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EUSTAS round-robin testing of steviol glycosides using an internal standard method

Jan M. C. Geuns[1,*] and Tom Struyf[2]

[1]Laboratory of Functional Biology, KU Leuven; [2]Laboratory of Molecular Plant Biology, Kasteelpark Arenberg 31, B-3001 Heverlee-Leuven, Belgium.

ABSTRACT

Our aim is to develop the best and cheapest methods for steviol glycoside (SVgly) analysis so that even small laboratories as well as food inspectors can easily check the purity of the steviol glycosides. The internal standard (IS) method is the best method for the analysis of samples. The 19-*O*-β-D-galactopyranosyl-13-*O*-β-D-glucopyranosyl-steviol was used as an IS, that was previously synthesised and the method was validated. The calibration curves can be easily reproduced by other laboratories and the analyses are very much simplified. The possible errors are much reduced by the IS method compared to the external standard method. The inter-laboratory relative standard deviation (RSD) for the analysis of all the SVglys present was about 1.75%, which is much better than what can be obtained by an external standard method. This value might still decrease after improvement of peak resolution and peak integration techniques in some laboratories. Indeed, the RSD found with the results of the best scoring laboratories was only 0.8%, but to be objective, we had to include all the results obtained. Our method made it possible to inject 5 times more of the same sample, resulting in a more accurate measurement of the small peaks, enhancing overall accuracy without the need of making new calibration curves. In the final protocol that was developed, all possible errors were excluded except for two: the drying and weighing of the analyte. In some laboratories, peak resolution and integration should be improved to increase the precision and accuracy of the analysis. A good chemist and/or company has to be found that can synthesise the IS in large quantities and can make calibration mixtures as well as vials with pre-weighed amounts of IS. This way, the only labour to be done for a SVgly analysis is the drying and weighing of an analyte.

KEYWORDS: analysis, steviol glycosides, internal standard method, HPLC, round-robin testing.

1. Introduction

In Europe and many other regions of the world, the purity of steviol glycosides has to be at least 95% on a dry wt. basis. This requires very accurate techniques and the inter-laboratory RSD should be as small as possible and preferably below 1%. Moreover, the accuracy of the method should be outstanding. The correct analysis of SVglys is a great challenge to the analytical chemist [1-3].

In previous round-robin testings of steviol glycosides, an external standard method was used and suggestions were given to improve the analysis. An external standard method implies that each participating laboratory needs to have very pure standards for the daily calibration of the High Performance Liquid Chromatograph (HPLC). Each step in the analysis needs to be carefully checked and many steps can have an influence on the quality of the analysis [4].

The internal standard (IS) method is the best method for the analysis of samples, as it is independent of errors in injection volume, changes in sample volumes, changes in sensitivity of the detector, etc.

*Corresponding author: Jan.Geuns@bio.kuleuven.be

Moreover, use of an internal standard automatically corrects for losses due to sample clean-up of complex samples (e.g., after extraction of foods). An ideal IS is a compound with properties very similar to, and that behaves in a similar way as the compounds to be analysed. Ideally, only in the last step of analysis (HPLC), should the IS be well separated from the compounds of the mixture to be analysed. We used the 19-*O*-β-D-galactopyranosyl-13-*O*-β-D-glucopyranosyl-steviol as an IS. This is the 19-galactosyl ester of steviolmonoside (13-*O*-β-D-glucopyranosyl-steviol) [5]. The testings were done in 2 stages: stage 1 was mainly intended to check the possibilities of reproducing the calibration curves in the different laboratories and to perform the analysis of 1 sample. The information collected from stage 1 was then used in stage 2 to write a protocol for the analyses in the different laboratories.

2. Stage 1: First round-robin testing with an IS

2.1. Aims of the first round-robin testing with an IS

The aims of the first round-robin testing with IS were to check if all the laboratories could reproduce the calibration curves, and to analyse 1 sample by the IS method. We also wanted to learn from this testing to improve the methods for further analyses. All the preparations were done by the Laboratory of Functional Biology, KU Leuven, Belgium.

2.2. Materials and Methods

2.2.1. Solvents and products

Solvents and water used were of HPLC quality. Other products were of Pro Analysis (PA) grade. Standards were crystallized to > 99% purity [6].

2.2.2. The internal standard (IS)

The IS (19-*O*-β-D-galactopyranosyl-13-*O*-β-D-glucopyranosyl-steviol) was made according to [7] (for details: see [5]). Each participating laboratory received samples of calibration mixtures with IS already added and dissolved, as well as two tubes containing 0.125 mg IS to which exactly 1 g of a sample solution had to be added.

2.2.3. Analytical HPLC of steviol glycosides and IS

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 acetonitrile (AcCN): 1 mM H_3PO_4 gradient (0–2 min: 34% AcCN; 2–10 min: 34 % → 42%; 10 - 16 min: 42 %; 16.1 min–25 min: 34%; 25 min: stop). UV-detection was at 200 nm (Shimadzu, SPD-6A). The injection volume was 20 µL.

Note: *After injection of about 500 samples, the columns might slightly deteriorate. To still obtain a good baseline separation of RebA and ST, the gradient is then started with 32% AcCN instead of 34%. Columns can also be rinsed with different solvents like hexane, acetonitrile, or methanol.*

2.2.4. Use of the IS

In a previous paper, it has been proved that the IS was well separated from the other steviol glycosides and that there were no interfering components present in the mixture without IS added. It was also proved that the peak ratios of SVglys over IS were constant before and after a Solid Phase Extraction (SPE) purification step, proving that SVglys and IS behaved in the same way and that there were no problems in using a purification step in the quantification of SVglys.

2.2.5. Calibration curves

The following dried and very pure standards (> 99% purity) were used to make calibration curves: RebA, ST, RebB and SB. The concentrations of RebA and ST varied between 0.032 and 0.517 mM and those of RebB and SB between 0.0162 and 0.259 mM, respectively. The concentration of the IS was fixed at 0.125 mg/mL (ethanol:water v/v; 50:50) and all dilutions were made with this concentration of IS. Each calibration curve was made using 5 different concentrations of standards. As RebB and SB normally occur in much smaller amounts, the calibration curves were made with smaller concentrations for these 2 compounds. These 4 standards are good model compounds for the different SVglys: 2 of them are neutral (RebA, ST) and 2 contain a carboxylic function (RebB and SB). Previously, it has been shown that all SVglys have similar molar extinction coefficients [8, 9].

An example of the separation of the standards and the internal standard is given in Figure 1. The fluctuation in the baseline between 5.1 and 7.5 min is due to the ethanol present in the solvent.

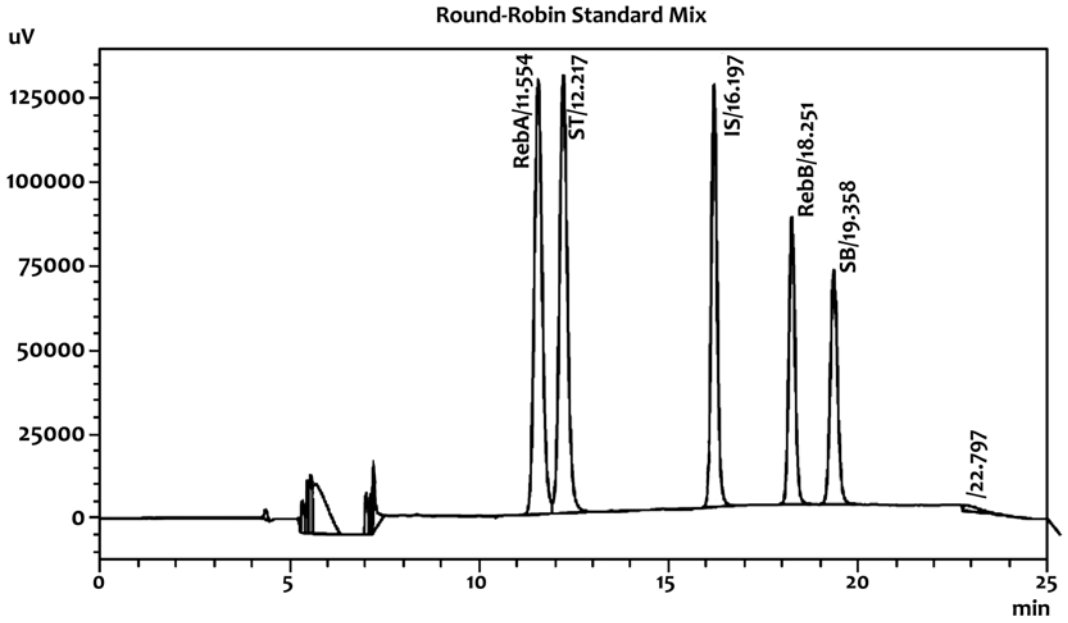

Figure 1. Order of elution of the different standards and the internal standard (RebA: at ± 11.5 min; stevioside: ± 12.2 min; IS: ± 16.2 min; RebB: ± 18.2 min; SB: ± 19.4 min).

The calibration plots were forced through zero and gave straight lines with $R^2 > 0.998$ [5]. The averaged trend line equations are y = 1.76x and y = 1.75x for RebA and ST, respectively. There is almost no difference between these two equations, as the SVgly concentrations are plotted as a function of their mM concentration. It has been shown earlier that the extinction coefficients of all SVglys are very similar; hence very similar calibration curves can be expected [9].

2.2.6. Exchange of calibration curves between laboratories

Is it possible to make calibration curves in one laboratory that can be used all over the world? If the answer is yes, the problem of making calibration mixtures in each laboratory, from now on, is no longer necessary. One important issue to test is the possible change of the SVgly over IS ratio by changing the wavelength of the detector. Therefore, measurements were done at 190, 200 and 210 nm.

There are small differences between the ratios of peak areas (compound/IS) when the measurements are done at 190, 200 or 210 nm (Table 1). This means that results will be slightly influenced by different wavelengths used in the participating laboratories. To evaluate the effect of the wavelength on the calibration curves, we analysed the calibration curves at 190, 200 and 210 nm (Tables 1 and 2).

For each compound analysed, the peak ratios slightly increased from 190 to 200 and 210 nm. The slopes of the calibration curves show better the changes at the different wavelengths (Table 2; Figure 2). The neutral SVglys ST and RebA behave in about the same way, and the slopes increase with increasing wavelength from 100% (190 nm) to about 106% (210 nm). The acid compounds RebB and SB both have a similar but much larger change in slopes from 100% (190 nm) to about 112% at 210 nm. This experiment shows that the extinction coefficients are slightly different at different wavelengths. Therefore, the wavelength of the detector should be well calibrated if calibraton curves are to be exchanged.

Analysis of SVglys at the smallest wavelength (190 nm) increases the sensitivity of the detector or the area of the peaks by about 3 times [9].

Note: *In the following round-robin testing, different calibration curves will be obtained as the amount of IS used will be different. To improve the accuracy, all the solutions were made on a weight basis too.*

Table 1. Measurements of the ratios of peak areas (compound/IS) when the analyses are done at 190, 200 or 210 nm.

UV	Conc. mM	0.517	0.2585	0.1292	0.0646	0.0321	0.0162
190 nm	ST	2.708	1.347	0.676	0.339	0.171	-
	RebA	2.708	1.355	0.681	0.344	0.174	-
	RebB	-	1.306	0.652	0.326	0.163	0.081
	SB	-	1.263	0.634	0.322	0.163	0.084
200 nm	ST	2.775	1.393	0.699	0.351	0.196	-
	RebA	2.758	1.381	0.691	0.347	0.177	-
	RebB	-	1.374	0.685	0.342	0.172	0.086
	SB	-	1.322	0.661	0.331	0.166	0.084
210 nm	ST	2.874	1.459	0.736	0.372	0.191	-
	RebA	2.845	1.445	0.728	0.367	0.186	-
	RebB	-	1.446	0.742	0.371	0.186	0.093
	SB	-	1.408	0.711	0.356	0.180	0.090

Table 2. Slopes (m) of the calibration curves measured at different wavelengths (190, 200 or 210 nm).

UV	ST	Reb A	Reb B	SB
190 nm	5.233 (100%)	5.242 (100%)	5.05 (100%)	4.895 (100%)
200 nm	5.376 (102.7%)	5.337 (101.8%)	5.31 (105.1%)	5.115 (104.5%)
210 nm	5.584 (106.7%)	5.528 (105.5%)	5.689 (112.7%)	5.462 (111.6%)

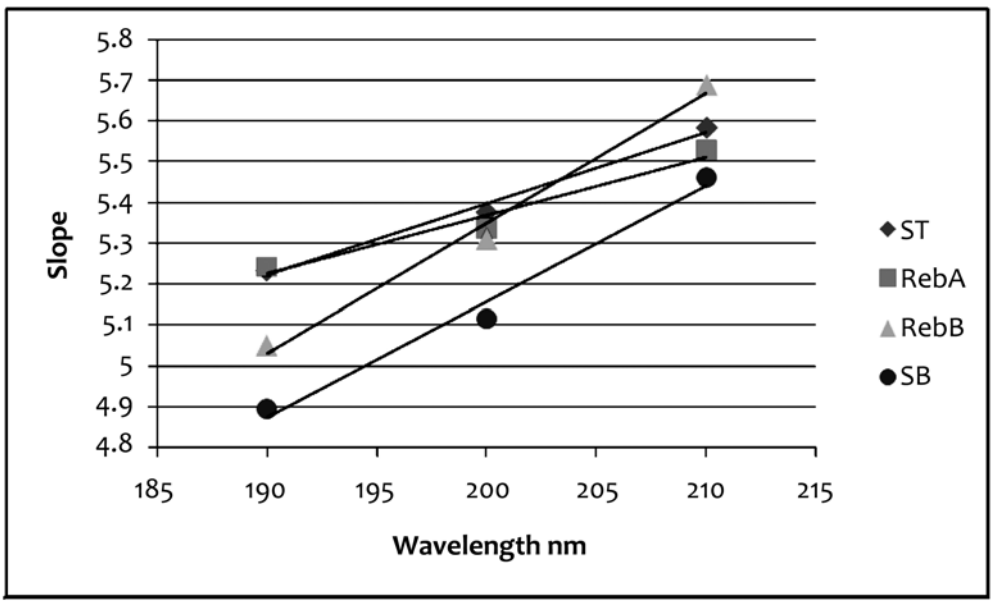

Figure 2. Changes of the slopes for different SVgly measured at different wavelengths.

