

Quantitation of Sweet Steviol Glycosides by Means of a HILIC-MS/MS-SIDA Approach

Caroline Well, Oliver Frank, and Thomas Hofmann*

Chair of Food Chemistry and Molecular Sensory Science, Technische Universität München, Lise-Meitner-Straße 34, D-85354 Freising-Weihenstephan, Germany

S Supporting Information

ABSTRACT: Meeting the rising consumer demand for natural food ingredients, steviol glycosides, the sweet principle of *Stevia rebaudiana* Bertoni (Bertoni), have recently been approved as food additives in the European Union. As regulatory constraints require sensitive methods to analyze the sweet-tasting steviol glycosides in foods and beverages, a HILIC-MS/MS method was developed enabling the accurate and reliable quantitation of the major steviol glycosides stevioside, rebaudiosides A–F, steviolbioside, rubusoside, and dulcoside A by using the corresponding deuterated 16,17-dihydrosteviol glycosides as suitable internal standards. This quantitation not only enables the analysis of the individual steviol glycosides in foods and beverages but also can support the optimization of breeding and postharvest downstream processing of *Stevia* plants to produce preferentially sweet and least bitter tasting *Stevia* extracts.

KEYWORDS: steviol glycosides, stevioside, rebaudioside A, LC-MS/MS, quantification

INTRODUCTION

The sweet-tasting leaves of the South American plant *Stevia rebaudiana* Bertoni (Bertoni) have been used for centuries by the native population in Paraguay and Brazil to sweeten and to increase the palatability of bitter-tasting herbal tea preparations.^{1,2} Within the past decades, various glycosides of the diterpenic 13-hydroxykaur-16-en-18-oic acid (steviol) were successfully isolated from *Stevia* leaves and identified as the key tasting principles of this so-called “sweet herb” (Figure 1). Stevioside, **1**, was found to be the most abundant steviol glycoside, followed by the rebaudiosides A–F, **2–7**, steviolbioside, **8**, and dulcoside A, **10**, present in somewhat lower concentrations and some trace glycosides proposed on the basis of mass spectrometric fragmentation patterns.^{3–9} Present in only trace amounts in *S. rebaudiana*,¹⁰ rubusoside, **9**, exhibiting a β -D-glucopyranosyl moiety at the hydroxyl group at position 13 as well as at the carboxy group at position 19, was isolated as the primary sweet stimulus from the Chinese herb *Rubus suavissimus* S. Lee.¹¹

Very recently, studies on the organoleptic properties of the most common steviol glycosides by an experimental approach combining human sensory studies and cell-based functional taste receptor expression assays revealed the glycosidic chain length, pyranose substitution, and C16 double bond as the structural features giving distinction to the sweet and bitter profile of the various steviol glycosides.¹² A comprehensive screening of the human TAS1R2/TAS1R3 sweet receptor as well as the 25 human TAS2R bitter taste receptors revealed rebaudioside D to exhibit the lowest sweet receptor threshold concentration of 2.2 μ M and the highest threshold of >400 μ M for activation of the bitter receptors TAS2R4 and TAS2R14. In comparison, dulcoside A was found as the steviol glycoside showing the highest sweet receptor threshold (38.8 μ M) and the lowest threshold for the activation of the bitter receptors hTAS2R4 (200 μ M) and hTAS2R14 (50 μ M).

For more than 40 years, crude steviol glycoside extract, containing stevioside, **1**, extracted from *S. rebaudiana*, has been permitted as a food additive and natural sweetener in Japan, South Korea, Brazil, Argentina, and Paraguay and is used as a dietary supplement in the United States.¹³ In 2008, purified rebaudioside A, **2**, gained generally recognized as safe (GRAS) status in the United States,¹⁴ and in the same year the Joint FAO/WHO Expert Committee on Food Additives suggested a temporary admissible daily intake (ADI) of 0–4 mg/kg body weight, expressed as steviol.¹⁵ This was followed by a positive safety opinion on steviol glycosides expressed by the European Food Safety Authority (EFSA) in 2010.¹⁶ In July 2011, the Standing Committee on the Food Chain and Animal Health (SCoFAH) voted in favor of the Commission proposal to authorize steviol glycoside. Finally, in November 2011, the European Union (EU) published a regulation permitting the sale and use of steviol glycosides as early as December 2, 2011.¹⁷

Due to regulatory constraints, sensitive analytical methods are needed to quantitate the individual sweet-tasting steviol glycosides in foods and beverages. Although capillary electrophoresis and high-performance thin layer chromatography (HPTLC) have been reported for stevioside analysis,¹⁸ high-performance liquid chromatography (HPLC) seems to remain the method of choice. Whereas reversed phase stationary phases exhibited only low separation capacity for the major steviol glycosides **1** and **2**¹⁹ in the past, recent publications showed a better separation capacity for **1** and **2**.^{20–22} Amino phases showed a high selectivity for steviol glycosides, but their applicability in routine analysis is limited by a low reproducibility, column bleeding, and long equilibration times.^{23–25} Recently,

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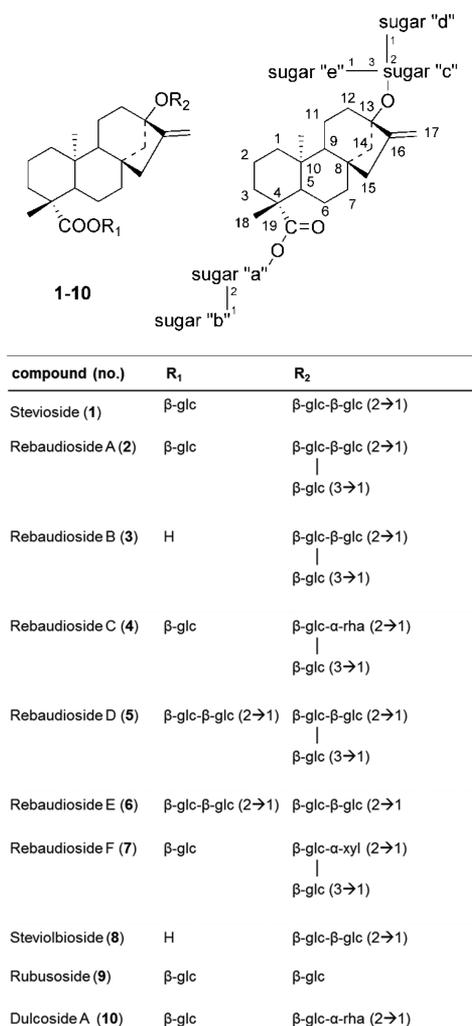


Figure 1. Chemical structures of steviol glycosides **1–10** (left) and numbering scheme for NMR analysis (right). glc, D-glucopyranosyl; rha, L-rhamnopyranosyl; xyl, D-xylopyranosyl.

hydrophilic liquid interaction chromatography was introduced for the separation of steviol glycosides **1–4** and **8–10**,^{26,27} but rebaudiosides **D–F**, **5–7**, were not considered. Although some HPLC-MS methods have been reported for quantitative analysis of steviol glycosides^{10,28,29} none of the published procedures allows for quantitation of all major steviol glycosides **1–10** in a single run; for example, RP-HPLC hyphenated with ultrahigh-performance LC-MS resulted in overlapping peaks for **1** and **2** as well as **4** and **10**,¹⁰ and a LC-ESI MS/MS method enabled the quantitation of the aglycone steviol and only five of its glycosides, namely, **1–4** and **8**.²⁵ Moreover, ¹H NMR spectroscopy has been reported for the analysis of the major steviol glycosides (**1–4**) in crude *Stevia* extracts,³⁰ but this method does not allow the quantitation of the steviol glycosides in food and beverage applications.

The objective of the present investigation was, therefore, to develop and validate a sensitive HPLC-MS/MS method enabling the accurate and reliable quantitative analysis of the major steviol glycosides **1–10** by means of a stable isotope dilution analysis (SIDA).

