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Identification and Quantification of Major Steviol Glycosides in *Stevia rebaudiana* Purified Extracts by ^{1}H NMR Spectroscopy

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Supporting Information

ABSTRACT: The use of ^{1}H NMR spectroscopy for the characterization of *Stevia rebaudiana* extracts is presented. The developed method allows qualitative and quantitative determination of the major steviol glycosides in purified extracts and fractions obtained from various stages of the purification process. Moreover, it proved to be a powerful tool to differentiate between glycosides which are naturally occurring in the stevia plant and artifacts formed in the course of the manufacturing process. Identification of steviol glycosides was achieved by the use of 2D NMR techniques, whereas quantification is based on qHNMR using anthracene as internal standard. The solvent mixture pyridine-d_5—DMSO-d_6 (6:1) enabled satisfactory separation of the signals to be integrated. Validation of the method was performed in terms of specificity, precision, accuracy, linearity, robustness, and stability. Quantitative results were compared to those obtained with the JECFA HPLC—UV method and were found to be in reasonable agreement. NMR analysis does not rely on the use of reference compounds and enables significantly faster analysis compared to HPLC—UV. Thus, NMR represents a feasible alternative to HPLC-based methods for the quality control of *Stevia rebaudiana* extracts.

KEYWORDS: *Stevia rebaudiana*, steviol glycosides, stevioside, rebaudioside A, qHNMR, quality control

INTRODUCTION

Steviol glycosides are *ent*-kaurene diterpene glycosides found in the leaves of *Stevia rebaudiana* Bertoni (Asteraceae), a plant native to Paraguay and Brazil. These constituents are responsible for the sweet taste of the leaves and extracts derived thereof. Purified *S. rebaudiana* extracts are used as natural noncaloric sweeteners and represent an alternative to sucrose and artificial sweeteners. Their regulatory status largely varies by country and determines the availability of *S. rebaudiana* purified extracts as food additive or dietary supplement. It was only very recently that a positive opinion on the safety of steviol glycosides was issued by the European Food Safety Authority (EFSA),[1] which will be of outmost importance in view of a future EU approval.

Known steviol glycosides include stevioside (St), rebaudioside A (RbA), rebaudioside B (RbB), rebaudioside C (RbC), rebaudioside D (RbD), rebaudioside F (RbF), dulcoside A (DuA), rubusoside (Rub), and steviolbioside (Stb) (Chart 1). The composition of purified Stevia extracts ultimately depends on the production approach employed by the manufacturer. Efforts to improve the sweetening properties of the final products resulted in the development of several manufacturing strategies.[2] While some of them have been directed toward the isolation of specific constituents, such as RbA, others have focused on the purification of the total glycosidic fraction, avoiding any modification of the native glycosidic composition while removing specific impurities.

Quality control procedures for Stevia sweeteners must be employed to ensure that standardization and safety requirements are met, as set by regulatory agencies. In addition, claims referring to consumer products as "natural extracts" or "natural sugar substitutes" must be substantiated as well. This is of critical importance, because consumers have specific expectations for products claimed as natural, which include the use of materials of natural origin, processed without the aid of methods that could potentially alter the naturally occurring substances. In this regard, quality control procedures should be able to detect the presence of chemicals such as residual solvents and artifacts formed during the manufacturing process that could modify the native glycosidic composition. Thus, the purification of steviol glycosides represents an excellent case study which combines the need for standardization, food safety, and the expectation of customers for natural products.

Quality assessment relies on the use of validated analytical methods. By far the most popular approach for the quantification of the individual steviol glycosides is LC in combination with UV or MS detection.[3−5] The recently revisited Joint FAO/WHO Expert Committee on Food Additives (JECFA) method[3] is based on RP-HPLC—UV and enables separation and quantification of nine steviol glycosides. This approach suffers from the intrinsic limitations of chromatography-based methods, namely, the need of standard compounds for analyte identification/quantification and relatively long analysis times. In addition, as column performance worsens over time, retention time drifts and insufficient separation may arise, which result in overall poor reproducibility.[6]

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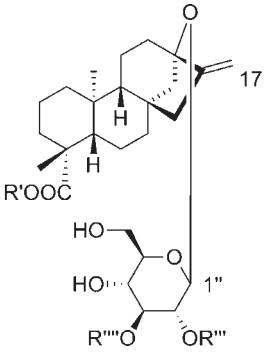
Chart 1. Structures of Stevioside, Rebaudioside A, Rebaudioside B, Rebaudioside C, Rebaudioside D, Rebaudioside F, Dulcoside A, Rubusoside, and Steviolbioside[a]



