

Inter-Laboratory Analysis of Steviol Glycosides by an External Standard Method

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Abstract: To optimise steviol glycoside analysis, several round-robin tests were organised by the European Stevia Association (EUSTAS). Seventeen laboratories participated in the testing. Only 8 laboratories have sent their results. In the first round-robin testing, 2 samples were analysed. The first sample had a purity of 96.2%. The second sample was a 4/5 dilution with NaHCO₃ of sample 1. This way, the drying process itself could be checked. The purity of sample 2 was 82.35%. The reported purities of sample 1 varied between 79.8 and 96.2%, those of sample 2 varied between 58.1 and 81.8%. The drying of sample 2 showed that weight loss was between 4.9 and 12.7%, demonstrating that not all laboratories dried the sample to a constant weight. In a second round-robin testing, the purity of the sample was 91.1%. The reported purities of the sample varied between 82.74 and 95.86%. About 3% purified RebB was added to the sample to check the quality of the analysis of this compound possessing a carboxylic group. The samples contained the following steviol glycosides: Reb D, Reb E, Reb A, ST, Reb F, Reb C, Dul A, Reb G, Rub, Reb B, SB and SM (1 lab). No SV was detected. The number of SVgly analysed in the different laboratories varied between 4 and 11. One lab only analysed ST and Reb A and gave a percentage composition of these compounds. To improve the accuracy of analysis, different suggestions are made, such as controlling the drying process of samples and standards, purity of standards, injection of sufficient material and use of solvent gradients to shorten the run time and to reduce integration errors. The results of this second round-robin tests are better than those of the first one.

Keywords: Steviol Glycosides, Round-robin Testing, Validation of Analytical Methods, HPLC

1. Introduction

A good validated method of analysis of steviol glycosides is of utmost importance for food inspectors as well as for the Stevia industry. The minimum purity requirement of 95% by JECFA and EFSA makes the analysis of steviol glycosides a very difficult task, as possible errors should be eliminated or at least be minimized. The result of a sample analysis should always be the same and totally independent of the laboratory that performed the analysis. As shown earlier, the proposed JECFA [3] method is not reliable and many analysts do not use it strictly as described in the protocol [1, 2]. The analysis of steviol glycosides by HPLC is essentially based on 2 different column types: adsorption (propylamine (-NH₂); HILIC) or reversed phases (well selected phases of Octadecyl silica gel (C₁₈)). The column dimensions are

usually 250 x 4.6 mm and particle size, 5 µm. Some people use HILIC columns [1, 4, 5]. To obtain a baseline separation, required for perfect peak integration, it might be necessary to use 2 C₁₈ columns in series or 1 ultra-high performance liquid chromatography column [6]. More literature on HPLC of steviol glycosides was cited in this last paper, but no baseline separation was obtained or not all the steviol glycosides were included in the analysis [7-10]. Usually, UV-detection at 210 nm is used, although some laboratories confirm the identity of the different compounds by LC-MS. To increase sensitivity and if the equipment is suited, measurements can be done at a lower wavelength (e.g. 190 nm). Solvents used normally are mixtures of acetonitrile: water (AcCN: H₂O) or AcCN in combination with diluted NH₄OAc or phosphoric acid. With NH₂-columns, an isocratic solvent AcCN: water phase (between 87:13 and 80:20) is often used. Under these conditions, the run time

may be as long as 65–75 min.

On C_{18} -type columns, a baseline separation can be obtained by use of 2 Grace Alltima C_{18} columns in series and a solvent gradient of AcCN: 0.1 mM phosphoric acid (see below). In theory, after a proper calibration, both types of steviol glycoside analyses should give similar results. The steviol glycoside analysis has previously been validated [11]. Recently, a review on analytical methods was published, but no detailed discussion was given on the accuracy of the different methods nor on the use of a method in different laboratories [12]. However, inter-laboratory accuracy should also be tested and therefore round-robin tests were organised.

In our round-robin testings, we advised the use of C_{18} columns as these can easily be rinsed with different solvents without damaging the columns.

As most of the laboratories do not have all the ultra-pure standards available for calibration, it is usually done only with stevioside and/or rebaudioside A. Previous work has shown that this is satisfactory as most of the steviol glycosides have a similar absorption coefficient [2, 11]. Therefore, the slopes of the calibration curves of all steviol glycosides are very similar as the calibration curves were made with mM concentrations [1, 2]. To express the results as weight by weight, (e.g. mg component/100 mg mixture) a correction has to be made for the different molecular masses [1]. Table 1 gives the slopes, molecular masses and conversion factors to obtain concentrations in mg/mL if calibration curves were made in mM concentrations (from [11]).

Table 1. Molecular weights and conversion factors are given to calculate concentrations in mg/mL of different steviol glycosides in a mixture after calibration of the HPLC with stevioside (5mg/5 mL = 1.243 mM), or RA respectively (5mg/5 mL = 1.035 mM).

Compound	Slopes $m \pm se$	Molecular weights	Conversion Factors (CF) to obtain concentration in mg/mL
Stevioside	4.52±0.11	804.38	0.80438
Rebaudioside A	4.18±0.04	966.43	0.96643
Rebaudioside C	4.32±0.02	950.44	0.95044
Dulcoside A	4.12±0.04	788.38	0.78838
Rubusoside	4.43±0.13	642.33	0.64233
Steviolbioside	2.30±0.05	642.33	0.64233
Rebaudioside B	3.10±0.11	804.38	0.80438
Rebaudioside D	nd	1128.48	1.12848
Rebaudioside E	nd	966.43	0.96643
Rebaudioside F	nd	936.42	0.93642

2. Method

EUSTAS Round-Robin testing

Seventeen laboratories agreed to participate in the analysis of 1 or 2 unknown samples using their own methods. The aim was to compare the different analyses and improve the cooperation between the laboratories to optimise analytical techniques. Analysis of steviol glycosides is not easy and not only the HPLC methods used are critical as a baseline separation is required, but also the whole set of good laboratory practices (GLP). Different aspects of analysis have to be considered such as: drying of the samples and standards to a constant weight, the purity of the standards, the weighing process itself, the dissolution of samples and standards, the injection and daily calibration of the HPLC, the identification of different compounds and the integration of all peaks and correction for differences in molecular mass. The results presented in this paper are the data sent to us by the different participating laboratories. The results were treated anonymously, and each lab received a number to compare its own results with those of the other laboratories.

3. Results

3.1. Theoretical Weight Loss and Purity of Sample 2 of the First Testing

An interesting result from the first round-robin testing is

the weight loss after drying to a constant weight. Heating NaHCO_3 at 105°C causes a decomposition of NaHCO_3 to Na_2CO_3 and loss of CO_2 and H_2O (total loss of 36.9%). As sample 2 contained 80% of sample 1 and 20% of NaHCO_3 , the total loss by heating can be calculated as follows:

The weight loss of sample 1 (only steviol glycosides) is 6%. If 100 mg of (“wet”) sample 2 is taken, it contains 80 mg steviol glycosides and when heated at 105°C, a loss of 4.8 mg can be expected (6%) plus a 36.9% weight loss of 20 mg bicarbonate = 7.38 mg. The total loss of 100 mg of sample 2 is thus $4.8 + 7.38 = 12.18$ mg or 12.18%. Dry matter content of sample 2 is thus 87.82%. The reported loss on drying of sample 2 varied between 4.9% and 12.7%. This demonstrates that not all the laboratories dried the sample to a constant weight.

The sample used in the second test had a purity of 91.1% and contained a mixture of 11 steviol glycosides. Below, only the results of the second round-robin testing are given.

3.2. Construction of Calibration Curves

Ultra-pure standards were prepared of the steviol glycosides [13] and calibration curves were made with ST, RebA and Rub. Drying to constant weight, weighing and handling were done using GLP. Automatic pipettes were avoided and solutions were made on a weight basis as this reduces possible errors (see below).

Figure 1 shows the calibration curves of ST, Reb A and Rub plotted as mM concentrations. Although the molecular

weights were totally different (804, 966 and 642 for ST, RebA and Rub, respectively), the slopes of the calibration curves plotted as mM concentrations were very similar (1429.6 ± 26.9). However, use of isocratic solvents prolongs

analysis time and, hence, the integration of small peaks eluting late might become problematic if calibration is done with early eluting compounds only. Therefore, solvent gradients were used.

