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ARTICLE

# Investigations on the Stability of Stevioside and Rebaudioside A in Soft Drinks

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The stability of the two steviol glycosides stevioside and rebaudioside A and the possible formation of the aglycon steviol in different soft drinks were analyzed in samples spiked with stevioside or rebaudioside A after 24, 48, and 72 h storage times at 80 °C. Degradation of up to 70% was observed, and stevioside was less stable than rebaudioside A. Stevioside and rebaudioside A and their degradation products were analyzed by high-performance liquid chromatography with ultraviolet detection (UV-HPLC) on a HILIC analytical column, and the identity of the degradation products was confirmed by liquid chromatography−electrospray ionization mass spectrometry (LC-ESI-$MS^n$) in negative mode. A UV-HPLC method was developed using a C18 analytical column to exclude the presence of the aglycon steviol, which gave a positive response in the forward mutation assay using the sensitive *Salmonella typhimurium* TM677 strain. The recoveries of steviol with this method ranged from 95.9 to 109.2%, and the calibration curves were linear from 1 to 100 $\mu$g/mL with $R^2 = 0.9999$. The limit of detection was 1 $\mu$g/mL. Confirmation by LC-ESI-$MS^n$ resulted in a LOD of 6 ng/mL. The absence of steviol in the degraded samples could be unambiguously confirmed by UV-HPLC and by LC-ESI-$MS^n$.

**KEYWORDS: Stevioside; rebaudioside A; steviol; stability in beverages; LC-MS analysis; degradation;**

## INTRODUCTION

Steviol glycosides, natural sweeteners from the leaves of *Stevia rebaudiana* Bertoni, are *ent*-kaurene type diterpene glycosides, the most abundant of which are stevioside and rebaudioside A (**Table 1**; **Figure 1**). The minor components of this plant include rebaudiosides C−F, rubusoside, and dulcoside A (1−2% in total) (*1*, *2*). Some evidence suggests that steviolbioside and rebaudioside B are not genuine constituents of *S. rebaudiana* but are instead formed by partial hydrolysis during the extraction process (*3*−*5*). Stevioside and rebaudioside A are 300−400 times more potent than sucrose, contain no calories, and are safe for diabetes (type II), phenylketonuria, and *Candida* patients (*2*). Nevertheless, steviol glycosides have not yet been authorized as food additives in Europe, with the exception of France (*6*), because of safety concerns. The rest of Europe is expected to authorize its use in 2011, following the European Food Safety Authority's (EFSA) April 2010 positive scientific opinion on the safety of steviol glycosides (*7*). The authorities' caution is partially based on the uncertainty of the safety of the degradation product and aglycon steviol. Steviol resulted in a positive response in the forward mutation assay using the sensitive *S. typhymurium* TM677 strain and in further in vitro tests (*8*−*10*). Steviol glycosides are converted in vitro to steviol by intestinal microflora (*11*−*14*). In vivo, steviol glucuronide was detected in human plasma (*15*−*18*) and free steviol in rats (*11*, *19*, *20*). Steviol glucuronide is excreted primarily via urine in humans and via bile in rats.

The use of steviol glycosides as food sweeteners requires detailed knowledge of their stability under different processing and storage conditions and awareness of whether degradation to steviol occurs. In the literature, the steviol glycosides in several foods are described as highly stable. No significant changes in stevioside and rebaudioside A concentrations could be observed at room temperature during long-term storage for at least 5 months in carbonated phosphoric and citric acidic beverages (*21*). Studies have shown that stevioside is stable in buffer systems in a pH range of 2−10 over 2 h at 60 °C (*22*). Stevioside and rebaudioside A are stable in acidic solutions at pH values of 2 and 3.5 after 3 days at room temperature and at pH 3.8 for 7 days at 80 °C (*23*) and also stable when exposed to sunlight until the level reached ca. 3000 langleys, which took approximately 1 week (*24*). Rebiana, a commercially available high-purity rebaudioside A, is stable in live yogurt for 6 weeks at 4 °C and in cake throughout the 5 day investigation period at room temperature (*5*). In contrast, remarkable losses in stevioside concentration of 22% (in citric acid, pH 2.1), 33% (in tartaric acid, pH 2.1), and even 75% (in phosphoric acid, pH 1.6) were observed after 4 months at room temperature (*22*).