St	R'=R'''=β-D-Glc, R''''=H
RbA	R'=R'''=R''''=β-D-Glc
RbB	R'=H, R'''=R''''=β-D-Glc
RbC	R'=R''''=β-D-Glc, R'''=α-L-Rha
RbD	R'=β-D-Glc-β-D-Glc(2→1)
	R'''=R''''=β-D-Glc
RbF	R'=R''''=β-D-Glc, R'''=β-D-Xyl
DuA	R'=β-D-Glc, R'''=α-L-Rha, R''''=H
Rub	R'=β-D-Glc, R'''=R''''=H
Stb	R'=H, R'''=β-D-Glc, R''''=H

[a]St = stevioside; RbA = rebaudioside A; RbB = rebaudioside B; RbC = rebaudioside C; RbD = rebaudioside D; RbF = rebaudioside F; DuA = Dulcoside A; Rub = rubusoside; Stb = steviolbioside; Glc = glucose; Rha = rhamnose; Xyl = xylose.

The increasing popularity of quantitative ^{1}H NMR (qHNMR) in the field of natural products analysis is well documented.[7] Contrary to chromatography-based methods, qHNMR represents a completely different approach which is not dependent on analyte separation. This enables faster analysis characterized by excellent reproducibility and robustness.[8] Analyte identification is supported by 2D NMR techniques, and quantification does not rely on external calibration, thus eliminating the need for standard compounds. This work deals with the development and validation of a qHNMR method for the analysis of *S. rebaudiana* purified extracts and fractions obtained from various stages of the manufacturing process. A critical comparison of NMR and HPLC−UV (JECFA method[3]) for the characterization of the extracts under investigation is presented herein.

MATERIALS AND METHODS

Chemicals and General Procedures. MeCN for HPLC applications was of gradient grade, and HPLC grade water was obtained using an Arium 611UV (Sartorius Stedim Biotech, Göttingen, Germany) water purification system. Technical grade solvents were distilled before use. Silica gel (VWR, Darmstadt, Germany) was used as stationary phase for CC. TLC was performed on silica gel 60 F_{254} plates (VWR, Darmstadt, Germany) using $CHCl_3$−MeOH−H_2O (60:40:8; v/v/v) as mobile phase. Detection was performed with H_2SO_4 (5% v/v methanolic solution). Residual water content was determined using a Perkin-Elmer (Wellesley, MA) TGA 7 thermogravimetric analyzer (heating rate 10 K/min). Liquid extracts were freeze-dried using a Thermo Scientific Heto PowerDry PL6000 freeze-dryer.

Samples and Reference Compounds. Purified *S. rebaudiana* extracts (SR-1 to SR-8) and liquid extracts from the different stages of the manufacturing process (SR-9 to SR-15) were obtained from Prodalysa LTDA. St (declared purity ≥98%), was purchased from Sigma Aldrich. RbA (95.0% purity by NMR against anthracene; residual water content 4.2% by thermogravimetry) was purchased from different suppliers. The amount of RbA used for recovery and linearity experiments was corrected using its purity as determined by NMR. Anthracene was purchased from Sigma Aldrich (declared purity >99%).

Isolation of RbB from Sample SR-7. Sample SR-7 (507 mg) was fractionated by CC over silica gel (300 g, 90 × 3.5 cm) using $CHCl_3$−MeOH−H_2O (60:40:8; v/v/v) as mobile phase (ca. 1.5 mL/min). Fractions were collected every three minutes and monitored by TLC. Fractions 137−150 were combined, and the resulting solution was evaporated under reduced pressure to yield 23 mg of RbB as determined by NMR and HPLC−MS investigations.

NMR. NMR spectra were acquired at 300 K with a Bruker (Bruker Biospin, Rheinstetten, Germany) Avance II 600 spectrometer equipped with a Bruker 5 mm TXI probehead with Z-gradient, using pyridine-d_5 (99.50%), DMSO-d_6 (99.90%, containing 0.03% TMS), deuterium oxide (99.90%), methanol-d_4 (99.8%), and acetonitrile-d_3 (99.80%), all purchased from Euriso-Top (Saint-Aubin, France). Data acquisition and processing were done with Bruker Topspin 2.1. ^{1}H NMR spectra were acquired using the Bruker *zg0* or *zg0pr* pulse programs using the following settings: relaxation delay (d_1) = 9 s, flip angle = 45°, acquisition time (AQ) = 2.66 s, FID data points = 64K, spectral width = 20 ppm, number of scans = 32. For experiments using presaturation the transmitter offset was manually set in order to achieve optimal suppression of the residual water signal. The acquired FIDs were Fourier transformed to yield spectra with 64K data points. Manual phase correction and automatic polynomial baseline correction were always used. Chemical shift values were referenced to the residual solvent signals or to the TMS signal. Signal integration was performed without inclusion of ^{13}C satellites. Inversion−recovery experiments were performed using the Bruker *t1ir* pulse program, with standard acquisition parameters. T1 values were calculated using the T1 relaxation routine (Topspin 2.1). 2D COSY, HSQC, and HMBC NMR spectra were acquired using the Bruker pulse programs *cosygpqf*, *hsqcedetgp*, and *hmbcgplpndqf* respectively, with standard acquisition parameters. HSQC experiments for samples SR-1 and SR-8 were performed by employing a reduced chemical shift window (92−106 ppm) in order to obtain better resolution of adjacent cross peaks in the anomeric region.[9] The acquired 2D data was Fourier transformed and manually phase corrected.

