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CHAPTER 14

ROS - Scavenging Capabilities of Steviol Glycosides and Derivatives Thereof

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ABSTRACT

Steviol glycosides (SVGlys) are well known as natural, non-caloric sweeteners. Not only their use as a sugar substitute is interesting, but also positive pharmaceutical effects to the human health were observed after intake of larger doses. These positive effects can be ascribed to the scavenging activity of SVGlys on reactive oxygen radicals.

Two aspects were examined with regard to the scavenging capabilities of SVGlys. The first relates to the fact that SVGlys are composed of sugars, while the other related to the steviol (SV) scaffold containing a double bond. In this project scavenging activities of rebaudioside A (RebA), stevioside (Stev) and leaf extract were compared to those of glucose (Glc) and sucrose (Suc). Also experiments concerning a possible correlation between scavenging activity and sugars or their number of OH-groups were done. To check if the double bond of the steviol scaffold is

responsible for the scavenging activity, experiments with the hydrogenated compound dihydro-stevioside (DH-Stev) were done. The scavenging activity was measured by means of a competitive reaction of OH-radicals from the Fenton reaction between terephtalic acid and the SVGly. The scavenging capability was expressed as the inhibitory concentration in mM (mmol/L) which reduces 50% of the radicals (IC_{50}).

Some optimization concerning temperature, reaction time, and volume of the reaction mixture were performed. Different scavenging capabilities were observed at 37°C and 70°C. At temperature of 70°C and an incubation time of 16 hours, reproducibility was ensured. SVGlys showed higher scavenging activities than sugars. The IC_{50} values of RebA, Stev and DH-Stev were similar. A good quadratic correlation between scavenging activity and the number of OH-groups was found.

The scavenging activity of SVGlys is apparently not caused to a major extent by the double bond in the steviol moiety, as the IC_{50} concentration for DH-Ste does not differ much from that of Ste. To further prove the correlation between scavenging activity and the number of OH-groups of sugars further experiments are suggested.

KEYWORDS

Dihydrostevioside, fluorescence detection, Fenton reaction, glucose, sucrose, OH-radical

ABBREVIATIONS

•OH: hydroxyl radical; ACN: acetonitrile; BPA: bisphenol A; DH-Stev: dihydrostevioside; DMF: dimethylformamide; DMSO: dimethylsulfoxid; Dulc: dulcoside; EDTA: ethylenediaminetetraacetic acid; Glc: glucose, HAc: acetic acid; HTPA: 2-hydroxy-terephthalic acid; IC50 : half maximal inhibitory concentration; MeOH: methanol; RebA: rebaudioside A; RebB: rebaudioside B; RebC: rebaudioside C; RebG: rebaudioside G; ROS: reactive oxygen species; Rub: Rubusoside; SB steviolbioside; Stev: stevioside; Suc: sucrose; SV: steviol; SVGlys: steviol glycosides; THF: tetrahydrofuran; TPA: terephthalic acid

INTRODUCTION

Steviol glycosides (SVGlys) are sweet tasting compounds. They are formed in leaves of *Stevia rebaudiana* (Bertoni), a plant that is native to South America (Paraguay and Brazil). The major compounds are stevioside (Stev) and rebaudioside A (RebA) which taste about 150 – 250 times and 200 – 300 times sweeter than sucrose (Prakash, Campbell, San Miguel, & Chaturvedula, 2012). In dried leaves the content of SVGlys has a range of 4 – 20% (Jan M. C. Geuns, 2003). Steviol glycosides, extracted from the leaves of *Stevia rebaudiana* and subsequently purified, have been approved for use as natural and non-caloric sweeteners (Jeppesen, 2011) since December 2011 in Europe (EU-commission, 2011).

Studies observe beneficial pharmacological effects of SVGlys such as anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-carcinogenic, anti-diarrheal, diuretic and immunomodulatory actions (Boonkaewwan & Toskulkao, 2008; Chatsudthipong & Muanprasat, 2009; Jeppesen, 2011). These effects only occur at higher doses; e.g. 750 mg/day (Chan et al., 2000). They are not claimed for doses that are typical for sugar replacement. