CHAPTER 5

Determination of Steviol Glycosides in Various Dairy Matrices and Soy Drink

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ABSTRACT

EFSA recently expressed the opinion that stevia extracts containing at least 95% rebaudioside A and/or stevioside are safe as a food additive. Approval for these stevia extracts as a food additive is expected very soon. It is therefore important that stevia extracts can be accurately and precisely determined in various food matrices.

The objective of this study was to determine steviol glycosides in dairy products and soy drink. Milk, ice-cream, fermented milk drink and soy drink sweetened with

steviol glycosides were analyzed. Fat from the food matrices was removed by centrifugation and proteins were precipitated with acetonitrile. The supernatant was concentrated on a SPE C_{18} column and analyzed with HPLC.

The results show an excellent recovery and very good agreement between the use of an external calibration curve, the standard addition method and the use of an internal standard which was added before the extraction.

KEYWORDS
Determination, extraction, internal standard, solid phase extraction, steviol glycosides, standard addition.

Introduction

In April 2010, the European Food Safety Authority (EFSA) expressed the opinion that stevia extracts consisting of no less than 95% rebaudioside A (RebA) and/or stevioside (Ste) are safe as a food additive. The Acceptable Daily Intake (ADI) is established at 4 mg steviol equivalents per mg bodyweight per day (EFSA, 2010). The final approval for steviol glycosides as a new food additive is expected very soon, so procedures for analysis of these compounds and their extraction must be optimized.

In order to assess the stability of the additives in food, their concentration must be followed over a certain period of time, depending on the food matrix. First, the added concentration of steviol glycosides must be determined in the food matrix, i.e. the recovery of the analytical method must approach 100 %. Next, the concentration of steviol glycosides must remain constant during the shelf life of the food, i.e. the steviol glycosides should not be degraded in or by the food matrix.

Most analysis of steviol glycosides are done by liquid chromatography, either on an amine or a reversed phase C18 column (Geuns, 2008; Geuns and Struyf, 2009; Hoekstra *et al.*, 2009; Kolb *et al.*, 2001). Detection is possible either with UV absorption or ELSD (Evaporative Light Scattering Detection). When detection is done in UV, the shortest possible wavelength should be used, to have the best sensitivity. So 200 or even 190 nm is preferred, rather than 210 nm. Other methods were also reported: high performance thin layer chromatography (Jaitak *et al.*, 2008) and capillary electrophoresis (Liu and Li, 1995).

The first results of a study that was undertaken in order to assess the stability of steviol glycosides in various matrices are presented. Specifically, milk, ice-cream,

fermented milk drink and soy drink with additions of a mixture of RebA and Ste were analyzed.

Methods and Materials

Sample processing:
Both semi-skimmed milk and non-aromatized soy drink were processed in a pilot plant (2-steps homogenization: 200 bar, 65 °C; indirect UHT-processing: 5 s, 140 °C; cooling to 20 °C; APV Paraflow Pilot) and aseptically filled in 0.25 L 3-HDPE bottles. Fermented milk drink containing 77.5 % yoghurt, 22.1 % water and 0.4 % pectin was also processed in the pilot plant (2-steps homogenization: 200 bar, 65 °C; indirect UHT-processing: 15 s, 95 °C; cooling to 20 °C; APV Paraflow Pilot). Ice-cream was prepared containing 22.65 % cream (35 % milk fat), 10.82 % skimmed milk powder, 48.53 % water, 11.82 % sucrose, 5.72 % glucose/maltose syrup (80-82 % DS, 36-40% DE), 0.45 % emulsifier/stabilizer and 0.01 % vanillin. After mixing the ingredients at 50 °C, the ice-cream mixes were homogenized (2-steps, 180 bar, 65 °C), pasteurized (15 s, 85 °C; APV Paraflow Pilot) and cooled down to 20 °C. After aging for 16 h at 4 °C, the ice-cream mixes were frozen in a continuous freezer (Gelmark Hoyer 160; Alfa-Laval) while an overrun of 100 % was aimed for. Ice-cream samples were hardened to -22 °C for at least 24 h and finally stored at -18 °C until analysis. A quantity of four different types of steviol glycosides was added to each of the samples. The composition of the tested steviol glycosides are given in Table 1. Sugar in the drinks was partly replaced by one of the four mixtures: (1) 100 % Reb A; (2) 100 % Ste; (3) 100 % of a commercial sample of steviol glycosides (SV); (4) a mixture of 80 % RebA and 20 % Ste. For ice-cream, sugar was partly replaced by one of the four mixtures in combination with oligofructose and erythritol or maltitol. All processed samples were analyzed within five working days after processing.