MATERIALS AND METHODS

Chemicals and Materials. Palladium on activated charcoal (10%), potassium hydroxide, ethanol, *n*-butanol, methanol, and ammonium acetate solution (5 M) were obtained from Sigma-Aldrich (Steinheim, Germany),

formic acid was from Merck KGaA (Darmstadt, Germany), deuterium (²H₂) and hydrogen gas were from Westfalen (Münster, Germany), deuterated solvents were from Euriso-Top (Gif-Sur-Yvette, France), HPLC grade solvents were from J. T. Baker (Deventer, The Netherlands), and membrane filter disks (0.45 μm) were from Satorius AG (Goettingen, Germany). Water used for chromatography was purified by means of a Milli-Q Advantage A10 water purification system (Millipore, Molsheim, France). Stevia extract A (rich in rebaudioside A), stevia extract B (rich in stevioside), and dried stevia leaves (SL1–SLS) were provided by Cargill (Minneapolis, MN, USA) or were purchased from a local market (Gröbenzell, Germany). A zero-calorie carbonated soft drink was purchased from a local retailer. Dried leaves of *R. suavissimus* (RL1) were purchased from Med Herbs (Wiesbaden, Germany).

Preparation and Purification of Reference Compounds. Stevioside, **1**, Rebaudioside A, **2**, and Rubusoside, **9**. A portion (1 g) of stevia extract A was dissolved in acetonitrile/water (80:20, v/v) and separated by means of medium-pressure liquid chromatography using an amino phase. Using water as solvent A and acetonitrile as solvent B (flow rate = 50 mL/min), chromatography started with 80% B for 5 min, followed by a linear gradient to 75% B within 10 min, maintaining 75% B for 15 min. Seven subfractions (A-I–A-VII) (Figure 2A) were collected,

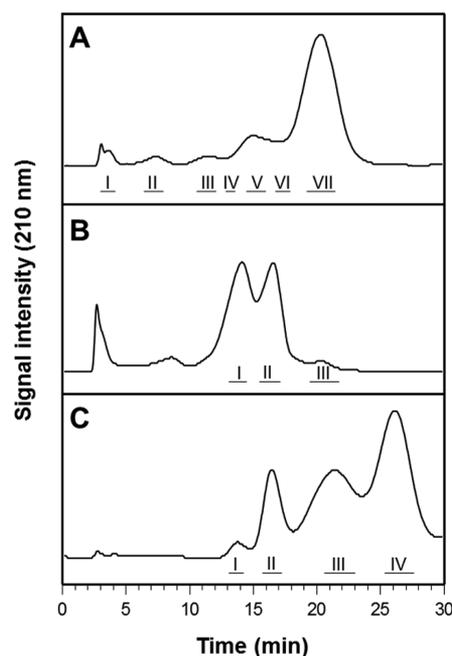


Figure 2. Medium-pressure liquid chromatographic separation of stevia extracts using an amino phase: (A) commercial rebaudioside A rich stevia extract A for isolation of **1**, **2**, **4**, **7**, **9**, and **10**; (B) commercial stevioside-rich stevia extract B for isolation of **5** and **6**; (C) rechromatography of fraction B-III for final purification of **5** and **6**.

and the solvent was removed under vacuum and lyophilized twice. LC-MS and NMR analyses (Figure 3) revealed stevioside, **1**, in fraction A-V, rebaudioside A, **2**, in fraction A-VII, and rubusoside, **9**, in fraction A-II, each as a white powder (purity > 98%, LC-MS, ELSD). Stevioside, **1**: UPLC-TOF-MS (ESI⁻), *m/z* 803.3695 ([M - H]⁻, measured), *m/z* 803.3701 ([M - H]⁻, calculated for C₃₈H₅₉O₁₈). Rebaudioside A, **2**: UPLC-TOF-MS (ESI⁻), *m/z* 965.4207 ([M - H]⁻, measured), *m/z* 965.4229 ([M - H]⁻, calculated for C₄₄H₆₉O₂₃). Rubusoside, **9**: UPLC-TOF-MS (ESI⁻), *m/z* 641.3165 ([M - H]⁻, measured), *m/z* 641.3173 ([M - H]⁻, calculated for C₃₂H₄₉O₁₃).

Rebaudioside C, 4, and Rebaudioside F, 7. An aliquot of fraction A-VI was dissolved in acetonitrile/water (80:20, v/v) and further separated by means of preparative RP-HPLC. Monitoring the effluent at 210 nm, chromatography was performed at a flow rate of 21 mL/min using water as solvent A and acetonitrile as solvent B. Starting the elution with 27% solvent B, the content of B was increased to 33% within 15 min.

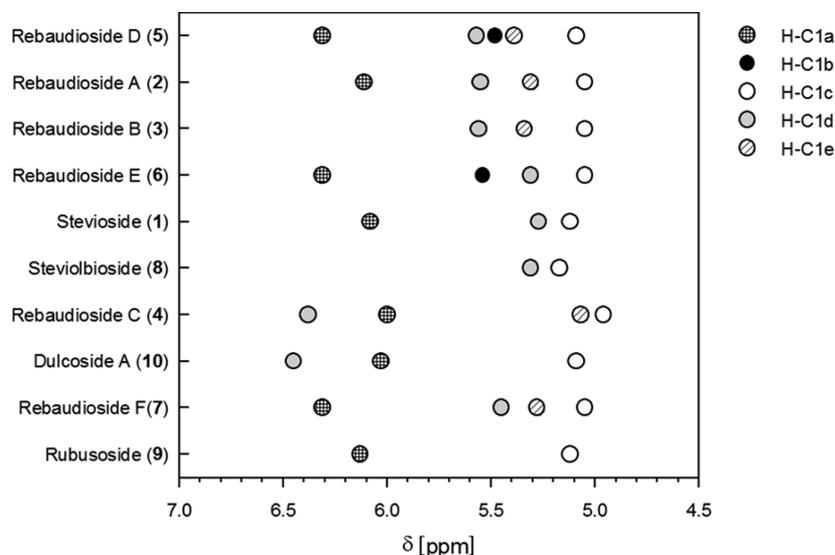


Figure 3. Comparison of chemical shifts (ppm) of anomeric protons of steviol glycosides 1–10.

Subfractions A-VI-1 and A-VI-2 were collected, freed from solvent in vacuum, and lyophilized twice to obtain rebaudioside C, 4, from fraction A-VI-2 and rebaudioside F, 7, from fraction A-VI-1, each in a purity of >98% (LC-MS, ELSD). Rebaudioside C, 4: UPLC-TOF-MS (ESI⁻), *m/z* 949.4263 ([M - H]⁻, measured), *m/z* 949.4280 ([M - H]⁻, calculated for C₄₄H₆₉O₂₂). Rebaudioside F, 7: UPLC-TOF-MS (ESI⁻), *m/z* 935.4091 ([M - H]⁻, measured), *m/z* 935.4124 ([M - H]⁻, calculated for C₄₃H₆₇O₂₂).

Dulcoside A, 10. An aliquot of fraction A-III, dissolved in acetonitrile/water (80:20, v/v), was separated by means of preparative RP-HPLC. Monitoring the effluent at 210 nm, isocratic chromatography was performed using water/acetonitrile (70:30, v/v) at a flow rate of 21 mL/min. The UV-absorbing fraction A-III-1 was collected, separated from organic solvent in vacuum, diluted with water, and lyophilized twice to obtain dulcoside A as a white powder (purity > 98%; LC-MS, ELSD). Dulcoside A, 10: UPLC-TOF-MS (ESI⁻), *m/z* 787.3755 ([M - H]⁻, measured), *m/z* 787.3752 ([M - H]⁻, calculated for C₃₈H₅₉O₁₇).