2.3. Analysis to be done by the participating laboratories

In the first round-robin testing, 24 laboratories participated. However, only 11 sent their results in due time. It was checked whether all the participating laboratories can reproduce the calibration curves with the following standards added: RebA, ST, RebB, SB, and of course, the IS. Thereafter, each laboratory had to analyse 1 sample by the IS method. The analysis also included the drying of the sample.

2.4. Results

2.4.1. Control of calibration curves with IS and analysis of 1 sample

In this round-robin testing, different vials containing samples to be tested were sent to the participating laboratories.

- Vials 1-5: solutions of calibration curves already containing the IS, all dissolved in ethanol/water (50:50).
- Vials 6-7: 0.125 mg IS (completely dried).
- Vial 8: unknown mixture of SVglys with an unknown water content.

2.4.1.1. Control of calibration curves

Vials 1-5 contained different concentrations of calibration mixtures (mM) of 4 SVgly standards as well as IS (0.125 mg/mL). The solvent is ethanol:water (50:50, v/v).

Table 3 gives the HPLC conditions used in the different participating laboratories. Most of them used apolar, mostly C_{18}-based columns.

The concentrations of the standards were given when the samples were sent to the participants (Table 4).

All the participants needed to do were: inject each calibration mixture twice in the HPLC (preferably with C_{18} columns), identify all the peaks and measure the peak areas of all peaks. They had to add these

Table 3. HPLC conditions used in the different laboratories.

Lab #	Column	Particle size	UV detector (wavelength)
1	Phenomenex Luna C_{18}	5 µM	UV 200 nm
5	2 x ODS Hypersil	5 µM	UV 200 nm
6	2 x Grace Alltima C_{18}	5 µM	UV 200 nm
9	?	?	UV 210 nm
15	2 x Grace Alltima C_{18}	5 µM	UV 200 nm
18	2 x Grace Alltima C_{18}	5 µM	UV 200 nm
19	2 x Grace Alltima C_{18}	5 µM	UV 200 nm
20	2 x Luna C_{18}	5 µM	UV 210 nm
21	Nucleodur HILIC 125x2 mm	3 µM	UV 210 nm
24	2 x Grace Alltima C_{18}	5 µM	UV 200 nm

Table 4. Concentration of IS (in mg/mL) and of the 4 standards (in mM).

Vial #	IS (mg/mL)	RebA	ST	SB	RebB
C1	0.125	0.517 mM	0.517 mM	0.259 mM	0.259mM
C2	0.125	0.259 mM	0.259 mM	0.130mM	0.130 mM
C3	0.125	0.130 mM	0.130 mM	0.065 mM	0.065 mM
C4	0.125	0.065 mM	0.065 mM	0.032 mM	0.032 mM
C5	0.125	0.032 mM	0.032 mM	0.016 mM	0.016 mM

values in a numbered and protected spreadsheet. The spreadsheet automatically plotted the ratios of standard over IS against the mM concentration of the standards. The trend line was fitted through zero and the trend line equations of the different standards were automatically calculated and printed on the graph. The results of the calibration curves obtained by the different laboratories are given in Table 5.

Nearly all laboratories were able to reproduce the calibration curves on their own HPLC system. The slopes of the calibration curves, plotted as the ratios of peak areas of standard over that of the IS against the mM concentration of the standards, are about the same for all the different SVglys. Laboratories 1, 5, 6, 15, 18, 19 and 24 measured at 200 nm. The averages of these laboratories were 5.364 ± 0.044, 5.343 ± 0.088, 5.399 ± 0.092, 5.213 ± 0.08 and 5.329 ± 0.057 for ST, RebA, RebB, SB and the average, respectively. The SD was small (0.057), an indication that the precision of the calibration curves was outstanding. The RSD

Table 5. Results of the calibration curves (y = m . x) plotted as ratios of the peak areas of standard over IS against the mM concentrations of the standards. The values given are the slopes (m) and the correlation values of the trend lines (R^2) forced through zero.

Lab #	ST	RebA	RebB	SB	Avg
1	5.3428	5.3191	5.3205	5.2974	5.32
R^2	1	1	1	0.8137	
5	5.2778	5.1873	5.3656	5.1907	5.2554
R^2	0.9986	0.9991	0.9994	0.9995	
6	5.376	5.3947	5.4646	5.2353	5.3677
R^2	0.9999	0.9998	1	1	
9	5.771	5.757	5.813	5.564	5.726
R^2	0.9991	0.9993	0.9994	0.9995	
15	5.3838	5.3165	5.4832	5.245	5.3568
R^2	1	0.9999	0.9997	1	
18	5.3842	5.3741	5.3495	5.1112	5.3048
R^2	0.9995	0.9999	1	1	
19	5.4147	5.4597	5.3584	5.1874	5.3551
R^2	1	1	0.9999	0.9999	
21*	5.4705	5.4703	5.5738	5.7874	5.5754
R^2	0.9993	0.9987	0.9989	0.9984	
22	5.7687	5.7982	5.9626	5.5789	5.777
R^2	0.9999	1	0.9999	0.9998	
23**	5.5316	5.6226	5.6269	5.4384	5.5549
R^2	1	1	1	.9999	
24	5.344	5.346	5.450	5.227	5.342
R^2	0.9999	0.9998	1	1	
Avg***	5.361	5.343	5.399	5.213	5.329
SD	0.044	0.088	0.092	0.08	0.057
RSD	0.82	1.65	1.70	1.53	1.08

*HILIC column; **:“hacked” spreadsheet; ***:average of laboratories measuring at 200 nm (1, 5, 6, 15, 18, 19, 24).

was only 1.08%. The RSDs of this inter-laboratory comparison are acceptable, as there was no control on all the parameters of the analysis. Laboratories 20 and 21 measured at 210 nm, but some laboratories did not report on this. Laboratory 21 used HILIC columns giving totally different separations. Therefore, the slopes of the calibration cannot be compared, but they are in the same order of magnitude.

Previously, it was shown that the extinction coefficients of the different SVglys were very similar and this explains the similarity of all the slopes. All participants also obtained very good correlation coefficients (R^2) of the trend lines (most of them above 0.999). The good reproducibility of the calibration curves in the different laboratories proves that it is possible to make calibration curves in one laboratory and to use these in other laboratories world-wide. Of course, all the laboratories should then use the same amount of IS (0.125 mg/vial) and measure the samples at the same calibrated wavelength (e.g., 200 nm). One laboratory (23) hacked the protected spreadsheet and therefore their results cannot be used in this study.

2.4.1.2. Measurement of water content of the mixture of steviol glycosides (vial 8)

Participants were asked to weigh 500 mg of vial 8 (unknown mixture of SVglys with an unknown water content) in a weighing vessel and to place the opened vessel in an oven at 105 °C. The lid should also be put in the oven and the sample should be dried to a constant weight or just overnight for about 16 h. The moisture content (loss of weight) had to be calculated as a percentage. This dried sample was not used for further analysis as degradation products of impurities might give extra peaks (see [9]). The results of the water content of the unknown sample are reported in Table 6.

Most of the laboratories have dried the sample properly and found moisture content of about 4%. One participant kept the dried sample in a desiccator for about 6 h and observed that the sample absorbed water again. Therefore, the protocol below now mentions that the dried samples should be weighed immediately after cooling to room temperature.

Table 6. Water content in the unknown sample as reported by the different participating laboratories.

Laboratory #	Amount before drying (mg)	Amount after drying (mg)	Weight Loss %
1	485.5	464.6	4.23
5	497.3	475.2	4.44
6	501.9	476.5	5.10
9	510.2	490.8	3.8
15	501.6	483.9	3.53
18	507	489.2	3.51
19	500.5	482.2	3.59
22	503.4	482.1	4.2
23	500	479.6	4.1
24	501.6	483.9	3.5
Average			4 ± 0.16

2.4.2. Analysis of 1 unknown sample

Participants were asked to weigh exactly 60 mg of the unknown mixture (vial 8) in a Falcon tube, to which 39.940 g of HPLC quality water must be added. All solutions were made on a weight basis, as this avoids errors due to possibly non-calibrated pipettes and solvent expansion at different temperatures. It is important to check that all SVglys are well dissolved. Subsequently, exactly 1 g of this solution must be added to vial 6 (or 7 in case the analysis will be repeated) containing 0.125 mg of IS. Heat the vial in a water bath at 40 °C or use sonication to be sure that the IS completely dissolves. Alternatively, about 0.1 mL of ethanol or methanol can be added to the vial with IS to easily dissolve the IS (important note: check first the quality of the ethanol or methanol used to avoid peaks of impurities!). After adding 1 g of solution of the unknown sample, thoroughly mix and inject 20 µL in the HPLC. Adding a small amount of alcohol does not influence the final result as the calculations in the IS method are done by peak area ratios.

Inject the unknown sample 6 times and calculate the relative standard deviation (RSD). This should be below 1 for the large peaks. The concentration of the IS was chosen in such a way, that the same sample can be used to inject, e.g., 5 times more for a better analysis of the smaller peaks present. Of course, the large peaks cannot be measured then, but analysis of the smaller peaks will be much better. Figure 3 shows the analysis of the unknown mixture to which IS was added.

Calculate the peak area ratios of the different compounds over that of the IS and use the calibration curves made under 1) to calculate the amounts in mM of SVgly present. Calculate the mmoles present in 40 g and convert the mmoles into mg steviol glycosides by using Table 7. Correct the results for the water content obtained in 2). Report all your results in the spreadsheet given and send it back ASAP. Those people who want to calculate the amount of steviol equivalents can use the last column of Table 7 to do this.

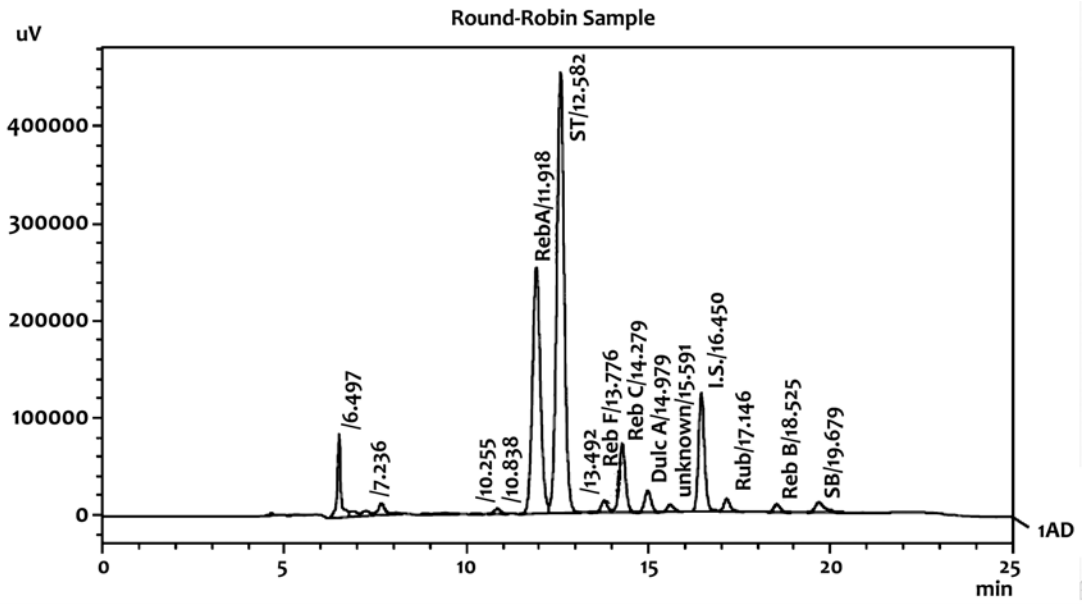

Figure 3. Example of the analysis of the unknown sample after the addition of IS. RebD: 7.236; RebE: 10.838; RebA: 11.918 min; stevioside: 12.582; RebF: 13.776; RebC: 14.279; DulA: 14.979; unknown: 15.991; IS: 16.450; rubusoside: 17.146; RebB: 18.525; steviolbioside: 19.679.

Table 7. Molecular masses (averages of all isotopes) and conversion factors to convert mg-amounts of SVgly into mg SVeq.