These results were achieved by applying HPLC with UV detection on Amino (*23*), C18 (*22*), HILIC (*25*), and carbohydrate columns (*21*). Mass spectrometry allows increased sensitivity in detection (*26*−*29*).

Quantification of steviol with a detection limit of 100 pg per injection was achieved after derivatization followed by fluorescence

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**Table 1.** Steviol Glycoside Sweeteners[a]

| name | R1 | R2 |
|---|---|---|
| steviol | H | H |
| steviolbioside | H | β-Glc-β-Glc(2−1) |
| rubusoside | β-Glc | β-Glc |
| stevioside | β-Glc | β-Glc-β-Glc(2−1) |
| rebaudioside A | β-Glc | β-Glc-β-Glc(2−1) β-Glc(3−1) |
| rebaudioside B | H | β-Glc-β-Glc(2−1) β-Glc(3−1) |
| rebaudioside C | β-Glc | β-Glc-α-Rha(2−1) β-Glc(3−1) |
| rebaudioside D | β-Glc-β-Glc(2−1) | β-Glc-β-Glc(2−1) β-Glc(3−1) |
| rebaudioside E | β-Glc-β-Glc(2−1) | β-Glc-β-Glc(2−1) |
| rebaudioside F | β-Glc | β-Glc-β-Xyl(2−1) β-Glc(3−1) |
| dulcoside A | β-Glc | β-Glc-α-Rha(2−1) |

[a] Glc, glucose; Rha, rhamnose; Xyl, xylose.

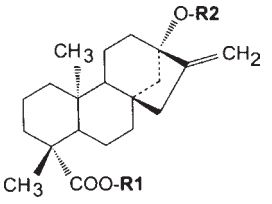


**Figure 1.** Structure of steviol glycosides.

detection (*30*), which is 20 times more sensitive than found using UHPLC-MS (*29*).

The purpose of this study was to investigate the stability of the main steviol glycosides, stevioside and rebaudioside A, in commercially available soft drinks using UV-HPLC and LC-ESI-$MS^n$. We conducted an accelerated shelf-life study with severe storage conditions to provoke the breakdown of the steviol glycosides and to get detailed information about the degradation products and possible steviol formation.

## MATERIALS AND METHODS

**Reagents.** HPLC grade acetonitrile and methanol were obtained from VWR International (Leuven, Belgium). Solid phase extraction (SPE) was carried out on a Baker 10 SPE system from Mallinckrodt Baker (Phillipsburg, NJ) using Strata C18-E cartridges (100 mg/mL, 55 μm, 70 Å) from Phenomenex (Aschaffenburg, Germany) and RC membrane filters (0.45 μm, Ø 25 mm) purchased from IVA (Meerbusch, Germany).

Stevioside and rebaudioside A with purities of ≥99.0% were obtained from Wako Chemicals (Neuss, Germany). Steviol with a purity of ≥98% (HPLC) was purchased from Sigma-Aldrich (Taufkirchen, Germany).

**Standard Solutions.** To prepare the stock solutions, the steviol glycosides were dried to constant weight at 103 °C, cooled, and stored in a desiccator until use. Ten milligrams of dried steviol glycosides was dissolved in water (10 mL). Spiking solutions were prepared by diluting aliquots of the stock solutions with water. Calibration solutions for HPLC analysis of stevioside and rebaudioside A were prepared by diluting the analytes with acetonitrile/water (8:2 v/v) to obtain final concentrations of 10−800 μg/mL.