Table 1: Composition of the steviol glycosides, added to the dairy samples and soy drink (data in m%)

Addition	RebA	Ste	RebF	RebC	DulA	Peak 6	Rub	RebB	SteB	TOTAL
RebA	96.29	0.09	0.31	0.25	0	0	0	0.29	0	97.23
Ste	6.33	83.54	0.57	0.47	0	0	0.55	0.63	0	92.09
SV glycosides	32.55	49.81	1.23	7.31	2.06	0.76	1.1	0.76	0.91	96.49

Sample preparation:
Fat from milk, soy drink and fermented milk drink was first removed by centrifugation (Biofuge Strator from Heraeus Instruments) of a sample of 25 mL

for 12 min at 15000 rpm (24000 g). The proteins were subsequently precipitated with acetonitrile, ACN (Acros, Beerse, Belgium). 7 mL water and 21 mL ACN was added to 7 mL supernatant of the first centrifugation. The addition of water was necessary in order to prevent the phase separation between the aqueous and organic phases. This mixture was stored for 10 min in the refrigerator. The proteins were then separated by a second centrifugation for 12 min at 15000 rpm. Ice-cream samples were melted first, and then processed in an analogous manner as the other samples. However, it was necessary to change some quantities. A sample of 25 mL melted ice-cream was centrifuged for 12 min at 15000 rpm (24000 g) in order to remove the fat. Next, 3 mL supernatant was mixed with 21 mL ultra pure (UP) water and 36 mL ACN. After 10 min cooling, the proteins were separated by a centrifugation for 12 min at 15000 rpm.

The supernatant was subsequently subjected to solid phase extraction (SPE) in order to concentrate the steviol glycosides. Because the high concentration of ACN in the supernatant would cause the steviol glycosides to elute immediately from the SPE column, a sample of 25 mL supernatant was diluted with 75 mL UP water for milk, soy drink and fermented milk drink. For ice-cream, 40 mL supernatant was diluted with 120 mL UP water. SPE columns (Hypersep C_{18}, 500 mg/3 mL from Thermo Scientific, Waltman, USA) were conditioned with 5 mL MeOH (Acros, Beerse, Belgium), and rinsed with 10 mL ultra pure water, made “in-house” using a Simplicity instrument from Millipore (Billerica, USA). After loading the sample, the column was rinsed with 10 mL UP water and 5 mL 20 % ACN. The steviol glycosides were finally eluted with 5 mL 60 % ACN.

Analysis:
All samples were analyzed with a HPLC apparatus (Thermo Scientific, Waltman, USA) consisting of an SCM1000 vacuum degasser, a P4000 pump, an AS1000 auto sampler with a fixed injection volume of 20 µl and an UV6000LP diode array detector with a flow cell of 10 µL and a path length of 5 cm. Separations were done on two ODS Hypersil Columns (each 20 x 0.3 cm; 5 µm) placed in series. All samples were eluted by a linear gradient using 25 mM H_3PO_4 (solvent A) and ACN (solvent B) as eluent, as follows: 0 min: 30 % B ; 10 min: 40 % B ; 20 min: 80% B ; 30 min, 80% B. UV Spectra were recorded between 195 and 360 nm for identification purposes, and the compounds were quantified at 200 nm. A sample chromatogram is shown in Figure 1. “Peak 6” in this chromatogram is a compound that has not yet been fully characterized. From a preliminary LC – MS experiment, it is known that the peak has a molecular weight of 804 $g.mol^{-1}$ and is tentatively assigned as Rebaudioside G (RebG) (Geuns, 2010)