To obtain the steviol equivalent of	Formula	Molecular weight Avg of all isotopes	Multiply the amount by:
Stevioside	$C_{38}H_{60}O_{18}$	804.88	0.396
Rebaudioside A	$C_{44}H_{70}O_{23}$	967.02	0.329
Rebaudioside C	$C_{44}H_{70}O_{22}$	951.02	0.335
Dulcoside A:	$C_{38}H_{60}O_{17}$	788.88	0.404
Rebaudioside G	$C_{38}H_{60}O_{18}$	804.88	0.396
Rubusoside	$C_{32}H_{50}O_{13}$	642.74	0.495
Steviolbioside	$C_{32}H_{50}O_{13}$	642.74	0.495
Rebaudioside B	$C_{38}H_{60}O_{18}$	804.88	0.396
Rebaudioside D	$C_{50}H_{80}O_{28}$	1129.16	0.282
Rebaudioside E	$C_{44}H_{70}O_{23}$	967.02	0.329
Rebaudioside F	$C_{43}H_{68}O_{22}$	937.00	0.340

Table 8 shows the results of the unknown sample. The values are given in mg/g of solution (values of laboratory 9 are not considered as they are extremely small (reason not known). Most of the laboratories reported the presence of at least 8 compounds present in the unknown mixture. Many laboratories did not report the amounts of RebE and D, as the quantities in the sample were very small, and possibly no reference compounds were present in the laboratory.

Table 8. Quantitative analysis of the unknown sample analysed by the different laboratories. Values are corrected for different molecular masses and for water content of the unknown sample. Values are given in mg/g solution.

Lab #	RebD	RebE	RebA	ST	RebF	RebC	DulA	Rub	RebB	SB
1		.0156	.518	.732	.020	.097	.0255	.0147	.013	.013
5			.477	.660	.0159	.093	.0285	.0147	.011	.0147
6	.016	.0094	.489	.679	.0183	.096	.0288	.0138	.011	.0168
9	*.022*		*.423*	*.583*	*.0184*	*.079*	*.0203*	*.0101*	*.008*	*.0115*
15	0	0	.500	.685	.018	.096	.029	.014	.011	.017
18	0	0	.503	.689	.019	.095	.029	.013	.011	.016
19	.018	0	.522	.727	.015	.095	.031	.012	.011	.014
22	.019		.505	.699	.018	.085	.023	.009	.010	.015
23		.01	.527	.754	.017	.091	.028	.013	.011	.015
24			.498	.689	.018	.098	.0294	.0141	.011	.0154
Avg	.018	.0117	.504	.702	.018	.094	.028	.013	.011	.015
SD	.001	.0034	.016	.03	.001	.004	.002	.002	.001	.001
RSD			3.19	4.26	8.63	4.15	8.47	13.57	7.04	8.41

Table 9. Reported purities of the unknown sample.

Lab #	Corrected conc. in mg/mL	Total SVglys in mg/g	Purity in %	Value to add	Corrected purity	Percentage
1	1.483	1.511	101.9	0.01615	103.0	106.6
5	1.433	1.314	91.7	0.0255	93.5	96.8
6	1.426	1.378	96.6	0	96.6	**100**
9	*1.445*	*1.175*	*81.3*	*0.0094*	*82.0*	*84.9*
15	1.454	1.370	94.3	0.0255	96.0	**99.3**
18	1.447	1.374	94.9	0.0255	96.7	**100.1**
19	1.447	1.447	100	0.0094	100.6	104.1
20			95.6	0.0094	96.4	**99.8**
22	1.4496	1.3798	95.2	0.0094	95.8	**99.2**
23	1.441	1.466	101.7	0.016	102.8	106.4
24	1.4416	1.373	95.2	0.0255	97.0	**100.4**
Avg	1.448	1.393	96.2		97.4	
SD	0.017	0.059	3.265		2.99	
RSD	1.172	4.269	3.393		3.07	

From Table 8, the total purity of the unknown sample could be calculated, and most of the laboratories reported a purity of about 96.2% (Table 9). The values of laboratory 9 (in italics) were not considered, as very small values were reported (the reason for this not known).

As not all laboratories reported values for RebD and E, a correction was made by adding the values obtained by laboratory 6 for these values (0.016153 and 0.0094 for RebD and E, respectively) to column 3 of Table 9. The "corrected purity" was than calculated and is given in the last column of Table 9 (97.4 ± 2.99, RSD: 3.07). After making this correction, the purity obtained for most of the laboratories is very near to the value of 96.6 which is considered the correct value as evidenced by the standard addition method as done before [5]. Six laboratories out of 11 found a purity which differed only between 0.1 to 0.8% from the value to be found, which is an excellent result (printed in bold). If the purity of 96.6% is considered 100%, the average of these laboratories is 99.8 ± 0.47 and the RSD is 0.47%, an excellent result which will be difficult to improve further, as this is an inter-laboratory comparison.

2.5. General conclusion of the first round-robin testing with IS

This round-robin testing using the IS method revealed that it is possible to reproduce the calibration curves in most of the laboratories using the same or similar reversed phase HPLC columns. This simplifies the analysis of steviol glycosides as, once good calibration curves are made in one laboratory, the mixture can be used in all laboratories world-wide. This is because the method is based on the peak ratios of standards over the IS. Moreover, for the same reason, it is not necessary to daily calibrate the HPLC. The method is also independent of the type or the sensitivity of the UV detector used. Errors due to changes in injection volume, failure of the equipment or to evaporation of solvent, belong to the past. To better dissolve all the SVglys and to prevent precipitation of analyte, it is possible to add a compatible solvent (ethanol, methanol) as evaporation of part of this solvent does not influence the final results. To improve the quantification of smaller peaks of unknown samples, the amount of IS was chosen in such a way that after a normal injection, larger amounts can be injected to measure the smaller peaks. This will improve the RSD of smaller peaks and it will not require additional calibrations.

3. Stage 2: Fine-tuning of the IS method

The results of the above round-robin testing were used to fine-tune the methods and to advise people about the analysis of SVglys. Items to be considered in stage 2:

- The drying process should be better described in the protocol.
- It has been shown that sending solutions to the participating laboratories is not ideal as there is no control on the temperature conditions of transport and some compounds may suffer from degradation, e.g. by radiation in the airport when scanning parcels.
- A weak point was also the preparation of vials containing exactly 0.125 mg/vial. Small errors in pipetting might have a huge influence on total amounts found (although most laboratories reported good values). If pipetting of IS is required, this should be done in syringes with the volume to be delivered trapped between 2 air bubbles (cfr. methods for quantitative injection in GC). This way, the exact volume can be checked and the syringe is rinsed with solvent that was first sucked into the syringe.
- To avoid spilling of IS and to further facilitate the analysis of SVglys, it is advisable to prepare a rather large amount of IS solution and to evaporate 1 ml fractions in different tubes, producing a stock of vials each containing exactly 0.125 mg of IS. When a new analysis has to be done, the analyte can then be dissolved in water and 1 g can be added to a vial with IS. As it is difficult to add exactly 1 g, the spreadsheet will be adapted, allowing a correction for slightly different weights of added SVgly solution.
- The protocol should be sufficiently detailed for each step in the analysis to avoid differences in methodology between the participating laboratories.

Before starting the analysis described below, people should carefully study the text as well as the protected spreadsheet. Once everybody understands the meaning of each step, it should be possible to obtain a very small RSD between the labs. We dream of a

value as small as possible, although a value of 1–2 % would already be acceptable as an inter-laboratory variance, certainly if all 10 steviol glycosides are being measured.

3.1. Protocol and round-robin testing of steviol glycosides by an internal standard method

In Table 10, different methods for analysis of steviol glycosides are compared with an indication of possible errors in different methods (non-exhaustive; given as +). A minus means that no errors are to be expected. The external standard method is given and compared with a normal IS method and with the EUSTAS protocol in which each step has been validated (validated calibration mixtures, validated vials with IS).

3.1.1. Description of the protocol

The participants received a vial with a dried validated calibration mixture and 2 vials with IS,

Table 10. Possible errors (+) in different methods for measurement of steviol glycosides.

Item	External standard	Internal standard	EUSTAS protocol
Standard itself			
Purity of standard	+	+	-
Water content of standard	+	+	-
Weighing process of standard	+	+	-
Calibration solution of standard	+	+	- (only 1 injection)
Analyte			
Drying process	+	+	+
Weighing	+	+	+
Analysis			
Injection volume standard is critical	+	-	-
Change of sensitivity of detector	+	-	-
Dissolution analyte	+	**(+)** co-solvent	-
Peak integration	+	+	+
Other issues			
Expansion of solvent	+	-	-
Inaccuracy of pipettes	+	-	-
Changes sample volume	+	-	-
Precipitation of analyte	+	- co-solvent	- co-solvent
Injection volume critical	+	-	-
Change of sensitivity detector	+	-	-
Daily calibration necessary	+	-	-
Costs of calibration standard	+	+	-
Calculation errors possible	+	+	-
Small peaks	+	-	-
Injecting 5x more	+ new calibration	-	-
New solution analyte (5x more)	+	-	-
Dissolution/precipitation	+	- solvents possible	- solvents possible
Co-solvent required/evaporation	+	-	-
Sample clean-up (e.g. food analysis)	+	-	-
Intra-lab RSD (10 components)	+	-	-
Inter-lab RSD (10 components)	+	**(+)**	-
Stress factor personnel	+	**(+)**	-

as well as an unknown sample to be analyzed (Figure 4). The second vial of IS was a "back-up" for possible mistakes when doing the analysis for the first time.

The unknown sample is a very interesting one as it shows that the method is also suitable even when a small unknown peak occurs at the position of the IS (See Figure 5). Moreover, a clear peak of RebD is present. Unfortunately, a good RebE peak is not present, but this is compensated for by the presence of RebG. The peak of DulA shows a shoulder, and this enables us to pay attention to the integration of this peak, although the result will not much influence the total purity of the sample as it concerns only a very small peak. We have chosen the amount of IS to allow the injection

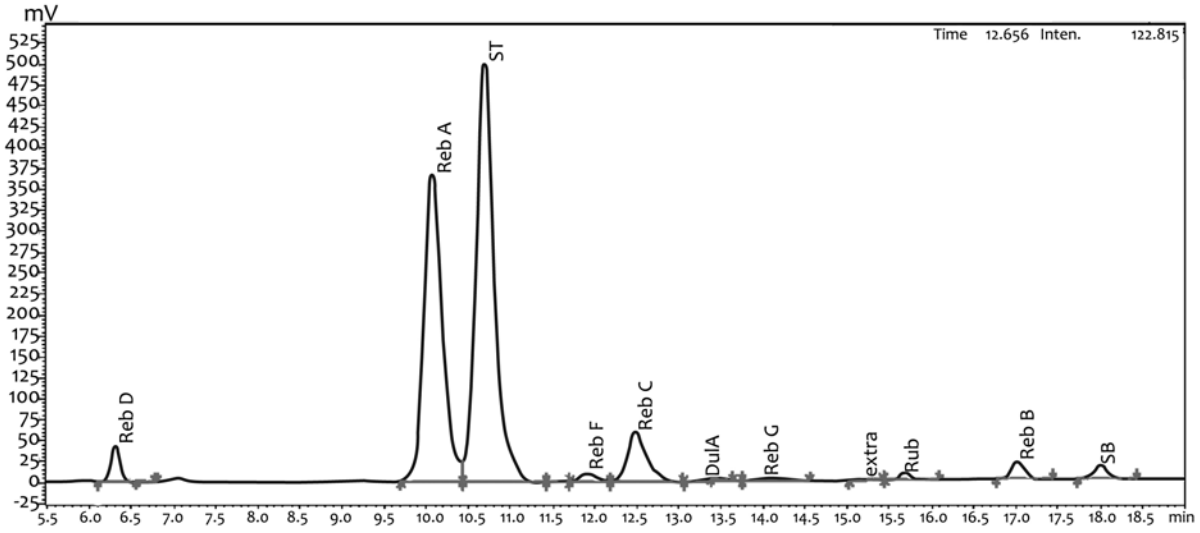

Figure 4. HPLC trace of an unknown sample to be analysed. Peaks to be identified and measured: RebD, RebA, ST, RebF, RebC, DulA, RebG, Rub, RebB, SB.