For steviol calibration, the stock solution (1.2 mg/mL) was diluted with methanol/water (9:1 v/v) to give final concentrations of 1−100 μg/mL.

**Sample Preparation.** The degradation of the steviol glycosides was analyzed in three commercially available soft drinks, a caffeinated soft drink, a lemon-lime flavored soft drink, and an energy drink. The degassed samples were added to volumetric flasks containing roughly 35 μg/mL of either stevioside or rebaudioside A. The flasks were filled to the mark, transferred to a screw-top 50 mL glass bottle, and weighed. The bottles were stored for up to 72 h in a drying oven at 80 °C. After 0, 24, 48, and 72 h, portions of each sample were removed for further analysis. Losses due to evaporation were compensated by the addition of water. The samples were cleaned by SPE as described in ref *25*, and the eluates were subjected to HPLC using a HILIC analytical column and UV detection. These degradation experiments were performed in duplicate for stevioside and rebaudioside A, respectively.

The processing method for steviol was validated using a commercially available caffeinated soft drink (1 mL) spiked with different volumes of steviol stock solution and analyzed using the method reported for dried stevia leaves (*25*). Aliquots of spiked samples were cleaned by SPE, and the eluates were subjected to HPLC using a C18 analytical column and UV detection. The precision and recovery were calculated by external calibration.

**HPLC Conditions.** Liquid chromatography under isocratic conditions was performed on a Varian system (Varian Deutschland GmbH, Darmstadt, Germany) consisting of the following components: a ProStar 230 pump, a ProStar 410 autosampler, a 335 diode array detector set to a wavelength of 210 nm, a four-channel degasser, a Metatherm column thermostat, and a Galaxie CDS chromatography data system.

Stevioside, rebaudioside A, and the degradation products were analyzed on a Luna HILIC column (250 × 4.6 mm) with the corresponding guard column (4 × 3.0 mm) purchased from Phenomenex (Aschaffenburg, Germany). The mobile phase consisted of acetonitrile/water (8:2 v/v) and was delivered with a flow rate of 1.0 mL/min at 36 °C. The injection volume was 20 μL.

For the specific detection of steviol, a Grace Alltima C18 5 μm column (250 × 4.6 mm) purchased from Alltech Grom (Rottenburg, Germany) was used at 36 °C. Isocratic elution was performed with a methanol/water mixture (9:1 v/v) at a flow rate of 1.0 mL/min. The injection volume was 20 μL.

**LC-MS Analysis.** An Agilent 1200 series quarternary HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of a G1311A quaternary pump with a G1322A vacuum degasser, a G1329A thermostated autosampler, a G1316A column oven set at 36 °C, and a G1314B VW detector set at 210 nm was used. MS detection was done with an HCTultra PTM Discovery Ion Trap System (Bruker Daltonik GmbH, Bremen, Germany) running in negative ESI mode. $N_2$ was used as nebulizing and drying gas and He as collision gas. Source conditions, nebulizing gas pressure, dry gas flow, and drying gas temperature, were set to 60 psi, 12 L/min, and 350 °C, respectively. The sampling, transfer, and focusing voltages (capillary, skimmer, octapoles, lenses) were optimized for a steviol standard solution (100 ng/mL) by ramping the most important parameters during direct infusion (DI) using a syringe pump (KD Scientific Inc., Holliston, MA) at a rate of 10 uL/min. The resulting parameters used were capillary voltage, 4500 V; skimmer, −25.0 V; Oct 1 DC, −8.41 V; Oct 2 DC, −0.50 V; lens 1, +3.3 V; lens 2, +71.5 V; capillary exit, −191.7 V. The mass traces of $m/z$ 317, 479, 641, 803, and 965 were recorded in extracted ion chromatogram (EIC) mode, and the identification of individual compounds was conducted by $MS^n$ fragmentation. The LC-MS instrument control and $MS^n$ data processing were performed with Bruker Daltonik Compass 1.3 HyStar software version 3.2-SR2 Built 44.0, integrating all necessary functions of the ChemStation Rev. B.01.03 SR2 (Agilent) software. The LC conditions for the separation of the steviol glycosides and steviol were the same as described above. The HPLC injection volume was 10 μL, respectively.