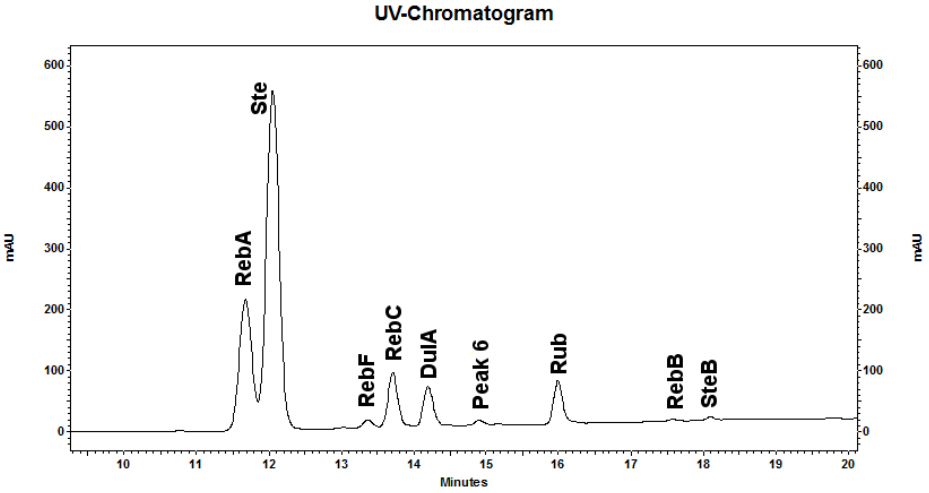

Figure 1: Sample chromatogram of steviol glycosides

Calibration:

All compounds were quantified, based on a single standard of RebA. Because every steviol glycoside has the same ester function as the chromophore, it is not surprising that the slope of the calibration curves of the different steviol glycosides is very similar if the concentration is expressed in mol.L^{-1} (Geuns and Struyf, 2009) (see also Figure 6 below). It is quite straightforward to apply correction factors based on molecular weights and to express the concentrations on a mass base, such as ppm.

A standard containing 97.07 mass % RebA was analyzed. The calibration curve was linear between 0 and 0.05 mmol.L^{-1} RebA, as shown by Figure 2. The standard was corrected for residual moisture.

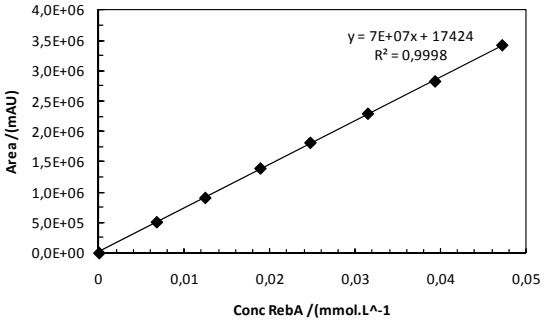

Figure 2: Calibration curve of RebA. with concentration in mmol.L^{-1}

Results and Discussion:

Recovery:
The recovery of RebA was tested in every type of tested food matrix. A known amount of RebA standard was added to the different matrices (without added stevia), prior to the extraction. The different concentrations of added RebA reflect the concentrations of steviol glycosides that will be used later in the processed samples. The results are shown in Table 2 and demonstrate an excellent recovery.

Table 2: Recovery of RebA in dairy samples

Sample	Added /(ppm)	Found /(ppm)	% RSD (n=3)	% Recovery
Milk drink	103.03	102	1.03	99.16
Fermented milk drink	115.6	114	0.97	98.45
Soy drink	64.23	63	2.03	98.23
Ice-cream	248.4	240	0.63	96.76

Standard Addition:
The general reliability of the analytical procedure can be tested by using standard addition. Moreover, this method can also be used to test for matrix effects. A matrix effect is defined as "*The combined effect of all components of the sample other than the analyte on the measurement of the quantity*" (IUPAC, 2009). This means that the response (or: sensitivity) of the analyte is different in the matrix from that in pure water.

