JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Stability of Steviol Glycosides in Several Food Matrices

Etienne Jooken,*[,†,‡] Ruis Amery,[†] Tom Struyf,[§] Barbara Duquenne,[∥] Jan Geuns,[§] and Boudewijn Meesschaert[†,‡]

[†]Faculty of Engineering Technology, Chemistry Department, KHBO, Zeedijk 101, B-8400 Oostende, Belgium

[‡]Centre for Surface Chemistry and Catalysis and Leuven Food Science and Nutrition Research Centre (LForCe), Department of Microbial and Molecular Systems, KU Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium

[§]Department of Biology, Laboratory of Functional Biology, KU Leuven, Kasteelpark Arenberg 31, B-3001 Leuven, Belgium

[∥]Technology and Food Science Unit, Institute for Agricultural and Fisheries Research (ILVO), Brusselsesteenweg 370, B-9090 Melle, Belgium

Supporting Information

ABSTRACT: As steviol glycosides are now allowed as a food additive in the European market, it is important to assess the stability of these steviol glycosides after they have been added to different food matrices. We analyzed and tested the stability of steviol glycosides in semiskimmed milk, soy drink, fermented milk drink, ice cream, full-fat and skimmed set yogurt, dry biscuits, and jam. The fat was removed by centrifugation from the dairy and soy drink samples. Proteins were precipitated by the addition of acetonitrile and also removed by centrifugation. Samples of jam were extracted with water. Dry biscuits were extracted with ethanol. The resulting samples were concentrated with solid-phase extraction and analyzed by high-performance liquid chromatography on a C18 stationary phase and a gradient of acetonitrile/aqueous 25 mM phosphoric acid. The accuracy was checked using a standard addition on some samples. For assessing the stability of the steviol glycosides, samples were stored in conditions relevant to each food matrix and analyzed periodically. The results indicate that steviol glycosides can be analyzed with good precision and accuracy in these food categories. The recovery was between 96 and 103%. The method was also validated by standard addition, which showed excellent agreement with the external calibration curve. No sign of decomposition of steviol glycosides was found in any of the samples.

KEYWORDS: *rebaudioside A, stevioside, milk, yogurt, soy drink, biscuits, jam*

INTRODUCTION

On November 11, 2011, the European Commission issued a regulation regarding the approval and regulation of the addition of steviol glycosides to food products for the European market.[1] This regulation took effect on December 2 and followed an opinion with positive advice by the European Food Safety Authority, EFSA, formulated in April, 2010.[2] In this opinion, the Acceptable Daily Intake (ADI) was set to 4 mg of steviol equiv per day and per kg bodyweight. Steviol glycosides (SVGly for short) can be used as food additives if they contain at least 95% of SVGlys, taken as the sum of the SVGlys rebaudioside A, B, C, D, E, and F; dulcoside A; rubusoside; steviolbioside; and stevioside (see Figure 1 for the structures and abbreviations of the names). Moreover, the sum of the main SVGlys, rebaudioside A and stevioside, should be at least 75%. SVGlys added to food should be labeled as "E 960".

The main advantage of SVGlys as food additive is that they are completely noncaloric and that they are about 200−300 times sweeter than sucrose.[3] Thus, they can help to reduce the daily caloric intake and counteract obesity, one of the diseases of the metabolic syndrome.[4,5] SVGlys are noncaloric because the sugars are connected to each other and to the steviol scaffold by β-glycosidic bonds (or a β-glycosidic ester bond at carbon 19; see Figure 1). As a consequence, the human digestive tract is unable to break down SVGlys.[6] The microbial flora of the colon will partly degrade the SVGlys to steviol. After being taken up by the gut, the steviol is then transported to the liver by the portal vein and converted to steviol glucuronide, which is excreted in the urine.[7]

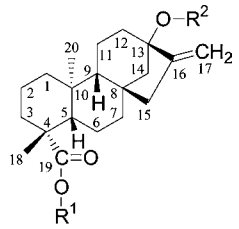
If SVGlys are to be used as food additives, then they should be stable in the food matrix both at process and at storage conditions and at terms relevant for that type of food. If they are not stable, at least the sweet taste will deteriorate, and this will of course lead to customer dissatisfaction. Moreover, the possibility exists that decomposition products might give rise to off-flavors in the food product or conduct to possible toxic products. So, it is imperative that the stability of the SVGlys in different food matrices is rigorously proven, and because of that, they need to be analyzable in the food matrix.

Studies about the stability of SVGlys in different foodstuffs are quite fragmentary and scarce and deal for the most part with the use of SVGlys in soft drinks. Chang and Cook[8] studied the stability of stevioside and rebaudioside A in carbonated drinks as early as 1983. They found some decomposition at elevated temperatures in solutions of citric and phosphoric acids (pH 2.4 or 2.6) or when the samples were irradiated by sunlight. However, if the solutions were kept at 4 °C or room