**Method Validation.** Selectivity in steviol analysis was determined by comparing the peaks in the chromatograms with those from standard solutions. Linearity was assessed by injecting from 1 to 100 μg/mL of steviol standard solutions in duplicate. Qualitative determination was achieved by comparing the retention times of the standard solution with those of the samples. Quantification was possible by applying the calibration plot equations calculated by the least-squares method. Precision was calculated in terms of intraday ($n$ = 4) and interday repeatability ($n$ = 3) by analyzing spiked caffeinated soft drink samples and was evaluated by calculating the relative standard deviation (RSD). The accuracy of the method was determined by calculating the recovery and the appropriate standard deviation (SD) in a caffeinated soft drink spiked with different amounts of steviol (**Table 2**). The limit of detection (LOD) was defined as a signal/noise ratio of 3:1 and the limit of quantification (LOQ) as a ratio of 6:1 in a spiked caffeinated soft drink after UV and MS detection ($n$ = 3).

The selectivity in the degraded samples was determined by comparing the chromatograms with those of standard solutions of stevioside and rebaudioside A; further degradation products were identified by MS evaluation via the corresponding molecular ions and MS/MS fragmentation. Linearity was assessed by injecting from 10 to 800 μg/mL of stevioside or rebaudioside A standard solution in duplicate. The degradation experiments were evaluated statistically by calculating the means and the standard deviations of two independent series.

## RESULTS AND DISCUSSION

**Sample Preparation.** HPLC of stevioside and rebaudioside A can be performed without sample preparation and without disturbing the interesting steviol glycoside detection, but the column lifetime is dramatically shortened and the appearance of the peaks and the baseline deteriorate. An analytical method including SPE (*25*) was used to avoid these issues. This method, which has been validated for stevioside and rebaudioside A in stevia leaves, was adapted for steviol determination in carbonated soft drinks.

**HPLC Analysis.** A good separation of stevioside and rebaudioside A and the degradation products can be achieved with the HILIC column (**Figure 2**). Furthermore, the HILIC column showed almost no bleeding and was suitable for use in LC-MS detection.

**Table 2.** Recovery of Steviol in the Spiked Caffeinated Soft Drink

| added ($\mu$g/mL) | detected ($\mu$g/mL) | recovery (%) | mean recovery (%) $\pm$ SD |
|---|---|---|---|
| 16.7 | 18.1 | 108.7 | 109.2 $\pm$ 1.2 |
| 16.7 | 18.4 | 110.2 | |
| 16.7 | 18.4 | 110.2 | |
| 16.7 | 18.0 | 107.7 | |
| 46.2 | 46.2 | 100.1 | 100.1 $\pm$ 0.1 |
| 46.2 | 46.2 | 100.1 | |
| 46.2 | 46.2 | 100.0 | |
| 46.2 | 46.2 | 100.0 | |
| 88.8 | 83.8 | 94.3 | 95.9 $\pm$ 2.2 |
| 88.8 | 86.5 | 97.4 | |
| 128.6 | 128.4 | 99.8 | 97.8 $\pm$ 2.4 |
| 128.6 | 128.5 | 99.9 | |
| 128.6 | 124.1 | 96.5 | |
| 128.6 | 122.2 | 95.0 | |
| 165.5 | 163.5 | 98.8 | 101.4 $\pm$ 2.9 |
| 165.5 | 165.4 | 99.9 | |
| 165.5 | 174.7 | 105.6 | |
| 165.5 | 167.3 | 101.1 | |

The detection of steviol is critical in the phase modus used on HILIC columns. Under the conditions described above, steviol is poorly retained with a retention time of 3.1 min, and coelution occurred with some unspecific matrix peaks. The elution order is quite different using a reversed phase column instead of a HILIC phase. Steviol was strongly retained, with a retention time of 5.1 min, under the conditions described above, and well-separated from other steviol glycosides or matrix peaks eluting within 4.1 min.