Known amounts of a pure standard were added to a sample. The sample was then analyzed before and after the additions. The concentration of the unknown was calculated from the increase in signal due to the addition of standard and was graphically evaluated by the intercept on the y-axis. The concentration calculated from the standard additions can then be compared to the concentration that is calculated from the external calibration graph. Standard addition is often used when matrix interferences are inferred.

We made four additions of pure RebA standard to a milk sample. The results are shown in Figure 3. The extrapolated straight line from the four addition points coincides almost perfectly with the area that was measured from the sample without the addition: the intercept from the graph of Figure 3 equals an area that differs only 0.75 % from the area of the sample without addition. Hence, we can conclude that the accuracy of the method is satisfactory.

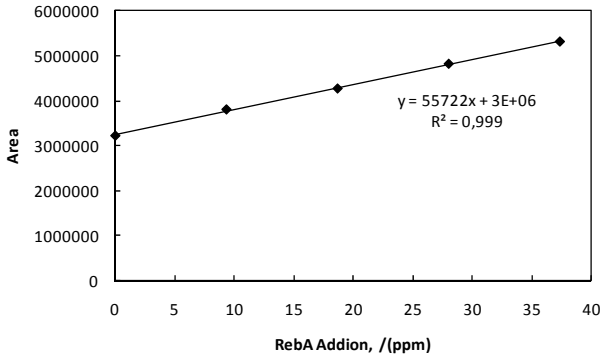

Figure 3: Standard Addition curve

A second conclusion that can be drawn from this experiment is that there are no significant matrix effects in this analysis. Matrix effects are frequently encountered in atomic absorption or emission spectroscopy, in mass spectrometry or X-ray fluorescence (Skoog *et al.*, 2007). In this analysis however, one would not expect matrix effects, because the analyte is separated from the matrix by SPE, and a second separation occurs on the HPLC column. The present results prove this point.

Internal standard:

Steviol-19-β-galactose-13-β-glucose, an isomer of rubusoside, shows all the requirements of an adequate internal standard (IS). The internal standard should match the chemical and physical properties of the analyte(s) as closely as possible, so possible losses of the analyte and the internal standard during the extraction are comparable. Moreover, the internal standard should elute in the chromatogram in an area where no other peak elutes. The structure of the internal standard is shown in Figure 4. Because this particular steviol glycoside is not a natural compound, it will never appear in any sample. On the other hand, it is expected the IS will behave similarly to other steviol glycosides in any extraction procedure.

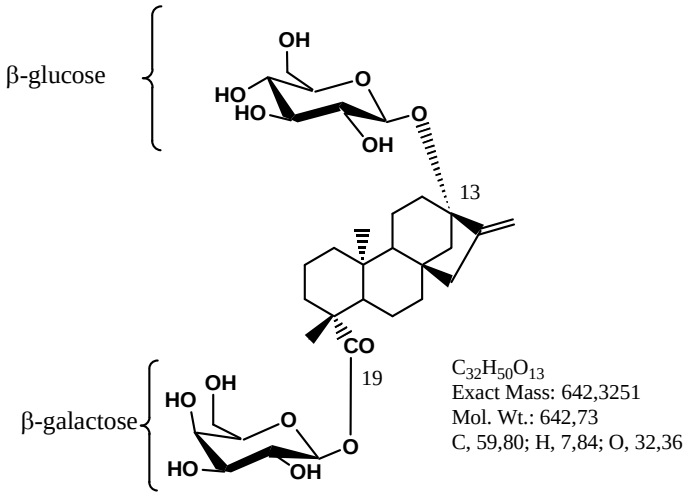

Figure 4: Structure of the internal standard, steviol-19-β-galactose-13-β-glucose, an isomer of rubusoside