Received: May 30, 2012
Revised: September 24, 2012
Accepted: September 29, 2012
Published: September 29, 2012

dx.doi.org/10.1021/jf302261j | J. Agric. Food Chem. 2012, 60, 10606−10612

Name (Abbreviation)	R^1	R^2
Rebaudioside A (RebA)	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Stevioside (Ste)	β-Glc	β-Glc-β-Glc(2→1)
Rebaudioside F (RebF)	β-Glc	β-Glc-β-Xyl(2→1) | β-Glc(3→1)
Rebaudioside C (RebC)	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)
Rebaudioside D (RebD)	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside E (RebE)	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)
Dulcoside A (DulA)	β-Glc	β-Glc-α-Rha(2→1)
Rebaudioside G (RebG)	β-Glc	β-Glc-β-Glc(3→1)
Rubusoside (Rub)	β–Glc	β-Glc
Rebaudioside B (RebB)	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Steviolbioside (SteB)	H	β-Glc-β-Glc(2→1)
Steviol (SV)	H	H



Glc: glucose Rha: rhamnose Xyl: xylose

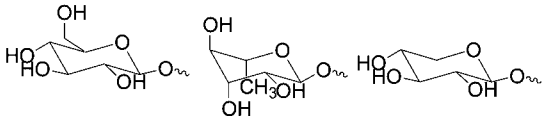


Figure 1. Structures of SVGlys.

temperature, the SVGlys were stable. The methods of analysis used by these authors were thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) with refractometric detection.

These results are contradicted by a more recent study by Clos et al.[9] Contrary to the results by Chang and Cook, Clos et al. found a good stability in cola- and citrus-like carbonated beverages at or near room temperatures, even under irradiation with sunlight. The reason for the discrepancy with the earlier results might be due to the analytical protocol used by Chang and Cook, which was probably more prone to error.

Kroyer investigated the thermal stability of stevioside in solid form and in acid solution.[10,11] He found that solid stevioside is stable at a temperature of 140 °C and for a period of 1 h. In solution, stevioside is stable in the pH interval of 2−10 at a temperature of 80 °C and for a period for up to 4 h. His results also indicate a lesser stability of stevioside in 1% phosphoric acid (pH ca. 1.6) as compared to other acids at the same concentration.

Wölwer-Rieck et al.[12] compared the stability of stevioside and rebaudioside A for use in soft drinks. According to their results, rebaudioside A is more stable than stevioside. Degradation of stevioside up to 70% was observed after 72 h of storage at 80 °C. The stability of the SVGlys increased with increasing pH, which is consistent with an acid-catalyzed hydrolysis of glucose units.

In a more general study, the feasibility of high purity rebaudioside A for use as a sweetener was investigated.[13] In line with the results by Wölwer-Rieck, Prakash et al. found rebaudioside A to be stable at a pH greater than 2. They found no evidence of decomposition when rebaudioside A was added to yogurt or white cake.

The present study is the first more or less extensive report on the stability of SVGlys in different food matrices. In close cooperation with the companies that contributed to this project, chosen were the following matrices: soy drink, semiskimmed milk, fermented milk drink, ice cream, skimmed and full-fat set yogurt, jam, and dry biscuits.

MATERIALS AND METHODS

Sample Processing. All food matrices were prepared containing sucrose as a reference product. This reference product was used to ascertain the absence of any matrix interference in the subsequent analysis of the SVGlys. In the real samples for analysis, the sucrose was in part or completely substituted with an appropriate amount of SVGlys (see below). All food products were stored as indicated below and analyzed periodically.

Both semiskimmed milk and nonaromatized soy drink were processed in a pilot plant (two-step 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. The samples were stored at two different temperatures, at 6 and 20 °C for 20 weeks. The reference products contained 8 and 5% sucrose by

Table 1. Concentrations of the Added SVGly Formulations

	concentration (mg kg^{-1})									
formulation	RebA	Ste	RebF	RebC	DulA	RebG	Rub	RebB	SteB	total
RebA formulation	96.29	0.09	0.31	0.25	ND[a]	ND	ND	0.29	ND	97.23
Ste formulation	6.33	83.54	0.57	0.47	ND	ND	0.55	0.63	ND	92.09
SG formulation	32.55	49.81	1.23	7.31	2.06	0.76	1.10	0.76	0.91	96.49
mix formulation[b]	78.30	16.78	0.36	0.29	ND	ND	ND	0.36	ND	96.09

[a]ND, not detected. [b]Mixture of 80% RebA and 20% Ste formulations.

weight, respectively. In the test samples, 30% of the sucrose was substituted by SVGlys.

Fermented milk drink was also processed in the pilot plant (two-step homogenization: 200 bar, 65 °C; indirect UHT processing: 15 s, 95 °C; cooling to 20 °C; APV Paraflow Pilot). The reference product contained 9% sucrose by weight. For the test samples, 30% of the sucrose was substituted by SVGlys. No flavors were added. Samples were stored at 6 and 20 °C for 20 weeks.

Ice cream was prepared containing cream (35% milk fat), skimmed milk powder, water, sucrose, glucose/maltose syrup [80−82% DS (dry substance), 36−40% DE (dextrose equivalent)], emulsifier/stabilizer, and vanillin. After the ingredients were mixed at 50 °C, the ice cream mixes were homogenized (two steps, 180 bar, 65 °C), pasteurized (15 s, 85 °C; APV Paraflow Pilot), and cooled down to 20 °C. After they were aged 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 the aimed. Ice cream samples were hardened to −22 °C for at least 24 h and finally stored at −18 °C until analysis. The reference product had a total sugar content of 22.8%, which was reduced to 16% by the addition of SVGlys and erytritol or maltitol.