Rebaudioside E, 6, and Rebaudioside D, 5. An aliquot (1 g) of stevia extract B, dissolved in acetonitrile/water (80:20, v/v), was separated by means of MPLC using the following gradient of water (solvent A) and acetonitrile (solvent B): 80% B for 3 min, followed by a linear decrease to 61% B within 15 min, and finally in 8 min to 60% B (flow rate = 50 mL/min). Three subfractions (B-I–B-III) (Figure 2B) were collected, separated from solvent under vacuum, and lyophilized twice. Whereas fractions B-I and B-II were found to contain mainly 1 and 2, respectively, fraction B-III was further separated by MPLC using water (solvent A) and acetonitrile (solvent B) as follows: 80% B for 4 min, followed by a linear gradient to 70% B within 8 min, maintaining 70% B for 9 min and, finally, decreasing solvent B to 60% B within 9 min. Four subfractions, namely, B-III-I–B-III-IV (Figure 2C) were collected, separated from solvent under vacuum, and lyophilized twice to afford rebaudioside A, 2, from fraction B-III-II, rebaudioside E, 6, from fraction B-III-III and, after a final RP-HPLC rechromatography, rebaudioside D, 5, from fraction B-III-IV as white, amorphous powders (purity > 98%; LC-MS, ELSD). Rebaudioside D, 5: UPLC-TOF-MS (ESI⁻), *m/z* 1127.4716 ([M - H]⁻, measured), *m/z* 1127.4758 ([M - H]⁻, calculated for C₅₀H₇₉O₂₈). Rebaudioside E, 6: UPLC-TOF-MS (ESI⁻), *m/z* 965.4230 ([M - H]⁻, measured), *m/z* 965.4229 ([M - H]⁻, calculated for C₄₄H₆₉O₂₃).

Rebaudioside B, 3, and Steviolbioside, 8. Following a literature protocol with some modifications,⁴ aliquots (0.3 mmol) of stevioside, 1, and rebaudioside A, 2, respectively, were dissolved in 10% ethanolic KOH (10 mL) and refluxed for 1.5 h at 110 °C. After cooling, the solution was acidified to pH 5.0 with acetic acid, the solvent was removed under vacuum, and, after addition of water (50 mL), the

mixture was extracted with *n*-butanol (3 × 30 mL). The organic layers were combined, and the solvent was removed under vacuum to afford rebaudioside B, 3 (90 mg), and steviolbioside, 8 (80 mg), respectively, which were purified by recrystallization from methanol (purity > 98%; LC-MS, ELSD). Rebaudioside B, 3: UPLC-TOF-MS (ESI⁻), *m/z* 803.3693 ([M - H]⁻, measured), *m/z* 803.3701 ([M - H]⁻, calculated for C₃₈H₅₉O₁₈). Steviolbioside, 8: UPLC-TOF-MS (ESI⁻), *m/z* 641.3163 ([M - H]⁻, measured), *m/z* 641.3173 ([M - H]⁻, calculated for C₃₂H₄₉O₁₃).

1D/2D NMR data for compounds 1–10 are given as Supporting Information (Tables S1–4).

Synthesis of 16,17-Dihydrostevioside and [²H_{3–5}]-16,17-Dihydrosteviol Glycosides. An aliquot (0.062 mmol) of a purified steviol glycoside, 1–4 and 8–10, dissolved in anhydrous methanol (50 mL), and catalytic amounts of palladium on activated charcoal (20 mg) were placed in a hydrogenation vessel, which was evacuated and flushed three times with nitrogen. Thereafter, the reaction mixture was vigorously stirred overnight under an atmosphere of hydrogen (for 16,17-dihydrostevioside) or deuterium (for [²H_{3–5}]-16,17-dihydrosteviol glycosides), respectively, at atmospheric pressure and room temperature. After removal of the catalyst by filtration, the solvent was removed under vacuum, the residue was taken up in water (10 mL) and freeze-dried to afford the diastereomeric mixtures (ratio ~10:1) of the target compounds as an amorphous powder (yield 87–90%, purity > 98%; LC-MS, ELSD). Spectral data of deuterated compounds refer to mixtures with different contents of deuterium.

16,17-Dihydrostevioside (major diastereomer), 16,17-dihydro-1: MS/MS (ESI⁻), *m/z* (%) 805.6 (15), 643.4 (100), 481.4 (10), 319.4 (5); ¹H NMR (500 MHz, pyridine-*d*₅, COSY), anomeric protons, δ 5.01 [d, 1H, ³J = 7.8 Hz, H-C(1c)], 5.23 [d, 1H, ³J = 7.7 Hz, H-C(1d)], 6.12 [d, 1H, ³J = 7.8 Hz, H-C(1a)]; ¹³C NMR (125 MHz, pyridine-*d*₅; HMQC, HSQC, 500 MHz), δ 40.8 [C(1)], 19.4 [C(2)], 38.5 [C(3)], 44.1 [C(4)], 57.4 [C(5)], 22.4 [C(6)], 42.7 [C(7)], 43.8 [C(8)], 55.3 [C(9)], 39.9 [C(10)], 20.0 [C(11)], 30.0 [C(12)], 85.9 [C(13)], 44.5 [C(14)], 46.9 [C(15)], 39.8 [C(16)], 13.9 [C(17)], 28.3 [C(18)], 177.2 [C(19)], 15.4 [C(20)], 95.8 [C(1a)], 98.1 [C(1c)], 106.5 [C(1d)], 74.0 [C(2a)], 84.0 [C(2c)], 77.4 [C(2d)], 62.1 [C(6a/6c/6d)], 62.4 [C(6a/6c/6d)], 63.1 [C(6a/6c/6d)], 71.0 [C(4a/4c/4d)], 71.3 [C(4a/4c/4d)], 72.6 [C(4a/4c/4d)], 77.7/77.9/78.0/78.9/79.2/79.3 [C(5c/3c/5d/3d/5a/3a)].

[²H₄]-16,17-Dihydrostevioside (major diastereomer), [²H₄]-16,17-dihydro-1: MS/MS (ESI⁻), *m/z* (%) 809.4 (30), 647.3 (100), 485.3 (25); ¹H NMR (500 MHz, pyridine-*d*₅), anomeric protons, δ 6.12 [d, 1H, ³J = 7.8 Hz, H-(C1a)], 5.02 [d, 1H, ³J = 7.7 Hz, H-(C1c)], 5.23 [d, 1H, ³J = 7.7 Hz, H-(C1d)]; ¹³C NMR (125 MHz, pyridine-*d*₅), δ 15.4, 15.6, 19.4, 20.0, 20.3, 22.4, 22.7, 28.3, 30.0, 38.5, 39.9, 40.8, 40.9,

41.4, 42.6, 42.6, 43.7, 43.8, 44.0, 44.5, 54.3, 55.3, 57.4, 62.1, 62.4, 62.7, 63.1, 71.0, 71.3, 71.6, 72.6, 74.0, 74.0, 77.2, 77.4, 77.7, 77.7, 77.9, 78.0, 78.9, 79.2, 79.3, 79.3, 84.0, 84.4, 85.1, 85.8, 85.9, 95.8, 98.0, 98.1, 106.5, 106.7, 177.1, 177.2.

[²H₃]-16,17-Dihydrorebaudioside A (major diastereomer), [²H₃]-16,17-dihydro-2: MS/MS (ESI⁻), *m/z* (%) 971.6 (25), 809.6 (100), 647.4 (30), 485.4 (30), 323.4 (30); ¹H NMR (500 MHz, pyridine-*d*₅), anomeric protons, δ 6.11 [d, 1H, ³J = 7.8 Hz, H-(C1a)], 4.97 [d, 1H, ³J = 7.9 Hz, H-(C1c)], 5.39 [d, 1H, ³J = 7.9 Hz, H-(C1d)], 5.25 [d, 1H, ³J = 7.8 Hz, H-(C1e)]; ¹³C NMR (125 MHz, pyridine-*d*₅), δ 15.1, 15.3, 19.1, 19.6, 20.1, 22.1, 22.5, 22.7, 28.1, 29.7, 38.2, 39.6, 39.6, 40.5, 40.7, 41.4, 42.3, 43.7, 43.8, 49.8, 54.1, 55.1, 57.2, 57.2, 61.8, 62.1, 62.1, 62.4, 62.5, 62.7, 70.7, 70.8, 71.3, 71.3, 71.4, 71.5, 73.6, 73.7, 74.9, 75.0, 76.2, 76.4, 77.0, 77.0, 77.8, 77.8, 78.2, 78.3, 78.3, 78.4, 78.8, 79.0, 79.1, 80.4, 81.2, 84.9, 85.8, 87.4, 87.5, 95.5, 98.0, 98.2, 104.3, 104.5, 104.7, 104.8, 176.9, 177.0.