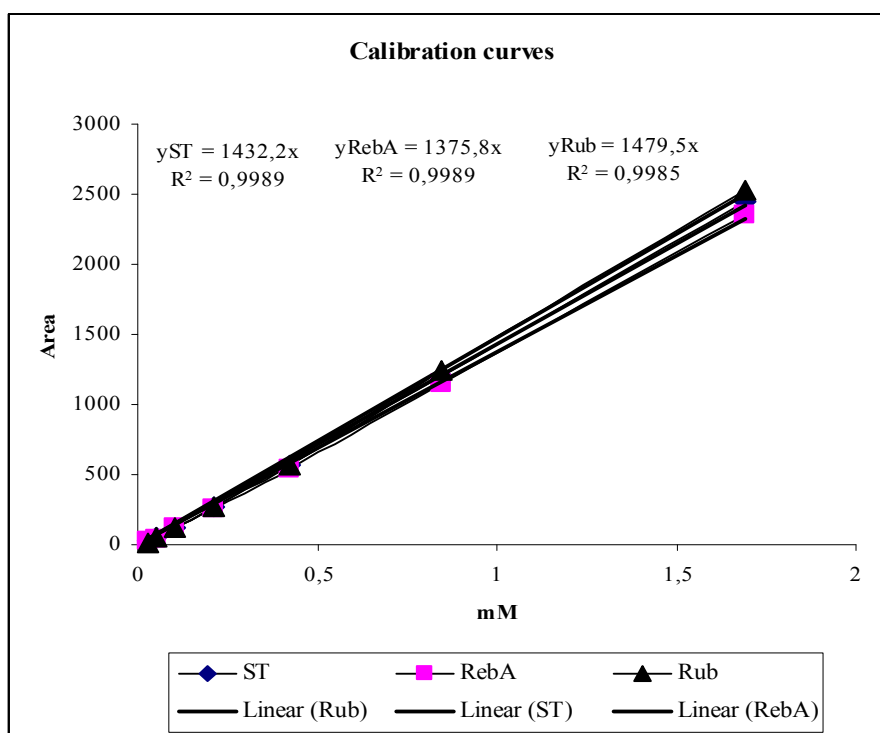


Figure 1. Calibration curves of ST, RebA and Rub plotted in mM concentrations.

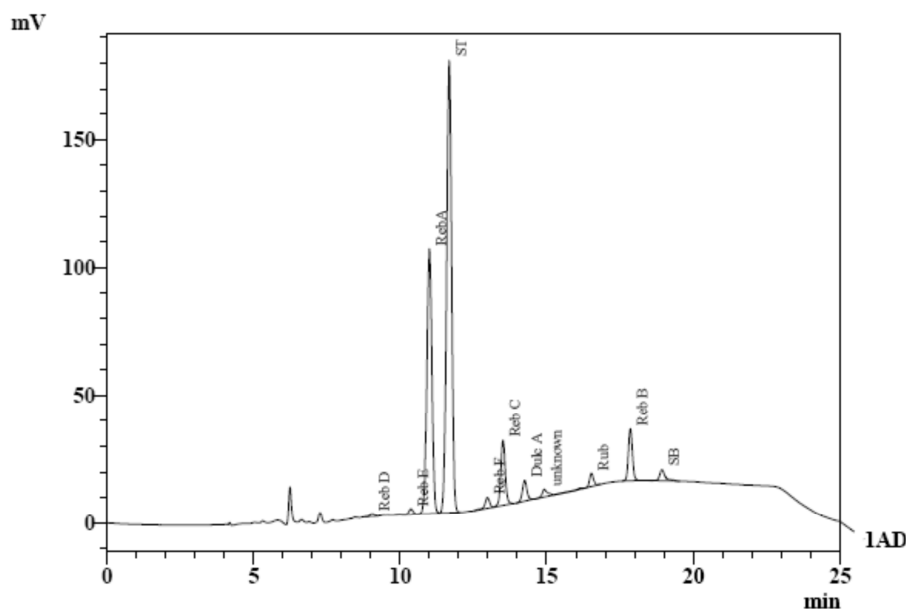


Figure 2. HPLC trace of the sample run on 2 Grace Alltima ODS-columns in series.