Full-fat and skimmed set yogurt were also processed in the pilot plant. The yogurt was packaged immediately after inoculation with the starter culture and incubated at 43 °C until pH 4.65 was reached. The samples were stored at 6 °C for 35 days. The reference product contained 7.3% sucrose, and 30% of this was substituted with SVGlys for the test samples, resulting in a sugar content of 5.1%.

The reference jam was prepared from frozen strawberries, sucrose, pectin, and potassium sorbate. The strawberry jam was heated for 15 min until a Brix value of 63−65° was obtained. The jam was stored in glass jars for 1 year at room temperature in the dark and in the light and at 4 °C. For the test samples, the sucrose was completely substituted with maltitol and an amount of SVGlys to obtain the same sweetness.

Dry biscuits containing margarine, $NaHCO_3$, flour, distilled water, and sucrose were prepared at the University College Ghent (Faculty of Biosciences and Landscape Architecture). The biscuits were baked at 185 °C for 14 min and stored for 4 weeks at room temperature. The reference biscuits contained 33% sucrose, which was totally substituted by SVGlys, maltitol, or isomalt and Fibersol-2 (a maltodextrin fiber).

For the sucrose substitution, one of four different types of SVGly mixtures was added to each of the samples. The compositions of these SVGly mixture formulations are given in Table 1. Sugar in the food matrices was replaced in part or entirely by one or more of the following four formulations of SVGlys: (1) "RebA" formulation, (2) "Ste" formulation, (3) a commercial sample of SVGlys (further referred to as the "SG" formulation), and (4) a mixture of 80% of the "RebA" mixture and 20% of the "Ste" mixture (referred to as the "mix" formulation). If necessary, a bulk additive (e.g., erytritol or maltitol) was added to improve the mass and mouth feel of the food. Oligofructose HSI and erytritol or maltitol were added to the ice cream. Maltitol was used for jam, and Fibersol-2 in combination with isomalt or maltitol was added to the dry biscuits.

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). To 7 mL of supernatant of the first centrifugation was added 7 mL of ultrapure (UP) water (Simplicity Millipore, Billerica) and 21 mL of ACN. The addition of water was necessary to prevent the phase separation between the aqueous and the 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. Samples of full-fat and semiskimmed set yogurt were diluted with an equal mass of water and treated in the same way.

Ice cream samples were melted first and then processed in an analogous manner as the previous samples. However, it was necessary to change some quantities. A sample of 25 mL of melted ice cream was centrifuged for 12 min at 15000 rpm (24000*g*) to remove the fat. Next, 3 mL of supernatant was mixed with 21 mL of UP water and 36 mL of ACN. After 10 min of cooling, the proteins were separated by a centrifugation for 12 min at 15000 rpm.

A sample of about 1 g of dry biscuits was ground in a mortar and extracted with about 20 mL of 95% ethanol at 40 °C for 30 min. The mixture was filtered over a fluted filter paper, and the ethanol was evaporated on a rotary evaporator. The residue was taken up in 50 mL of 10% ACN and centrifuged for 12 min at 15000 rpm to remove the fats and proteins. The supernatant was finally filtered over a filter paper.

Samples of about 20 g of jam were weighted in a 50 mL falcon tube. A volume of 30 mL of water was added, and the mixture was well vortexed and placed in an ultrasonic bath for 15 min. After centrifugation (8000*g*, 15 min), the supernatant was transferred to a measuring cylinder. The pellet was extracted a second time with 30 mL of water and a third time with 20 mL of water. The volume of the combined supernatants was recorded. A sample of 1 mL was transferred to a microcentrifuge tube and centrifuged (10000*g*; 5 min); 250 μL hereof was used for HPLC analysis.

Solid-Phase Extraction (SPE). All samples, except those from jam, were further treated by SPE to concentrate the SVGlys. Because the high concentration of ACN in the supernatant would cause the SVGlys to elute immediately from the SPE column, a sample of 25 mL of supernatant was diluted with 75 mL of UP water for milk, soy drink, fermented milk drink full-fat, and semiskimmed set yogurt. For ice cream, 40 mL of supernatant was diluted with 120 mL of UP water. The final filtrate of the biscuits was used without dilution.

SPE columns (Hypersep C_{18}, 500 mg/3 mL from Thermo Scientific, Waltman, United States) were conditioned with 5 mL of MeOH (Acros, Beerse, Belgium) and rinsed with 10 mL of UP water. After the sample was loaded, the column was rinsed with 10 mL of UP water and 5 mL of 20% ACN. The SVGlys were finally eluted with 5 mL of 60% ACN.

Analysis. All samples were analyzed with a HPLC apparatus (Thermo Scientific, Waltman) 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. The analysis was done according to the recommendations made by Geuns.[3] Separations were done on two ODS Hypersil Columns (each 20 cm × 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 the eluent, as follows: 0 min, 30% B; 10 min, 40% B; 20 min, 80% B; and 30 min, 80% B. UV spectra were recorded between 195 and 360 nm for identification purposes, and the compounds were quantitated at 200 nm. A sample chromatogram is shown in Figure S1 in the Supporting Information.