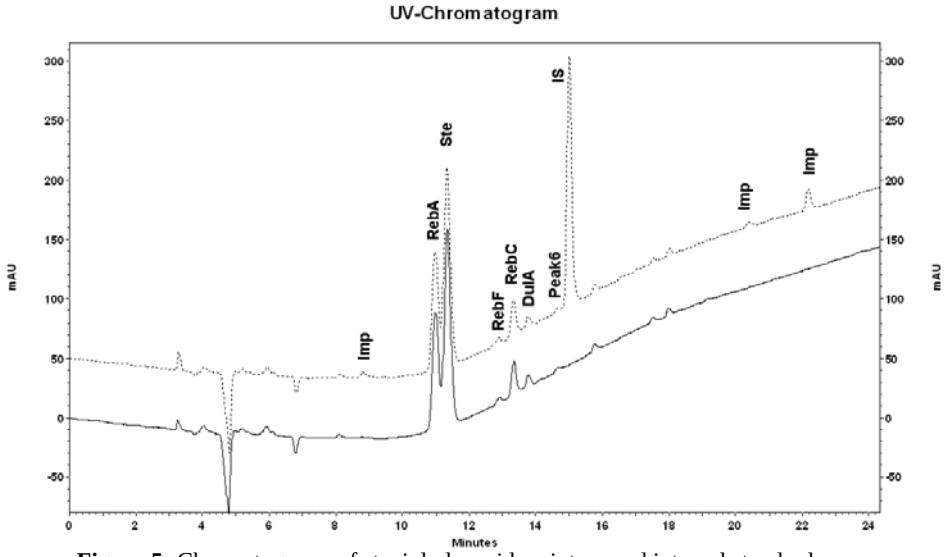

Figure 5: Chromatograms of steviol glycoside mixture and internal standard
full line: mixture of steviolglycosides; dotted line: mixture of steviolglycosides and internal standard

Figure 5 shows a chromatogram of a mixture of steviol glycosides (full line) and the same mixture to which the internal standard has been added (dotted line). As

shown in the chromatogram, the internal standard elutes with a retention time of around 15 min, well separated from the other steviol glycosides. The small peaks marked “Imp” (impurities) in the upper trace are apparently impurities in the internal standard, which are not present in the sample of steviol glycosides. However, judged from the relative peak areas, the internal standard is chemically very pure.

In a preliminary experiment, a calibration curve from the IS was measured and compared with calibration curves from RebA and Ste. Because the concentration is assessed with UV absorption and the chromophores of these three compounds are basically identical, very similar slopes are expected when the concentration is plotted in mol.L^{-1} (Geuns and Struyf, 2009). This is indeed confirmed for RebA and Ste, but the IS has a somewhat lower slope, as indicated in Figure 6.

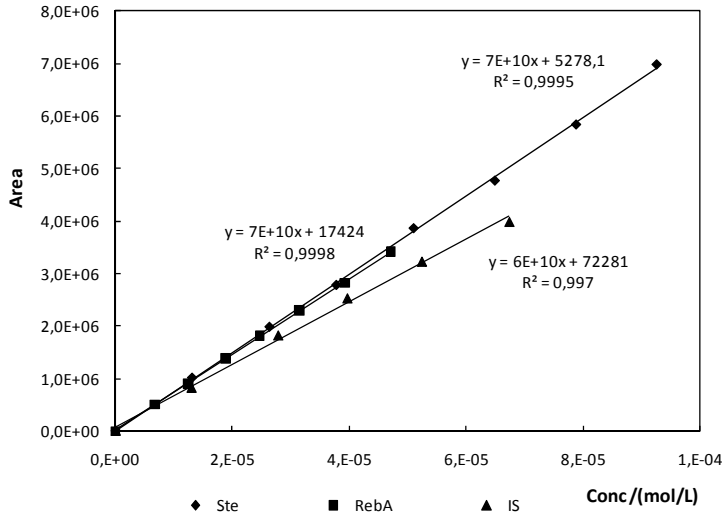

Figure 6: Comparison of the slopes of the calibration curves between RebA, Ste and IS

All samples were well dried, and the concentrations corrected for residual moisture. The reason for the slightly deviating slope might be due to a few impurities remaining from the synthesis (Figure 5). However, these impurities do not influence the analytical results.