3.3. Analysis of the Sample of the Second Testing

A baseline separation was obtained by use of 2 Grace Alltima C₁₈ columns in series and a solvent gradient of AcCN: 0.1 mM phosphoric acid starting at 34: 66 (1 - 4 min) going to 41.6: 58.4 at 10 min (4.1 - 10 min). After 6 min at this solvent strength (10.0 - 16.0 min), the columns are rinsed

with the first solvent (16.1 - 25 min). Total analysis time is less than 30 min (Figure 2). The detection was at 197 nm.

The average drying loss of the sample was about 4.4% (n=6). Although the Karl Fischer method measures water content more precisely, this method is not retained as it is expensive. Moreover, JECFA [3] suggested that samples be

dried to a constant weight.

Table 2 gives the analysis values by a validated external

standard method of the sample of the second round-robin testing.

Table 2. Percentage composition and mg of each component per 100 mg dry wt. of the mixture (corrected for water loss, 4.4%).

Name	Retention time(min)	% of SVgly	mg/100 mg dry sample
Reb D	9.043	0.36	0.32
Reb E	10.37	0.58	0.53
Reb A	10.99	34.02	30.83
ST	11.73	47.75	43.26
Reb F	12.98	1.17	1.06
Reb C	13.51	7.16	6.48
Dul A	14.25	1.92	1.74
Reb G	14.92	0.87	0.79
Rub	16.53	0.86	0.78
Reb B	17.86	4.37	3.96
SB	18.94	0.94	0.85
Purity			90.6

The results of the analyses of this sample by the different laboratories are given in Table 3. Most of the laboratories used NH₂ columns, except those marked with an asterix (* = C₁₈; ** = HILIC).

Table 3. Analyses of the sample by the participating laboratories. Values are expressed on a dry wt. basis.

Lab	% loss	RebD	RebE	RebA	ST	RebF	RebC	DulA	RebG	Rub	RebB	SB	SM
2**	3.64			30.82	45.22		6.21	5.92					
5*	?			30.89	47.17	1.19	7.25	2.05	0.60	1.08	4.62	0.90	0.09
6*	4.4	0.33	0.52	30.9	43.5	1.1	6.50	1.70	0.80	0.80	4.0	0.9	
9	4.37	1.39		31.22	43.8	1.05	6.27	1.31		0.79	4.37	0.82	
11	3.3			27.32	43.4		5.51	1.24		0.78	3.95	0.54	
15	2.98			30.30	42.5	1.1	6.5	1.8	0.7	0.7	4.5	0.8	
16	3.15			31.19	44.66	0.93	6.59	1.83	0.58	0.73	4.36	0.93	
17				36.69	54.78								

* Using C₁₈ columns

** Using HILIC columns bold and italics: only percentage composition was given

Table 4 lists the purity of the sample as reported by the different laboratories. As some laboratories did not measure all the smaller peaks, values were added to obtain a “corrected purity”.

Table 4. Purity of the sample obtained by different laboratories (only those reporting results).

Lab	Purity (% of dry wt)	# compounds analysed	Correction from labs 5 and 6	Corrected purity
2**	88.2	4	8.54	96.74
5*	95.86	10	0.85	96.71
6*	91.1	117	0.09	91.19
9	91.01	9	1.41	92.42
11	82.74	7	2.84	85.58
15	88.9	9	0.94	89.84
16	91.8	9	0.94	92.74
17	nd	2	nd	nd