Calibration. All compounds were quantitated with an external calibration curve, based on a single standard of rebaudioside A of high

Table 2. Relative Concentrations of SVGly in the Samples of Semiskimmed Milk with "SG" Formulation and Stored at 20 °C (Open Squares in Figure 5) Expressed as Percentage

week	RebA	Ste	RebF	RebC	DulA	RebG	Rub	RebB	SteB	total
0	33.74	51.62	1.28	7.57	2.13	0.78	1.14	0.79	0.95	100.00
1	30.93	54.86	1.27	7.70	2.34	0.57	1.35	0.51	0.49	100.00
5	32.95	53.95	1.21	7.60	2.22	0.23	1.15	0.33	0.36	100.00
9	32.13	52.75	1.26	7.89	2.28	0.66	1.40	0.77	0.86	100.00
15	32.27	52.03	1.19	7.98	2.67	0.87	1.34	0.87	0.78	100.00
20	32.22	52.55	1.06	8.41	2.38	1.10	0.83	0.71	0.75	100.00

purity. Because every SVGly has the same ester function as the chromophore, it is not surprising that the slopes of the calibration curves of the different SVGlys are very similar if the concentration is expressed in mol L^{-1}.[14] It is quite straightforward to apply correction factors based on molecular weights and to express the concentrations on a mass base, such as mg kg^{-1}, as we do in the present study.

RESULTS AND DISCUSSION

Validation and Recovery. The preferred method of analysis for SVGlys is of course HPLC. The columns that are most commonly used are NH_2 and C_{18}; HILIC columns are reported less frequently.[14] UV detection at 200 nm is used mostly. For our analysis, we needed a protocol of analysis that is relatively fast and robust. We chose two C18 columns, coupled in series, to obtain sufficient resolution between the critical pair, RebA and Ste (see Figure S1 in the Supporting Information). This method has been validated by EUSTAS, the European Stevia Association.[15]

The analytical procedure was validated using standard addition. In this procedure, several additions of pure RebA standard were added, prior to the extraction, to the food matrix already containing SVGlys. As an example, the standard addition curve of full-fat set yogurt is shown in Figure S2 in the Supporting Information. The standard addition yielded a concentration of RebA of 47.85 mg kg^{-1}, while the external calibration curve gave 46.84 mg kg^{-1}. Because the difference is only 1.43%, we can be assured that our procedure gives accurate results. Relative standard deviations (RSD) of the major SVGlys (RebA and Ste) were in general less than 5% and always less than 10%.

The recovery for every food product was tested by adding a known amount of standard RebA to every matrix. As can be seen in Table 3, the recovery was excellent and varied between 95 and 103%.

Heat Stability of SVGly. The stability of the SVGlys was evaluated by assessing the concentrations of the nine individual SVGlys over the total storage time. Any sign of decomposition should be indicated by either a decrease in concentration of the major SVGlys (i.e., RebA and Ste) and/or an increase of the "lighter" minor SVGlys (i.e., RebB, SteB, etc.). This is because the decomposition of SVGly will include hydrolysis of the sugar moieties, attached to the steviol scaffold.

To enforce the thermal hydrolysis of the SVGly, their heat stability was tested in an independent experiment. The results are shown in Figure 2. In this experiment, a sample of the SG formulation was kept in an oven at 180 °C. At regular time intervals, a portion of about 30 mg of the sample was removed from the oven, dissolved in a proper quantity of water, and analyzed by HPLC. As can be clearly seen, the concentrations of RebA and Ste decrease over time, and this decrease is mirrored by an increase in the concentrations of RebB and SteB. For clarity, only these four SVGlys are shown. It is also important to note that the total SVGly concentration decreases. This is partly because some other peaks in the chromatogram, which have not yet been identified, increase in size. By lack of identification, it is of course impossible to quantitate their contribution to the total amount of SVGlys. Moreover, because we record concentrations on a mass basis (i.e., as mg kg^{-1}), the total mass of SVGly per unit of the mass of food will decrease if an amount of a major SVGly is decomposed into an equivalent amount of a smaller one. So, it is clear that as the decomposition progresses, the total amount of SVGly that we analyze decreases.

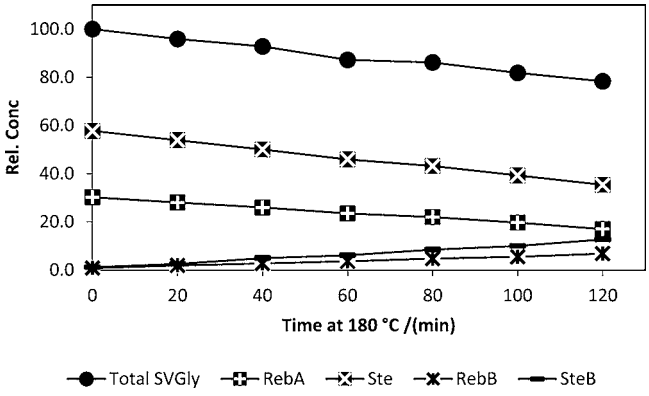


Figure 2. Forced thermal decomposition of a dry SG formulation sample at 180 °C. Conditions: see text.