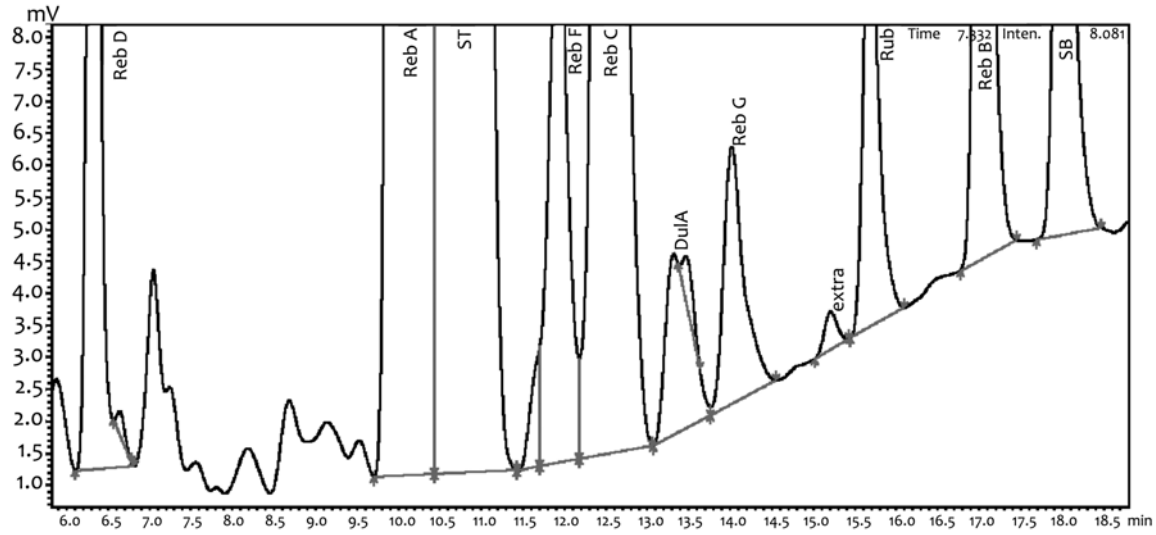

Figure 5. Details of part of the chromatogram shown in Figure 4. The peak eluting after DulA should be considered as a shoulder on DulA because the inclination of the line going up is much slower than of a normal peak. Ahead of the peak of Rub a small peak of an unknown ("extra") occurs (area to be filled in under [1] of the spreadsheet).

of a 5 x larger amount of the same sample to get better RSD of the small peaks (explained in the protocol).

This protocol should give the right purity value for an unknown sample. The accuracy of the method has been tested before by the standard addition method [5].

3.1.2. Definitions

1) Purity = percentage of sum of the 10 authorised steviol glycosides present on dry wt. of mixture.
2) Percentage composition: percentage of each sweetener in the mixture. Meaning of 97% RebA is: of the mixture with a purity of at least 95%, 97% is RebA.

3.1.3. Requirements before a successful analysis can be done

1) A good analytical balance is required with a resolution of at least 0.1 mg. The balance should be calibrated on a regular basis and be placed on a stable balance table, weighing > 100 kg to absorb the energy of vibrations. To reduce the uncertainty and eventually to be able to obtain a RSD value around 1%, amounts of at least 50 mg should be weighed.
2) Vial containing a completely dried calibration mixture, containing 0.125 mg IS, 0.550 µmoles each of RebA and ST, and 0.250 µmoles each of RebB and SB.
3) Vials containing calibrated amounts of IS (0.125 mg/vial).
4) Mixture of steviol glycosides to be analyzed (analyte).
5) HPLC conditions:
   - Reversed phase columns, *e.g.*, 2 Grace Alltima C18 columns in series; each 250 x 4.6 mm, 5 µm particles. Other columns giving a baseline separation of the most critical pair (RebA and ST) can also be used, *e.g.*, Phenomenex Luna; Phenomenex Kinetex UHPLC-column [10].
   - HPLC equipment with the possibility of running solvent gradients.
   - UV detector suitable for use at 200 nm or even at 190 nm and having a small detector cell with a light path of 10 mm.
   - Solvent: gradient of acetonitrile: 1 mM phosphoric acid at 1 mL/min. Conditions: (0–2 min: 34% AcCN; 2–10 min: 34% → 42%; 10–16 min: 42%; 16.1 min–25 min: 34%; 25 min: stop).
   - Use zero-dead-volume connections to avoid peak broadening and to simplify peak integration.

Notes: *(1) The solvent flow to be used is of course dependent upon the column size. (2) After injection of about 500 samples, the columns might slightly deteriorate. To maintain a good baseline separation of RebA and ST, the gradient is then started with 32% AcCN instead of 34%.*

6) Check of the HPLC equipment
   - Start the HPLC and run the gradient to be used without injecting anything. Check the baseline stability.
   - Inject a blank, *i.e.* solvent without sample, to check the quality of the solvent used and the possible changes in the baseline.
   - Inject a sample containing RebA and ST (the calibration mixture can be used for this purpose). Adapt the gradient to obtain a perfect baseline separation between RebA and ST. When using older HPLC equipment, it might be helpful to check for possible dead volumes originating from, *e.g.*, too large tube diameters, too large flow-cells, or lack of zero-dead-volume connections.
   - Always inject a sample (20 µL) of the steviol glycosides to be analyzed before the addition of IS to check the absence of any peaks running ahead of Rub at the place where the IS is supposed to elute. The sample used is the same as prepared under point 5.2.2 below: solution of an analyte (60 mg SVglys/40 g solution).
   - If a small peak of an unknown compound is present just ahead of rubusoside, its area should be introduced on the spreadsheet and the area of the IS will be corrected by deducing this value from the area of the IS [**1**]. This peak is certainly not one of the authorized sweeteners and therefore we can subtract its area from that of the IS.

3.2. Analysis of steviol glycosides using the IS Method

Please do not try to change the protected part of the spreadsheet as this will influence the results obtained.

3.2.1. Water content

Aim: To determine the water content after weighing an exact amount of the analyte of about 500 mg before and after drying to a constant weight (or overnight, 16 h at 105 °C).

Note: *The Karl Fischer method measures water content more precisely. However, this method is not retained as it is expensive. Moreover, the Joint Expert Committee for Food Additives (JECFA) suggested that samples be dried to a constant weight.*

Procedure:

1) Weigh an empty and dry weighing vessel with lid (value **A**).
2) Exactly weigh about 500 mg of the unknown sample of SVglys in the weighing vessel with lid (value **B**).
3) The amount of wet sample is: **C = B – A**. Add this value in the spreadsheet provided [**2**].
4) Dry the opened vessel with wet mixture of the analyte to a constant weight or overnight (16 h at 105 °C; Figure 6). Do not forget to place the lid in the oven to avoid expansion/ contraction problems when cooling down the closed vessel.
5) After the drying period, place the lid on the hot vessel in the oven and allow it to cool in a desiccator for about 15 min.
6) Weigh the vessel with the dried sample immediately after cooling down (value **D**).
7) The dry weight of the unknown sample is **E = D – A** (mg dry wt.). Add this value in the spreadsheet provided [**3**].

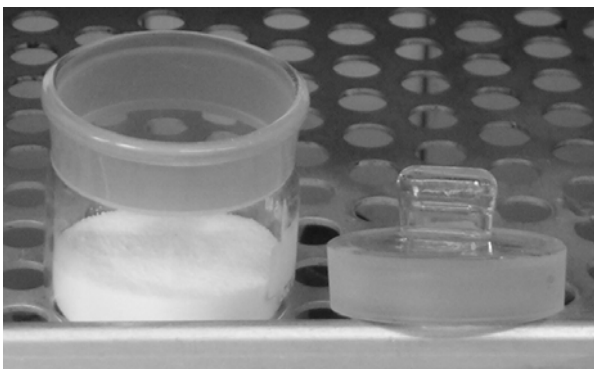

Figure 6. Weighing vessel with lid in the drying oven.

8) The percentage dry weight is: **F = E/C x 100** (times 100 to present it as a percentage) (Automatically calculated in the spreadsheet provided) [**4**].
9) The water content in percentage is: **G = 100 – F** (Automatically calculated in the spreadsheet provided) [**5**].
10) This dried sample is not used anymore for the analysis of SVglys, as during the drying process some impurities might have been degraded giving rise to extra-peaks in the chromatogram. The value of (**F**) is used to correct the analysis of the analyte.

3.2.2. Solution of an analyte

Aim: To make a solution of 60 mg SVglys/40 g solution, corrected for water content.

1) Weigh a clean 50 ml Falcon tube (value **H**).
2) Weigh exactly about 60 mg of wet analyte in the pre-weighed Falcon tube (Value **I**).
3) The exact amount of wet sample is: **J = I – H**. Add this value in the spreadsheet provided [**6**].
4) Add 39.94 g of water (value **K**). The exact amount of added water is: **L = K – I** (in g). Add this value in the spreadsheet provided [**7**]. Close the tube and warm to dissolve the sample. Alternatively, use a sonication bath at 50 °C. After dissolution, store the tube for further use. Cool it down to the laboratory temperature.
5) Calculate the exact concentration per gram solution (mg/g): **M = J/(J + L)** (Automatically done in the spreadsheet provided) [**8**].
6) Correct the solution for the water content of the analyte.
   Corrected concentration **N = M x F /100 (mg/g)** (Automatically done in the spreadsheet provided) [**9**].
7) Thoroughly mix the cooled sample and inject 20 µL of the solution to check the quality of the HPLC analysis (see above) and to check that no peaks occur at the position of the IS (just ahead of Rub). If a peak elutes before that of Rub (see Figure 5), its area should be recorded in the spreadsheet under number [**1**].

3.2.3. Calibration of the HPLC: use of the calibration mixture

1) Add 1 mL of solvent to vial 2 containing the calibration mixture.

Notes: *(1) Water can be used, or ethanol or methanol. Carefully check the purity of the ethanol or methanol used! If alcohol is used, the solvent can be easily evaporated under a flow of nitrogen while heating at 50 °C. In this way, one vial of calibration mixture can be used for at least 1 month. As the calibration is done using the peak ratios, loss of part of the calibration mixture due to several injections is not important. (2) To save calibration mixture, after dissolving the calibration mixture in 1 mL solvent, it can be divided by putting small fractions of 100 µL in inserts used in HPLC injectors. Evaporate the solvent and use the inserts when needed to calibrate the HPLC.*

2) Perform 3 injections of the calibration mixture, each time 20 µL.
3) Record the peak areas and calculate the ratios of area SVgly over area IS. Add the peak areas in the spreadsheet provided [**10**]. Peak ratios are automatically calculated and calibration curves are plotted in the spreadsheet as a function of the mM concentrations. The slopes are also given.
4) Plot the "calibration curves" for the different standards as a function of the mM concentrations.

Note: *In a previous round-robin testing of steviol glycosides using the IS method, all participating laboratories could perfectly reproduce the calibration curves made with 5 concentrations and the trend lines were forced through zero (R^2 > 0.999). When only the IS is injected, no peaks appear at the position of the standards. Calculation of the amounts of steviol glycosides using calibration curves forced through zero or not, did not give significant differences (differences between 0.2–0.5%). Therefore, a simplified calibration curve can be used consisting of only 2 calibration points, i.e., zero and the greatest concentration used.*

5) Zero is used as second calibration value. The slopes of the trend lines (y = m.x) will be used to calculate the amounts of SVglys present in the analyte (in mM concentration) [**11**]. The average slopes of ST and RebA are also calculated in the spreadsheet.

Note: *The slopes of ST, RebA, RebB and SB are used to calculate the amounts of these compounds. The average of the slopes of ST and RebA is used for the calculation of the other neutral SVglys.*

3.2.4. Analysis of the analyte

1) Add a known amount (1 g = value **O**) of the prepared analyte solution (section 5.2.2) to a vial containing 0.125 mg IS. Add this value in the spreadsheet provided [**12**]. Now 0.1 g of ethanol or methanol is added to better dissolve the IS. Thoroughly mix in an ultrasonic bath at 50 °C. This addition of alcohol does not influence the result of the final analysis. However, by adding 0.1 g of solvent, there is a small correction needed for the area of a possible unknown peak eluting ahead of rubusoside, as now 20 µL out of 1.1 g will be injected (correction automatically done in the spreadsheet).
2) If the added amount under 1) above is different from the expected 1 g to be added, a correction has to be made by adapting the slope of the calibration curves made according to section 5.2.3.
   The equation of the calibration curve becomes: **y = (m × 1/Og) × x = m' × x** with m' = corrected slope (Automatically corrected in the spreadsheet provided) [**13**].
3) Perform 6 injections of 20 µL of the sample into the HPLC. This gives an idea of the variation of the integration process itself (can be deduced from the raw data in the spreadsheet).
4) Register all the peak areas and calculate the ratios of the area SVgly/area IS. Add the peak areas in the spreadsheet provided [**14a**].
5) Use the corrected slopes m' of the calibration curves to calculate the amounts of the different SVglys present (in mM). Unknown concentration of each SVgly (mM) = peak ratio/m' (Calculations automatically done in the spreadsheet provided) [**15**].
6) Convert the values of mM into mg SVgly present using the molecular weights given in Table 7.
   The amount SVglys of *e.g.*, 0.504 mM Reb A is 0.504 mmol/kg x 967.02 mg/mmol = 487.378 mg/kg or 0.487 mg/g solution (All calculations are done automatically in the spreadsheet provided) [**16**].
7) Calculate the sum **Q** of all SVglys found: **Q** = sum of all SVgly (mg/g) (All calculations are done automatically in the spreadsheet provided) [**17**].

8) Purity (**P**) of the analyte is: **P = Q/N x 100** (times 100 to present it as a percentage) (All calculations are done automatically in the spreadsheet provided) [**18**].

Note: *This purity has been automatically corrected for water content of the analyte and for the exact amount of sample added to the IS.*

9) Calculate the total amount of SVglys in 1 g of dry analyte:
Total amount is: 1 g × P /100 (All calculations are done automatically in the spreadsheet provided) [**19**].