NMR Method Validation. All NMR spectra for method validation were acquired using pyridine-d_5−DMSO-d_6 (6:1) containing anthracene as internal standard (1.5 mg/mL). Sample SR-1 was used for determination of precision, accuracy, and robustness. For these experiments, 10 mg of sample SR-1 was accurately weighed and dissolved in 700 μL of the NMR solvent. After vortexing for 1 min, 600 μL portions were transferred to the NMR tubes. Samples were stored in the dark until analysis, which was always completed within 48 h from the preparation of the solvent. For determination of intraday and interday precision, sample SR-1 was investigated on three consecutive days. Each day, five replicates were prepared and analyzed three times each. Repeatability was determined by comparing results from multiple spectra acquisition of the same sample. Results of precision and repeatability experiments were expressed as relative standard deviation. Recovery experiments for accuracy determination were performed by spiking sample SR-1 with known amounts of RbA, respectively at the

19%, 50%, and 91% levels. Recovery rates were expressed as percentages relative to the theoretical total amount of RbA in the samples. Robustness was investigated by deliberate variations of shim, flip angle, and phase. All robustness experiments were performed with the sample set used for determination of precision on day 3. To investigate shim robustness, the samples were first shimmed appropriately, and then the *Z* axis shim was modified until appreciable distortion of the signals was detected (see Supporting Information). Flip angle robustness was investigated by using a 50° flip angle instead of 45°. Variations of phase were performed after optimal manual phase correction, and consisted of modification of the first order phase of +1°, with the pivot fixed at 0 ppm (see Supporting Information). For determination of linearity, five solutions of RbA with concentrations ranging from 0.2 to 19.3 mg/mL were prepared and analyzed in duplicate. Two signals of RbA (δ 5.20 and 5.57) and that of anthracene (δ 8.11) were integrated, and calibration curves were constructed using the signal ratio. Limit of detection (LOD) and limit of quantification (LOQ), respectively defined as 3C/S/N and 10C/S/N,[10] were calculated for both signals of RbA (δ 5.20 and 5.57).

NMR Sample Preparation. Ten milligrams of samples SR-1 to SR-8 were accurately weighed, dissolved in 700 μL of the NMR solvent, and vortexed for 1 min. Liquid extracts (SR-9 to SR-15) were freeze-dried. Ten milligrams of the resulting powder was accurately weighed and dissolved in the NMR solvent. Sample SR-15 was insoluble in the NMR solvent and had to be dissolved in deuterium oxide. Samples SR-9 and SR-10 were centrifuged at 10000 rpm for 5 min to remove insoluble materials. 600 μL portions of the resulting solutions were transferred to the NMR tubes.

HPLC. HPLC analyses were carried out using an HP 1100 system (Agilent, Waldbronn, Germany) equipped with autosampler, DAD and column thermostat. Separations were performed on a Capcell Pak C18-MGII column (250 × 4.60 mm i.d., 5 μm) (Shiseido Co.Ltd., Tokyo, Japan) fitted with a Merck LiChrospher C18 (5 μm) guard column (Darmstadt, Germany). The mobile phase consisted of a mixture of MeCN−phosphate buffer (10 mM, pH = 2.6) (32:68 v/v, isocratic elution). The detection wavelength was 210 nm, and the thermostat was set at 40 °C. The injection volume was 5 μL; the flow rate was 1 mL/min. For quantitative analysis, samples and standard compounds (St and RbA) were accurately weighed and dissolved in MeCN−H_2O (30:70 v/v) to obtain a final concentration of about 2 mg/mL. Each sample was injected in triplicate. The amounts of individual steviol glycosides in the samples were calculated as reported in the JECFA monograph.[3] Values were expressed as percentages on the dried weight basis as determined by thermogravimetry. For LC−ESI-MS experiments, the HPLC system was coupled to a Bruker (Bruker Daltonics, Bremen, Germany) Esquire 3000^{plus} iontrap (split ratio 1:5), using MeCN−0.3% formic acid in H_2O (32:68 v/v, isocratic elution). The MS parameters were as follows: ESI positive mode; nebulizer gas, 40 psi; drying gas flow rate, 10.00 L/min; *m*/*z* range, 100−1500.