All concentration changes in Figure 2 are more or less linear over the time frame of 120 min. The slope for the (linear) decay of RebA is −0.11. This is somewhat less than the slope for Ste, which is −0.18. This confirms the observation in the literature that RebA is more stable than Ste.[12]

Stability of SVGly in Soy Drink. Samples of soy drink were prepared as indicated in the Materials and Methods. All four formulations of SVGly were added to the soy drink (see Table 1). The reason for using several different formulations

Table 3. Recovery of Reb A from Different Food Matrices

sample	added (mg kg^{-1})	found (mg kg^{-1})	% RSD (n = 3)	% recovery
semiskimmed milk	103.03	102	1.03	99.16
fermented milk drink	115.55	114	0.97	98.45
soy drink	64.23	63	2.03	98.23
ice cream	24.84	24	0.63	96.76
full-fat set yogurt	93.40	96	2.25	102.95
skimmed set yogurt	93.40	95	1.14	101.82
dry biscuits	63.88	61	1.96	94.80

was that, concurrent with the chemical analysis, a trained panel also tasted all of the samples and evaluated sweetness and overall taste. The results of this research will be published elsewhere. The samples were stored at 6 and 20 °C for 20 weeks.

In Figure 3, the concentrations of SVGlys, added to soy drink, are plotted as a function of time. It is clear that neither

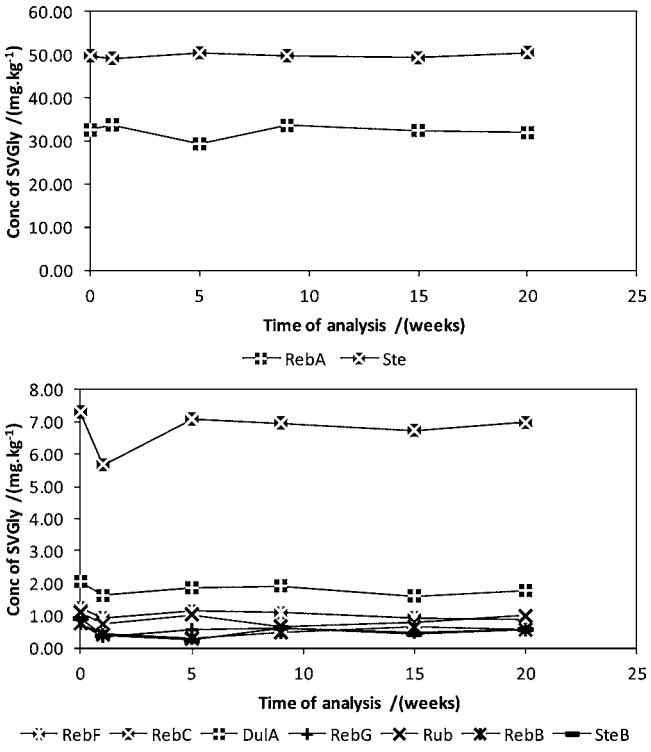


Figure 3. Concentrations of major (top) and minor (bottom) SVGly in soy drink over time. Samples were stored at 20 °C, and the SG formulation was used for this experiment.

the major SVGlys are hydrolyzed nor the minor ones increase in concentration. As this observation was found in every sample that was analyzed for the present study, we will from now on show only the total SVGly concentration over time. This will give us enough indication about the SVGly stability, because we demonstrated in the previous section that the total SVGly concentration will drop upon decomposition.

The total SVGly concentrations in the soy drink samples, for the four used SVGly formulations and both storage conditions, are presented in Figure 4. As is evident from this figure, the SVGlys remain perfectly stable in all conditions.

Semiskimmed Milk. The storage conditions and SVGly formulations added to semiskimmed milk were identical to those for the soy drink. The results are shown in Figure 5. The first data point, the analysis of week 1, seems to be anomalous. The total SVGly concentration decreases and recovers in the next weeks. However, if we look at the relative concentrations of the individual SVGly for each data point, we do not see a systematic decrease of the concentrations of the major SVGly, nor is an increase of the smaller SVGly apparent. These data, for the SG formulation and storage at 20 °C, are collected in Table 2. Given the fact that for every data point in Figure 5 a fresh bottle was opened, these data do not show an abnormal and certainly not a systematic variation. So, probably the decrease in total concentration can be attributed to the sample

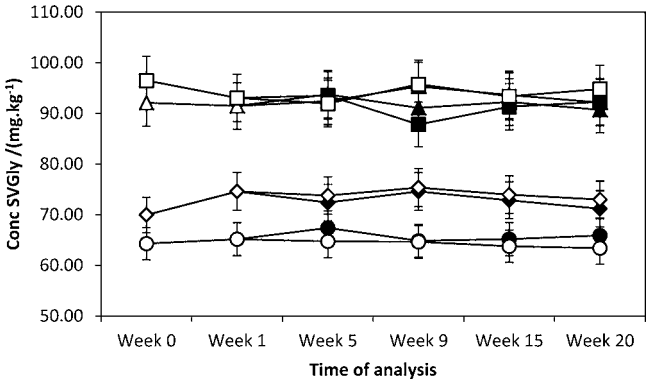


Figure 4. Total concentration of SVGly in soy drink as a function of time. Closed symbols refer to storage at 6 °C; open symbols refer to storage at 20 °C. Circles, triangles, squares, and diamonds refer to RebA, Ste, SG, and mix formulations, respectively.