10) Accurate measurement of small peaks. The same sample as used in 3) above can be used to measure the small peaks in the chromatogram more accurately. Completely evaporate or freeze dry the sample. Add 200 µL of ethanol or methanol. Close the vial and thoroughly mix. Pour the solution into an insert suitable for containing small sample volumes. Inject the sample again (20 µL). Now the peak areas of the smaller peaks can be measured more accurately as they will be about 5 times larger. Do not try to measure the larger peaks of RebA and ST as these will probably be too large. Add the peak areas of the small peaks as well as that of the IS in the spreadsheet provided [**14b**] (Automatically, all peak ratios and corrected slopes are calculated in the spreadsheet provided). The RSD of small peaks should decrease by this second injection.

Note: *When developing the IS method, the amount of IS to be added to each sample (0.125 mg) was chosen to enable the evaporation of solvent for measuring the smaller peaks more accurately.*

The tedious and daily calibration of the HPLC with an external standard is no longer necessary.

It was observed that problems might exist with the integration of peaks. Therefore, we asked to inject the calibration mixture thrice and the unknown sample 6 times.

We also try to improve the measurement of small peaks (can be shown by their smaller RSD).

3.3. Analyses to be done by the participating laboratories

The work load of the participating laboratories was reduced to drying, weighing and dissolving of an unknown sample. The calibration mixture could be used to optimise the separation between ST and RebA and to construct calibration curves with the following standards added: RebA, ST, RebB and SB. We asked to inject the calibration mixture thrice and the unknown sample 6 times. This way, it was possible to obtain an idea about the peak integration process itself.

3.4. Results of the second round-robin testing with an IS

3.4.1. Control of calibration curves with IS and analysis of 1 sample

In this round-robin testing, different vials containing samples to be tested were sent to the participating laboratories.

- Vial 1: calibration mixture already containing the IS (completely dried).
- Vials 2–3: 0.125 mg IS (completely dried).
- Vial 4: unknown mixture of SVglys with an unknown moisture content.

3.4.2. Control of calibration curves

Vial 1 had the calibration mixture, containing calibrated amounts of 4 SVgly standards (0.489, 0.494, 0.219, 0.189 mM for RebA, ST, RebB and SB, respectively) as well as IS (0.125 mg/mL).

Table 11 gives the HPLC conditions used in the different participating laboratories. Most of them used apolar, mostly C_{18}-based columns.

All the participants needed to do was injecting the calibration mixture thrice in the HPLC (preferably with C_{18} columns), identifying all the peaks and measuring their areas. They had to add these values in a numbered and protected spreadsheet. The spreadsheet automatically plotted the calibration curves. The trend line was fitted through zero and the trend line equations of the different standards were automatically calculated and printed in the spreadsheet.

The results of the calibration curves obtained by the different laboratories are given in Table 12.

In each laboratory, the slopes of the calibration curves, plotted as the ratios of peak areas of standard over IS against the mM concentration of the standards, are about the same for all the different SVglys. Lab 27* reported a bad resolution between ST and RebA. Therefore, the slopes are totally

Table 11. HPLC conditions used in the different laboratories.

Laboratory	Column type and size	Particle size	UV detector (wavelength)
1	Luna C_{18}; 250 x 4.6 mm +	5 µM	UV 200 nm
	Kinetex C_{18}; 75 x 4.6 mm	2.6 µM	UV 200 nm
5	Kinetex C_{18}; 150 x 4.6 mm	2.6 µM	UV 200 nm
6	2 x Grace Alltima C_{18}; 250 x 4.6 mm	5 µM	UV 200 nm
7	Kinetex C_{18}; 150 x 4.6 mm	2.6 µM	UV 205 nm
18	2 x Grace Alltima C_{18}; 250 x 4.6 mm	5 µM	UV 200 nm
19	2 x Grace Alltima C_{18}; 250 x 4.6 mm	5 µM	UV 200 nm
21	--------	------	------
24	2 x Grace Alltima C_{18}; 250 x 4.6 mm	5 µM	UV 200 nm
25	Waters T3; 150 x 2.1 mm	1.8 µM	UV 200 nm
27	2 x Zorbax SB-C_{18}; 250 x 4.6 mm	5 µM	UV 205 nm
28	2 x Luna C_{18}; 250 x 4.6 mm	5 µM	UV 210 nm
30	2x Teknokroma; C-18 250*4.6 mm	5 µM	UV 203 nm
31	2 x Grace Alltima C_{18}; 250 x 4.6 mm	5 µM	UV 200 nm
32	2 x Grace Alltima C_{18}; 250 x 4.6 mm	5 µM	UV 200 nm

Table 12. Results of the calibration curves (y = m . x) plotted as ratios of the peak areas of standard over IS against the mM concentrations of the standards. The values given are the slopes (m).

Lab #	RebA	ST	RebB	SB	Avg
1	6.062	6.122	6.113	6.102	6.092
5	5.652	5.762	5.994	6.071	5.707
6	5.922	5.976	5.949	5.954	5.949
7	6.332	6.311	6.516	6.510	6.321
19	6.039	6.037	6.066	6.061	6.038
21	---	---	---	---	---
24	5.936	6.108	5.996	5.993	6.022
25	---	---	---	---	---
27*	*5.512*	*8.183*	*6.439*	*6.904*	*6.847*
28	6.257	6.287	6.378	6.364	6.272
30	6.058	6.105	6.317	6.293	6.082
31	5.956	6.015	6.051	6.049	5.985
32	5.319	5.325	5.426	5.206	5.322
Average	5.953	6.005	6.081	6.060	5.979
SD	0.29	0.28	0.30	0.35	0.29
RSD	4.9	4.7	4.9	5.8	4.8

different for ST and RebA. This certainly had a negative influence on the analysis of the SVglys, and hence, their results were printed in italics and were not used for the calculation of averages.

The averages of all laboratories were 5.953 ± 0.29, 6.005 ± 0.28, 6.081 ± 0.30, and 6.060 ± 0.35 for ST, RebA, RebB and SB, respectively. Previously, it was shown that the extinction coefficients of the different SVglys were very similar and this explains the similarity of all the slopes. Of course, as the wavelength of the detector influences the slopes (see above), the RSD between different laboratories is rather large. However, as each laboratory uses its own calibration curves, there is no problem for the subsequent quantification of the different steviol glycosides. Laboratories 21 and 25 used their own external standard method and did not report on calibration curves (laboratory numbers in bold italics). Of course, as these laboratories did not follow the protocol, their results cannot be used in the calculations.

3.4.3. Measurement of water content of the mixture of steviol glycosides (vial 4)

Participants were asked to weigh 500 mg of vial 4 (the mixture of steviol glycosides) in a weighing vessel and to place the opened vessel in an oven at 105 °C. The lid should also be put in the oven while drying overnight for about 16 h. The moisture content was calculated as a percentage of wt. loss. This dried sample was not used for further analysis as degradation products of impurities might give extra peaks [9].

The results of the water content of the unknown sample are reported in Table 13. Laboratory 21 did not report the water content, and laboratories 7, 25 and 27 did not follow the protocol.

Most of the laboratories have dried the sample in the correct way and found water content of about 3.2%. Not many conclusions can be drawn from the results of the water content. This might vary much by the atmospheric conditions in the laboratory of the participant when opening the vial and

Table 13. Water content in the unknown sample as reported by the different laboratories.

Laboratory #	Amount before drying (mg)	Amount after drying (mg)	Weight Loss %
1	502.3	482.4	4.02
5	503.4	487.2	3.218
6	500.8	484.3	3.295
7	60.7	58.6	3.526
19	498.2	483.9	2.87
21	---	---	---
24	501.1	486.7	2.873
25	---	---	*6.38*
27	50	47.8	4.39
28	365	353.8	3.068
30	500	486.6	2.68
31	498.6	484.2	2.888
32	501.6	483.9	3.529
Average			3.20
SD			0.41
RSD			12.7

weighing the sample. Laboratory 7 and 27 dried small amounts and this might give wrong water content as weighing errors might be greater.

3.4.4. Analysis of the unknown sample

Participants were asked to weigh exactly 60 mg of the unknown mixture (vial 4) in a Falcon tube, to which 39.940 g of HPLC quality water had to be added. All solutions were made on a weight basis, as this avoids errors due to possible non-calibrated pipettes and solvent expansion at different temperatures. It is important to check that all SVglys are well dissolved. Subsequently, exactly 1 g of this solution must be added to vial 2 (or 3 in case the analysis will be repeated) containing the IS (0.125 mg). Add 0.1 mL of ethanol or methanol to easily dissolve the IS (important note: check first the quality of the ethanol/methanol used!). Then thoroughly mix or sonicate at 50 °C and inject 20 µL in the HPLC. Adding a small amount of alcohol does not influence the final result as the calculations in the IS method are done by peak area ratios.

Inject the unknown sample 6 times and calculate the relative standard deviation (RSD). The concentration of the IS was chosen in such a way that the same sample can be used to inject, e.g., 5 times more for a better analysis of the smaller peaks present. Of course, the large peaks probably cannot be measured then, but analysis of the smaller peaks will be much better. Figure 7 shows the analysis of the unknown mixture to which IS was added.

After filling in the peak areas in the spreadsheet provided, the peak area ratios of the different compounds over that of the IS were automatically calculated. The calibration curves made under 5.4.2 were used to calculate the amounts in mM of SVglys present (see spreadsheet). The mmoles present in 40 g were calculated and the mmoles were converted into mg steviol glycosides by using Table 7. The results were corrected for the water content obtained in 5.4.3. The amounts of steviol equivalents (SVeqs) were also calculated on both a dry and fresh wt. basis by using conversion factors of Table 7.

Table 14 shows the results of the unknown sample. The values are given in mg/g of solution. Most of the laboratories reported at least 8 compounds present in the unknown mixture. Many laboratories did not report the amounts of RebE and D, as the

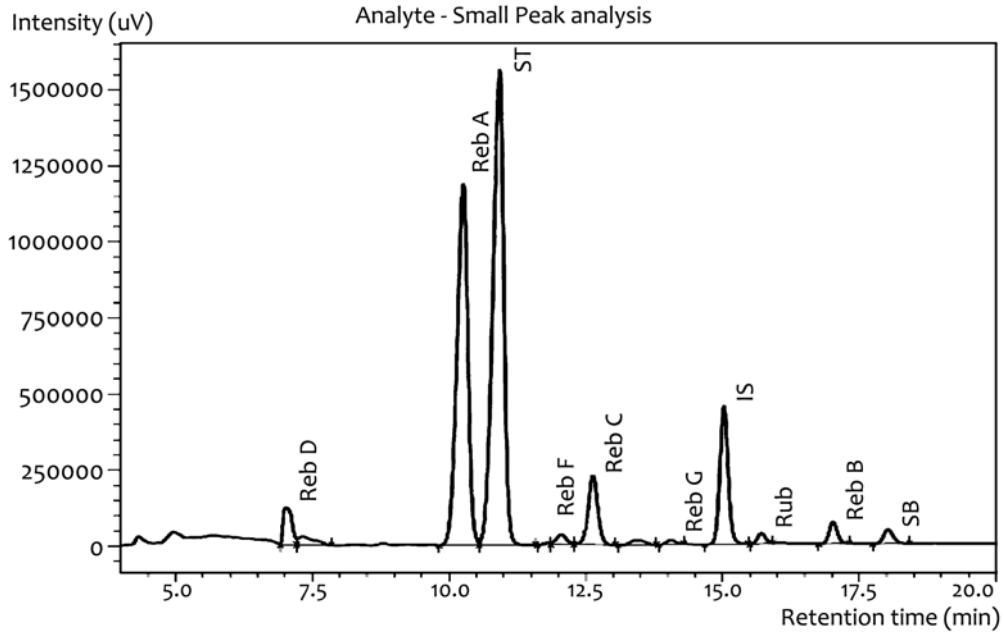

Figure 7. Example of the analysis of the unknown sample after the addition of IS. (RebD: 7.2); RebA: 10.25 min; stevioside: 11.0; RebF: 12.1; RebC: 12.6; DulA: 13.5; Reb G: 14.1; IS: 15.1; rubusoside: 15.7; RebB: 17.1; steviolbioside: 18.1.

Table 14. Quantitative analysis of the unknown sample. Values are corrected for different molecular masses and for water content of the unknown sample. Values are given in mg/g solution.

Lab #	RebA	ST	RebF	RebC	DulA	RebG	Rub	RebB	SB
1	0.560	0.620	0.015	0.092	0.002	0.004	0.006	0.021	0.012
5	0.563	0.626	0.016	0.099	0.004	0.003	0.007	0.022	0.012
6	0.555	0.619	0.014	0.094	0.005	0.005	0.006	0.021	0.013
7	0.560	0.623	0.014	0.091		0.001	0.006	0.022	0.013
19	0.589	0.663	0.016	0.098	0.005	0.004	0.006	0.022	0.013
21	---	---	---	---	---	---	---	---	---
24	0.576	0.621	0.013	0.096	0.005	0.006	0.006	0.021	0.013
25	---	---	---	---	---	---	---	---	---
27	0.543	0.612	---	0.112	---	---	0.016	0.021	0.013
28	0.587	0.654	0.015	0.095	0.002	0.004	0.006	0.021	0.010
30	0.579	0.642	0.017	0.097	0.006	0.003	0.006	0.021	0.013
31	0.572	0.640	0.015	0.096	0.005	0.005	0.008	0.021	0.013
32	0.501	0.692	0.018	0.109	0.029	---	0.014	0.011	0.016
Avg	0.566	0.637	0.015	0.098	0.005	0.004	0.008	0.021	0.013
SD	0.023	0.022	0.002	0.006	0.002	0.001	0.003	0.003	0.001
RSD	4.10	3.46	10.18	5.79	33.26	31.20	41.25	14.13	9.87

quantities in the sample were very small, and possibly no reference compounds were present.