4. Discussion

One laboratory (17) analysed only the percentage composition of 2 SVgly and 1 laboratory (2) analysed only 4 compounds. The other laboratories analysed between 7 and 11 SVglycosides of the mixture. None of the laboratories reported the occurrence of SV, which is not expected in the mixture of these relatively polar SVgly. Only 1 lab (6) reported the values for Reb E (0.52) and 2 laboratories (6, 9) the values for Reb D (0.33, 1.39). The values recorded on a dry wt. basis varied between 27.32 and 31.19 and between 42.5 and 47.17 for Reb A and ST, respectively. Reb F was reported by 5 laboratories and its value was between 0.93 and 1.19. Reb C varied

between 5.51 and 7.25. A much larger spread was found for Dul A: between 1.24 and 5.92. An unidentified compound in the mixture with MW 804, tentatively named Reb G, was analysed by 4 laboratories (between 0.58 and 0.80). This compound is not yet fully characterised by NMR (Geuns, unpublished). The values for Rub, Reb B and SB were measured by 6 laboratories and their amounts varied between 0.7 and 1.08, 3.95 and 4.62 and between 0.54 and 0.93 for Rub, Reb B and SB, respectively. Only 1 laboratory (5) analysed SM (0.09). The weight loss after drying of the sample varied between 2.98% and 4.4% (not all laboratories reported the weight loss). The purity of the sample obtained by the addition of all compounds analysed, is given in Table 4 (second column). The purity of the sample was 91.1%, checked by the

standard addition method of 3 SVgly as described by [2] (Reb A, ST and Reb B). Three laboratories obtained a value of about the expected purity (91.01 – 91.8). Laboratory 5 overestimated the purity (95.86%) whereas the other laboratories reported purities between 88.2 and 88.9%. As not all laboratories analysed all the compounds present in the mixture, a correction was made by adding the values of non-analysed compounds taken from laboratories 5 (SM only) and 6. After the addition of the “lacking” values, the “corrected” purities varied between 85.58 and 96.74% (Table 2). In this way, 4 laboratories (6, 9, 15 and 16) obtained acceptable values only differing from the expected values by no more than 1.5%. Laboratory (11) underestimated the purity and 2 labs overestimated the purity by about 6%.

This second round-robin testing has shown that it is difficult

Table 5. RSD calculated on the averages of the first and second round-robin testing of steviol glycosides.

	RebA	ST	RebF	RebC	duA	RebG	Rub	RebB	SB	%
first	12.7	6.4	24.5	19.5	38.1	8.3	2.4	87.5	84.2	5.6
second	4.5	3.5	8.8	8.1	72.3	15.1	16.7	6.3	17.7	4.5

The accuracy of analysis of the most important sweeteners (Reb A, ST, Reb C) was much improved too. The participants who participated for the first time had access to the guidelines of the previous testing [2]. This certainly helped them to improve their analysis. To further improve the quality of the analyses, the different laboratories should try to identify and quantify all the SVgly present in a sample. Probably 2 injections will be necessary to improve the quantification of the smaller peaks, one injection as usual, and one injection of a 5 or 10 x more concentrated solution. To better dissolve the samples and to avoid precipitation, a good co-solvent might be required. This might pose problems with the external standard method that, so far, is used for the quantitation of SVgly, as small amounts of the co-solvent might evaporate, leading to faulty results. Impurities of the co-solvent might also show up.

The required purity of steviol glycosides is 95%. The mean RSD of 4.5% obtained is still rather large, as it means that then purities of 95 ± 4.5 have to be accepted, i.e. purities between 90.5 and 99.8%. Is it possible to increase the accuracy and to reduce the RSD? How can it be done?

The listing below is a non-exhaustive list of possible items enhancing the degree of errors influencing the accuracy of measurement of steviol glycosides by the external standard method.

Item

External Standard Itself

- Purity of standards:

High purity standards are required and were made [8]. The purity of commercial standards is sometimes far from the claimed purity values.

- Water content of standard:

Standards may contain up to 5% water and should be dried to a constant weight before use.

- 1) Weigh an empty and dry weighing vessel with lid (value A).

to motivate the participating laboratories to send their results before the deadline although about 2 months were given for performing 1 analysis. However, there are a number of positive points to report. Most of the laboratories did analyse many more compounds than they did the first time. Moreover, the differences for the compounds analysed were less than during the first round-robin testing, certainly for the main compounds present. This can be evaluated by the values of the relative standard deviation (RSD, Table 5). The RSD's were calculated on the average values provided by the different laboratories. Reb D and E were not included as the number of measurements was too small. In the first round-robin testing, the RSD's were always about 50% larger than those calculated on the results of the second one. This means that the quality of the different analyses has improved.

