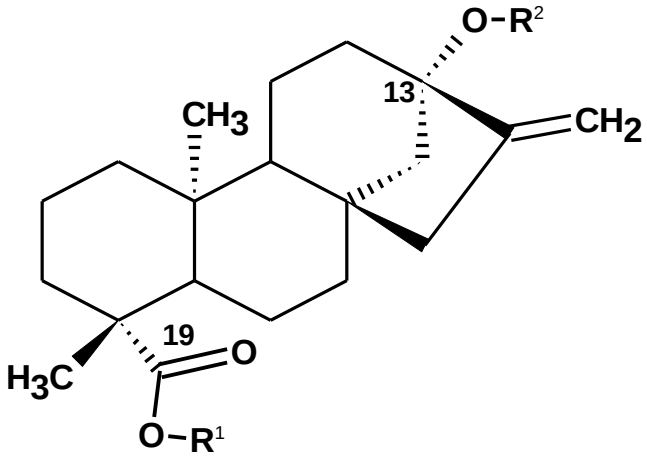
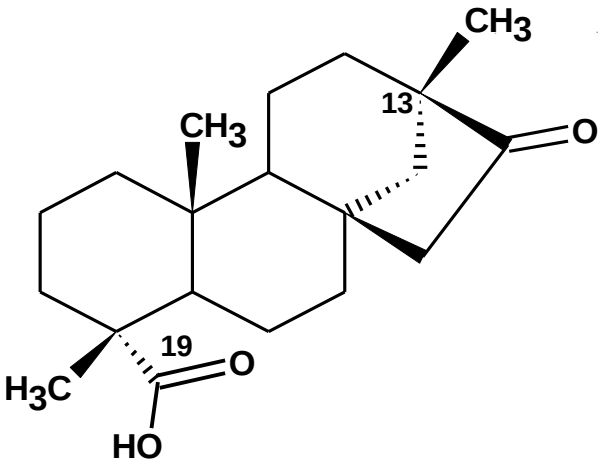
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A4 | 2017 | 0.2±0.006 | 0.1±0.004 | 0.2±0.005 | 0.2±0.006 | 5.8±0.2 | 0.6±0.02 | 0.2±0.006 | 4.6±0.2 | nf | nf | nf | 11.9±0.5 |
| A5 | 2017 | nf | nf | nf | nf | 0.3±0.001 | nf | nf | 17.2±0.5 | nf | nf | nf | 17.5±0.6 |
| A6 | 2016 | 0.02±0.0006 | 0.1±0.003 | 0.3±0.01 | nf | 0.8±0.02 | 0.1±0.003 | nf | 2.5±0.08 | nf | nf | nf | 3.8±0.1 |
| A7 | 2016 | nf | nf | nf | nf | 0.1±0.003 | nf | nf | 3.8±0.1 | nf | nf | nf | 3.9±0.1 |
| B1 | 2017 | 0.008±0.0003 | 0.02±0.0005 | 0.07±0.0002 | 0.1±0.05 | 0.4±0.001 | 0.4±0.01 | 0.05±0.002 | 0.6±0.02 | 0.4±0.01 | 0.06±0.002 | 0.05±0.002 | 2.7±0.1 |
| B2 | 2017 | 0.01±0.003 | 0.02±0.0007 | 0.08±0.0003 | 0.2±0.006 | 0.4±0.001 | 0.5±0.02 | 0.06±0.002 | 0.8±0.03 | nf | 0.07±0.002 | 0.06±0.002 | 2.2±0.1 |
| B3 | 2017 | nf | nf | nf | nf | nf | nf | nf | 1.5±0.05 | nf | nf | nf | 1.5±0.1 |