From Table 14, the total purity of the unknown sample could be calculated, and most of the laboratories reported a value of about 92% purity (Table 15). The weak point in this round-robin testing was the delivery of completely dried IS in small tubes. If, after the addition of 1 g of unknown sample, not all of the IS dissolves, this gives an overestimation of the amounts of SVglys present. To prevent this happening, participants were asked to add 100 µL of ethanol or methanol after adding the unknown sample to the tube with IS.

Although the sample seemed to contain RebD, further analysis revealed that the peak occurring at the same Retention Time (RT) was not RebD. Therefore, all the reported values for RebD were omitted in Table 14.

In the chromatograms (e.g., Figure 7), RebD eluted at the start. However, it is not certain that this peak consists of only RebD as the resolution between the polar compounds at the beginning of the chromatogram is insufficient. It seems rather impossible to separate all 10 SVglys in only one chromatographic system, which suggests the necessity of the combination of and/or switching between reversed phase and normal phase columns.

Laboratories 21 and 25 used their own external standard method. Therefore, the purity reported is printed in italics. Their results clearly show that by use of an external standard method, a difference of 25% between laboratory 21 and 25 was found for the total purity of the mixture of the unknown sample, proving the superiority of this EUSTAS protocol (92.211 ± 1.618) having an inter-laboratory RSD of only 1.75%. Of course, an external standard method should give exactly the same purity value. However, by using an external standard method, many more parameters are not under control thus giving rise to a huge inter-laboratory RSD as exemplified in the above results of laboratories 21 and 25 and previous results with an external standard method [4].

The results of laboratories 30 and 32 were studied in more detail. It was found that the peak areas of

Table 15. Reported purities of the unknown sample.

Lab #	Reported conc. in mg/mL	Expected value mg/mL	Purity in %	SVeqs (mg/g dry wt.)	SVeqs (mg/g wet wt.)
1	1.356	1.456	92.445	333.018	319.634
5	1.352	1.445	93.550	340.855	329.886
6	1.332	1.453	91.672	344.300	323.285
7	1.329	1.458	91.269	332.561	320.836
19	1.417	1.522	93.101	339.614	329.866
21	---		*76.28*		
24-1	1.366	1.473	92.736	328.245	328.245
24-2	1.350	1.458	92.593	337.678	327.925
24.3	1.312	1.455	92.172	335.824	326.174
24.4	1.426	1.592	89.573	333.179	323.605
24.5	1.378	1.492	92.359	336.844	327.165
25	---		*95.42*		
27	1.316	1.430	92.028	336.174	321.416
28	1.394	1.541	90.461	329.246	319.143
30	1.383	1.456	94.986	346.167	336.890
31	1.375	1.511	90.999	331.838	322.254
32	1.390	1.439	95.205	343.838	343.517
Avg			92.211	336.63	326.66
SD			1.618	5.48	6.66
RSD			1.75	1.63	2.04

the IS during the analysis of the unknown sample were significantly smaller than those of the calibration curves. This might explain the greater purity found as probably part of the IS was not completely dissolved after the addition of 1 g of the analyte solution.

Laboratory 24 did 5 independent analyses and all the results were included in Table 15.

The steviol equivalents (SVeqs) were also calculated in the spreadsheet and are given as mg/g dry wt. or as mg/g wet wt. of the mixture of SVglys (Table 15). This simplifies the analysis of mixtures of SVglys in different recipes.

3.4.5. Analysis of small peaks in the unknown sample

The amount of IS in the sample vial permitted the evaporation of the solvent and the dissolution of the residue again in 5 times less solvent (methanol or ethanol; Figure 5). If again 20 µL were injected, the integration of smaller peaks should be better. Table 16 shows the % RSD for small peaks obtained in the different laboratories that performed this extra analysis (the % RSD is compared between the first injection (first row) and after injection of 5 times more (second row) in Table 16.

Table 16 shows that the % RSD significantly decreases when 5 times more of the unknown mixture is injected. This means that the accuracy of the analysis of small peaks was much increased. Unfortunately, only a few laboratories performed this task. The RSD's for the major peaks were already small for the first injection (averages of 0.282, 0.261 and 0.406 % for RebA, ST and RebC, respectively). These peaks became too large to be integrated when 5 times more was injected.

More information is given in the additional information including a practical exercise.

4. General conclusion

As calibration curves could be forced through zero without any problems, the HPLC can be calibrated by the injection of 1 calibration mixture only (highest concentration). It was shown that it is possible to reproduce the IS calibration curves of provided calibration mixtures in most of the participating laboratories using the same or similar reverse phase HPLC columns. This simplifies the analysis of steviol glycosides. The method is based on the peak ratios of standards over the IS. Moreover, for the same reason, it is not required to daily calibrate the HPLC. The method is also independent of the type or the sensitivity of the UV detector used. Errors due to changes in injection volume, failure of the equipment or to evaporation of solvent, belong to the past. To better dissolve all the SVglys and to prevent precipitation of analyte, it is possible to add a suitable solvent (ethanol, methanol) as evaporation of part of this solvent does not influence the final results. To improve the quantification of smaller peaks of unknown samples, after a normal injection, larger amounts can be injected to measure the smaller peaks. This improved the RSD of smaller peaks and it did not require additional calibrations.

In this round-robin testing using vials with validated amounts of IS, an inter-laboratory RSD of 1.75% was found. We hope that this can still be improved, when all laboratories try to follow the protocol as prescribed. Indeed, the RSD found with the results of the best scoring laboratories was only 0.8%, but to be objective, we had to include all the results obtained by the different participants. This value might still decrease after improvement of peak resolution and peak integration

Table 16. Percentages of RSD of the amounts (mg/g solution) for small peaks. The upper row of each laboratory is for the first injection, the lower row for injection of 5 times more.

Lab #	RebA	ST	RebF	RebC	DulA	RebG	Rub	RebB	SB
1	0.322	0.182	1.330	0.556	6.022	0.033	3.926	0.769	2.326
	---	---	0.187	---	0.938	0.012	0.835	0.758	1.424
28	0.367	0.427	1.539	0.289	5.172	0.081	4.779	0.476	2.710
	0.239	0.235	0.432	0.291	1.388	0.002	0.596	0.124	0.235
31	0.157	0.174	1.581	0.374	1.919	0.075	6.932	0.158	0.333
	0.214	0.081	1.656	0.051	0.517	0.056	1.935	0.147	0.346

Table 17. Percentages RSD for 4 compounds after 3 injections of the calibration mixture. The values were obtained from the ratios of standards over IS.

Lab #	RebA	ST	RebB	SB
1	0.126	0.138	0.236	0.232
5	0.238	0.158	0.344	0.423
7	0.186	0.794	0.678	1.551
19	0.710	0.741	0.123	0.256
24	0.063	0.082	0.027	0.070
27	0.790	0.744	0.671	1.134
28	0.079	0.046	0.135	0.135
30	0.196	0.099	0.548	0.180
31	0.254	0.412	0.130	0.121
32	0.010	0.010	0.012	0.015

techniques in some laboratories as evidenced by thoroughly analysing the raw data given in the spreadsheets.

Table 17 shows the percentages RSD of the different laboratories after 3 injections of the calibration mixture. The values were obtained from the ratios of standards over IS. The peaks of the calibration mixture were relatively large. Therefore, the peak integration was relatively easy. However, only 2 participants (24, 32) obtained very small RSD. Analysis of all the results demonstrated that the variation was not due to differences in injection volume, but only to differences in peak integration. The differences in peak integration are not necessarily due to differences in the equipment or the integration software. The results of 24 and 31 were obtained in the same laboratory. The analyses of number 24 were done by an experienced technician, and those of 31 by a student who had to setup the whole HPLC equipment on his own. In an analysis, much more attention should be given to peak integration as this will certainly influence the overall RSD.

The results of this round-robin testing can be used to further fine-tune the methods and to advise people about the analysis of SVglys. It should be possible to obtain an inter-laboratory RSD below 1%. All participants should carefully try to obtain a base-line separation between ST and RebA and check the peak integration process itself. Use of ultra-high performance liquid chromatography columns might help to obtain a better peak resolution and facilitate peak integration [10-11].

5. The future of steviol glycoside analysis

An exact analysis of steviol glycosides is a big challenge for the analytical chemist. We developed a protocol that works and if analysts take care of a good peak resolution and integration, the tedious work of analysis is reduced to drying and weighing of the analyte. A good chemist and/or company has to be found that can synthesise the IS and can make calibration mixtures as well as vials with pre-weighed amounts of IS. This might lead to a world-wide correct analysis of steviol glycosides. Those only interested in total amounts of steviol equivalents present in different foods might use a validated internal standard method [12].

The spreadsheets required for a practical exercise can be downloaded from: https://www.dropbox.com/sh/z9xx6grsebcnoqh/AADkdgGkZgOQNCgySnM9uyO7a?dl=0

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ADDITIONAL INFORMATION

Analysis of steviol glycosides using the IS method: practical aspects including a practical exercise at the end

If the above protocol has been followed, the calculations can be done in the following way (transcribed from a spreadsheet):

The spreadsheets can be downloaded from:

https://www.dropbox.com/sh/z9xx6grsebcnoqh/AADkdgGkZgOQNCgySnM9uyO7a?dl=0

[**1**] Area of a small peak possibly overlapping with IS (if no overlap occurs, enter 0): (enter peak area) [**1**]

1. Water content

- Did you zero the balance before measuring? Y/N
- Give the weight of the empty drying vessel with lid (value A): mg
- Add 500 mg analyte and measure the weight of the vessel with lid + sample (value B): mg
  [**2**] The amount of "wet" sample is: Value (B – A) = mg [**2**]
- After drying to a constant weight, give the weight of vessel with dried sample + lid (Value D) mg
  [**3**] The dry weight of the unknown sample is: value (D – A) = mg [**3**]. Discard the dried sample.
  [**4**] The percentage dry weight (given as %) is: Value ([3]/[2])*100 = [**4**] %
  [**5**] The water content in percentage = 100 – value [**4**] = %

2. Solution of an analyte

- Did you zero the balance before measuring? Y/N
- Weigh an empty Falcon tube of 50 mL (Value H)
- Weigh about 60 mg of (wet) analyte (Value I)
  [**6**] The exact amount of wet sample is: Value (I – H) = mg [**6**]
- Zero the balance and add 39.94 g water to the Falcon tube (exact amount is Value K)

[**7**] The exact amount of added water is ... g (K)
[**8**] Calculate the concentration per g solution Value [**6**]/([**7**]+[**6**]) in mg/g [**8**]
[**9**] Calculate the corrected concentration by taking the water content into consideration ([**4**] * [**8**])/100 = mg/g [**9**]

3. Calibration of the HPLC

- Inject the calibration mixture 3 x and report the peak areas in Table 1 AI

Calculate the ratios of SVglys over IS (Table 2 AI)

Calculation of the slopes of the calibration mixtures in mM (Table 3 AI)

Write the equations of the calibrations with the trend line forced through zero (y = m*x). For the un-calibrated peaks, the average of the calibrated peaks is taken (Table 4 AI).

Table 1 AI. Peak areas of the calibration mixture.

Areas [10]	Reb A	ST	Reb B	SB	IS
Injection 1	RebA1	ST1	RebB1	SB1	IS1
Injection 2	RebA2	ST2	RebB2	SB2	IS2
Injection 3	RebA3	ST3	RebB3	SB3	IS3

Table 2 AI. Ratios of the peak areas of SVglys over IS for the 3 injections. Also the average is calculated of the 3 injections.

Ratio (SVgly/IS)	**Reb A**	**ST**	**Reb B**	**SB**
Injection 1	RebA1/IS1	ST1/IS1	RebB1/IS1	SB1/IS1
Injection 2	RebA2/IS2	ST2/IS2	RebB2/IS2	SB2/IS2
Injection 3	RebA3/IS3	ST3/IS3	RebB3/IS3	SB3/IS3
Average	**Sum values/3**	**Sum values/3**	**Sum values/3**	**Sum values/3**

Table 3 AI. Calculation of the slopes (m) [**10a**] – [**10 d**] of the trend lines (y = m*x) plotted through zero of the different SVglys in mM of the calibration mixture. (m = y/x) in which "y" is the peak ratio (Table 2 AI) and "x" is the given concentration of the SVgly (see Table).

Conc. in mM	**0.489 (Reb A)**	**0.494 (ST)**	**0.219 (Reb B)**	**0.189 (SB)**
Reb A	[**10a**]			
ST		[**10b**]		
Reb B			[**10c**]	
SB				[**10d**]

Table 4 AI. Equations of the calibration lines forced through zero (y = m*x).

Reb A	y =	[**10a**]	x
ST	y =	[**10b**]	x
Reb B	y =	[**10c**]	x
SB	y =	[**10d**]	x
Average for not calibrated SVglys	y =	[**10e**]	x

The average of the slopes [**10e**] of the calibrated SVglys is calculated as: ([**10a**]+[**10b**]+ **[10c**]+[**10d**])/4.