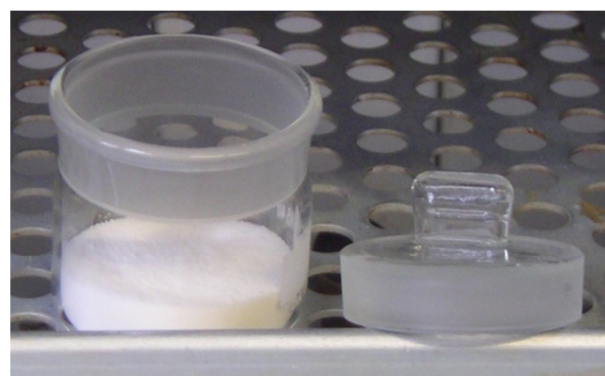


Figure 3. Example of a weighing vessel with lid also placed in the oven.

- 2) Exactly weigh about 500 mg of the standard or unknown sample of SVglys in the weighing vessel with lid (value B).
 - 3) The amount of wet sample is: $C = B - A$.
 - 4) Dry the opened vessel with wet mixture of standard or analyte to a constant weight or overnight (16 h at 105°C). 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 dried sample (value D).
 - 7) The dry weight of the unknown sample is $E = D - A$ (mg dry wt.).
 - 8) The percentage dry weight is: $F = E/C \times 100$ (times 100 to present it as a percentage).
 - 9) The water content in percentage is: $G = 100 - F$.
- Weighing process of the standards:

A sufficient large amount of standard should be weighed to decrease the percentage of uncertainty. Even by using an analytical balance with a resolution of ± 0.1 mg, weighing only

2 mg gives an uncertainty of 10%, i.e., $2 \text{ mg} \pm 0.1 \text{ mg}$ or $(0.2/2) \times 100 = 10\%$. To reduce the possible error beneath 1%, at least 50 mg of standard should be weighed $(0.2/50) \times 100 = 0.4\%$. Taking each time 50 mg of very pure standards of the compounds occurring in small amounts would become very expensive, and reuse of standards might introduce impurities. For this reason, preliminary research has been done and it was shown that calibration with only 1 standard is sufficient for the measurement of steviol glycosides (see Figure 1). Calibration curves should be made in mM concentrations.

- Calibration solution of standard:

Calibration curves should be made on a weight basis starting with at least 50 mg of the standard. Much uncertainty can be introduced even by using calibrated pipettes as observed by the author who unexpectedly tested calibrated pipettes in an analytical laboratory by weighing 1 ml water and found deviations up to 10% (results not presented). If pipetting of standards would be 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.

Analyte

- Drying process: the product should be dried to a constant weight (see above)

- Weighing: at least 50 mg should be weighed to avoid weighing errors (see above). In case of analysis of plant material (leaves) at least 200 mg should be taken for analysis to avoid a bad sampling. In many papers in the scientific literature, only 20 mg leaf tissue is analysed and this is a too small amount as 20 mg might come from only 1 leaf. It is known that leaf position on the plants has a huge influence on the steviol glycoside amount [14]. The analysis of only 20 mg leaf tissue might also over- or underestimate the yield per hectare when results of this kind of analyses are extrapolated to know the yield per ha.

- Purification of crude extracts:

Purification of water extracts by SPE techniques is certainly advantageous for maintaining good column conditions. However, each purification step can add a degree of uncertainty to the analysis as losses will certainly occur. Several SPE methods are given for the purification of different extracts of food samples [11].

Analysis

- The injection volume of standard or analyte is very critical, even with an automatic injector. This can be checked by injection of different volumes of water and collecting and weighing the volume that was delivered by the injector. In this way, possible errors can also be detected if different amounts would be injected to increase the size of small peaks (e.g., 20 μl versus 100 μl injections).

- Change of sensitivity of the detector:

The sensitivity of a detector might change after the light bulb has been used for a while. This should be checked at regular moments.

- Dissolution of analyte:

Some compounds of the mixture might be less soluble and be extruded from the mixture. Adding a solvent (e.g. EtOH or

MeOH) might prevent this, but part of the EtOH or MeOH might evaporate from the injection vials and this might contribute to errors.