**Figure 1**




**Steviol (ST)**




**Isosteviol (iST)**

Figure 2

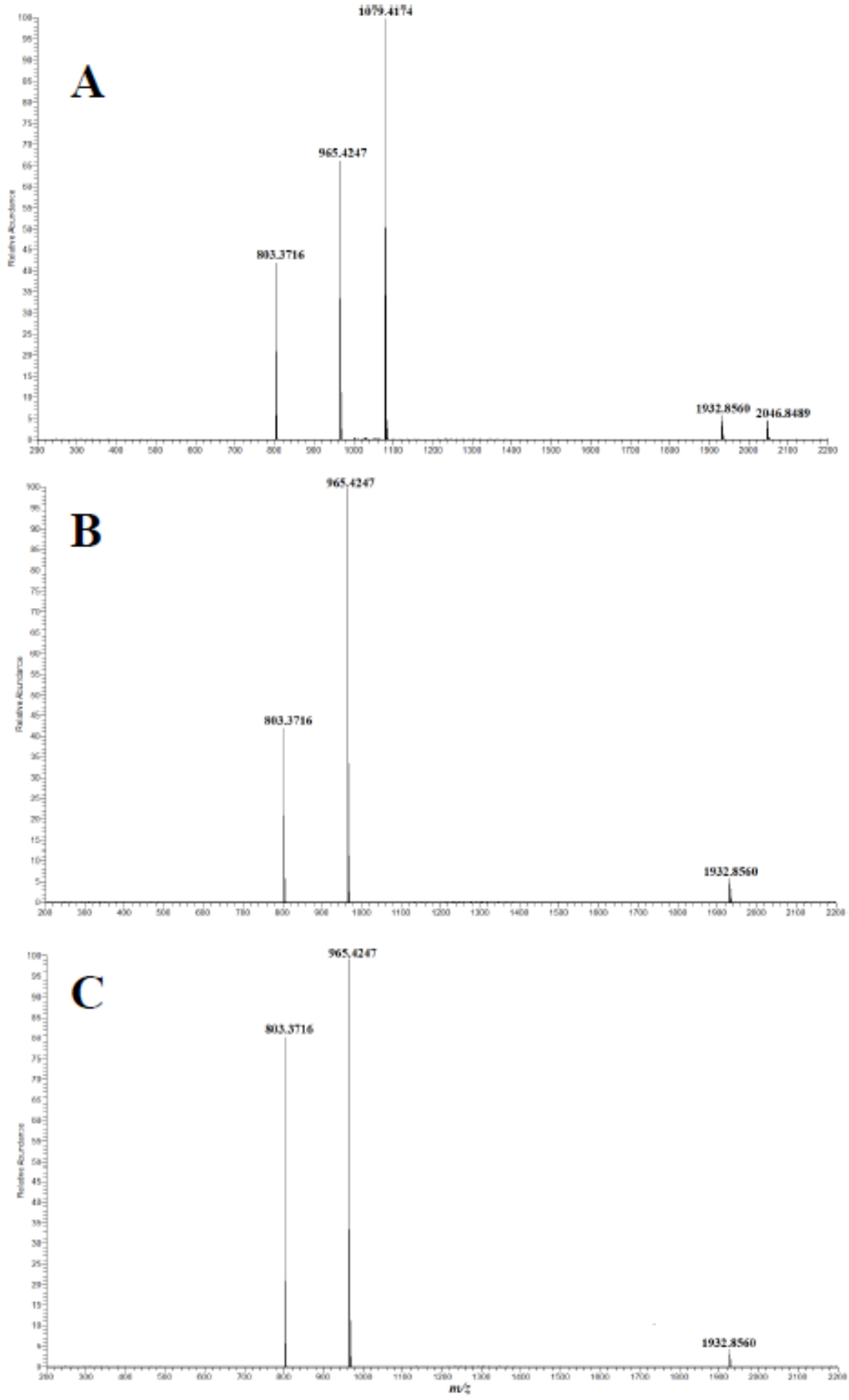
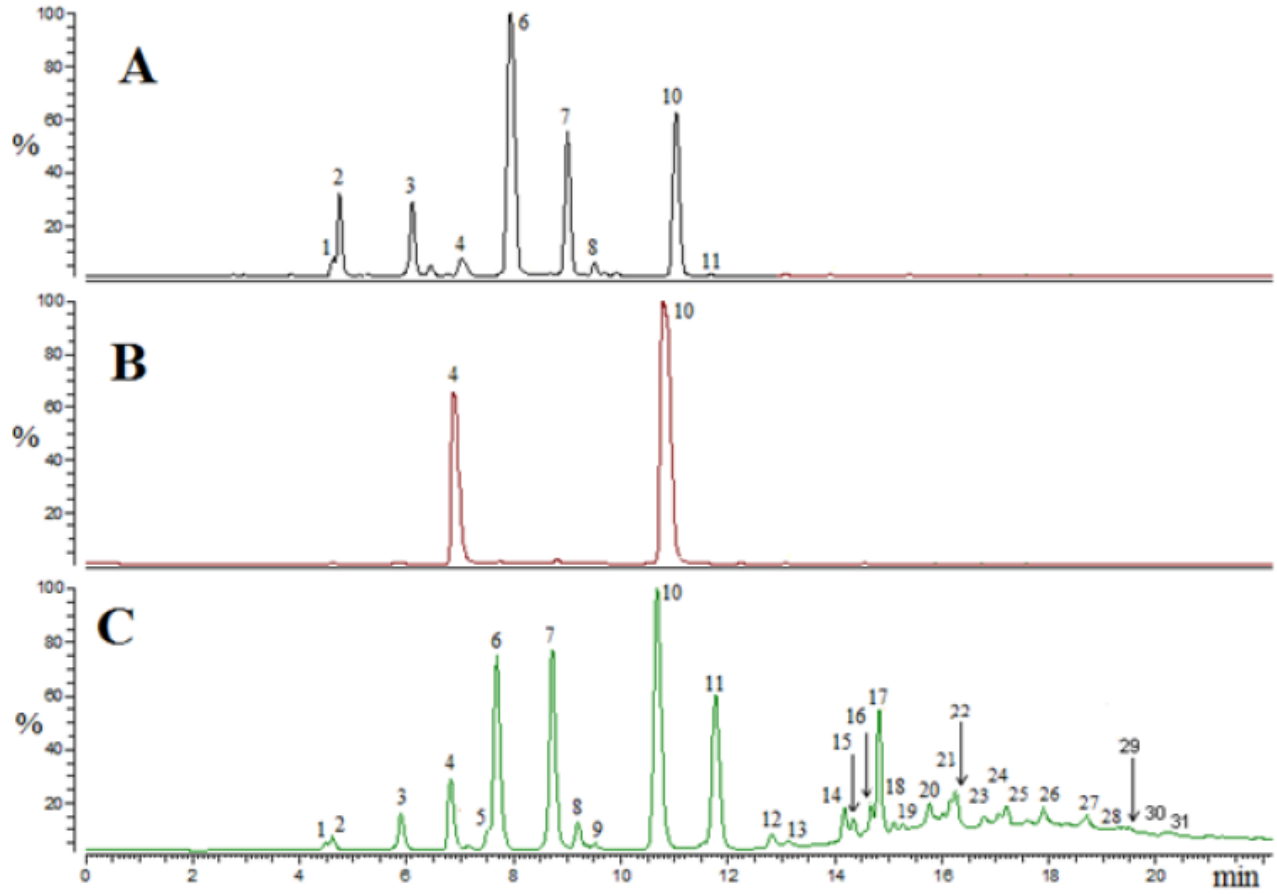