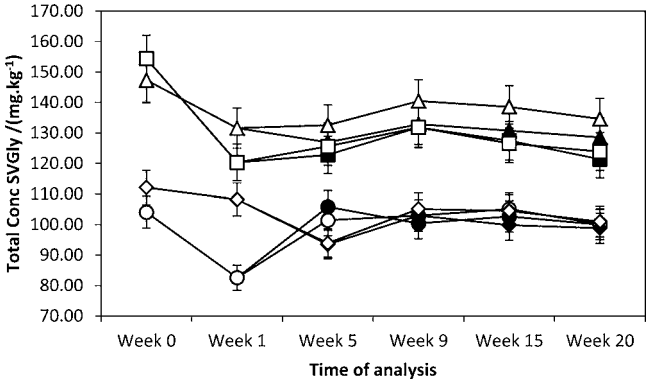


Figure 5. Concentration of SVGlys in semiskimmed milk. The symbols have the same meaning as in the caption of Figure 4.

processing, where somewhat less SVGlys were added than intended. Hence, we can conclude that no decomposition of SVGlys occurs in these samples.

Fermented Milk Drink. The storage conditions for the samples of fermented milk drink were again identical to the conditions used for the soy drink samples. Also, all four SVGly formulations were added to the fermented milk drink samples, as for the soy drink. The results are presented in Figure 6. It is clear that there is no sign of decomposition in these samples.

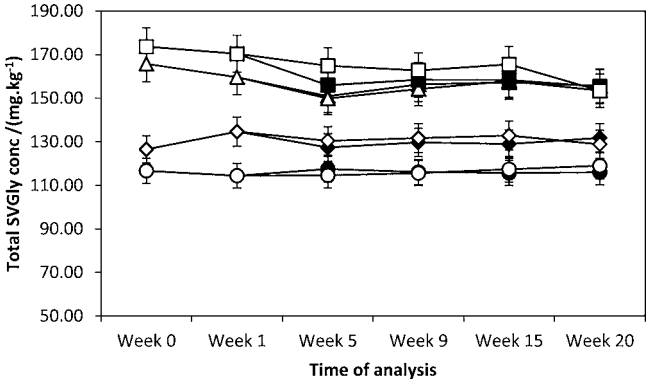


Figure 6. SVGly concentration as a function of time for fermented milk drink. For the meaning of the symbols, see the caption of Figure 4.

dx.doi.org/10.1021/jf302261j | J. Agric. Food Chem. 2012, 60, 10606−10612

Ice Cream. Samples of ice cream were—obviously—stored at −18 °C. The role of sugar in normal ice cream is of course more than just adding sweetness; it also improves the body and texture of the mix. Because this is very important for the typical sensation, it is not possible to produce an ice cream with an attractive taste and mouth feel wherein the sugar is completely substituted by SVGlys (or any other high intensity sweetener). In our samples, the sugar content was reduced from 22.8 to 16%, and even then, a substitute had to be provided for a better body and texture of the ice cream. Erytritol and maltitol were chosen as the additives. Apart from body and texture, the sugar alcohols also add to the sweetness. The ice cream samples were analyzed in the beginning and at the end of the storage period of 12 weeks. Figure 7 shows no sign of decomposition, as can reasonably be expected for samples that were stored at such a low temperature.

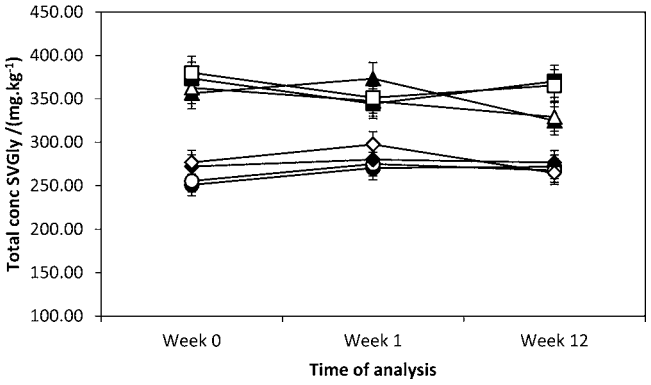


Figure 7. Total SVGly concentrations in ice cream samples. Closed symbols refer to samples with maltitol, and open symbols refer to samples with erytritol. Circles, triangles, squares, and diamonds refer to RebA, Ste, SG, and mix formulations, respectively.

Skimmed and Full-Fat Set Yogurt. Set yogurt contains live microorganisms that obviously have β-galactosidase enzymes, because they hydrolyze lactose.[15,16] The question that needed answering is whether these microorganisms also have β-glycosidase activity. Moreover, the pH of set yogurt is rather low. Although unlikely according to the literature,[10,11,13] one cannot rule out the possibility that this acid environment might contribute to the hydrolysis of the ester and acetal β-bonds. So, there were ample reasons to include skimmed and full-fat set yogurt in the present study.

