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Validation of Steviol Glycoside Analysis by an External Standard

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Abstract

Very pure steviol glycoside standards were used for the validation of the methods of analysis. UV spectra of steviol glycosides and of steviol were measured in water, EtOH, 80% AcCN and 35% AcCN (5 mg/100 ml). The maximum absorption was around 196, 205, 199 and 197 nm in water, EtOH, 80% AcCN and 35% AcCN, respectively. The calibration curves of steviol glycosides and of steviol were rectilinear between 15 and 1243 µM. The UV signal was monitored at 190 and 210 nm. It was shown that it is possible to make calibration curves with one single pure standard (e.g., stevioside or rebaudioside A). The amounts of the other steviol glycosides present in commercial mixtures were calculated by use of conversion factors that compensated for the different molecular weights. Calculation of steviol equivalents has also been done. The detection limit (S/N = 5) of steviol glycosides is about 25 ng, that of steviol after derivatisation to its (7-methoxy-4-coumarinyl) methyl ester about 100 pg. Limits of quantification (S/N=10) are 50 ng and 200 pg for steviol glycosides, and steviol, respectively. The range of measurements is between 0.025 and 1000 µg/mL for steviol glycosides (or between about 15 and 1250 µM) and between 0.05 and 50 µg/mL for steviol as its fluorescent derivative (or between 15 nM and 160 µM). Although the separation of stevioside and rebaudioside A is easier on propylamino silica gel columns, not all the steviol glycosides are well separated. As JECFA suggested the use of reversed phase columns, analyses were done on 2 reversed columns in series. The analysis of crude samples can also be done on C18 columns that can easily be rinsed when becoming dirty. Methods are summarized for the analysis of steviol glycosides and steviol in leaves and different foods. Methods for SPE purification are also given.

Keywords: External Standard Method; HPLC; SPE Purification; Steviol; Steviol Equivalents; *Stevia rebaudiana*; Steviol Glycosides; Validation of Analytical Methods

Abbreviations

Using SV for steviol allows the use of the following abbreviations:

SVgly(s):		Steviol glycoside(s)
SVeq(s)	:	Steviol equivalent(s)
SVglu	:	Steviol glucuronide
SM	:	Steviol monoside
SVE	:	Steviol 19-ester
SVglu	:	Steviol glucuronide
ST	:	Stevioside
RebA-G	:	Rebaudioside A-G
SB	:	Steviolbioside
DulA	:	Dulcoside A
Rub	:	Rubusoside

Introduction

Steviol glycosides are the sweeteners found in the leaves of *Stevia rebaudiana* Bert. (Bert.) [1]. The purity of the mixture, comprising the most abundant sweeteners present, stevioside and rebaudioside A, should be ≥ 95 % on a dry weight basis. High purity rebaudioside A (>95 %) can also be found on the market. It has a somewhat better taste profile than stevioside and the other sweeteners. In some countries the mixture of steviol glycosides is called "Steviosides". However, this term is confusing and should be avoided as stevioside is only 1 specific chemical compound of the mixture [2-5]. The purity of steviol glycosides is defined as the sum of all the authorized steviol glycosides present in a mixture and is expressed on a dry weight basis. A purity of >95 % means that the sum of the steviol glycosides makes up at least 95 % of the dry weight of a sample. The correct dry weight of a sample is ob-

tained after drying to a constant weight in special weighing vials. In Stevia extracts, over 30 sweeteners have already been identified, although most occur in very minute amounts and these compounds are not yet authorized [6]. All Stevia sweeteners have different molecular weights, and are degraded to steviol by the bacteria of the colon [7]. Therefore, JECFA and EFSA used the term "Steviol Equivalents" to propose an ADI of 0-4 mg steviol equivalents/kg body weight; i.e., 10 mg stevioside or 12 mg rebaudioside A/kg body weight, respectively.

The minimum purity requirement of 95 % by JECFA and EFSA makes the analysis of steviol glycosides a very difficult task, as possible errors should be eliminated or at least be minimized. Preliminary results in different laboratories have shown very large differences for the purity determination (RSD between 4.5 and 8 %). The large RSD means that then purity would be 95 ± 8, or between 87 and 103 % which seems unacceptable. The result of a sample analysis should always be the same and totally independent of the laboratory that performed the analysis. As shown earlier, the proposed JECFA method is not reliable and many analysts do not use it strictly as described in the protocol [1,8]. A good validated method of analysis of steviol glycosides is of utmost importance for food inspectors as well as for the Stevia industry. The analysis of steviol glycosides by HPLC is essentially based on 2 different column types: adsorption (propylamine (-NH_2); HILIC)) or reversed phases (well selected phases of Octadecyl silica gel (C_{18})). The column dimensions are usually 250 x 4.6 mm and particle size, 5 µm. Some people use HILIC columns [8-15]. Usually, UV-detection at 210 nm is used, although some laboratories confirm the identity of the different compounds by LC-MS. To increase sensitivity and if the equipment is suited, measurements can be done at a lower wavelength (e.g. 190 nm).

Solvents used normally are mixtures of acetonitrile:water (AcCN:H_2O) or AcCN in combination with diluted NH_4OAc or phosphoric acid. With NH_2-columns, an isocratic solvent AcCN: water phase (between 87:13 and 80:20) is often used. Under these conditions, the run time may be as long as 65-75 min. As JECFA advised the use of reversed phase columns, our work was done using selected C_{18} phases. Work has been done to improve this JECFA method [12-16]. However, the resolution was not sufficient for exact peak integration or not all the authorized SVglys were analysed. To obtain a baseline separation, required for perfect peak integration, it might be necessary to use 2 C_{18} columns in series or 1 ultra-high performance liquid chromatography column as recently shown [16]. However, nothing is known about the robustness of this kind of columns when injecting crude plant extracts. On C_{18}-type columns, a baseline separation can be obtained by use of 2 Grace Alltima C_{18} columns in series and a solvent gradient of AcCN: 0.1 mM phosphoric acid (see below). One of the advantages of C_{18} columns are that they can easily be rinsed with different solvents without damaging the columns.