- Intra-laboratory and Inter-laboratory RSD of the 10 components of the mixture should be as small as possible and for the required purity level of steviol glycosides ($\geq 95\%$) should be below 2%. This way, a claimed purity of 95% would lie between 93 and 97% which seems acceptable.

Volume Aspects that might Influence Accuracy

- If there is no control on the temperature of the samples to analyse, expansion or contraction of solvent (e.g., water) might increase uncertainty. This can give changes of sample and injection amounts.

- Inaccuracy of pipettes: pipettes used are possibly not recently calibrated and solvent expansion or contraction also has an influence. Therefore, weighing of all solvents is the best way of handling this problem.

- Precipitation of analyte: when concentrated solutions are used, some compounds might precipitate. The addition of co-solvents might help reduce this problem (keep out for evaporation, see above).

- Daily calibration necessary:

The external standard method requires a daily calibration of the whole equipment and this is time-consuming and expensive (very pure standards are required).

- Calculation errors possible:

Even with well-trained personnel, calculation errors are possible and all analyses should be double-checked to avoid human errors.

- Small peaks:

Small peaks make peak integration very difficult and erroneous. The injection of 5 x more sample is not always possible, the more as then new calibration curves have to be made for the higher amounts injected. It cannot be done to inject 5 x more and divide then the peak areas by 5. This would introduce more uncertainty. The best way of handling is to make solutions that are 5 x more concentrated and stick to the same injection volume. However, then there might be a problem of precipitation of some less soluble material. Adding a co-solvent might circumvent this, but then problems of evaporation might occur or additional peaks appearing in the chromatogram due to impurities in the co-solvent.

Stress Factor of the Personnel

Although the personnel of the laboratory were well trained, the huge list of demands for perfect performances throughout steviol glycoside analysis nearly drove them to madness. Therefore, an internal standard method should be developed that will prove its value, not only for solvent losses as above, but also for losses of sample during sample clean-up when extracting complex food matrices, as well as for differences in injection volume and a possible change of detector sensitivity. Work is in progress to circumvent all the problems of an external standard method. However, for the moment being, when people are following the above guidelines, nearly perfect analyses can be obtained as shown by 4 laboratories that reported results only differing by 1.5% from the target value.

5. Conclusion

JECFA [3] suggested the use of reverse phase columns for the analysis of steviol glycosides. A baseline separation of the most important steviol glycosides can be obtained by use of 2 reverse phase columns in series (e.g., 2 Grace Alltima C₁₈ columns) and a solvent gradient of AcCN: 0.1 mM phosphoric acid starting at 34: 66 (1 - 4 min) going to 41.6: 58.4 at 10 min (4.1 - 10 min). After 6 min at this solvent strength (10.0 - 16.0 min), the columns are rinsed with the first solvent (16.1 – 25 min). Total analysis time is less than 30 min (Figure 2). The detection can be done at 200 nm if the equipment is suited for this. Recently, the use of 1 ultra-high performance liquid chromatography column was reported also giving a baseline separation [6]. Without baseline separation, the peak integration becomes more difficult and it might become impossible to obtain an inter-laboratory RSD of around 1%. The correct analysis of steviol glycosides is a real challenge for the analytical chemist and many pitfalls exist. Taking into account all the above suggestions and GLP, it might be possible to do correct steviol glycoside analyses by an external standard method. Then an inter-laboratory RSD of about 1.5% can be obtained as shown by 4 laboratories that took care of all the suggestions made. In this way, a purity of 95% [15] means that the interval of uncertainty is rather small (95% purity means that values between 93.5 and 96.5% might be accepted). However, the analysis remains a difficult issue and the use of an internal standard might eliminate most of the tedious points in the analysis.

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Abbreviations

Using SV for steviol allows the use of the following abbreviations: SVgly: steviol glycosides; SVEq: steviol equivalents; SVglu: steviol glucuronide; SM: steviol monoside; SVE: steviol 19-ester; SVglu: steviol glucuronide; ST: stevioside, RebA - G: rebaudioside A – G; SB: steviolbioside; DulA: dulcoside A; Rub: rubusoside.

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