RESULTS AND DISCUSSION

Qualitative NMR Analysis. Preliminary investigations were performed in order to select the optimal solvent for NMR analysis of the steviol glycosides in purified *S. rebaudiana* extracts (SR-1 to SR-8). Solvents or solvent mixtures which enabled full dissolution of the samples included deuterium oxide, DMSO-d_6, methanol-d_4, acetonitrile-d_3−deuterium oxide (80:20), and pyridine-d_5. The obtained ^{1}H NMR spectra were characterized in each case by three distinct groups of signals, corresponding to the protons of the steviol glycosides backbone, nonanomeric protons of the sugar moieties, and anomeric protons together with the olefinic protons at C-17 (see Supporting Information). The latter spectral region represented the most convenient area for both

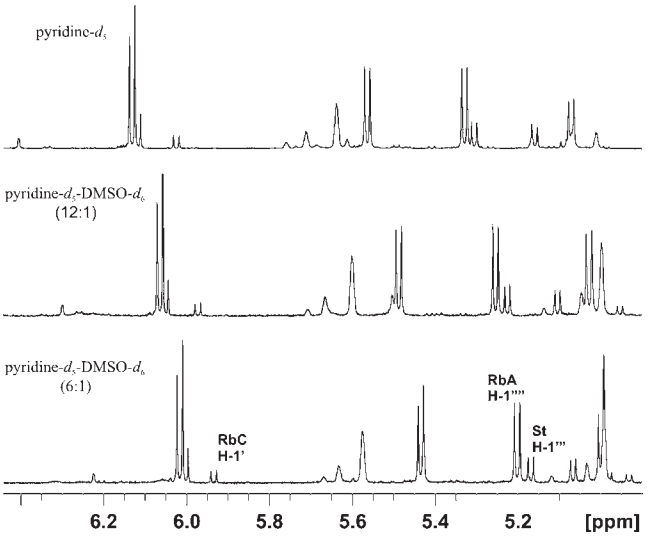


Figure 1. ^{1}H NMR spectra (anomeric region) of sample SR-1 recorded in different solvents.

Table 1. Diagnostic ^{13}C NMR Data for St, RbA, RbB, and RbC in Pyridine-d_5[a]

	St	RbA	RbB	RbC[b]
δ C-1′ lit.	95.6	95.6		96.1
δ C-1′ found	95.4	95.4		95.7
δ C-1″ lit.	97.7	97.9	97.8	97.7
δ C-1″ found	97.6	97.9	97.6	97.3
δ C-1‴ lit.	106.5	104.5	104.4	104.2
δ C-1‴ found	106.4	104.5	104.2	103.8
δ C-1⁗ lit.		104.5	104.6	102.1
δ C-1⁗ found		104.5	104.4	101.8
δ C-17 lit.	104.5	104.5	104.6	106.1
δ C-17 found	104.2	104.2	104.5	105.8

[a] Assignments based on 2D HSQC and HMBC spectra recorded at 150 MHz. Spectra were referenced to the residual solvent signal at δ 123.9. Literature values derived from refs 11,12. [b] Differences in chemical shift attributed to calibration mismatch.

qualitative and quantitative investigations. Thus, solvent was chosen based on the obtained signal dispersion in the anomeric spectral region. In this regard, pyridine-d_5 provided a nearly satisfactory separation of key anomeric resonances (Figure 1, first spectrum). Moreover, since most NMR literature data of steviol glycosides has been recorded using pyridine-d_5,[11,12] direct comparison of ^{13}C resonances for analyte identification purposes was possible. Acquisition of COSY, HSQC, and HMBC 2D NMR spectra enabled the observation of diagnostic NMR correlations which confirmed the identity of the steviol glycosides (Table 1). HSQC experiments were performed using a reduced chemical shift window (92−106 ppm) in order to obtain better resolution of adjacent cross peaks in the anomeric region (see Supporting Information). This approach enabled the identification of four steviol glycosides in the purified extracts, namely, St, RbA, RbB, and RbC. Samples SR-1 to SR-6 were mixtures of three major steviol glycosides (RbA, St, and RbC), whereas in samples SR-7 and SR-8 RbA represented the main steviol glycoside, accompanied by RbB as a minor compound (Figure 2). RbB might be a