As most of the laboratories do not have all the ultra-pure standards available for calibration, it is usually done only with stevioside and/or rebaudioside A. Previous work has shown that this is satisfactory as most of the steviol glycosides have a similar absorption coefficient [1]. Therefore, the slopes of the calibration curves of all steviol glycosides will be very similar as the calibration curves are made in mM concentrations. To express the results as weight by weight (e.g. mg component/g mixture), a correction has to be made for the different molecular masses [8]. As people might also be interested in the calculation of steviol equivalents, these calculations were also done and a method is given for directly measuring the SVeqs.

Methods and Material

Solvents and Products

Solvents and water used were of HPLC quality. Other products were of PA grade. Standards were crystallized to > 99 % purity [17].

Analytical HPLC of Steviol Glycosides

All SVgly samples were analysed using analytical HPLC (Shimadzu Prominence) on two Grace Alltima C_{18} columns in series (250 mm x 4.6 mm, particle size 5 µm) using an AcCN: 0.1 % H_3PO_4 gradient (0 - 2 min: 34 % AcCN; 2 -10 min: 32 % 42 %; 10 - 16 min: 42 %; 16.1 min: 34 %). UV-detection was at 200 nm (Shimadzu, SPD-6A). The injection volume was 10 µl.

Measurement of Molar Extinction Coefficients

The molar extinction coefficients of the most important steviol glycosides and steviol were measured in EtOH, water, 80% AcCN and 35% AcCN. As UV detection in HPLC is usually done in AcCN-water mixtures, it was relevant to measure the extinction coefficients in the eluents that are often used in HPLC (80% AcCN for propylamino columns and 35% AcCN for octadecyl silica gel columns). The extinction coefficients were measured at compound concentrations of 31 and 62 µM and at the wavelength of maximum absorption in the solvent used.

Calibration Curves

Calibration curves were constructed for the following steviol glycosides: stevioside, rebaudioside A, B, C, dulcoside A, rubusoside, steviolbioside, as well as for steviol, that could be purified to > 99 % purity by crystallization. The concentrations used were between 0.0625 and 5 mg/5 mL, but these concentrations were converted and plotted as mM concentrations. The solubility of steviolbioside and of steviol in water is rather low and, therefore, solutions and dilutions were made in MeOH. This does not influence the separation by HPLC.

Precision

The intra-day and inter-day precision of the method was tested by repeating the analyses on the same day (intra-day, n=3) or on different days (inter-day (n=3).

Accuracy

To measure the accuracy, samples with a known concentration of stevioside and rebaudioside A were spiked with 3 different amounts of stevioside or rebaudioside A, giving the concentrations as in Table 2.

Measurement of Steviol in Stevia Leaves, Commercial Steviol Glycoside Mixtures or Foods

Methods for sensitive measurement of Steviol (SV) were published by Minne et al. [18]. To avoid losses due to adsorption of SV to active glass surfaces, all manipulations were done using polypropylene tubes and pipette tips. To 100 mg of freeze-dried powdered plant material, 4 µg internal standard (dihydro-isosteviol, DHISV) in acetone solution was added. After evaporation of the acetone, the residues were extracted three times with 2 % KOH in MeOH (0.5 mL) in Eppendorf conical flasks. During extraction, the tubes were kept at 4°C in the dark and vortexed continuously. Extraction with different organic solvents was found insufficient for the extraction of steviol. After centrifugation, the extracts were collected and water (5 mL) was added to the combined extracts. The apolar lipids were removed by three extractions (5 mL each) with diethyl ether. The water phase was acidified to pH 6 by the addition of acetic acid (6 N). Acidification with HCl to lower pH values (pH ≈ 3) was avoided to prevent the conversion of steviol into isosteviol. The SV was extracted three times with equal volumes of peroxide free diethyl ether. The combined ether fractions were evaporated to dryness under a stream of N_2 and the water-free residue was derivative by esterification of the free carboxyl groups with the alkylating reagent 4-(bromomethyl)-7-methoxycoumarin. This reaction was done in an aprotic solvent (dry acetone or N, N-dimethylformamide, DMF) in the presence of 1 µL N, N-diisopropylethylamine. No traces of water should be present (details: [8]. The reaction mixture was heated at 70°C for 20 min and 2 µL of the resulting sample solution could be injected in the HPLC or purified as described below.

After derivatisation, the acetone was evaporated under a stream of N_2 and the residue dissolved in 1 mL of $CHCl_3$. Chromatographic sample clean-up was done using small columns of 250 mg silica gel applied to 1 mL pipette tips containing a plug of glass wool. The solution containing the (7-methoxy-4-coumarinyl) methyl ester derivatives of SV and DHISV was run through the silica gel column. The column was rinsed, first with 2 mL of $CHCl_3$, and then with 1 mL $CHCl_3$-MeOH (98:2), and finally the mixture of ester derivatives was eluted with 1 mL of $CHCl_3$-MeOH (80:20). The eluate could be injected directly on to the HPLC column or further concentrated under a stream of N_2 for further sample clean-up by TLC and $CHCl_3$: MeOH (98:2) as solvent see [18]. The resulting ester derivatives were separated on 1 ODS column (250 × 4.6 mm i.d., 5 µm particle size) using fluorescence detection with excitation at 321 nm and emission at 391 nm. The mobile phase consisted of AcCN-water (80:20 v/v) with a flow rate of 1 $mL.min^{-1}$. A linear relationship was observed for concentrations between 0.5 and 50 µg/mL SV and the detection limit was 100 pg.