4. Analysis of the analyte and measurement of the smaller peaks more accurately

[**12**] Add a weighed amount of about 1 g (= value **O**) of the prepared analyte solution (see step 5.2.4 in the protocol) to a vial containing 0.125 mg IS. Warm and shake the vial to better dissolve the IS. Remember that in an unknown sample there might be a small peak just ahead of the IS and overlapping with the IS (value [**1**] see above). The value of [**1**] should be corrected for as otherwise the area of the IS becomes somewhat larger, by which there is an underestimation of the peaks of SVglys. If there is no peak [**1**], this correction is not necessary. Corrections are done automatically in the spreadsheet (see additional information). If alcohol (e.g., 0.1 g = x g alcohol) was added to better dissolve the IS, its value will influence the amount of peak [**1**] to be deduced from the peak of IS. A correction is automatically done in the spreadsheet.

Corrections to be done:

1) If a small unknown peak [**1**] occurs, the area of the IS in an analysis should be corrected for by deducing the value [**1**] from the area of the IS found in the analysis. If [**1**] is zero, there is no influence. If 0.1 g of alcohol was added to better dissolve the IS, the amount of [**1**] to be deduced from the IS becomes: [**1**]/(1+0.1 g alcohol) (calculations automatically done in the spreadsheet).
2) If the amount of added solution of analyte is not exactly 1 g (value **O**), a correction should be included to obtain values of SVglys per g of solution. This can be done by multiplying the slopes by a correction factor CF= 1/**O** (Table 6 AI).

Inject the analyte with IS 6 times and report the peak areas in Table 5AI. If there is a peak [**1**], calculate the corrected IS by subtracting the value [**1**]/(1+ x g alcohol). Always use the corrected IS.

If value O ≠ 1 g, calculate the corrected slopes by multiplying the slopes [10a] – [T4] by 1/O.

[**13**] Equations of the calibration curves with corrected slopes (Table 6 AI):

Table 5 AI. Areas of all the peaks after injection of the unknown sample 6 times.

AREAS	RebD	RebE	RebA	ST	RebF	RebC	DulA	RebG	IS	Corr IS	Rub	RebB	SB
Inj. 1													
Inj. 2													
Inj. 3													
Inj. 4													
Inj. 5													
Inj. 6													

Table 6 AI. Equations of the calibration curves with corrected slopes [**13a**] – [**13e**].

				equations with corrected slopes
Reb A:	Y RebA =	[**10a**] * CF	x	y = [**13a**] * x
ST	Y ST =	[**10b**] * CF	x	y = [**13b**] * x
Reb B	Y Reb B =	[**10c**] * CF	x	y = [**13c**] * x
SB	Y SB =	[**10d**] * CF	x	y = [**13d**] * x
Not calibrated SVGlys	Y SVGlys =	[**10e**] * CF	x	y = [**13e**] * x

Calculate the peak ratios of SVgly over IS (Table 7 AI).

Calculate the averages of the 6 injections of the different SVglys; calculate the Standard Deviation and the Relative Standard Deviation (best to be done in a spreadsheet).

Calculate the concentration of SVglys in mM [**15**] by dividing the ratio of peak area SVgly by the corrected slope value of each SVgly from Table 6 AI (see [**13**]) and put the values in Table 8 AI.

Calculate the concentration of each SVgly in 1 g solution in mg/g [**16**] and report the results in Table 9 AI. Calculate also the average, the SD and RSD.

Example: Concentration Reb A: 0.1 mM, i.e. 0.0967 mg/g solution; ST conc. = 0.4 mM = 0.32195 mg/g solution.

Calculate the total amount of SVglys in 1 g solution in mg/g [**17**], which is obtained by the sum of all the averages of each SVgly. Also calculate the Standard Deviation and the Relative Standard Deviation (best done in a spreadsheet).

The purity of the analyte in percentage [**18**] is obtained by the following calculation: ([**17**]/[**9**]) * 100 = % [**18**]

The total amount of SVglys in mg per 1 g dry analyte [**19**] is obtained by the following calculation: [**18**] * 1000/100 = mg/g [**19**]

Table 7 AI. Peak areas of the different SVglys over corrected IS [**14a**].

AREAS [14b]	**RebD**	**RebE**	**RebA**	**ST**	**RebF**	**RebC**	**DulA**	**RebG**	**Rub**	**RebB**	**SB**
Injection 1											
Injection 2											
Injection 3											
Injection 4											
Injection 5											
Injection 6											
Average	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**
SD											
RSD											

Table 8 AI. Concentrations of SVglys in mM [**15**] obtained by dividing the values of Table 7 AI by the corresponding slopes from the calibration mixture from Table 5 AI.

Conc. SVgly in mM [15]	**RebD**	**RebE**	**RebA**	**ST**	**RebF**	**RebC**	**DulA**	**RebG**	**Rub**	**RebB**	**SB**
Injection 1											
Injection 2											
Injection 3											
Injection 4											
Injection 5											
Injection 6											
Molecular Mass (all isotopes)	**1129.16**	**967.02**	**967.02**	**804.88**	**937.0**	**951.02**	**788.88**	**804.88**	**642.74**	**804.88**	**642.74**

Table 9 AI. Concentration of the different SVglys in mg/g solution of analyte [**16**].

Conc. SVgly in mM [15]	**RebD**	**RebE**	**RebA**	**ST**	**RebF**	**RebC**	**DulA**	**RebG**	**Rub**	**RebB**	**SB**
Injection 1											
Injection 2											
Injection 3											
Injection 4											
Injection 5											
Injection 6											
Average (mg/g)	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**
SD											
RSD											

Table 10 AI. SV eq in 1 g solution (mg/g) [**20**].

	RebD	**RebE**	**RebA**	**ST**	**RebF**	**RebC**	**DulA**	**RebG**	**Rub**	**RebB**	**SB**
[**16**] Avg conc SVglys (mg/g)	D	E	A	S	F	C	dulA	G	Rub	B	SB
Conversion factors (CF)	0.282	0.329	0.329	0.396	0.340	0.335	0.404	0.396	0.495	0.396	0.495
Sveq (mg/g)											

5. Total amount of steviol equivalents per gram dry weight of the analyte

The average concentration of SVglys (in mg/g solution) from Table 9 AI is transferred into Table 10 AI. (Values D, E, A etc). The amount of SVeq of each SVgly (in mg/g solution) is obtained by multiplying the values D, E etc. by the respective conversion factor (row 2).

The total amount of SVeq in 1 g solution is obtained by the sum of all the SVeq of the different SVglys (mg/g solution) [**21**].

Practical exercise

Where necessary, values will be provided and the reader should try to follow the protocol and do all the calculations to obtain the purity of an unknown sample as given at the end.

[**1**] Area of unknown. In this sample, there was not a peak just in front of the IS and co-eluting with it.
[**2**] Amount of "wet" analyte: 500.8 mg
[**3**] Amount of dry analyte: 484.3 mg
[**4**] Calculate % dry weight
[**5**] Calculate percentage dry weight
[**6**] Amount "wet" sample: 60.1
[**7**] Exact amount of added water 39.940 (in g)
[**8**] Calculate the concentration (in mg/g)
[**9**] Calculate the corrected concentration (mg/g)

Calculate the peak ratio of areas of SVglys over IS and report values in Table 2 AI and calculate the average.

Note: *Obviously, the injection volumes were not the same by this automatic injector. However, the peak ratios to be calculated will be very similar.*

Calculate the slopes of the trend lines of the calibration mixtures in mM and report them in Table 3 AI

Write the equations of the calibrations with the trend line forced through zero (y = m*x). For the un-calibrated peaks, the average of the calibrated peaks is taken (Table 4 AI).

[**12**] Exact amount of solution added: O = 1.000 g.

Did you add 0.1 g alcohol to better dissolve the IS? In that case, report the exact amount, e.g., 0.1 g.

Calculate the value of the area of IS if a small peak occurs that co-elutes with the IS (correction is done for [**1**] and care is also taken for possibly added alcohol).

If the amount **O** was not exactly 1 g, a correction is also made for this difference to obtain exact values for the concentrations in mg/g solution. This correction is done by multiplying the slopes [**10a**] – [**10d**] by a correction factor CF = (1/**O**).

Report the peak areas of all the peaks of SVglys. Calculate the corrected IS if [**1**] ≠ 0.

Table 1 AI. Peak areas of the calibration mixture.

AREAS [10]					
	Reb A	ST	Reb B	SB	IS
Injection 1	4562118	4646188	2054026	1774631	1570312
Injection 2	3967826	4050439	1780785	1540544	1367346
Injection 3	3644063	3728932	1643978	1420926	1266683

Table 2 AI. Ratios of the peak areas of SVglys over IS for the 3 injections. Also the average is calculated of the 3 injections.

Ratio (SVgly/IS)	**Reb A**	**ST**	**Reb B**	**SB**
Injection 1				
Injection 2				
Injection 3				
Average				

Table 3 AI. Calculation of the slopes (m) [**10a**] – [**10 d**] of the trend lines (y = m * x) plotted through zero.

Conc. in mM	0.489 (Reb A)	0.494 (ST)	0.219 (Reb B)	0.189 (SB)
Reb A	[**10a**]			
ST		[**10b**]		
Reb B			[**10c**]	
SB				[**10d**]

Table 4 AI. Equations of the calibration lines forced through zero (y=m*x).

Reb A	y =		x
ST	y =		x
Reb B	y =		x
SB	y =		x
Average for not calibrated SVglys	y =		x

[**13**] Report the equations of calibration curves with corrected slopes in Table 6 AI:

Calculate the peak ratios of SVgly over IS (Table 7 AI) If [**1**] ≠ 0, then use the corrected IS of Table 6 AI.

Calculate the averages of the 6 injections of the different SVglys; calculate the Standard Deviation and the Relative Standard Deviation (best to be done in a spreadsheet).

[**15**] Calculate the concentration of SVglys in mM and report the values in Table 8 AI.

[**16**] Calculate the concentration of SVglys in 1 g solution (in mg/g) [**16**] and report the results in Table 9 AI. Calculate also the average, the SD and RSD.

[**17**] Calculate the total amount of SVglys in 1 g solution (in mg/g): sum of all SVglys in 1 g solution

[**18**] Calculate the purity in % ([**17**] divided by [**9**] * 100).

[**19**] Calculate the amount of SVglys in mg per g dry analyte (= [**18**] * 10)

Table 5 AI. Areas of all the peaks after injection of the unknown sample 6 x. Note: Reb E was absent and omitted in all the Tables so far. However, to complete the Tables in the exercise, fictive values were added.

AREAS	**RebD**	**RebA**	**ST**	**RebF**	**RebC**	**DulA**	**RebG**	**Rub**	**RebB**	**SB**	**IS**	**Corr IS**
Inj. 1	402309	5050341	6823684	134207	870485	60095	54708	85617	231553	172205	1505711	1505711
Inj. 2	401216	5032900	6801123	135330	870856	59198	51561	85808	229642	172718	1503238	1503238
Inj. 3	398081	4967123	6714092	134068	856640	57209	50868	85078	226614	172684	1483752	1483752
Inj. 4	396356	4953850	6705174	133651	855751	57191	52188	86319	226997	172954	1481547	1481547
Inj. 5	410182	4938258	6679257	137565	849546	59730	52967	82420	222196	170494	1473843	1473843
Inj. 6	265149	3419270	4634693	87155	583190	40715	33896	53384	153617	119791	991181	991181

Table 6 AI. Equations of the calibration curves with corrected slopes.

				equations with corrected IS
Reb A:	Y RebA =	[**10a**] * CF	x	y = ... * x
ST	Y ST =	[**10b**] * CF	x	y = ... * x
Reb B	Y Reb B =	[**10c**] * CF	x	y = ... * x
SB	Y SB =	[**10d**] * CF	x	y = ... * x
Not calibrated SVGlys	Y SVGlys =	[**10e**] * CF	x	y = ... * x

Table 7 AI. Peak areas of the different SVglys over IS [**14a**].

AREAS [14b]	**RebD**	**RebA**	**ST**	**RebF**	**RebC**	**DulA**	**RebG**	**Rub**	**RebB**	**SB**
Injection 1										
Injection 2										
Injection 3										
Injection 4										
Injection 5										
Injection 6										
Average	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**	**Sum/6**
SD										
RSD										

Calculation of Steviol equivalents (SVeqs).

Copy-paste the values of the average concentrations of SVglys (in mg/g solution) from Table 9 AI into Table 10 AI.

[**20**] Calculate the amount of steviol equivalents of each SVgly present (mg/g solution) by multiplying the amount of SVgly by the conversion factor given in Table 10 AI (row 2).

Table 8 AI. Concentrations of SVglys in mM [**15**].

Conc. SVgly in mM [15]	**RebD**	**RebA**	**ST**	**RebF**	**RebC**	**DulA**	**RebG**	**Rub**	**RebB**	**SB**
Injection 1										
Injection 2										
Injection 3										
Injection 4										
Injection 5										
Injection 6										
Molecular Mass (all isotopes)	**1129.16**	**967.02**	**804.88**	**937.0**	**951.02**	**788.88**	**804.88**	**642.74**	**804.88**	**642.74**

Table 9 AI. Concentration of the different SVglys in mg/g solution of analyte [**16**].