Only the RebA and SG formulations were added to the set yogurt samples. The samples were stored at 6 °C for 35 days. The pH remained constant at about 4.65 during this period (results not shown). Figure 8 indicates that the SVGly remain perfectly stable over the period investigated. This is in line with the results by Prakash et al.,[13] and the period of follow-up of the samples was similar (34 days in our study and 6 weeks, or 42 days, in Prakash's study)

Jam. Strawberry jam was included in this study as an example of a fruit matrix. It is clear from Figure 9 that there is no breakdown of SVGlys in any of the used storage conditions. So, we can conclude that SVGlys are also stable in conditions of ambient light, as is in agreement with the results by Clos,[9] but disagrees with Chang et al.[8] Unless a mechanism of sensitization is at hand (i.e., the light is absorbed by another compound and transferred to the SVGly), it is hard to envision

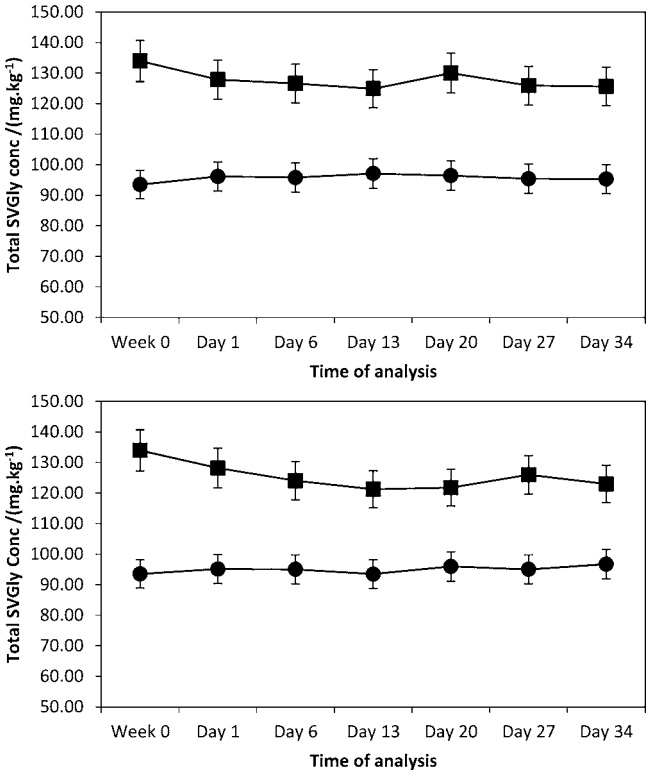


Figure 8. SVGly concentrations in full-fat (top) and skimmed (bottom) set yogurt. Circles and squares refer to RebA and SG formulations, respectively.

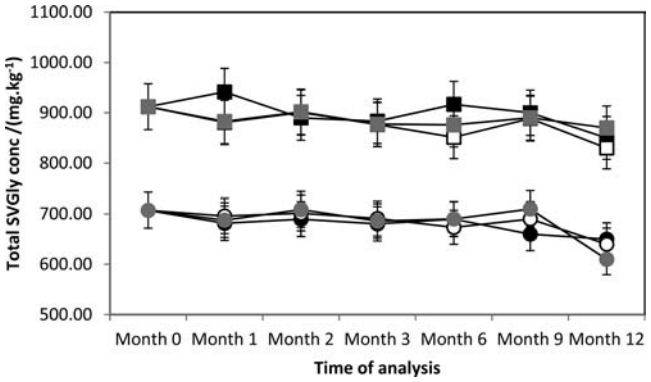


Figure 9. Total SVGly in jam samples. Black symbols refer to storage at 4 °C, gray symbols refer to storage at room temperature in the dark, and white symbols refer to storage at room temperature in light. Circles and squares refer to RebA and SG formulations, respectively.

photochemical reactions of SVGlys, because they absorb only extremely short wavelength UV radiation.

Dry Biscuits. Initially, there were some reservations about the stability of SVGlys in baked confectionery. Our dry biscuits were baked at 185 °C, where the reported upper temperature limit for SVGlys is 140 °C.[10,11] Therefore, only one formulation, RebA, was added. This formulation contains more than 95% rebaudioside A, which is the most stable of the prominent SVGlys according to the literature[12] and according to our own observations (see Figure 2). Apparently, the actual temperature inside the biscuits during baking does not reach the set oven temperature (presumably due to the presence of liquid water), because according to Figure 10, there is no sign of the decomposition of the RebA. This result corroborates

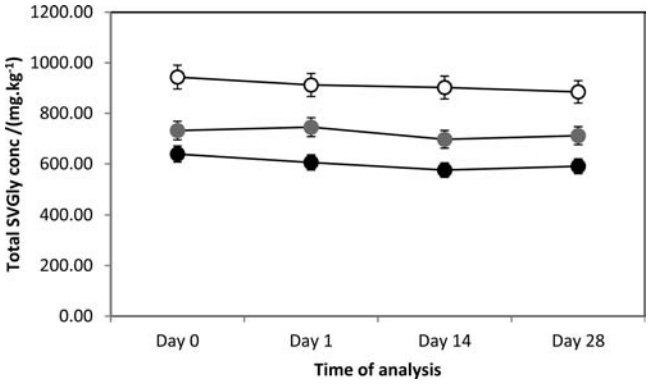


Figure 10. Total SVGly concentration in dry biscuits as a function of time. RebA formulation was added to all samples. Black, gray, and open circles refer to, respectively, 80% maltitol + fibersol-2, 70% maltitol + fibersol-2, and 70% isomalt + fibersol-2.

with the result of Prakash for white cake.[13] In our study, the baking temperature was somewhat higher (185 °C as compared to 176 °C in Prakash's study), and the storage time was longer (28 vs 5 days).