Having proven that the calibration graph of the internal standard is linear up to a concentration of about 45 ppm, a new calibration graph was determined, in which a varying concentration of RebA was combined with a fixed concentration of the

internal standard. The ratio of the area of the RebA signal to the area of the IS was plotted against the concentration of RebA. The resulting calibration curve is shown in Figure 7.

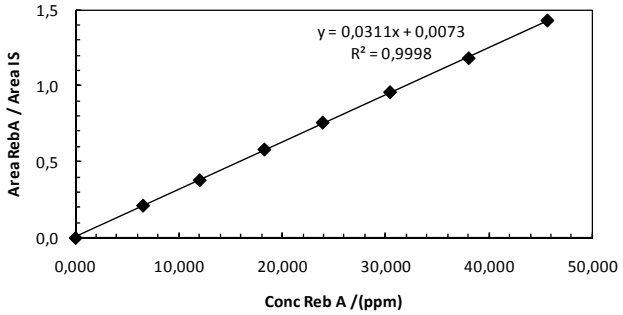

Figure 7: Calibration curve with the use of the internal standard

As a final test to the accuracy and reliability of our extraction and analysis, the internal standard was added in a known concentration to a sample of milk. The IS was added prior to the extraction. Using the external calibration graph (Figure 2) and the area of the RebA peak, a concentration of 44,8 ppm of RebA was found. The concentration of RebA was also calculated using the internal standard by taking the proportion of the area of the RebA peak over those of the IS peak and using the calibration graph of Figure 7. This yielded an average concentration of 44,9 ppm, which is only 0,4 % more. This can be regarded as a deviation that is normal for this type of analysis. This experiment was done in duplicate.

The results of these experiments and the results about the standard addition indicate that the accuracy and the precision of the analytical result cannot be substantially improved by using any of these techniques, at least for this extraction scheme. The internal standard method, however, has the advantage that possible losses during (more elaborate) extractions will not influence the result, because it is – correctly – assumed that the same losses will occur in the internal standard. Therefore, this method will improve both precision and accuracy of the method.

Analysis of processed samples:
Three samples were analyzed for each addition to milk and soy drink. For ice-cream and fermented milk drink, two samples were analyzed for each addition. The results are shown in Table 3.

Table 3: Results of the analysis of dairy samples and soy drink. To the samples, RebA, Ste, a commercial steviol glycoside or a 80:20 mixture of RebA and Ste were added, for the composition of the RebA, Ste and the commercial sample, see Table 1. For the different steviol glycosides the added amount is given as well as the amount found back using the method of external calibration based on RebA (units in mg/kg)