**Method Validation.** The external calibration curve of steviol standard solutions was linear between 1 and 100 $\mu$g/mL with $R^2 > 0.999$. The equation of the calibration curve was $y = 8.0797x - 5.154$ with $y$ = peak area of the UV detection signal and $x$ = concentration of steviol in $\mu$g/mL. The intraday precision ($n = 4$) of the steviol-spiked samples ranged from 1.1 to 2.5% RSD and the interday precision ($n = 3$) from 1.9 to 4.4% RSD. In **Table 2** recoveries from 95.9 to 109.2% at four different concentration levels are reported. As the caffeinated soft drink showed the highest degradation rates for stevioside and rebaudioside A (see below), this matrix was used for explicit steviol method evaluation. Nevertheless, we determined the recovery in a lemon-lime flavored soft drink and an energy drink ranging from 91 to 100%.

The LODs and LOQs calculated for steviol were as follows: The LOD/LOQ after UV detection with 1 and 2 $\mu$g/mL, respectively, were, as expected, higher than those after MS detection (6 or 12 ng/mL). With a LOD of 6 ng/mL corresponding to 60 pg injected after LC-MS, steviol can be detected more sensitively than reported by Minne (*30*), who calculated a LOD of 100 pg per injection.

The external calibration curves of stevioside and rebaudioside A were linear between 10 and 800 $\mu$g/mL as described (*25*).

**Stability of Stevioside and Rebaudioside A in Soft Drinks.** The pH values in the degassed soft drinks ranged from 2.4 in the caffeinated drink to 2.7 in the lemon-lime flavored drink to 3.5 in the energy drink. We conducted an accelerated shelf-life study with severe storage condition (80 °C) to provoke a thermal breakdown of the steviol glycosides. We determined the stevioside and rebaudioside A concentrations in duplicate. The recovery data in relation to the accordant sample at time 0 are presented in **Figure 3** replenished by the standard deviation.