In conclusion, our results show that SVGlys can be analyzed in food products, as diverse as dairy products (including ice cream), jam, and biscuits, with good precision and accuracy. The sample preparation included the removal of fat and proteins and concentration with SPE. The HPLC analysis was done on a double C18 column using a single RebA standard. Recoveries ranged between 95 and 103%.

The stability of several SVGlys was tested in a diverse range of food categories. No sign of decomposition was found under any of the investigated circumstances. So, one can be assured that the addition of SVGlys to food will not alter the quality or the normal shelf life of the food.

■ ASSOCIATED CONTENT

Supporting Information

Sample chromatogram and standard addition curve of full-fat set yogurt. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +32 59 56 90 00. Fax: +32 59 56 90 01. E-mail: Etienne.jooken@khbo.be.

Funding

We thank the IWT-Vlaanderen (Instituut voor de Aanmoediging van Innovatie door Wetenschap en Techniek; IWT-Flanders, The Institute for the Promotion of Innovation by Science and Technology) for Grant IWT 080728.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Flanders FOOD (ir. A. Vandamme) for coordination of the project. We also express our gratitude to the companies that contributed to the project.

■ REFERENCES

(1) EU Commission. Commission Regulation (EU) No. 1131/2011. *Official J. Eur. Union* **2011**, *L295*, 205−211.

(2) EFSA. EFSA Panel on Food Additives and Nutrient Sources (ANS); Scientific Opinion on the safety of steviol glycosides for the proposed uses as a food additive. *EFSA J.* **2010**, *8*, 1537.

(3) Geuns, J. M. C. *Stevia and Steviol Glycosides*; Euprint: Heverlee, 2010; pp19−50.

(4) Cornier, M. A.; Dabelea, D.; Hernandez, T. L.; Lindstrom, R. C.; Steig, A. J.; Stob, N. R.; Van Pelt, R. E.; Wang, H.; Eckel, R. H. The Metabolic Syndrome. *Endocr. Rev.* **2008**, *29*, 777−822.

(5) Geeraert, B.; Crombé, F.; Hulsmans, M.; Benhabilès, N.; Geuns, J.; Holvoet, P. In *Steviol Glycosides and Obesity, the Metabolic Syndrome and Atherosclerosis*; Proceedings of the 4th Stevia Symposium 2010 organized by EUSTAS: Stevia: science, no fiction, Leuven, 2010; Geuns, J. M., Ed.; Euprint: Leuven, 2010; pp 151−152.

(6) Geuns, J. M. C.; Buyse, J.; Vankeirsbilck, A.; Temme, E. H. M. Metabolism of stevioside by healthy subjects. *Exp. Biol. Med.* **2007**, *232*, 164−173.

(7) Geuns, J. M. C.; Buyse, J.; Vankeirsbilck, A.; Temme, E. H. M.; Compernolle, F.; Toppet, S. Identification of steviol glucuronide in human urine. *J. Agric. Food Chem.* **2006**, *54*, 2794−2798.

(8) Chang, S. S.; Cook, J. M. Stability Studies of Stevioside and Rebaudioside-A in Carbonated Beverages. *J. Agric. Food Chem.* **1983**, *31*, 409−412.

(9) Clos, J. F.; DuBois, G. E.; Prakash, I. Photostability of rebaudioside A and stevioside in beverages. *J. Agric. Food Chem.* **2008**, *56*, 8507−8513.

(10) Kroyer, G. T. The low calorie sweetener stevioside: Stability and interaction with food ingredients. *LTW—Food Sci. Technol.* **1999**, *32*, 509−512.

(11) Kroyer, G. T. Stevioside and Stevia-sweetener in food: Application, stability and interaction with food ingredients. *J. Verbraucherschutz Lebensmittelsicherh.* **2010**, *5*, 225−229.

(12) Wölwer-Rieck, U.; Tomberg, W.; Wawrzun, A. Investigations on the Stability of Stevioside and Rebaudioside A in Soft Drinks. *J. Agric. Food Chem.* **2010**, *58*, 12216−12220.

(13) Prakash, I.; DuBois, G. E.; Clos, J. F.; Wilkens, K. L.; Fosdick, L. E. Development of rebiana, a natural, non-caloric sweetener. *Food Chem. Toxicol.* **2008**, *46*, S75−S82.

(14) Geuns, J. M. C.; Struyf, T. In *EUSTAS Round Robin Testing of Steviol Glycosides*, Proceedings of the 3rd Stevia Symposium, Leuven, 2009; Geuns, J. M. C., Ed.; Euprint: Leuven, 2009; pp 35−48.

(15) Montanari, G.; Zambonelli, C.; Grazia, L.; Benevelli, M.; Chiavari, C. Release of beta-galactosidase from lactobacilli. *Food Technol. Biotechnol.* **2000**, *38*, 129−133.

(16) Ustok, F. I.; Tari, C.; Harsa, S. Biochemical and thermal properties of beta-galactosidase enzymes produced by artisanal yoghurt cultures. *Food Chem.* **2010**, *119*, 1114−1120.