Results and Discussion

As steviol glycosides may contain water (in some samples up to 6%), all our experiments were done after drying at 105°C for at least 2 h. Steviol glycosides are stable at this temperature [1].

Molar Extinction Coefficients of Steviol Glycosides and Steviol

To evaluate the possibility of calibrating the HPLC with only one standard (e.g., ST or RebA), the molar extinction coefficients of the most important SVglys and SV were measured in EtOH, water, 80% AcCN and 35% AcCN (Table 1). As UV detection in HPLC is usually done in AcCN-water mixtures, it was relevant to measure the extinction coefficients in the eluents that are often used in HPLC (80% AcCN for propylamino columns and 35% AcCN for octadecyl silica gel columns). The extinction coefficients were measured at compound concentrations of 31 and 62 µM and at the wavelength of maximum absorption in the solvent used (Table 1).

Extinction coefficient ε = A/l.c	A: absorption, l = 1 cm light pad; c= concentration in M.

The wavelength of maximum absorption was 196 in water, 205 in EtOH, 199 and 197 in 80% or 35% AcCN, respectively. The extinction coefficients of the different compounds did not differ significantly. It was found that the extinction coefficients in water and AcCN solutions (around 7500) are much larger than in EtOH (around 4000). As steviol glycosides are often measured at 210 nm, the extinction at that wavelength is also given in Table 1. It shows that measurement at 210 nm is less sensitive than at lower wavelengths.

Compound	water 196 nm	EtOH 205 nm	EtOH 210 nm	80% AcCN 199 nm	35% AcCN 197 nm
Stevioside (n=13)	7657 (197)	3572 (270)	2931 (173)	7872 (187)	8379 (188)
Reb A (n=9)	7010(172)	4078 (92)	3434 (389)	7010 (172)	7858 (388)
Reb B (n=3)	6312 (462)	5679 (1043)	1828 (14)	7835 (1120)	6660 (370)
Reb C (n=4)	8094 (156)	3535 (270)	2901 (264)	5043 (387)	8897 (10)
Dulc A (n=5)	7601 (506)	5635 (1133)	3535 (563)	7638 (1298)	7973 (159)
Rubusoside (n=7)	7943 (118)	4848 (605)	3120 (424)	7818 (848)	8788 (560)
SB (n=3)	5404 (373)	5289 (1207)	3657 (917)	6256 (894)	5331(126)
Steviol (n=3)	-	5672 (673)	3830 (483)	8065 (795)	-

Table 1: Extinction coefficients (± se) of different SVglys and SV measured at concentrations between 31 and 62 µM (Dulc A= dulcoside A; SB= steviol bioside).

Calibration Curves of Steviol Glycosides: Linearity and Range of Measurements

Calibration curves were constructed for the following steviol glycosides: ST, RebA - C, DulA, Rub, SB, as well as for SV, that could be purified by crystallization to over 99 % purity. The concentrations used were between 0.0625 and 5 mg/5 mL. The solubility of SB and of SV in water is rather low and, therefore, solutions and dilutions were made in MeOH. This does not influence the separation by HPLC.

The calibration curves of the SVglys and SV were linear up to 1 mg/mL (or about 1.25 mM). Higher concentrations were not tested to avoid precipitation of some of the compounds. The measurement range was between 0.25 and 10 µg/10 µL injected or between 25 and 1000 µg/mL (or between about 15 and 1250 µM). The calibration curves were extrapolated through zero and the equation of the calibration curves was y = m.x. The peak area obtained for each SVgly is a function of its extinction coefficient, which itself is a function of the molar concentration of the compound. Therefore, molar concentrations were plotted in Figure 1. This allowed us to better compare the slopes of the different curves. After analysis, a correction should be made for the molecular weight of each compound. As there were no significant differences between the extinction coefficients of the different SVglys, it could be expected that the calibration curves plotted as µM concentrations, would have the same values of their slopes. Indeed, this was the case as shown in Table 2, that gives the slopes m of the curves, together with the molecular weights and the conversion factors of the different molecules for calibration with ST. Multiplication of the molar concentration with this conversion factor gives the SVgly expressed as mg/mL. As all SVglys have similar extinction coefficients and similar slopes, it followed that it was possible to make calibration curves with one single pure standard (e.g., ST, or RebA) and that the amounts of the other SVglys present could be calculated by taking into account the different molecular weights of the compounds.