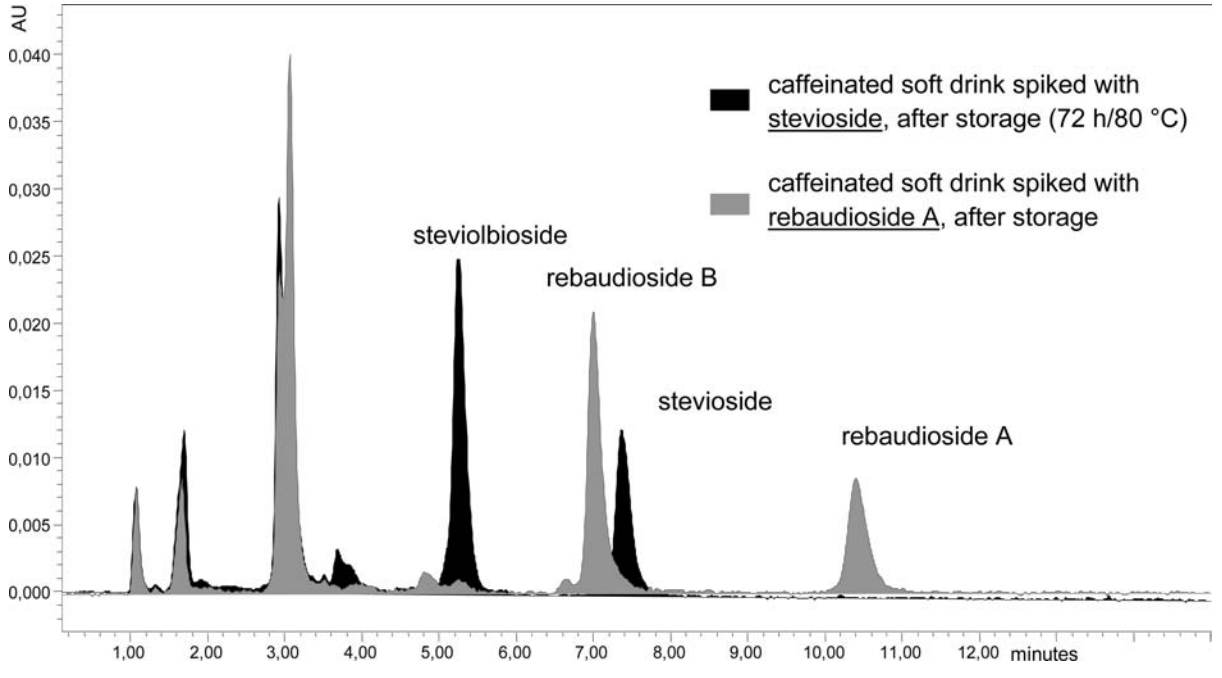


**Figure 2.** HPLC chromatograms of the spiked and stored caffeinated soft drink on the HILIC column.

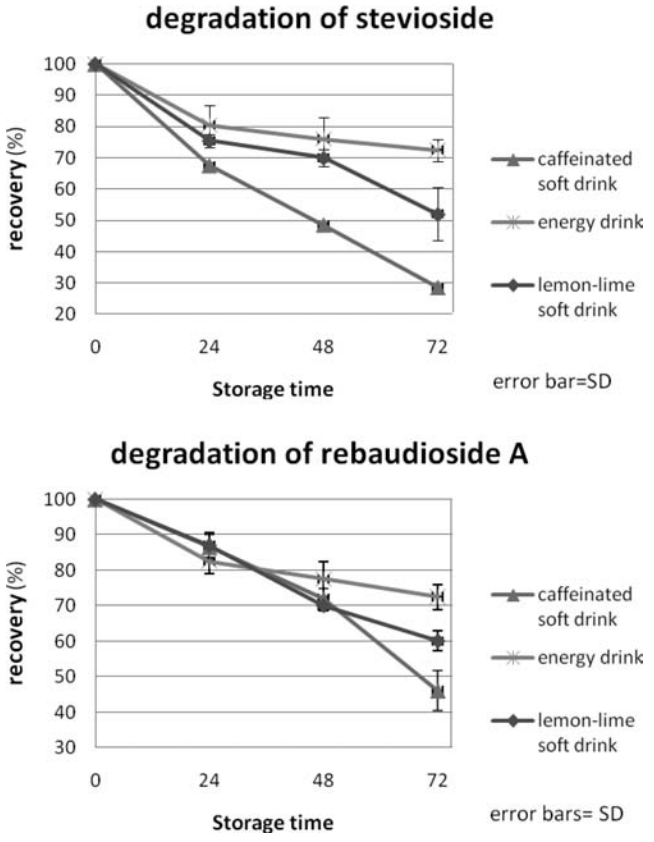


**Figure 3.** Degradation rates of stevioside and rebaudioside A in the different soft drinks after several storage times.

*Stevioside*. There is convincing evidence that stevioside was strongly degraded, confirming preliminary investigations (*23*). After 24 h, the stevioside concentration decreased in the caffeinated soft drink by nearly 32% (**Figure 3**). The highest degradation of 71% was observed in the caffeinated lemonade after 72 h, whereas the energy drink was decomposed to only 27%. This confirms similar investigations (*22*) detecting degradation rates in stevioside concentration of 75% in phosphoric acid (pH 1.6) after 4 months at room temperature.

*Rebaudioside A*. Again, a remarkable degree of degradation occurred. The highest degradation, nearly 54%, was observed in the caffeinated soft drink after 72 h, and the lowest one again in the energy drink (**Figure 3**).