[²H₄]-16,17-Dihydrorebaudioside B (major diastereomer), [²H₄]-16,17-dihydro-3: MS/MS (ESI⁻), *m/z* (%) 809.6 (100), 647.6 (25), 485.4 (20), 323.2 (30); ¹H NMR (500 MHz, pyridine-*d*₅), anomeric protons, δ 5.01 [overlapping signal, H-(C1c)], 5.45 [d, 1H, ³J = 7.9 Hz, H-(C1d)], 5.32 [d, 1H, ³J = 7.9 Hz, H-(C1e)]; ¹³C NMR (125 MHz, pyridine-*d*₅), δ 16.0, 16.1, 19.9, 20.3, 23.2, 29.3, 29.4, 35.1, 38.8, 39.8, 41.1, 41.2, 42.3, 42.6, 43.8, 43.9, 44.0, 44.7, 50.2, 54.6, 57.1, 62.5, 62.9, 63.0, 69.9, 70.0, 71.7, 71.8, 71.9, 75.2, 75.3, 76.4, 77.4, 78.2, 78.4, 78.5, 78.6, 78.7, 81.5, 85.3, 86.2, 88.0, 98.2, 104.8, 105.0, 180.3.

[²H₃]-16,17-Dihydrorebaudioside C (major diastereomer), [²H₃]-16,17-dihydro-4: MS/MS (ESI⁻), *m/z* (%) 955.6 (20), 793.4 (100), 631.4 (15), 485.4 (30), 323.2 (15); ¹H NMR (500 MHz, pyridine-*d*₅), anomeric protons, δ 5.91 [d, 1H, ³J = 8.5 Hz, H-(C1a)], 4.91 [d, 1H, ³J = 7.8 Hz, H-(C1c/C1e)], 6.48 [m, 1H, H-(C1d)], 5.05 [d, 1H, ³J = 7.8 Hz, H-(C1c/C1e)]; ¹³C NMR (125 MHz, pyridine-*d*₅), δ 15.1, 15.6, 18.7, 19.0, 19.9, 20.3, 22.3, 22.7, 28.0, 28.3, 32.6, 35.3, 38.5, 38.7, 39.9, 40.0, 40.8, 40.9, 42.4, 42.5, 42.6, 42.7, 42.9, 43.9, 44.0, 44.0, 54.3, 56.1, 57.5, 57.7, 62.0, 62.1, 62.4, 62.4, 62.6, 63.2, 69.5, 69.9, 70.0, 70.3, 71.0, 71.1, 71.4, 71.6, 71.8, 72.5, 72.5, 72.7, 74.0, 74.3, 74.5, 75.0, 75.2, 75.8, 77.3, 77.5, 78.0, 78.3, 78.6, 78.6, 78.8, 79.3, 79.3, 79.8, 85.6, 86.7, 89.9, 90.3, 95.3, 95.7, 96.4, 97.9, 101.6, 102.6, 104.5, 104.6, 177.2, 177.4.

[²H₄]-16,17-Dihydrorebaudioside (major diastereomer), [²H₄]-16,17-dihydro-8: MS/MS (ESI⁻), *m/z* (%) 647.4 (100), 485.4 (20), 323.2 (15); ¹H NMR (500 MHz, pyridine-*d*₅), anomeric protons, δ 5.07 [d, ³J = 7.7 Hz, H-(C1c), overlapping signal], 5.22 [d, ³J = 7.7 Hz, H-(C1d) overlapping signal]; ¹³C NMR (125 MHz, pyridine-*d*₅), δ 16.0, 16.0, 20.0, 20.3, 23.0, 23.2, 29.4, 31.2, 38.9, 39.8, 41.2, 42.1, 42.7, 43.5, 43.6, 43.7, 44.0, 44.1, 44.8, 54.6, 55.7, 57.2, 62.6, 62.7, 62.8, 71.5, 71.6, 71.6, 71.7, 77.0, 77.2, 77.7, 77.7, 77.8, 78.0, 78.1, 78.1, 78.6, 78.7, 84.1, 84.5, 85.2, 86.0, 97.7, 97.8, 106.3, 106.6, 180.6.

[²H₄]-16,17-Dihydrorebaudioside (major diastereomer), [²H₄]-16,17-dihydro-9: MS/MS (ESI⁻), *m/z* (%) 647.4 (15), 485.4 (100), 323.2 (10); ¹H NMR (500 MHz, pyridine-*d*₅), anomeric protons, δ 6.15 [d, ³J = 7.8 Hz, H-(C1a), overlapping signal], 5.01 [d, ³J = 7.8 Hz, H-(C1c), overlapping signal]; ¹³C NMR (125 MHz, pyridine-*d*₅), δ [ppm]: δ 15.5, 15.7, 19.4, 19.5, 20.0, 20.4, 22.4, 22.7, 28.3, 28.4, 30.5, 36.1, 38.5, 39.8, 39.9, 40.8, 40.9, 41.1, 42.6, 42.7, 43.5, 43.6, 43.6, 44.0, 44.1, 44.7, 54.4, 55.5, 57.5, 57.5, 62.1, 62.1, 63.2, 63.4, 71.1, 71.1, 72.5, 72.8, 74.0, 75.4, 75.5, 77.8, 78.1, 78.9, 79.2, 79.3, 79.4, 85.2, 85.6, 95.8, 95.9, 99.4, 100.0, 177.0, 177.2.

[²H₂]-16,17-Dihydrodulcoside A (major diastereomer), [²H₂]-16,17-dihydro-10: MS/MS (ESI⁻), *m/z* (%) 794.4 (10), 632.4 (100), 486.4 (25), 324.2 (5); ¹H NMR (500 MHz, pyridine-*d*₅), anomeric protons, δ 5.94 [d, 1H, ³J = 8.5 Hz, H-(C1a)], 4.95, overlapping signal, H-(C1c)], 6.29 [m, 1H, H-(C1d)]; ¹³C NMR (125 MHz, pyridine-*d*₅), δ 15.1, 15.5, 18.7, 18.9, 19.4, 19.5, 19.9, 20.3, 22.3, 22.7, 28.0, 28.3, 32.5, 35.5, 38.5, 38.7, 39.9, 39.9, 40.8, 40.9, 42.5, 42.6, 42.9, 43.0, 43.9, 44.0, 54.3, 56.1, 57.5, 57.7, 62.0, 62.1, 62.9, 63.5, 69.4, 69.9, 70.3, 71.0, 71.1, 72.3, 72.3, 72.6, 72.6, 72.7, 73.3, 74.0, 74.2, 74.3, 74.3, 76.7, 77.5, 78.0, 78.6, 78.8, 79.3, 79.7, 80.1, 80.2, 85.4, 86.3, 95.7, 96.2, 98.2, 101.6, 102.7, 177.2, 177.3.

Stability of [²H₃₋₅]-16,17-Dihydrosteviol Glycosides in Aqueous Solution. An aliquot of a freshly prepared solution of [²H₃₋₅]-16,17-dihydrosteviol glycosides (26.5 μmol/L [²H₄]-16,17-dihydro-1, 22.8 μmol/L [²H₃]-16,17-dihydro-2, 30.8 μmol/L

[²H₄]-16,17-dihydro-3, 28.0 μmol/L [²H₃]-16,17-dihydro-4, 34.8 μmol/L [²H₄]-16,17-dihydro-8, 40.6 μmol/L [²H₄]-16,17-dihydro-9, 35.5 μmol/L [²H₃]-16,17-dihydro-10) in acetonitrile/water (80:20, v/v) was analyzed by UPLC-TOF/MS before and after storage for 2 days at 20 °C and for 14 weeks at 6 °C, respectively.