A
1079.4174
965.4247
803.3716
1932.8560
2046.8489
B
965.4247
803.3716
1932.8560
C
965.4247
803.3716
1932.8560
m/z

**Figure 3**


A
B
C
%
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
min
1
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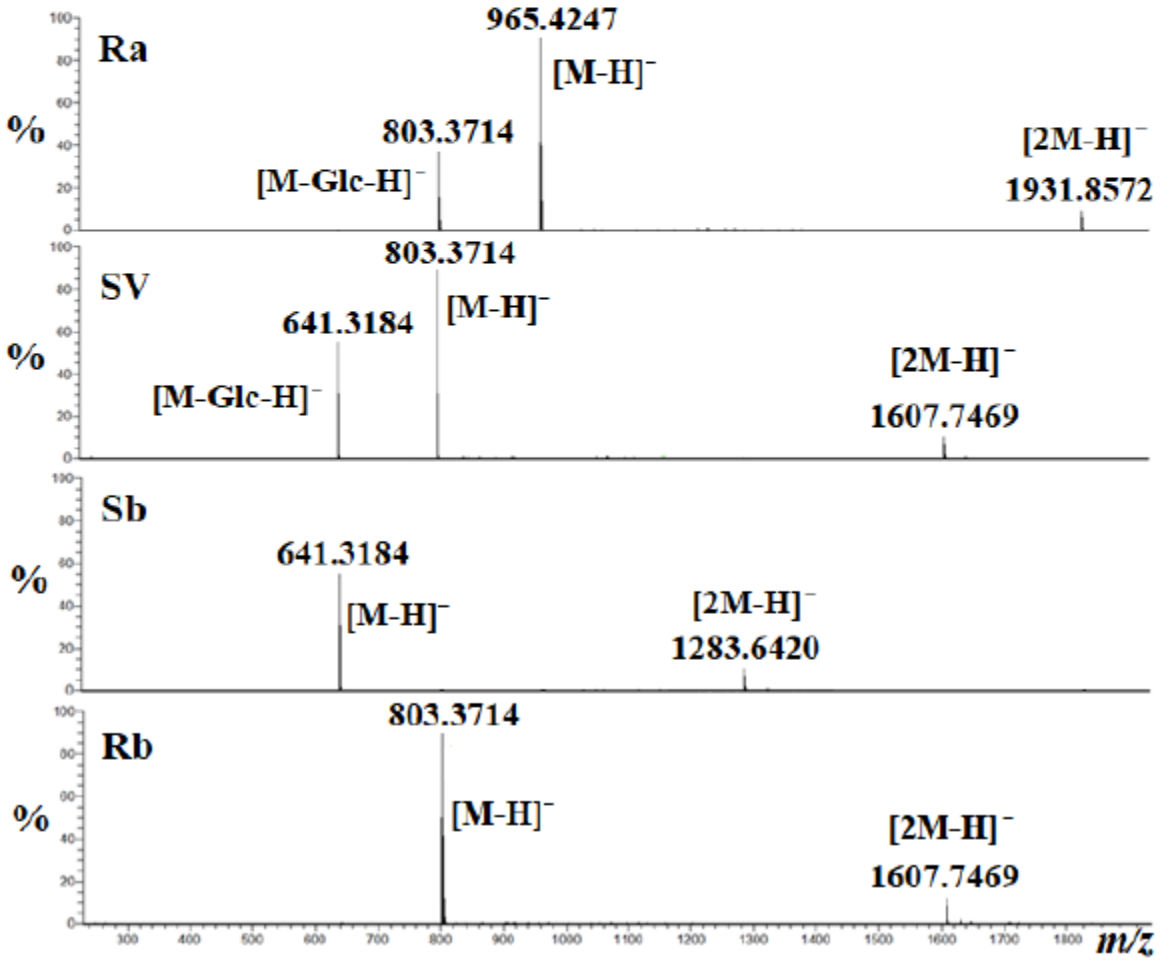
## Supplementary Fig. S1

**Steviol-glycosides behavior in ESI source.**

Compounds with glucose residues at C19, such as Ra and SV, spontaneously lose one glucose moiety. On the contrary, in the chromatograms of those with no residues at C19, such as Rb and Sb, the deglucosylated form $[M\text{-}glc\text{-}H]^-$ was not detected.



## Supplementary Fig. S2

**Ions detected in the sample B1 (tea), B2 (tea) and B3 (drink).**

The detected ions differed by 342.1164 u, which corresponds to a sucrose residue. Thus, these samples contained a series of oligomers of glucose (n=16), probably maltodextrin.