Conc. SVgly in mM [15]	**RebD**	**RebA**	**ST**	**RebF**	**RebC**	**DulA**	**RebG**	**Rub**	**RebB**	**SB**
Injection 1										
Injection 2										
Injection 3										
Injection 4										
Injection 5										
Injection 6										
Average (mg/g)										
SD										
RSD										

Table 10 AI. SVeq in 1 g solution (mg/g) [**20**].

	RebD	**RebE**	**RebA**	**ST**	**RebF**	**RebC**	**DulA**	**RebG**	**Rub**	**RebB**	**SB**
[**16**] Avg conc SVglys (mg/g)	D	E	A	S	F	C	dulA	G	Rub	B	SB
Conversion factors (CF)	0.282	0.329	0.329	0.396	0.340	0.335	0.404	0.396	0.495	0.396	0.495
Sveq (mg/g)											

[**21**] Calculate the total amount of SVeq in 1 g solution (mg/g solution) (sum of all the SVeq).

[**22**] Calculate the total amount of SVeq per g analyte (mg/g dry analyte). Total amount of SVeq [**21**] divided by the corrected concentration of SVglys [**17**] (mg/g solution) times 1000.

[**23**] Calculate the total amount of SVeq per g "wet" analyte (mg/g wet analyte). Total amount of SVeq [21] divided by the amount of SVgly/g solution [**16**] times 1000.

If no value of [**1**] is given (or 0) and if [**12**] **O** = 1, then a purity of 95.5% should be obtained. Play along by changing the value of [**1**] (e.g., 10,000) and see what happens with the corrected IS and total purity. You can also change the amount of [**12**], (e.g., **O** = 0.5 or **O** = 2) and keep an eye on the purity then reported. Note that the peak areas reported in all the tables were obtained with the following values: [**1**] = 0; **O** = 1. In the spreadsheets to be downloaded, simulations are included for the IS peaks if other amounts of **O** are used, i.e.: **O** = 2 (in this case, the concentration of the IS is 50% which is too small, and hence a correction factor is needed (1/**O** = 0.5). If **O** = 0.5, the IS peaks are too large and the correction factor is then 1/**O** = 2). By using the right **O**-values with the right peak areas of IS, a purity of 95.5% should be found.

With a purity of 95.5%, the amount of SVeqs is 341.6 mg/g dry analyte and 330.4 mg/g wet analyte.

Steviol glycoside analysis by a validated IS method

Is there a peak just in front of rubusoside? If not, [1] = 0, otherwise give its area in cell C6

1. Water Content

[1] unknown peak	[1] =	0		cell C6 is area of peak in front of IS
Did you zero the balance		Y/N		
[2]Wet sample amount	[2] =	500,8	mg	Cell C8 is wet sample amount
[3]Dry sample amount	[3]	484,3	mg	Cell C9 is dry sample amount
[4] % dry wt. Calculation	**[4] =**	96,71	%	cell C10=Cell C9/C8*100
[5] Water content % Calculation	**[5] =**	3,29	%	Cell C5=100-C10

2. Solution of Analyte

[6] Amount of wet analyte	[6] =	60,1	mg			
[7] Amount added water	[7] =	39,94	g =	**39940**	mg of added water	Calculation
[8] Conc. per g solution Calculation	**[8] =**	1,502	mg/g solution		Cell C16 = C14/C16	
[9] Corrected Conc Calculation	**[9] =**	1,453	mg/g solution		Cell C17= C10*C16/100	

3. Calibration of the HPLC

Table 1 AI: Peak areas

Report values

RA	ST	RB	SB	IS
4562118	4646188	2054026	1774631	1570312
3967826	4050439	1780785	1540544	1367346
3644063	3728932	1643978	1420926	1266683

Table 2 AI: Ratio peak SVgly/IS

Calculation	2,905	2,959	1,308	1,130
Calculation	2,902	2,962	1,302	1,127
Calculation	2,877	2,944	1,298	1,122
Average	**2,895**	**2,955**	**1,303**	**1,126**

Table 3 AI: Calculation of slopes		slope	Corr.slope	note mM conc of the standards	cells D32 - D36: corrected slopes if **O≠1**
average slope Reb A Calculation	**A**	5,922	**5,922**	**0,489** mM conc RebA	Cell C32/C$65
average slope ST Calculation	**ST**	5,976	**5,976**	**0,494** mM conc ST	
average slope Reb B Calculation	**B**	5,949	**5,949**	**0,219** mM conc RebB	
average slope SB Calculation	**SB**	5,954	**5,954**	**0,189** mM conc SB	
average slope A-SB Calculation	**T/4**	5,950	**5,950**		
	SD	0,022			
	RSD	0,373 %			

Write the equations of the different calibration curves (y=m*x):

Table 4 AI: Equations of the calibratin curv			With corrected slopes		
	RebA	y = 5,922 x	RebA	y =	5,922 x
	ST	y = 5,976 x	ST	y =	5,976 x
	RebB	y = 5,949 x	RebB	y =	5,949 x
	SB	y = 5,954 x	SB	y =	5,954 x
	Not calibr	y = 5,950 x	Not calit	y =	5,950 x

4. Analysis of the analyte

[12] Add a weighed amount of 1 g of the analyte solution to a vial with IS.

The exact amount of **O** =	**O** =	1,000 g	Cell C50 is value of added solution in g
Did you add 0.1 g alcohol to dissolve IS?	Alcohol=	0,100 g	Cell C51 is amount of alcohol added in g
Denominator:	**Denom.**	1,1	Cell C52 is the denominator (1+ g alcohol added)
Correction of IS: Corr. IS = IS - [1]/([1]+c56)=	**CF for IS**	0,9090909	Cell C53 is the Correction Factor for slopes if alcohol was added
Corrected amount to be deduced from IS	**Corr Area**	0	Corrected area to be substracted from IS if there is a peak of [1] and after the addition of alcohol

Table 5 AI: Peak areas of all SVglys

This sample had no Reb E

D	E	A	ST	F	C	DulA	G	IS	**Corr. IS**	Rub	B	SB
402309	29708	5050341	6973684	134207	870485	60095	54708	1505711	**1505711**	85617	231553	172205
401216	26561	5032900	6951123	135330	870856	59198	51561	1503238	**1503238**	85808	229642	172718
398081	25868	4967123	6864092	134068	856640	57209	50868	1483752	**1483752**	85078	226614	172684
396356	27188	4953850	6855174	133651	855751	57191	52188	1481547	**1481547**	86319	226997	172954
410182	27967	4938258	6829257	137565	849546	59730	52967	1473843	**1473843**	82420	222196	170494
265149	8896	3419270	4784693	87155	583190	40715	33896	991181	**991181**	53384	153617	119791

If the added water **O** ≠ 1, a Correction Factor has to be made to correct the slopes with.

Correction factor for added solution is 1/O **CF =** 1 Cell C65 = 1/**O**, the correction for the volume of added solution. Multiply the slopes by this value.

[13] The slopes should be multiplied by this factor (done in Table 3 AI)

Table 6AI: Molucular masses (all isotopes)	RebD	RebE	RebA	ST	RebF	RebC	DulA	RebG	IS	Rub	Reb B	SB
	1129,16	**967,02**	**967,02**	**804,88**	**937**	**951,02**	**788,88**	**804,88**		**642,74**	**804,88**	**642,74**

[14a] Table 7: Peak areas over IS

use corrected IS	D	E	A	ST	F	C	DulA	G	IS	Rub	B	SB
Cell B57/K57 etc.	0,2671887	0,0197302	3,3541237	4,63148904	0,089132	0,578122	0,0399	0,03633		0,056862	0,1537832	0,1143679
	0,2669012	0,0176692	3,3480394	4,62410011	0,0900257	0,57932	0,0394	0,0343		0,057082	0,1527649	0,1148973
	0,2682935	0,0174342	3,3476774	4,62617203	0,0903574	0,577347	0,0386	0,03428		0,05734	0,1527304	0,1163833
	0,2675285	0,0183511	3,3437009	4,62703782	0,0902104	0,577606	0,0386	0,03523		0,058263	0,1532162	0,1167388
	0,2783078	0,0189756	3,3505998	4,6336394	0,0933376	0,576416	0,0405	0,03594		0,055922	0,1507596	0,1156799
	0,2675082	0,0089752	3,4496928	4,82726465	0,0879305	0,588379	0,0411	0,0342		0,053859	0,1549838	0,1208568
Average	**0,269288**	**0,0168559**	**3,365639**	**4,66161718**	**0,0901656**	**0,579532**	**0,0397**	**0,03505**		**0,056554**	**0,1530397**	**0,1164873**
SD	0,004	0,004	0,041	0,081	0,002	0,004	0,001	0,001		0,002	0,001	0,002
RSD	0,02	0,23	0,01	0,02	0,02	0,01	0,03	0,03		0,03	0,01	0,02

Table 8 conc in mM [15]

	D	E	A	ST	F	C	DulA	G	IS	Rub	B	SB
divide peak ratios by corrected slopes	0,045	0,003	0,566	0,775	0,015	0,097	0,007	0,006		0,010	0,026	0,019
	0,045	0,003	0,565	0,774	0,015	0,097	0,007	0,006		0,010	0,026	0,019
	0,045	0,003	0,565	0,774	0,015	0,097	0,006	0,006		0,010	0,026	0,020
	0,045	0,003	0,565	0,774	0,015	0,097	0,006	0,006		0,010	0,026	0,020
	0,047	0,003	0,566	0,775	0,016	0,097	0,007	0,006		0,009	0,025	0,019
	0,045	0,002	0,583	0,808	0,015	0,099	0,007	0,006		0,009	0,026	0,020

Table 9: conc. In mg/g solution [15]	D	E	A	ST	F	C	DulA	G	IS	Rub	B	SB
Multiply conc. In mM by molecular mass/1000	0,051	0,004	0,548	0,624	0,014	0,092	0,005	0,005		0,006	0,021	0,012
MM in Table 6 AI	0,051	0,003	0,547	0,623	0,014	0,093	0,005	0,005		0,006	0,021	0,012
	0,051	0,003	0,547	0,623	0,014	0,092	0,005	0,005		0,006	0,021	0,013
	0,051	0,003	0,546	0,623	0,014	0,092	0,005	0,005		0,006	0,021	0,013
	0,053	0,004	0,547	0,624	0,015	0,092	0,005	0,005		0,006	0,020	0,012
	0,051	0,002	0,563	0,650	0,014	0,094	0,005	0,005		0,006	0,021	0,013
Average	**0,051**	**0,003**	**0,550**	**0,628**	**0,014**	**0,093**	**0,005**	**0,005**		**0,006**	**0,021**	**0,013**
SD	0,0008	0,0007	0,0067	0,0109	0,0003	0,0007	0,0001	0,0001		0,0002	0,0002	0,0003
RSD	1,65	23,45	1,23	1,74	2,00	0,77	2,58	2,66		2,69	0,91	1,99
% composition SVgly	3,68	0,23	39,60	45,24	1,02	6,67	0,38	0,34		0,44	1,49	0,91

[17]= Total SVgly in 1 g solution (mg/g)	**1,388**		Cell B106 is the total amount of SVgly in 1 g solution
[18] purity	**95,5**	%	Cell B107 is the purity expressed as a %
[19] SVgly/g dry wt (mg/g)	**955**	mg/g	cell B108=B107*10

5. Calculation of Steviol equivalents per g dry weight of analyte

	D	E	A	ST	F	C	DulA	G	IS	Rub	B	SB	
Paste values of conc. SVgly in mg/g solution		0,051	**0,003**	0,550	0,613	0,014	0,093	0,005	0,005		0,006	0,021	0,013
Conversion factors		**0,282**	**0,329**	**0,329**	**0,396**	**0,34**	**0,335**	**0,404**	**0,396**		**0,495**	**0,396**	**0,495**
SVeq in mg/g solution (B113*B114) etc.		0,014	0,001	0,181	0,243	0,005	0,031	0,002	0,002		0,003	0,008	0,006

[21] Total amount of SVeq per g solution	0,4964 mg/g solution	Cell B117= Sum(B113:N113)	
[22] Total SVeq per g dry weight of analyte	**341,6** mg/g dry weight	Cell(B117=B117/C17)*1000	
[23] Total SVeq per g wet weight of analyte	**330,4** mg/g wet weight	cell (B121=B117/C16)*1000	

Simulation of IS peak areas if different amounts of **O** are taken

with 1 g solution O	with 2 g solution	with 0.5 g solution
IS	IS	IS
1505711	752856	3E+06
1503238	751619	3E+06
1483752	741876	3E+06
1481547	740774	3E+06
1473843	736922	3E+06
991181	495591	2E+06

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

ABBREVIATIONS

Using SV for steviol, allows the use of the following abbreviations: SVgly(s) for steviol glycosides, SVeq for steviol equivalents, SVglu for steviol glucuronide, SM: steviol monoside, SVE: steviol-19-ester, ST: stevioside, RebA–G: rebaudioside A–G, SB: steviolbioside, DulA: dulcoside A, Rub: rubusoside; IS: internal standard (19-*O*-β-D-galactopyranosyl-13-*O*-β-D-glucopyranosyl-steviol).

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