Quantitation of Steviol Glycosides. Sample Workup. A portion (10 mg) of finely powdered, dried stevia leaves was placed in a volumetric flask (50 mL), and aliquots (600 μL) of a stock solution of the corresponding internal standards (50 μmol/L [²H₄]-16,17-dihydro-1), 50 μmol/L [²H₃]-16,17-dihydro-2, 25 μmol/L [²H₄]-16,17-dihydro-3, 25 μmol/L [²H₃]-16,17-dihydro-4, 50 μmol/L ([²H₄]-16,17-dihydro-8, 50 μmol/L [²H₄]-16,17-dihydro-9, 50 μmol/L [²H₃]-16,17-dihydro-10) in acetonitrile/water (80:20, v/v) and acetonitrile/water (80:20, v/v; 40 mL) were added. After ultrasonication for 40 min at room temperature, the mixture was made up to 50 mL with acetonitrile/water (80:20, v/v), equilibrated for 30 min, and centrifuged (13000 rpm) at 20 °C, and, after appropriate dilution with acetonitrile/water (80:20, v/v), the supernatant was analyzed by means of LC-MS/MS.

Calibration. The purity of each analyte was determined prior to analysis by quantitative NMR spectroscopy. The analytes were mixed with the [²H₃₋₅]-16,17-dihydrosteviol glycosides used as internal standards in molar concentration ratios from 10 to 0.1 (10:1; 3:1; 1:1; 0.3:1; 0.1:1 for 1–4, 8–10) and from 5.0 to 0.05 (5:1; 1.5:1; 0.5:1; 0.15:1; 0.05:1 for 5–7) in acetonitrile/water (80:20, v/v). Calibration was performed by plotting the ratios of peak areas of analyte/internal standard versus the concentration ratios of analyte/internal standard. The following calibration functions were obtained by linear regression: 1/[²H₄]-16,17-dihydro-1 (*y* = 0.395*x* - 0.038; *R*² = 0.9990); 2/[²H₃]-16,17-dihydro-2 (*y* = 1.114*x* + 0.046; *R*² = 0.9998); 3/[²H₄]-16,17-dihydro-3 (*y* = 2.402*x* - 0.045; *R*² = 1.0000); 4/[²H₃]-16,17-dihydro-4 (*y* = 0.561*x* - 0.025; *R*² = 0.9998); 5/[²H₄]-16,17-dihydro-1 (*y* = 3.468*x* - 0.050; *R*² = 0.9999); 6/[²H₄]-16,17-dihydro-1 (*y* = 0.828*x* + 0.002; *R*² = 0.9984); 7/[²H₄]-16,17-dihydro-1 (*y* = 0.586*x* - 0.015; *R*² = 0.9997); 8/[²H₄]-16,17-dihydro-8 (*y* = 3.184*x* - 0.165; *R*² = 0.9998); 9/[²H₄]-16,17-dihydro-9 (*y* = 1.203*x* - 0.098; *R*² = 0.9998); 10/[²H₃]-16,17-dihydro-1 (*y* = 10.794*x* - 0.506; *R*² = 0.9997).

Recovery. A portion (10 mg) of dried, ground leaves of *R. suavissimus* was placed in a volumetric flask (50 mL) and spiked with a stock solution (600 μL) of the purified steviol glycosides 1–8 and 10 at three concentration levels. After the addition of acetonitrile/water (80:20, v/v; 40 mL), 800 μL of the corresponding [²H₃₋₅]-16,17-dihydrosteviol glycosides (50 μmol/L [²H₄]-16,17-dihydro-1, 50 μmol/L [²H₃]-16,17-dihydro-2, 25 μmol/L [²H₄]-16,17-dihydro-3, 25 μmol/L [²H₃]-16,17-dihydro-4, 50 μmol/L [²H₄]-16,17-dihydro-8, 50 μmol/L [²H₄]-16,17-dihydro-9, and 50 μmol/L [²H₃]-16,17-dihydro-10) in acetonitrile/water (80:20, v/v) were added as the internal standards, the mixtures were ultrasonicated for 40 min at room temperature, made up to 50 mL with acetonitrile/water (80:20, v/v), and centrifuged for 10 min (13000 rpm) at 20 °C, and, after appropriate dilution with acetonitrile/water (80:20, v/v), the supernatant was analyzed by means of LC-MS/MS to give mean recovery rates of 101% (1), 110% (2), 96% (3), 110% (4), 99% (5), 100% (6), 96% (7), 93% (8), and 101% (10).

In addition, a stevioside-free zero-calorie carbonated beverage was ultrasonicated, and 20 mL was spiked with defined amounts of the purified steviol glycosides 1–10 at three concentration levels, followed by the addition of 700 μL of the corresponding internal standards (50 μmol/L [²H₄]-16,17-dihydro-1, 50 μmol/L [²H₃]-16,17-dihydro-2, 25 μmol/L [²H₄]-16,17-dihydro-3, 25 μmol/L [²H₃]-16,17-dihydro-4, 50 μmol/L [²H₄]-16,17-dihydro-8, 50 μmol/L [²H₄]-16,17-dihydro-9, 50 μmol/L [²H₃]-16,17-dihydro-10) in acetonitrile/water (80:20, v/v). After adjustment of the pH to 5.0 and dilution with acetonitrile to a final acetonitrile content of 80%, the samples were analyzed by means of LC-MS/MS to give mean recovery rates of 102% (1), 98% (2), 101% (3), 96% (4), 90% (5), 102% (6), 97% (7), 108% (8), 103% (9), and 106% (10).

Medium-Pressure Liquid Chromatography (MPLC). MPLC was performed on a preparative Sepacore system (Büchi, Flawil, Switzerland) consisting of two pump modules (C-605), a control unit (C-620), a fraction collector (C-660), a manual injection port equipped with a 20 mL loop, and an UV detector (C-635). Monitoring the effluent at 210 nm, chromatography was performed

on a 150 × 40 mm i.d. polypropylene cartridge (Büchi) filled with Septra NH₂, 50 μm, 65 Å, bulk material (Phenomenex, Aschaffenburg, Germany).

High-Performance Liquid Chromatography (HPLC). Preparative HPLC was performed on a 250 × 21.2 mm i.d. 5 μm RP-18 column (ThermoHypersil, Kleinostheim, Germany) using a HPLC system (Jasco, Gross-Umstadt, Germany) consisting of two PU-2087 pumps (flow rate = 21 mL/min), a 7725i injection valve (Rheodyne, Bensheim, Germany), a DG-2080-53 type solvent degasser (Uniflows Co., Tokio, Japan), and an MD-2010 Plus detector.

High-Performance Liquid Chromatography–Mass Spectrometry (HPLC-MS/MS). For HPLC-ESI-MS/MS analysis, a Dionex UltiMate 3000 HPLC-System (Dionex, Idstein, Germany), consisting of a binary pump (HPG-3400SD), a degasser (SRD-3400), an autosampler (WSP-3000TSL), and a thermostable column compartment (TCC-3000SD), was connected to an API 4000 QTRAP mass spectrometer (AB Sciex Instruments, Darmstadt, Germany) operating in the negative electrospray ionization (ESI) mode. Data acquisition was performed with Analyst 1.5 software (AB Sciex, Darmstadt, Germany). After sample injection (10 μL), chromatography was performed on a 150 × 2.0 mm i.d., 3 μm, TSKgel NH₂-100 column (Tosoh Bioscience, Stuttgart, Germany) using a binary gradient (flow rate = 200 μL/min) comprising 5 mM ammonium acetate and 0.05% formic acid in acetonitrile/water (95:5, v/v) as solvent A and 5 mM ammonium acetate and 0.05% formic acid in acetonitrile/water (10:90, v/v) as solvent B: starting with solvent A/B (95:5; v/v), solvent B was increased to 12% within 5 min, then to 20% within 7 min, and to 50% within an additional 8 min, thereafter maintaining for 2 min. After the separation, the column was flushed back to starting conditions within 1 min, followed by a column equilibration period of 10 min under starting conditions (A/B, 95:5, v/v). For HPLC-MS/MS, the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode running in the ESI mode with an ion spray voltage of −4500 V. Zero grade air served as nebulizer gas (60 psi) and as turbo gas (400 °C) for solvent drying (50 psi). Nitrogen served as curtain (20 psi) and collision gas (4.5 × 10^{−5} Torr). Each of the selected mass transitions was monitored for a duration of 10 ms and, together with the MS parameters, are compiled in Table 1.