Rebaudioside A was more stable against acid hydrolysis than stevioside. Chang reported a 36% loss in stevioside concentration in beverages after 4 months at 37 °C (*21*), whereas rebaudioside A was degraded to only 25%. The stability of rebaudioside A, not only after acid hydrolysis in food, is reported in the literature. In vitro transformation of stevioside and rebaudioside A after incubation with human microflora (*12*) under strict anaerobic conditions led to a complete degradation of stevioside to steviol after 10 h of incubation, whereas rebaudioside A was completely degraded after 24 h. In vitro tests with rat intestinal microflora (*31*) showed a complete degradation of stevioside within 2 days, whereas the conversion of rebaudioside A required 6 days.

It is obvious that the stability of the steviol glycosides mainly depends on pH and increases with rising pH values. Similar results were reported by Kroyer (*22*), who observed a total decomposition of stevioside at pH 1, whereas no degradation could be observed at higher pH values.

**Identification of the Degradation Products by LC-MS.** Comparison of the HPLC chromatograms from the stability investigations showed that new peaks appeared during the storage period.

**Table 3.** Molecular Ions of the Detected Steviol Glycosides

| name | $[M-H]^-$ | name | $[M-H]^-$ |
|---|---|---|---|
| steviol | 317 | stevioside | 803 |
| steviolmonoside | 479 | rebaudioside B | 803 |
| steviolbioside | 641 | rebaudioside A | 965 |
| rubusoside | 641 | | |

ESI-MS in negative ion mode was used to further characterize the reaction products because the analyte signals should be about 10 times higher than in positive mode (*26*, *29*). **Table 3** indicates the molecular ions of the identified degradation products, whereas in MS/MS fragmentation these compounds were readily confirmed through subsequent glycosidic losses of fragments of 162 Da.

In the soft drinks spiked with stevioside, the molecular ion of stevioside ($m/z$ 803) and two new peaks ($m/z$ 641 and 479) assigned to steviolbioside or rubusoside and steviolmonoside were detected.

The degradation pattern in the samples spiked with rebaudioside A was different. Besides the molecular ion for rebaudioside A ($m/z$ 965), the mass ($m/z$ 803) representing stevioside or rebaudioside B appeared and, additionally, steviolmonoside ($m/z$ 479) was detected, but no steviolbioside or rubusoside ($m/z$ 641). Distinguishing between stevioside/rebaudioside B and steviolbioside/rubusoside by LC-MS alone is not possible even with ion trap MS/MS detection (*27*). However, by comparison of the UV chromatograms of the samples stored for 72 h and spiked with stevioside and rebaudioside A, the presence of rebaudioside B instead of stevioside could be identified due to its shorter retention time (**Figure 2**). Otherwise, it is well-known from the literature (*5*, *21*, *23*, *32*) that steviolbioside is a degradation product of stevioside and rebaudioside B a degradation product of rebaudioside A.

As shown before, the absence of steviol in the degraded samples could not safely be verified using the HILIC column due to the interfering matrix. However, no peak corresponding to steviol ($m/z$ 317) was detected in the MS spectra within the 4 min retention time.

Using the C18 analytical column, the presence of steviol in the degraded samples could reliably be excluded. No steviol peak in the UV and EI chromatograms after a retention time of 5.1 min could be detected, whereas the same sample spiked with steviol standard gave a well visible and separated signal, respectively.

A LOQ of 12 ng/mL after MS detection indicates that, if steviol was present, the concentrations were $< 80\ \mu$g/L soft drink.

In conclusion, stevioside and rebaudioside A were degraded when added to different carbonated soft drinks and stored for up to 72 h at 80 °C. Among the degradation products, only those products resulting from the successive elimination of glucose were detected, but the formation of steviol, the aglycon of the steviol glycosides, could reliably be excluded.

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