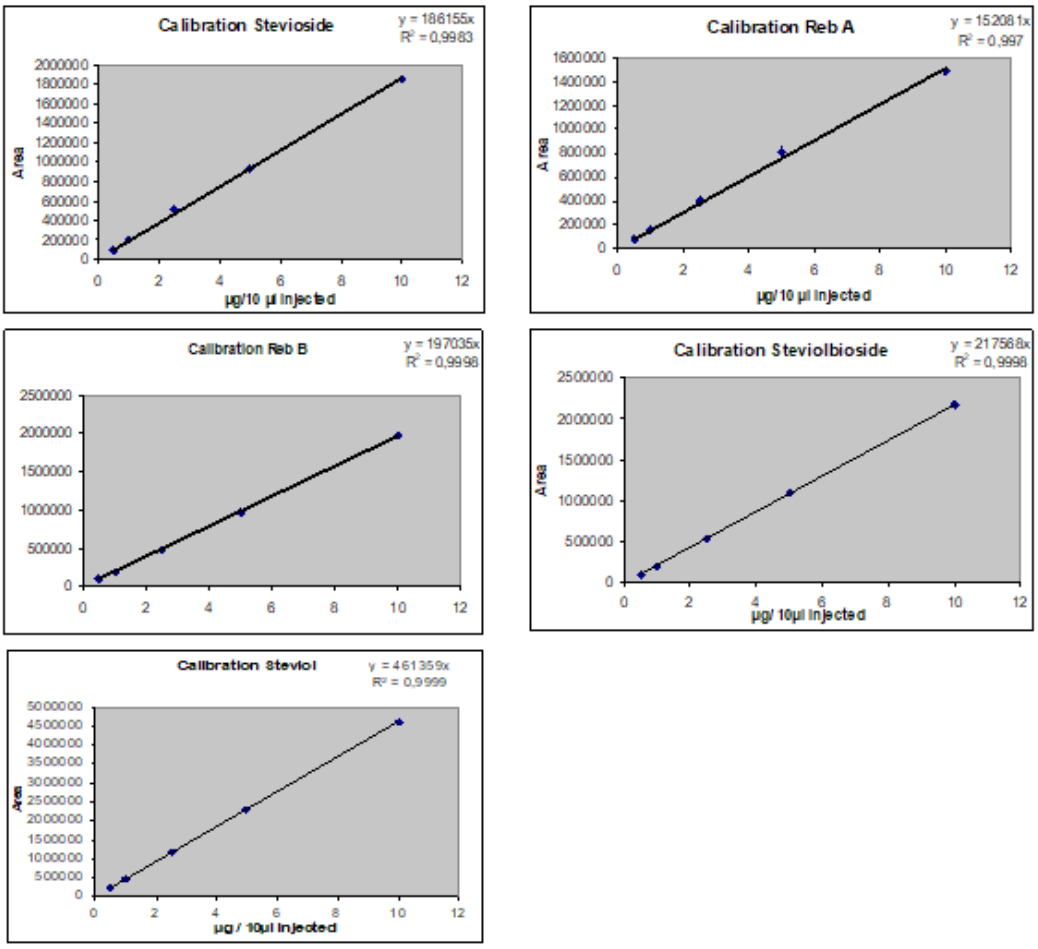


Figure 1: Calibration curves of the different SVglys and of SV.

Compound	Slopes m ± se	Molecular masses	Conversion Factors (CF) to obtain concentration in mg/mL
Stevioside	4.52±0.11	804.38	0.80438
Rebaudioside A	3.89±0.09	966.43	0.96643
Rebaudioside C	4.32±0.02	950.44	0.95044
Dulcoside A	4.12±0.04	788.38	0.78838
Rubusoside	4.43±0.13	642.33	0.64233
Steviolbioside		642.33	0.64233
Rebaudioside B		804.38	0.80438
Rebaudioside D		1128.48	1.12848
Rebaudioside E		966.43	0.96643
Rebaudioside F		936.42	0.93642

Table 2: Slopes of calibration curves, molecular masses and conversion factors are given to calculate concentrations in mg/mL of different SVglys in a mixture after calibration of the HPLC with ST (5mg/5 mL = 1.243 mM).

All the above is exemplified by the following calculations:

Calibration of the HPLC by injection of 10 µL of a ST solution (5 mg/5mL = 1.243 mM) might produce a peak area of 5000. If, after injection of a solution of RebA with an unknown concentration, a peak area of 2500 is found, its molar concentration can be calculated by dividing its peak area by that of ST, in this case giving 0.5 times the concentration of ST, or 0.6215 mM. The concentration of RebA in mg/mL is then obtained by multiplying the molar concentration by the conversion factor of Table 2, or 0.6215 x 0.966 = 0.6 mg/mL. If calibration with 10µL of a RebA solution (5mg/5 mL= 1.035 mM) gives a peak area of 5000, the same peak area of an unknown ST concentration means a molar ST concentration of 1.035 mM or 0.832 mg/mL (1.035 x 0.8804).

SV was not included in this section, as it is a carboxylic acid without attached sugar units, which behaves somewhat differently under the chromatographic conditions used. However, to measure steviol quantitatively, that possibly is occurring in very small amounts in Stevia leaves [18], a method including the use of an internal standard (dihydroisosteviol) and fluorescent derivative formation was developed and published [18]. For those not having the internal standard (dihydroisosteviol) and wishing to measure steviol, a calibration curve has to be made using an external standard. However, in that case, there is no correction for losses that possibly occur during sample clean-up due to the acidic nature of the relatively apolar steviol.

Analysis of samples

As the measurement of SVglys was based on an external standard method, for each series of samples, the HPLC was calibrated by injection of 10 µL of a pure ST standard solution (5 mg/5 mL = 1.243 mM). After HPLC analysis, the molar concentration of the different SVglys was obtained by dividing the peak area of the SVglys (PASG) by the area of the standard (PAS; ST) and multiplying with the molar concentration of the standard ST (1.243 mM).

Molar concentration SVglys = PASG/PAS x 1.243 mM

Multiplying the mmolar concentration of each SVgly with the conversion factor (CF) of Table 2 gives the concentration in mg/mL. Multiplying this value with the total volume (in mL) of extracts provides the total amount of each steviol glycoside (TASG).

TASG = concentration in mg/mL x total volume (mL)

The methods described were used in the analysis of commercial SVgly mixtures of different qualities. All samples were labelled over 95% purity. However, two samples were of poor quality (total SVglys less than 95 %) and one sample was of good quality (purity over 95%; Table 3).