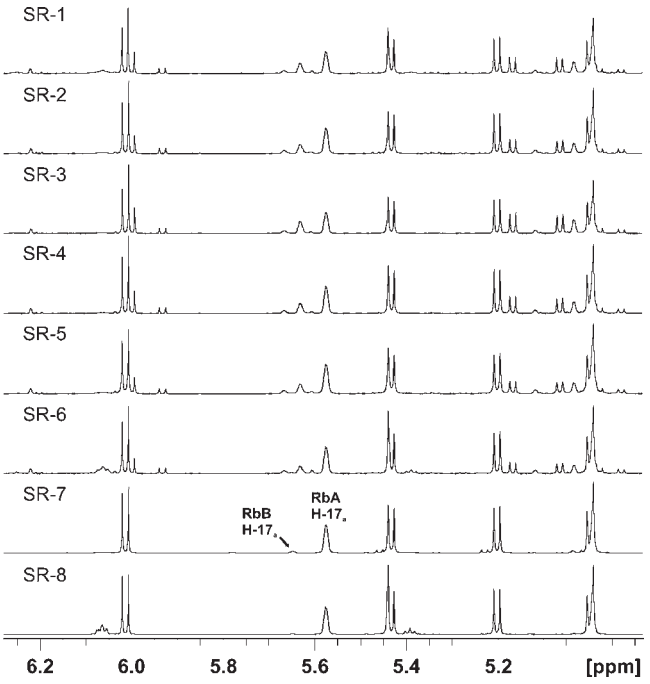


Figure 2. ^{1}H NMR spectra (anomeric region) of samples SR-1 to SR-8 acquired under the optimized conditions (pyridine-d_5—DMSO-d_6 (6:1), 600 MHz).

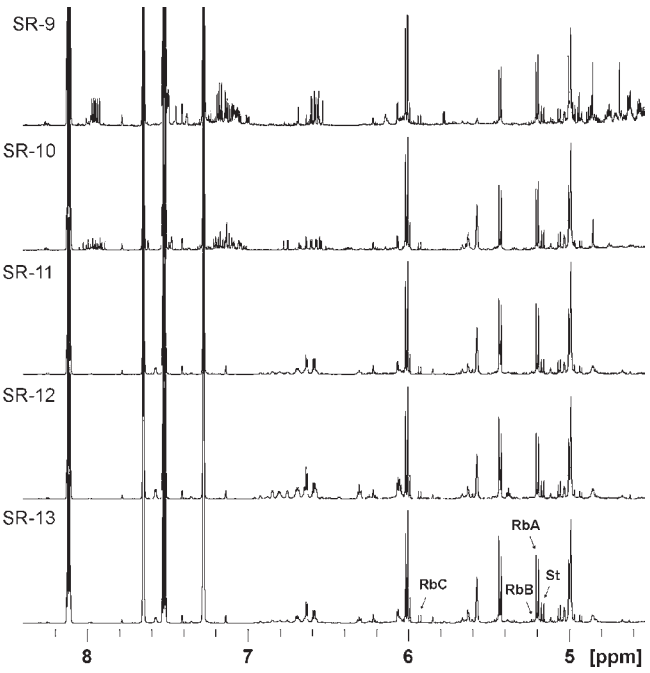


Figure 3. ^{1}H NMR spectra (4.6 to 8.2 ppm) of samples SR-9 to SR-13 acquired under the optimized conditions (pyridine-d_5—DMSO-d_6 (6:1), 600 MHz).