		RebA addition		Ste addition		SV glycosides		Mixture	
Sample	Compound	Added	Found	Added	Found	Added	Found	Added	Found
Milk drink	RebA	103.03	82.35	10.13	8.01	52.09	37.21	83.87	75.34
	Stev	0.10	nd	133.66	121.12	79.70	65.99	26.81	31.46
	RebF	0.33	nd	0.91	0.34	1.97	1.53	0.45	0.32
	RebC	0.27	nd	0.75	0.40	11.69	9.27	0.36	0.32
	DulA	0.00	nd	0.00	nd	3.29	2.81	0.00	nd
	Peak 6	0.00	nd	0.00	nd	1.21	0.68	0.00	nd
	Rub	0.00	nd	0.88	1.09	1.76	1.62	0.18	0.22
	RebB	0.31	0.21	1.01	0.60	1.22	0.61	0.45	0.41
	SteB	0.00	nd	0.00	nd	1.46	0.59	0.00	0.13
Fermented milk drink	RebA	115.55	113.03	11.39	10.59	58.60	57.55	94.72	94.33
	Stev	0.11	nd	150.37	146.46	89.66	88.70	30.16	38.57
	RebF	0.37	0.77	1.03	0.52	2.22	2.43	0.50	0.69
	RebC	0.30	0.29	0.85	0.63	13.15	13.01	0.41	0.33
	DulA	0.00	nd	0.00	nd	3.70	3.75	0.00	nd
	Peak 6	0.00	nd	0.00	nd	1.36	0.96	0.00	nd
	Rub	0.00	nd	0.99	1.15	1.98	1.79	0.20	nd
	RebB	0.35	0.31	1.13	0.32	1.37	0.87	0.51	0.73
	SteB	0.00	nd	0.00	nd	1.64	1.33	0.00	nd
Soy drink	RebA	64.23	64.76	6.33	5.49	32.55	33.75	52.30	52.53
	Stev	0.01	nd	83.54	84.41	49.81	49.18	16.76	21.42
	RebF	0.02	0.07	0.57	0.61	1.23	0.93	0.28	nd
	RebC	0.02	0.11	0.47	0.09	7.31	5.67	0.23	0.24
	DulA	0.00	nd	0.00	nd	2.06	1.62	0.00	nd
	Peak 6	0.00	nd	0.00	nd	0.76	0.37	0.00	nd
	Rub	0.00	nd	0.55	0.60	1.10	0.74	0.11	0.24
	RebB	0.02	0.22	0.63	0.14	0.76	0.41	0.28	0.15
	SteB	0.00	nd	0.00	0.12	0.91	0.37	0.00	nd
Ice-cream	RebA	248.4	268.0	24.5	19.8	126.0	110.6	203.6	197.2

Sample	Compound	RebA addition		Ste addition		SV glycosides		Mixture	
		Added	Found	Added	Found	Added	Found	Added	Found
Ice-cream (Cont'd)	Stev	0.20	nd	323.3	349.2	192.8	190.7	64.8	82.2
	RebF	0.80	0.60	2.2	1.0	4.8	4.0	1.1	nd
	RebC	0.60	0.50	1.8	1.5	28.3	26.4	0.90	0.60
	DulA	0.00	nd	0.00	nd	8.0	6.7	0.00	nd
	Peak 6	0.00	nd	0.00	nd	2.9	0.9	0.00	nd
	Rub	0.00	nd	2.1	1.6	4.3	3.4	0.40	nd
	RebB	0.70	1.2	2.4	0.30	2.9	0.90	1.1	nd
	SteB	0.00	nd	0.00	nd	3.5	0.8	0.00	nd

As indicated in Table 3, all the concentrations are expressed in mg/kg and were measured with the method of the external calibration graph. The entry "nd" means not detected, indicating that the concentration was too low to be measured accurately. It is clear that the agreement between the added and found concentrations is in general satisfactory for fermented milk drink, soy drink and ice cream. The results are somewhat less satisfactory for milk drink.

All analyses were done in duplicate or in triplicate. RSD values were not included in this table. For the major compounds (RebA and Ste), RSD values were always below 10 %, and very often around 5 %. Because the concentration of the minor compounds was small, the analysis for these compounds was not so good.

Conclusions

Our results on the analysis of steviol glycosides in dairy products and soy drink show that these steviol glycosides can be analyzed with good precision and accuracy and excellent recovery and reproducibility. The agreement between the different methods used to quantify the analytes, i.e. external calibration, standard addition and internal standard method was excellent.

For the simple routine analysis of dairy products and soy drink, results of an acceptable accuracy will be obtained using only an external calibration curve. Of course, this calibration curve must be obtained using a standard of high and known purity. Because the slopes of the calibration curves of all steviol glycosides are almost identical (if the concentration of the curve is expressed in $mol.L^{-1}$) a single standard will be sufficient for the analysis of all steviol glycosides.

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