Sample	505171	Ip0906	Nv2060301
Loss on drying	2.30%	2.80%	5%
Stevioside	31.29%	52.44%	56.44%
Reb A	14.67	23.93	28.22
Reb C	4.89	7.21	6.51
Dulcoside A	1.63	2.95	3.26
Other	0.65	0.98	3.26
Total = Purity	53.13%	87.51%	97.68%

Table 3: Analysis of 3 commercial SVgly mixtures with different qualities.

Measurement of Steviol Glycosides in the Presence of Impurities

An analysis was done with a sample containing stevioside (92%) and rebaudioside A (6%) in the presence or in the absence of glucose. Compounds like glucose are not detected by an UV detector. HPLC conditions: Column: C_{18}, 250mm x 4.6 mm, solvent: AcCN:water (35:65); flow: 1 ml/min, UV detection at 210 nm. Blanks were also run to identify the solvent peaks. Calibration curves should be made with >99.5 % pure standards of the SVglys, as demonstrated above. Solvent blanks should always be run to correct for the solvent (or water) peaks.

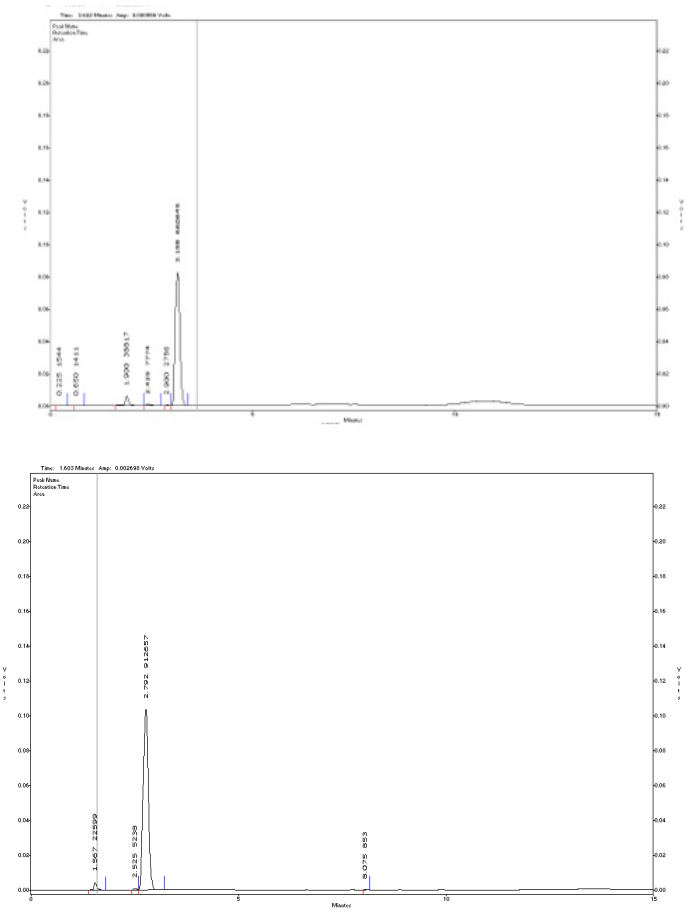
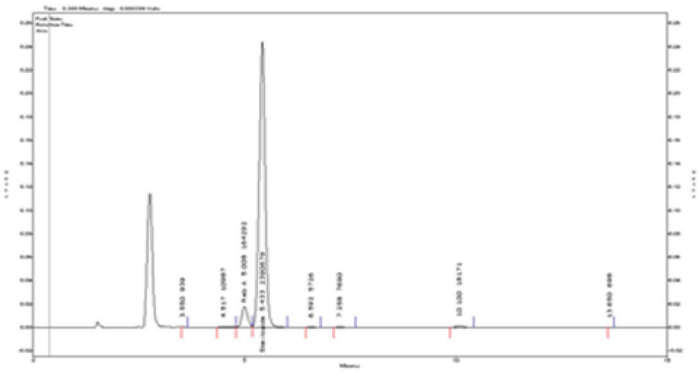


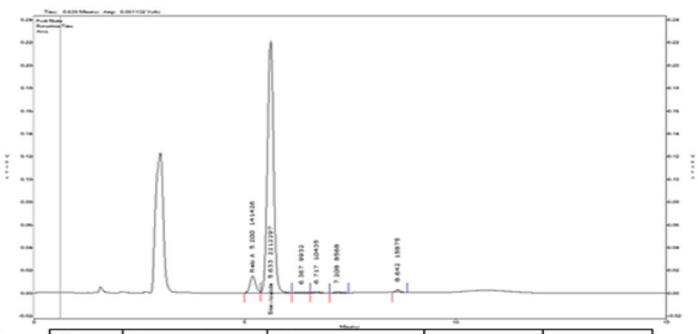
Figure 2: above: H_2O blank, showing "Solvent" peaks in the beginning of the chromatogram; below: 1 % glucose blank, showing "Solvent" peaks in the beginning of the chromatogram.

In the following experiment, a solution was made of a SVgly mixture in water (5 mg/5mL). The second solution contained the same SVgly mixture but this time dissolved in 1 % glucose solution (5 mg/5mL 1 % glucose). Although the HPLC chromatograms (Figures 3 and 4) looked very similar, the second one was in fact of a sample of which the purity was lower than that Figure 3. This is because glucose as impurity was not seen by the UV-detector and because the solution was made in 1 % glucose.



Pkno	Retention Time	Compound	Area	Area %
1	3.550		939	0.036
2	4.517		10987	0.423
3	5.008	Reb A	164292	6.321
4	5.433	ST	2390679	91.978
5	6.592	Reb C	5726	0.220
6	7.258	DulA	7690	0.296
7	10.100		18171	0.699
8	13.650		698	0.027
Total				100

Figure 3: HPLC chromatogram of a steviol glycoside sample in water (5 mg /5 ml H_2O).