degradation product of RbA formed during manufacturing or storage, in agreement with the fact that the C-19 ester linkage appears to be the most heat-sensitive bond in RbA.[13] Identification of RbB was accomplished directly by acquisition of 2D NMR spectra (see Supporting Information) of sample SR-7. Correct identification was supported by structure elucidation of the pure compound isolated from sample SR-7. Its NMR spectroscopic data was in good agreement with published literature data for RbB.[12] Fractions obtained from various stages of the manufacturing process of *S. rebaudiana* purified extracts were also investigated (Figure 3), particularly in order to assess which steviol glycosides conserved their naturally occurring relative concentrations along the process. Sample SR-9 was the crude leaf extract obtained by extraction with water. Its ^{1}H NMR spectrum revealed the presence of RbA, St, and RbC as main steviol glycosides. It is interesting to observe that no RbB was detected in this sample. This is a relevant finding because it allows conclusions to be drawn concerning the origin of RbB in more purified fractions. The presence of this compound in such fractions could be regarded as the result of processing methods which alter the native glycosidic composition found in the starting plant material. Not unexpectedly, a series of additional resonances could be observed, especially in the sugar region (3.50−5.79 ppm) and in the low field region (6.50−8.00 ppm). Analysis of the HSQC spectrum showed the characteristic anomeric ^{1}H and ^{13}C resonances of glucose ($\delta_{H\alpha}$ 5.72, J = 3.7 Hz, $\delta_{H\beta}$ 5.14, J = 7.7 Hz, $\delta_{C\alpha}$ 93.1, $\delta_{C\beta}$ 97.8). Sample SR-10 was a fraction obtained from the crude extract, enriched in steviol glycosides but still containing additional constituents. Interestingly, glucose could not be detected in this sample, but only in the by-stream fraction SR-14 (see Supporting Information), which contained low-molecular weight constituents removed from the crude extract. Similarly, fraction SR-15 was a by-stream fraction derived from purification of the crude extract which was found to contain high-molecular weight constituents, as the very broad signals in the ^{1}H NMR spectrum suggested (see Supporting Information). Finally, in the last steps of purification (samples SR-11, SR-12, and SR-13, respectively collected at the start, middle, and end of final purification process), only RbA, St, and RbC as major constituents could be detected. The composition of these fractions was very similar to that of the final products SR-1 to SR-6. This is in agreement with the fact that only minor modifications in composition take place in the last purification steps, often only affecting the color of the extract. It is interesting to note that small amounts of RbB could be detected in samples SR-10, SR-11, and SR-13 as demonstrated by the resonance at δ 5.23 (d, 7.8 Hz). This was not the case for final products SR-1 to SR-6, and possibly indicates the use of milder conditions in the manufacturing of the latter samples. In addition, close inspection of the ^{1}H NMR spectra of both final products (SR-1 to SR-6) and samples from the last steps of purification (SR-11 to SR-13) revealed the presence of several minor unidentified resonances, which could arise from further glycosides or impurities in the extracts.

Quantitative NMR Analysis. The application of quantitative ^{1}H NMR (qHNMR) requires that at least one non-overlapping signal for each molecule to be quantified is available for integration.[14] Although the use of pyridine-d_5 was appropriate in terms of qualitative analysis of the samples, quantification was not possible because of partial overlap of H-1′′′′ of RbA and H-1′′′ of St in samples SR-1 to SR-6 and SR-9 to SR-13. One of the experimental parameters which can be modified to obtain sufficient signal dispersion is the modification of the solvent composition.[14] Figure 1 depicts the effect of adding increasing amounts of DMSO-d_6 to the sample dissolved in pyridine-d_5. The best results in terms of signal separation were obtained with the mixture pyridine-d_5—DMSO-d_6 (6:1). In the resulting spectra (SR-1 to SR-6 and SR-9 to SR-13), the doublets of

Table 2. Chemical Shift of the Signals Used for Quantification of Steviol Glycosides (Pyridine-d_5−DMSO-d_6 (6:1), 600 MHz)[a]

	δ H (J in Hz)
St	5.17, d (7.7)
RbA	5.20, d (7.9)[b]
	5.57, brs[c]
RbB	5.65, brs
RbC	5.93, d (8.3)

[a] Spectra were referenced to the TMS signal at δ 0.00. [b] Used for samples SR-1 to SR-6 and SR-10 to SR-13. [c] Used for samples SR-7 and SR-8.

H-1′′′′, H-1′′′, and H-1′ of, respectively, RbA, St, and RbC can be integrated because no major signal overlapping takes place. In the spectra of samples SR-7 and SR-8, H_a17 of RbA and RbB showed the lowest degree of overlap and were thus chosen for quantification (Table 2).

Internal Standard. Quantification of individual constituents by means of qHNMR relies on the use of one internal standard (IS). Anthracene was used because its multiplet at δ 8.11 (4H) does not overlap with signals arising from constituents or solvents in the sample (see Supporting Information). Anthracene is available at high purity, nonhygroscopic, and soluble in the NMR solvent. This polycyclic aromatic hydrocarbon is also known to degrade under the effect of light.[15] Thus, NMR samples were stored in the dark until analysis, which was always completed within 48 h. Analysis of a blank sample consisting of anthracene in the NMR solvent showed that under these experimental conditions no appreciable degradation of the IS takes place (see Supporting Information). Stability of the samples under these experimental conditions was also assured (see Supporting Information).