Table 1. Optimized Mass Spectrometric Parameters for the Quantitative Analysis of Steviol Glycosides 1–10

compound ^a	Q1 mass (Da)	Q3 mass (Da)	DP ^b (V)	CE ^b (V)	CXP ^c (V)
1	803.4	641.3	−150	−42	−9
[² H ₄]-16,17-dihydro-1	809.4	647.3	−140	−50	−31
2	966.5	804.5	−160	−46	−13
[² H ₃]-16,17-dihydro-2	971.4	809.4	−160	−52	−41
3	803.4	641.2	−225	−64	−31
[² H ₄]-16,17-dihydro-3	809.5	647.5	−200	−66	−21
4	950.5	788.5	−175	−52	−13
[² H ₃]-16,17-dihydro-4	955.6	793.6	−160	−48	−51
5	1127.5	803.5	−180	−68	−25
6	965.5	641.5	−235	−70	−19
7	935.5	773.2	−175	−46	−43
8	641.4	479.1	−220	−58	−21
[² H ₄]-16,17-dihydro-8	647.4	485.4	−195	−60	−15
9	641.4	479.3	−160	−38	−7
[² H ₄]-16,17-dihydro-9	647.3	485.4	−135	−48	−17
10	787.4	625.3	−140	−30	−9
[² H ₃]-16,17-dihydro-10	794.4	632.4	−140	−30	−29

^aNumbering refers to Figures 1 and 4. ^bDeclustering potential, collision energy. ^cCell exit potential.

UPLC-Time of Flight Mass Spectrometry (UPLC-TOF/MS). Exact masses of the isolated compounds were measured on a Waters Synapt G2 HDMS mass spectrometer (Waters, Manchester, UK) coupled to an Acquity UPLC core system (Waters) consisting of a binary solvent manager, sample manager, and column oven. Analytes were dissolved in methanol, and aliquots (1–5 μL) were injected into the UPLC-TOF/MS

system equipped with a 2 × 150 mm, 1.7 μm, BEH C18 column (Waters). Chromatography was performed at a flow rate of 0.3 mL/min at elevated temperature (40 °C) using the following solvent gradient: starting with a mixture (5:95, A/B; v/v) of acetonitrile (A) and aqueous formic acid (B; 0.1%, pH 2.5), the acetonitrile content was increased to 95% within 3 min and, finally, maintained at 95% for 1 min.

A solution of sodium formate (0.5 mM) in 2-propanol/water (9:1, v/v) was used for calibration of the mass spectrometer. Measurements were performed using negative ESI, and the resolution mode consisted of the following parameters: capillary voltage, −3.0 kV; source temperature, 150 °C; desolvation temperature, 450 °C; cone gas, 30 L/h; and desolvation gas, 850 L/h. Data processing was performed by using MassLynx 4.1 software (Waters). All data were lock mass (leucine enkephalin) corrected: [M + H]⁺ (*m/z* 556.2771) and [M − H][−] (*m/z* 554.2615).

Nuclear Magnetic Resonance Spectroscopy (NMR). The ¹H, ¹³C, COSY, DEPT, HSQC, and HMBC spectroscopic experiments were performed on a 400 MHz Avance III and a 500 MHz Avance III NMR spectrometer, respectively, both from Bruker (Rheinstetten, Germany). Steviol glycosides were dissolved in pyridine-*d*₅. Data processing was performed using Topspin version 2.1 or 3.1 (Bruker); the individual data interpretation was done using MestReNova 5.1.0-2940 (Mestrelab Research S.L., Santiago de Compostela, Spain).

Quantitative Nuclear Magnetic Resonance Spectroscopy (qNMR). The ¹H NMR spectra were acquired at 300 K using a Avance III 500 MHz spectrometer (BrukerBiospin, Rheinstetten, Germany) equipped with a Bruker 5 mm TCI probe. Bruker Topspin 2.1 was used for data acquisition and processing. Solutions (600 μL) of the steviol glycosides in pyridine-*d*₅ (Euriso-Top, Gif sur Yvette Cedex, France) were analyzed in 5 mm × 7 in. NMR tubes (Bruker, Faellanden, Switzerland). The NMR probe was tuned and matched with the sample in place, and ¹H NMR spectra were acquired using Bruker zg pulse programs. To ensure good-quality spectra, the NMR probe was manually tuned and matched to 50 Ω resistive impedance to minimize RF reflection, with the sample in place. After automatic optimization of the lock phase, each sample was shimmed (*z*¹ − *z*⁵, *xyz*, *z*¹ − *z*⁵), and the 90° pulse width was determined individually for each sample using the AU program “pulsecal sn” (Bruker Topspin 2.1). All spectra were recorded in the *baseopt* mode acquiring a maximum of 8 scans of 64K complex data points (corresponding to an acquisition time of 3.98 s at a sweep width of 8223 Hz) and a relaxation delay of 40 s. The FID was multiplied with a 0.3 Hz exponential line-broadening factor and zero-filled prior to Fourier transformation. If the automatic phase correction use was not proper enough, a careful manual zero- and first-order phase correction was performed. Baseline correction was performed automatically using the command *abs*. Integration was done manually and, whenever required, adjustment of the integrals was performed by the software functions SLOPE and BIAS. ¹³C satellites were not included in integration. The PULCON (pulse length based concentration determination) was used for quantitation. Benzoic acid (NIST 350b, purity = 99.9978 ± 0.0044%), dissolved in D₂O, served as reference compound by determining the area of known concentrations by applying the *quant_zgcal* experiment. With the same experimental settings (*quant_zg*), the area of a signal, referring to a known number of protons (anomeric proton), was determined to calculate the concentration of the analyte.

RESULTS AND DISCUSSION

To develop a highly selective and sensitive LC-MS/MS method for the accurate quantitation of sweet-tasting steviol glycosides in stevia products, sweetened foods and beverages, first, reference materials of the individual steviol glycosides 1–10 (Figure 1) were needed to be prepared in high purity.

Preparation of Reference Material of Steviol Glycosides. To obtain reference compounds as analytical standards, steviol glycosides 1, 2, 4–7, 9, and 10 were isolated from commercially available stevia extracts, namely, a rebaudioside A-rich extract A (Figure 2A) and a stevioside-rich extract B (Figure 2B,C) by means of MPLC using an amino-bulk material as stationary phase, followed by rechromatography by RP-HPLC.

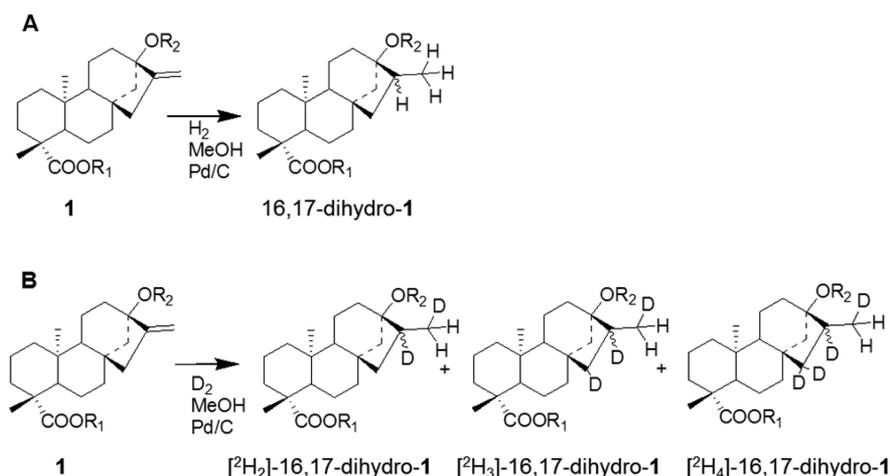


Figure 4. Hydrogenation (A) and deuteration (B) of steviol glycosides using palladium on charcoal to afford 16,17-dihydrosteviol glycosides and $[\text{2H}_{2-4}]$ -16,17-dihydrosteviol glycosides, respectively.