Pkno	Retention Time	Compound	Area	Area %
1	5.200	RebA	141426	5.896
2	5.633	ST	2212297	92.235
3	6.367	RebF	9932	0.414
4	6.717	RebC	10435	0.435
5	7.208	DulA	10435	0.357
6	8.642	SB	15875	0.357
Total				100 %

Figure 4: HPLC analysis of 5 mg steviol glycoside/5 ml 1% glucose; i.e. this solution contains 1 mg stevioside and 10 mg glucose per ml.

However, by weighing and dissolving equal amounts of 2 different samples of the SVglys (5 mg/5mL), one pure sample and the other one with 90 % glucose as impurity), would result in totally different chromatograms as the impure sample would only give small peaks for ST and RebA of which the amount is only 0.5 mg/5 mL. This proves that the method can be used to measure the purity of SVgly mixtures in the presence of impurities similar to glucose or other compounds that do not absorb UV-light.

Detection Limit, Limits of Quantification, Range of Measurement

- The detection limit of SVglys by a UV detector at 210 nm was about 25 ng (Knauer Smartline UV detector 2500: noise ratio 5:1).

- The limit of detection of SV after derivatisation was 100 pg (Signal: noise ratio 5:1, [18]).

- The limits of quantification were 50 ng and 200 pg for SVglys and SV, respectively (signal/noise ratio: 10/1).

- The range of measurements was between 0.25 and 1000 µg/mL for SVglys (or between about 15 and 1250 µM) and between 0.05 and 50 µg/mL for SV as a fluorescent derivative (or between about 15 nM and 160 µM; [18]).

Precision

The precision of the method was tested by both intra-day (n=3) and inter-day (3 d, n=3) repeatability. The intra-day variation was about 1.9 and 2 % for ST and RebA, respectively. The inter-day variation was of a similar magnitude. For SV, the intra-day (n = 9) and inter-day (n = 9) variations were 0.64 and 0.88 %, respectively [18]. The precision of injection was measured by 5 consecutive injections of a 1mg/mL RebA solution. The standard error was 0.4 % (peak area 3698 ± 14.9).

Accuracy

To measure the accuracy, samples with a known concentration of ST and RebA were spiked with 3 different amounts of ST or RebA, giving the concentrations as in Table 3. The measurement of the unspiked samples using the calibration curves of Figure 2 gave the values 1.01 and 0.99 for ST or RebA, respectively, as expected (Table 4). Extrapolation of the trend line of the spiked samples gave an intercept with the y-axis and this was the same value as found for the unspiked samples, proving the accuracy or trueness of the method 0.98 ± 0.023 and 1.01 ± 0.005 for ST and RebA, respectively.

Unspiked	1	1.01 ± 0.06	1	0.99 ± 0.05
Spiked with	0.2		0.2	
	0.5		0.5	
	1		1	
Value found by extrapolation		0.98 ± 0.023		1.01 ± 0.005

Table 4: Analysis of samples after spiking with known amounts of ST or RebA, respectively (n=3).

Choice of Columns: C_{18} versus NH_2 Based Separations

As it was possible to make good separations on C_{18} columns (250 mm x 4.6 mm; solvent, AcCN:H_2O, 35:65), these columns were preferred, as crude water extracts can be injected, avoiding possible losses by sample purification. These columns can easily be washed with different solvents (acetonitrile, acetone, hexane, methanol) to remove accumulating impurities, thus prolonging column life. Propylamino columns, although giving very good separations, cannot be used for the injection of crude extracts without sample clean-up.

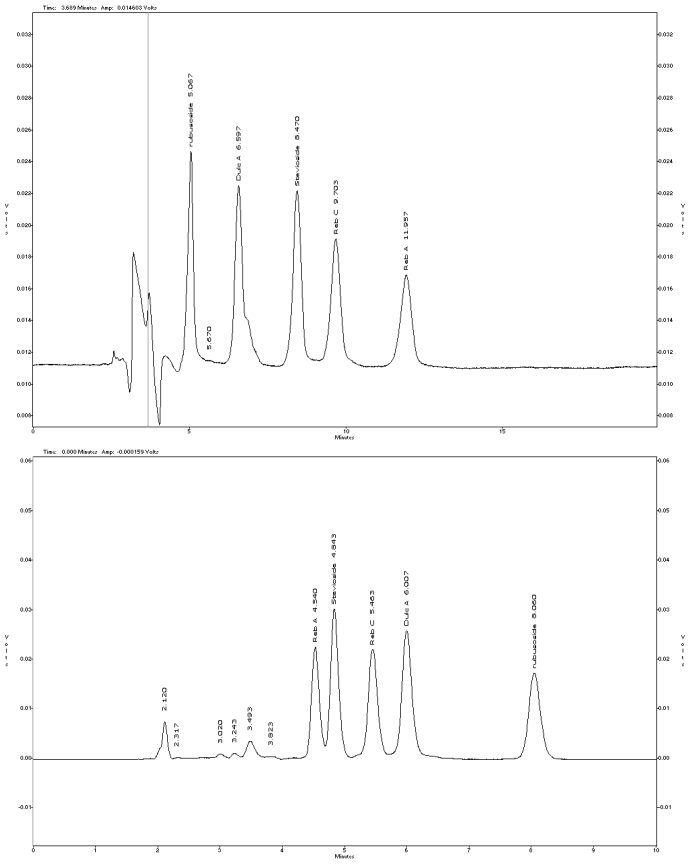


Figure 5: Upper part: mixture of standards on 1 NH_2 column (L 250mm; ID 4.6 mm; solvent 72 % AcCN); order of peaks: Rub, DulA, ST, RebC, RebA; below: mixture of standards on 1 C_{18} column (L 250mm; ID 4.6 mm; solvent 28 % AcCN); order of peaks: RebA, ST, RebC, DulA and Rub.