Experiment Optimization. Published qHNMR methods which employ anthracene as IS include the determination of ginkgolic acids in *Ginkgo biloba*[16] and cannabinoids in *Cannabis sativa*.[17] However, these works do not describe the relaxation behavior (T1) of anthracene, nor discuss the optimization of important experimental parameters such as relaxation delay (d_1) and flip angle. As part of method validation, we determined the T1 relaxation values for the IS and for H-1′′′′ (1.0 s) and H-17_a (0.9 s) of RbA, representative for the steviol glycosides to be quantified. The T1 calculated for the signal of anthracene was 4.2 s and thus represented the protons with the longest T1 in the sample. In order to obtain full relaxation of this signal, an interpulse delay = 5*T1 = 21 s would have to be used (for a 90° flip angle). However, shorter interpulse delays can be employed to shorten the analysis time, according to the target accuracy of the method.[18] In order to choose a suitable relaxation delay, a series of ^{1}H NMR spectra of sample SR-1 were acquired by systematically increasing d_1 (range 1−19 s, 2 s steps), using a fixed flip angle of 45° and a fixed acquisition time (AQ) of 2.7 s. A d_1 = 9 s enabled a 99.3% recovery of the IS signal compared to the reference value obtained with d_1 = 19 s (corresponding to an interpulse delay of 21.7 s) and was thus chosen as a good compromise between analysis time and accuracy of the method (see Supporting Information). The suppression of the residual water signal was obtained by employing a presaturation pulse sequence, which enabled the obtainment of a flatter baseline by dampening signals of exchangeable protons (e.g., OH). For samples SR-1 to SR-8 and SR-10 to SR-13, the suppression of the residual water signal had no effect on the intensity of the signals to be integrated (see Supporting Information). However, for the crude extract (sample SR-9) a significant area reduction was observed, which was caused by the vicinity of the residual water signal to the signals to be integrated. Thus, in this particular sample quantification was not performed. 32 scans were acquired, yielding a total analysis time of about 7 min per sample. Under these experimental conditions, the limit of quantification calculated for RbA (δ 5.57, brs) was 0.16 mM, corresponding to 1.09% and thus appropriate for the quantification of the major steviol glycosides.

Table 3. Intraday and Interday Precision[a]

	intraday (n = 5)			
	day 1	day 2	day 3	interday
RbA	53.85 (0.35)	53.43 (0.52)	51.82 (0.80)	53.03 (2.02)
St	20.99 (0.49)	20.79 (0.45)	20.10 (0.87)	20.61 (2.27)
RbC	7.47 (1.95)	7.61 (2.11)	7.02 (1.89)	7.37 (4.11)

[a] Results expressed as percentage values on the dried weight basis. Relative standard deviation in parentheses.

Method Validation. Validation of the ^{1}H NMR method was done in terms of specificity, precision, accuracy, robustness and linearity. Specificity was assured by the use of 2D NMR experiments (COSY, HSQC, HMBC), which enabled unambiguous assignment of characteristic resonances of the major steviol glycosides. Assignments were verified by ^{1}H NMR spectra comparison with available reference substances. Signals chosen for integration did not show any major overlapping with extraneous resonances, including signals from the same molecule, related constituents, solvents or internal standard. Intraday precision, interday precision, and repeatability were respectively within 2.11%, 4.11%, and 4.11% (Tables 3 and 4), all expressed as relative standard deviations. Recovery rates for accuracy determination were within 101.9 and 95.5%. Robustness was investigated by deliberate modification of shim, flip angle, and phase (see Supporting Information). A maximum deviation of 1.0% from the reference values was observed, which confirmed the robustness of the method. Linearity of the method could be confirmed in the concentration range 0.2−19.3 mg/mL for RbA by constructing calibration curves using the signals of H-1′′′′ and H-17_a (R^2 = 0.9995 and R^2 = 0.9997 respectively). Quantitative results for the analyzed samples are presented in Table 4.

Comparison with HPLC. The JECFA HPLC−UV method[3] was employed as a reference method for NMR investigations. The tentative identification of steviol glycosides in HPLC chromatograms was done by HPLC−MS experiments, performed under the same experimental conditions as HPLC−UV. The elution order of the identified constituents was in good accordance with that reported in the JECFA monograph.[3] In addition, peak assignment for St, RbA, and RbB was verified by comparison with the pure compounds. Quantitative HPLC−UV results for St, RbA, RbB, and RbC were found to be in reasonable agreement with the NMR results (Table 4). Importantly, HPLC allowed the quantification of steviol glycosides in the crude extract (sample SR-9), whereas NMR only allowed a qualitative analysis of this kind of sample. The use of HPLC−MS also enabled the tentative identification of further minor steviol glycosides in some of the purified extracts, namely, RbD, RbF,

Table 4. Quantification of RbA, St, RbC, and RbB in Samples SR-1 to SR-13 as Determined by NMR and HPLC−UV[a]