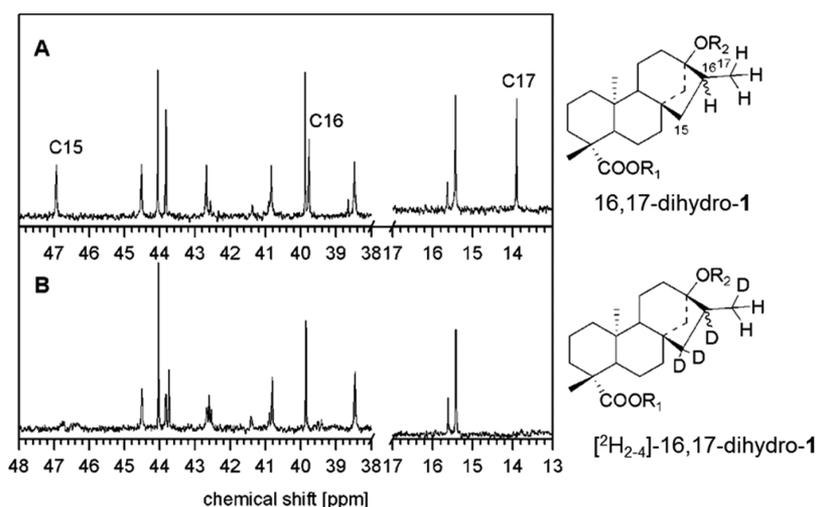


Figure 5. Section of ^{13}C NMR spectra (13–48 ppm) of (A) 16,17-dihydrostevioside (16,17-dihydro-**1**) and (B) $[\text{2H}_{2-4}]$ -16,17-dihydrostevioside ($[\text{2H}_{2-4}]$ -16,17-dihydro-**1**).

In addition, compounds **3** and **8** were obtained by alkaline hydrolysis of **2** and **1**, respectively. The structures of the steviol glycosides **1–10** were verified by means of LC-MS/MS, UPLC-TOF/MS, and 1D- and 2D-NMR, in particular, by using the chemical shift differences of the anomeric protons in steviol glycosides **1–10** (Figure 3) and by comparison with literature data.^{4–8,11,31} Quantitative NMR spectroscopy revealed a purity of >98% for each steviol glycoside.

Synthesis of $[\text{2H}_{3-5}]$ -16,17-Dihydrosteviol Glycosides as Internal Standards for LC-MS/MS Analysis. To obtain internal standards for mass spectrometric analysis of steviol glycosides, the exocyclic double bond at carbon atom C(16) of steviol glycosides was hydrogenated with palladium on activated charcoal under a hydrogen atmosphere. A similar synthesis has been described previously.³² For example, hydrogenation of stevioside, **1**, led to the diastereomeric mixture of 16,17-dihydrostevioside (16,17-dihydro-**1**) (Figure 4A) showing an expected increase of the molecular weight of 2 Da. However, as the natural isotopic pattern of **1** revealed the $[\text{M} + 2 - \text{H}]^-$ ion to account for a frequency of 11.9% (data not shown), 16,17-dihydro-**1** was not considered a suitable internal standard due to a significant spectral overlap with the analyte **1**.

To overcome this challenge by increasing the molecular weight difference between analyte and internal standard, the steviol glycosides **1–4** and **8–10** were deuterated to afford diastereomeric mixtures of $[\text{2H}_{3-5}]$ -16,17-dihydrosteviol glycosides (Figure 4B). As displayed in Figure 5 for deuterated stevioside ($[\text{2H}_4]$ -16,17-dihydro-**1**), deuteration of the target molecules was found to take place at C(15), C(16), and C(17) as indicated in the ^{13}C NMR spectrum by the much lower signal intensity of deuterated carbon atoms when compared to hydrogenated carbons.^{33,34} Internal standards were obtained as mixtures with different contents of deuterium. As LC-TOF/MS analysis revealed no spectral overlap between the analytes and the candidate internal standards, as exemplified for $[\text{2H}_4]$ -16,17-dihydro-**1** (see Supporting Information Figure S1), and storage (20 °C/2 days, 4 °C/14 weeks) of freshly prepared aqueous solutions of $[\text{2H}_{3-5}]$ -16,17-dihydrosteviol glycosides did not indicate any deuterium/proton re-exchange, the $[\text{2H}_{3-5}]$ -16,17-dihydrosteviol glycosides were considered candidate internal standards for quantitation of steviol glycosides by means of LC-MS/MS.

LC-MS/MS Quantitation of Steviol Glycosides **1–10.** To investigate the suitability of $[\text{2H}_{3-5}]$ -16,17-dihydrosteviol glycosides as candidate internal standards, a robust high-throughput

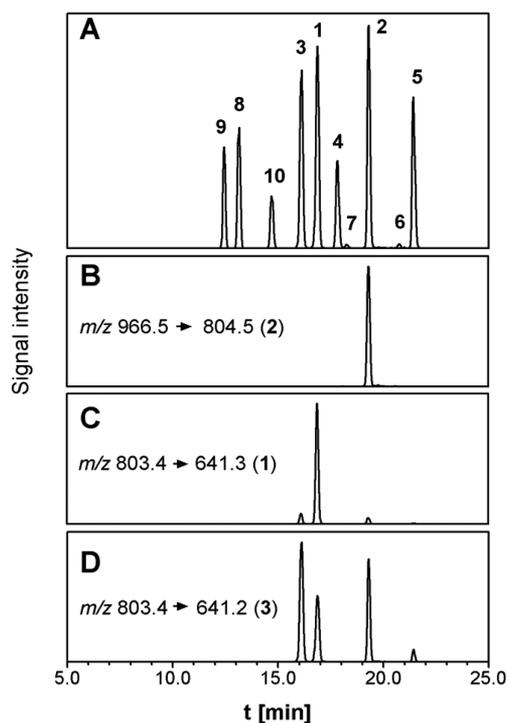


Figure 6. HILIC separation of steviol glycosides using a TSKgel NH₂-100 stationary phase (A) and mass traces of the steviol glycosides 2 (B), 1 (C), and 3 (D).

method needed to be developed and validated for the accurate quantitative analysis of steviol glycosides 1–10 in food samples. First, the analytes including the isobaric pairs 8/9, 1/3, and 6/2 were successfully separated by means of hydrophilic liquid interaction chromatography (HILIC) using a TSKgel NH₂-100 stationary phase (Figure 6A). To analyze the target molecules with high selectivity by using tandem mass spectrometry operating in the MRM mode, solutions of the analytes (1–10) as well as the deuterated internal standards were individually infused into the ESI[−] source of the MS/MS system with a

constant flow by means of a syringe pump to optimize ionization parameters and collision-induced fragmentation (Table 1). By means of the excellent chromatographic separation of the individual steviol glycosides, the problems of “in source fragmentation” of the glycosides were overcome, as even the isobaric compounds 1 and 3, 2 and 6, and 8 and 9 were separated. The need to separate these isobaric compounds for the quantitation of the individual steviol glycosides 1–10 is illustrated for the mass transitions of 2, 1, and 3 (Figure 6B–D).