Conversion of Steviol Glycosides tnto Steviol Equivalents and Vice-Versa

The amounts of SVglys authorized in different foods are given in steviol equivalents (SVeqs). Table 5 gives the conversion factors to convert the amount of SVglys to SVeqs and vice-versa.

To obtain the steviol equivalent of	Molecular weight	Multiply the amount by:
Stevioside	804.38	0.395
Rebaudioside A	966.43	0.329
Rebaudioside C	950.44	0.334
Dulcoside A:	788.38	0.4
Rubusoside	642.33	0.496
Steviolbioside	642.33	0.496
Rebaudioside B	804.38	0.395

Rebaudioside D	1128.48	0.282
Rebaudioside E	966.43	0.329
Rebaudioside F	936.42	0.34
To obtain the steviol glycoside	Molecular weight	Multiply steviol equivalent by:
Stevioside	804.38	2.532
Rebaudioside A	966.43	3.039
Rebaudioside C	950.44	2.994
Dulcoside A:	788.38	2.5
Rubusoside	642.33	2.016
Steviolbioside	642.33	2.016
Rebaudioside B	804.38	2.532
Rebaudioside D	1128.48	3.546
Rebaudioside E	966.43	3.039
Rebaudioside F	936.42	2.941

Table 5: Conversion factors to convert the amount of SVgly to SVeq and *vice-versa*.

Example: 1 g of dry 95 % pure RebA contains 950 mg RebA and this is 312.55 mg SVeqs. With mixtures it becomes more complicated, e.g., 95 % pure SVglys with 60 % RebA and 40 % ST, the amounts are: Total SVglys = 950 mg, of which 570 mg is RebA or 187.53 mg SVeq from RebA (= 570 * 0.329) and 380 mg ST or 150 mg SVeq from ST (380 * 0.395).

Extraction of Stevia Leaves

Analysis of steviol glycosides

Dried leaves were pulverized to a fine powder with a ball mixer (Retsch mixer mill MM200). After drying at 105˚C for 2 h, a weighed amount (500 mg) was extracted with water (30 mL) in closed Falcon tubes by boiling for 10 min in a water bath. After centrifugation and cooling, the supernatant was collected and the residue was extracted a second and third time each with water (30 mL). After centrifugation and cooling, the supernatants were combined, thoroughly mixed and samples were taken for HPLC analysis. With the volumes given, there was no risk of measuring outside the calibration curves. If working with more concentrated samples, however, they should be diluted before analysis (Figure 6).

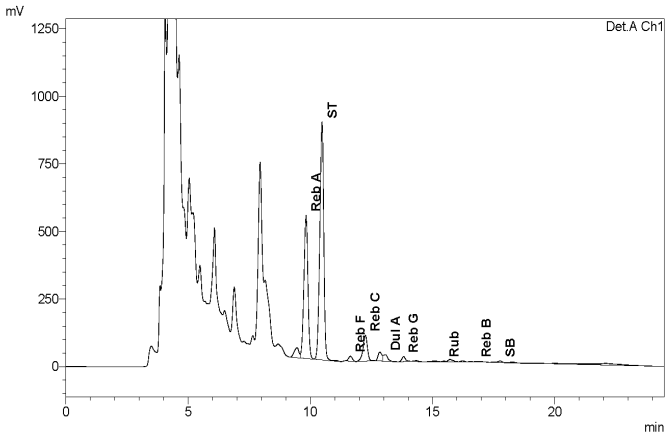


Figure 6: Example of the separation of a crude Stevia leaf extract without SPE purification step.

HPLC was done without purification of the extracts to prevent losses. HPLC conditions: Alltech C_{18} column, 4.6mm ID, 25 cm, 5 µm particle size; AcCN:H_2O: 35:65; detection: UV 210; Injection volume: 10 µL. For each series of samples, the HPLC was calibrated by injecting 10 µL of >99.5 % pure stevioside (5 mg/5 mL).

Purification of Samples

If purification of samples is wanted, it can be done with C_{18} SPE columns of 0.5 or 1 g depending on the capacity required. Crude extract (up to 3 mL) can be purified on a 1 g SPE column, preconditioned with MeOH (5 mL) followed by water (5 mL). After the application of the sample, the columns were rinsed with 20 % acetonitrile in water (5 mL) and the steviol glycosides were eluted with 50 % acetonitrile in water (5 mL).

Notes

- As the analyses were based on an external standard method, all the volumes used were carefully controlled and possible dilutions taken into account.

- The adsorption/elution capacities of C_{18} SPE columns of different brands can differ significantly. Therefore, some samples and standards should be run to check their quality and possibly adapt the composition of the rinse and elution solvents accordingly.

- If available, LC-MS should be done to identify the different steviol glycosides.

Analysis of Steviol

To 1 g of sample, internal standard, (dihydroisosteviol -DHISV) (4 µg) was added and then the sample was extracted three times with 2% KOH in MeOH (0.5 mL) in Eppendorf conical flasks. The analysis was done as summarised above [18]. Although many pigments were remaining in the basic 2% KOH solution after extraction with diethyl ether, and, after acidification, were co-extracted together with the (7-methoxy-4-coumarinyl) methyl ester derivatives of SV and DHISV, most of the pigments could be removed by the purification step on silica gel columns. It was also possible to further purify the esters by TLC [18].