	RbA		St		RbC		RbB	
	NMR	HPLC	NMR	HPLC	NMR	HPLC	NMR	HPLC
SR-1	54.10 (0.66)	53.74 (1.74)	21.13 (0.75)	21.33 (1.86)	7.59 (2.66)	6.81 (0.03)	nd	nd
SR-2	59.64 (0.17)	60.87 (0.77)	17.96 (0.30)	17.66 (0.86)	7.01 (0.52)	7.07 (0.32)	nd	nd
SR-3	51.53 (0.30)	53.32 (1.27)	25.08 (0.20)	26.30 (2.22)	6.85 (0.93)	6.98 (2.12)	nd	nd
SR-4	57.88 (0.11)	59.43 (1.57)	18.89 (0.22)	19.32 (0.95)	6.19 (0.58)	6.77 (1.75)	nd	nd
SR-5	60.88 (0.55)	63.18 (0.50)	16.32 (0.71)	16.38 (2.74)	6.46 (2.42)	6.98 (1.58)	nd	nq
SR-6	62.50 (0.19)	62.66 (0.82)	14.47 (0.42)	13.99 (0.34)	7.10 (1.34)	6.95 (0.41)	nd	nq
SR-7	90.95 (0.17)	92.02 (0.22)	nd	nq	nd	nd	4.74 (1.76)	5.07 (1.33)
SR-8	96.02 (0.25)	97.43 (0.74)	nd	nq	nd	nd	1.46 (4.11)	1.54 (1.76)
SR-9	nq[b]	24.29 (0.51)	nq[b]	4.69 (2.47)	nq[b]	nq	nd	nq
SR-10	40.56 (0.67)	44.14 (0.47)	10.48 (1.58)	9.10 (1.23)	4.67 (2.98)	4.49 (1.33)	nq	nq
SR-11	59.26 (0.14)	63.88 (0.43)	11.09 (0.45)	10.83 (0.47)	6.78 (0.56)	7.28 (0.76)	nq	1.35 (2.24)
SR-12	56.92 (0.20)	59.65 (0.24)	13.44 (0.50)	12.86 (1.65)	7.07 (0.98)	6.58 (1.57)	nd	0.92 (1.59)
SR-13	58.28 (0.23)	59.06 (1.76)	14.29 (0.44)	13.55 (0.68)	6.32 (0.83)	6.53 (1.07)	nq	1.93 (1.88)

[a] Results expressed as percentage values on the dried weight basis. Relative standard deviation in parentheses; nd, not determined; nq, not quantified. [b] Partial signal suppression as result of water signal suppression.

DuA, Rub, and Stb (see Supporting Information). However, quantification of these minor constituents by HPLC−UV was not always possible because of their very low content in the investigated samples or because of peak overlap. The identification of these minor constituents by means of NMR failed because of their very low content in the investigated extracts, which did not allow the acquisition of diagnostic 2D NMR data. Still, NMR enabled quantification of the major steviol glycosides about four times faster compared to HPLC−UV. It must be noted that the analysis time can be further reduced by lowering the number of scans if quantification of only the major constituent (e.g., RbA, more than 50% in the purified extracts) has to be carried out. This is normally not possible with HPLC, where complete separation of the constituents must occur before the next chromatographic run can begin. A further advantage of NMR is the fact that analyte identification and quantification do not rely on the use of standard compounds. This results in faster analysis, because external calibration is not needed. Identification of the major steviol glycosides is straightforward because it is based on ^{13}C NMR data comparison, which can be regarded as being highly reproducible. Exemplary is the fact that the literature NMR data used as reference for analyte identification dates back to 1976.[11] On the contrary, HPLC as a chromatographic method is subjected to retention time drifts and stationary phase degradation with time, which weaken reproducibility and specificity of the method. Finally, the use of NMR to monitor the purification process offered precious insight into the composition of the intermediate fractions obtained. HPLC performed in a similar manner in regard to the steviol glycosides; however, it was not possible to detect glucose in the samples. This underlines the importance of NMR as universal detection method, capable of simultaneous analysis of heterogeneous constituents. The detection of glucose by HPLC would have required phase switch to normal phase and a different detector (e.g., ELSD). Thus, NMR can be regarded as a feasible alternative to HPLC for the analysis of the major steviol glycosides in *S. rebaudiana* extracts. The advantages of NMR must be however evaluated in view of higher costs related to the purchase of the spectrometer, its maintenance and the use of deuterated solvents. Such costs might be acceptable if a fast analysis is required, as in case of high-throughput applications.

■ ASSOCIATED CONTENT

Supporting Information. Selected 1D and 2D NMR data; HPLC−MS and HPLC−UV chromatograms; analysis of the minor steviol glycosides by HPLC−UV. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

St, stevioside; RbA, rebaudioside A; RbB, rebaudioside B; RbC, rebaudioside C; RbD, rebaudioside D; RbF, rebaudioside F; Rub, rubusoside; Stb, steviolbioside; S/N, signal-to-noise ratio; d_1, relaxation delay; AQ, acquisition time; IS, internal standard

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