The [²H_{3–5}]-16,17-dihydrosteviol glycosides synthesized eluted slightly before their corresponding steviol glycosides (Figure 7). Typically, the most intensive mass transition of the analyte and that of the most intense isotopologue of each internal standard were used for quantitation of the target analytes (Table 1). The mixtures with different contents of deuterium were used as internal standards. For quantitation of compounds 1–10, calibration functions with correlation coefficients of >0.998 were determined by adding the peak areas of both diastereomers of each internal standard (Table 1). Calibration functions refer to the most intense isotopologue of each internal standard. To check the accuracy of the developed method, recovery experiments were performed with dried leaves of *R. suavissimus*, which had been confirmed in preliminary LC-MS/MS experiments to contain no steviol glycoside other than rubusoside (9) (data not shown). To achieve this, the dried, ground leaves were spiked with defined amounts of the steviol glycosides 1–8 and 10 and the [²H_{3–5}]-16,17-dihydrosteviol glycosides in three different concentration levels, extracted with acetonitrile/water (80:20, v/v), and, then, analyzed by means of LC-MS/MS. The mean recovery rates were between 93% (7) and 110% (2, 4). As the lower limits of calibration, the signal-to-noise (S/N) ratio was determined for each steviol glycoside in the least concentrated calibration solution.³⁵ By injection of 10 μL, we found the following parameters: S/N of 88 (1; 34 nmol/L), S/N of 28 (2; 42 nmol/L), S/N of 19 (3; 15 nmol/L), S/N of 46 (4; 20 nmol/L), S/N of 1090 (5; 20 nmol/L), S/N of 6 (6; 1.2 nmol/L), S/N of 460 (7; 13 nmol/L), S/N of 494 (8; 33 nmol/L), S/N of 91 (9; 41 nmol/L), and S/N of 290 (10; 31 nmol/L).

Table 2. Concentration of Steviol Glycosides 1–10 in Dried Leaves of *S. rebaudiana* (SL1–SL5) and *R. suavissimus* (RL1)

sample	concn ^a (mg/g dried leaves) ± sd ^b									
	1	2	3	4	5	6	7	8	9	10
SL1	55.6 1.3	60.1 6.2	0.9 <0.1	7.6 0.8	2.3 0.2	1.3 0.2	0.3 <0.1	0.4 0.1	0.3 <0.1	0.2 <0.1
SL2	120.3 4.6	57.9 0.8	0.6 <0.1	9.4 0.6	4.5 <0.1	2.6 0.4	0.3 <0.1	0.5 <0.1	0.6 <0.1	0.9 0.1
SL3	71.8 5.9	18.6 0.6	0.3 <0.1	4.9 <0.1	0.5 <0.1	6.7 0.3	1.0 0.1	0.4 <0.1	0.2 0.3	1.1 <0.1
SL4	60.8 3.9	14.7 0.9	0.1 <0.1	7.3 0.1	1.1 0.1	1.7 0.3	0.3 <0.1	nd 0.5	0.4 0.5	0.8 0.2
SL5	47.4 2.1	23.4 1.4	0.1 <0.1	5.9 1.1	0.4 <0.1	4.7 0.1	0.9 <0.1	0.1 <0.1	0.5 0.6	0.8 <0.1
RL1	nd	nd	nd	nd	nd	nd	nd	nd	22.1 0.3	nd

^aConcentrations are given as the mean of two samples and three analytical replicates. nd, not detectable. ^bNumbering refers to Figure 1.

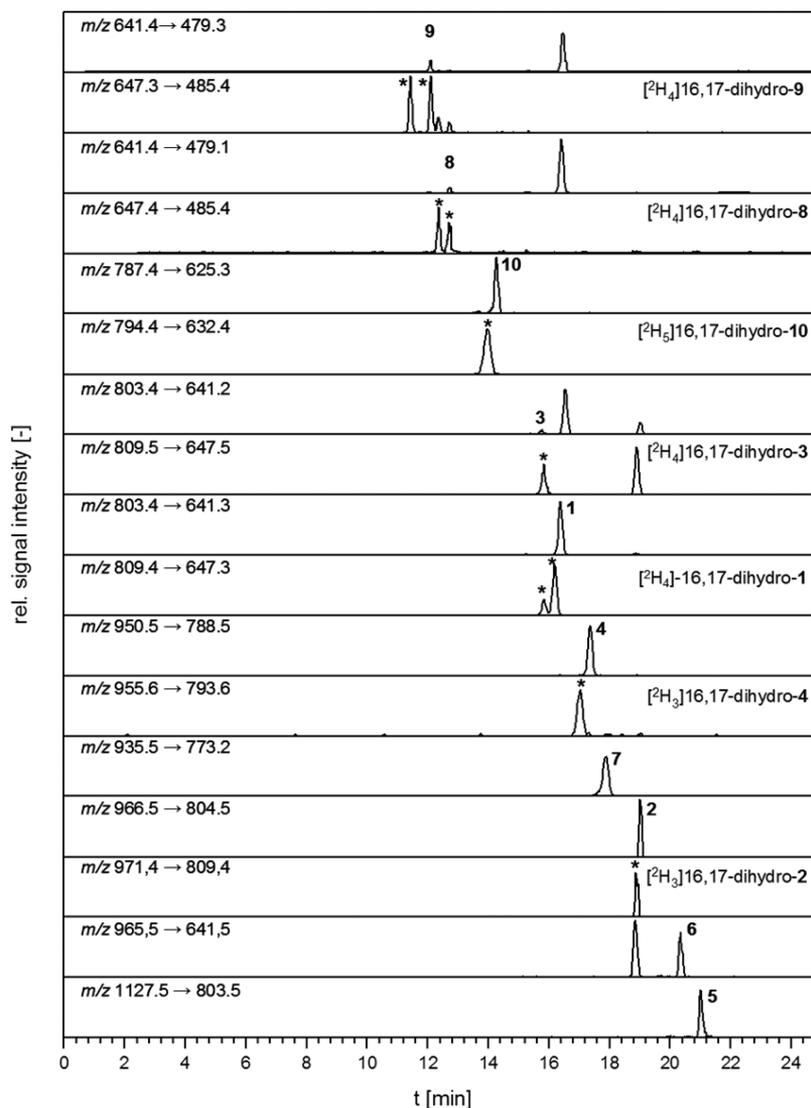


Figure 7. HILIC-MS/MS analysis of representative *Stevia* leaf sample showing mass traces of steviol glycosides 1–10 and the corresponding $[^2\text{H}_{2-5}]$ -16,17-dihydrosteviol glycosides (diastereomers marked with asterisks). Signal intensity of each mass transition is normalized.

Additional recovery experiments were performed in a commercial, stevia-free, low-calorie carbonated soft drink by spiking the beverage with steviol glycosides 1–10 and the corresponding internal standards in three different concentrations. Mean recovery rates ranging from 90% (5) to 108% (8) were found, thus demonstrating the developed LC-MS/MS method to be a robust and accurate tool for the quantitation of steviol glycosides in foods.

Quantitation of 1–10 in Dried Stevia Leaves. Using the developed HILIC-MS/MS method, steviol glycosides were quantitatively determined in different samples of dried leaves of *S. rebaudiana* and *R. suavissimus*, respectively (Table 2). Stevioside, 1, rebaudioside A, 2, and rebaudioside C, 4, were found as the most abundant steviol glycosides with concentrations of 4.7–12.0% (1), 1.5–6.0% (2), and 0.5–0.9% (4) of the dried leaf. These data are well in line with previous literature data reporting stevioside contents of $6 \pm 1.6\%$,¹⁵ 5–10%³⁶ and 4–14%,²⁸ and 9%,²³ respectively, and rebaudioside A contents between 2 and 4% of dry weight.^{15,23,28,36} The levels of dulcoside A, 10, were comparatively low (0.08% on average), even when compared to literature data ($0.5 \pm 0.4\%$).¹⁵ The leaves of *R. suavissimus* were found to contain rubusoside, 9, as the predominant glycoside with concentrations of 2.2%, which is

somewhat lower than the previously published levels of 5% determined by means of a HPLC-PDA method.³⁷

In conclusion, an accurate and robust method for the HILIC-MS/MS quantitation of sweet steviol glycosides 1–10 was developed using the corresponding $[^2\text{H}_{3-5}]$ -16,17-dihydrosteviol glycosides as suitable internal standards. This reliable quantitation tool can help to optimize breeding and postharvest downstream processing of stevia plants to produce preferentially sweet and least bitter tasting *Stevia* extracts.

■ ASSOCIATED CONTENT

Supporting Information

Tables S1–S6 and Figure S1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*(T.H.) Phone: +49 8161-71-2902. Fax: +49 8161-71-2949. E-mail: thomas.hofmann@tum.de

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Notes

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