Methods of Analysis in Food: General Guidelines

For the time being, all SVgly measurements have been done by an external standard method. Work is in progress to further improve the analyses by inter-laboratory analyses and by searching for a good internal standard. This will simplify the quantitative aspect of the analyses.

Methods

- **Steviol glycosides:**

Each day, a calibrated sample should be injected for quantitative work (external standard method). As the analyses are based on an external standard method, all the volumes used have to be carefully controlled by weighing.

- **Steviol**

To 1 g of sample, 4 µg internal standard (DIHSV) was added and then the sample was extracted with 2 N KOH and analyzed as described above, following the method of Minne et al. [18].

Steviol equivalents can also be measured with an IS method after carefully hydrolysing the food. To demonstrate the specificity of the developed method we compared a HPLC chromatogram of low-fat milk sample spiked with SVglys and an unspiked low-fat milk sample (Figure 7). Samples were incubated with 2.5 % H_2SO_4 at 80°C for 15 h and were subsequently derivative as described in Materials and Methods. No interfering peaks were observed in the unspiked milk sample. The chromatogram of the spiked milk sample showed only one extra peak. This peak had the same retention time as derivatised ISV standard and was well separated from the internal standard peak. Based on these findings we can conclude that this method is specific for a post-derivative analysis of ISV, formed by acid hydrolysis of SVglys, using DHISV as internal standard after Bartolomees et al. [19].

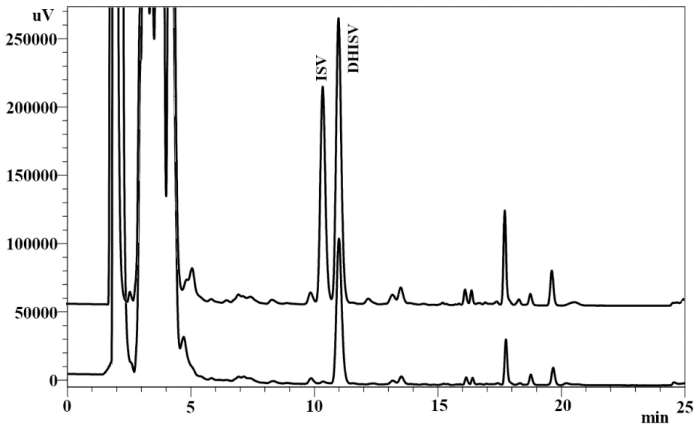


Figure 7: High performance liquid chromatographic analysis with fluorescence detection of a spiked (upper) and unspiked (lower) low-fat milk sample.

Analysis of Beverages Without Alcohol

- **Analysis of Steviol Glycosides**

- Depending on the percentage of sugar replaced, and depending upon the beverage itself, the amounts of steviol equivalents of sweeteners found may vary between 4 and 16 mg/100 ml.

- Centrifuge (of filtrate) 50 ml to remove possible particles.

- Take 3 samples of 10 ml each, and run the sample over a 1 ml bed volume preconditioned C_{18} cartridge (precondition: rinse cartridge with MeOH (5 ml), followed by 10 ml HPLC quality water).

- Elute the polar compounds with HPLC water (10 ml) and discard (this fraction contains sugars and salts).

- Rinse the SPE columns with 20% acetonitrile in water (5 ml), and discard.

- Elute the SVglys with 50% acetonitrile in water (5 ml). This fraction can be injected (eg. 10 µl) on to the HPLC column after filtration on a 0.2 µm filter. If necessary, the fraction may be concentrated by evaporation under nitrogen at about 80°C and the residue dissolved in a known volume of water (slightly heat in closed vessels to dissolve).

- Run the HPLC and calculate amounts, taking into consideration the different molecular weights of the SVglys (for conversion factors, see Table 2).

- After the previous step, the elution of the C_{18} SPE columns with 5 ml 0.1% acetic acid in methanol will elute free steviol (if present at all).

- **Analysis of Steviol:** Use the methods as described by Minne et al. [18].

Beverages Containing Alcohol

Methods are similar to a). However, make sure to dilute the sample to an alcohol percentage below 20% to enable the steviol glycosides to bind quantitatively to the SPE columns.

Extraction of Complex Foods

- **Steviol glycosides**

- A sample (2 g) is weighed exactly and mixed in water (30 ml). Boil for 10 min in closed Falcon tubes. Centrifuge and collect the water fraction. Repeat the extraction of the residue 3 times. In this way, the 2 g of food will have been extracted with 120 ml water. Combine the water fractions and centrifuge (or filter).

- Take 3 samples (20 ml each) and concentrate and analyses the steviol glycosides on C_{18} SPE cartridges as described in a).

As steviol glycosides are very stable under neutral conditions, they remain unchanged for several years and can be extracted again after more than 3 y, as demonstrated by the extraction of beer containing stevioside [18].

- **Steviol**

Extract and analyses 2 g DW with 2N KOH as described above.

Conclusion

The external standard method presented can be used to analyse mixtures of commercial SVglys, as well plant material or different foods. Care should be taken to obtain a base-line separation of the critical pair RebA and ST. This is required for obtaining accurate peak area integration. All the solvents used should be of top quality and the standard for calibration should be >99 % purity and dried to a constant weight before each use and sufficient material should be weighed to avoid weighing errors. It has been shown that the use of 1 standard is acceptable if the calibration curves are made in mM concentrations. All volumes should be carefully checked and it is advisable to weigh all products and solvents to minimize errors. As it is an external standard method, calibration of the HPLC should be done on a daily basis. Care should be taken in purification processes, e.g., by SPE columns as there is no control on possible losses. In a next step, inter-laboratory analyses will be done to exchange basic information between the participants and to improve SVglys analyses.

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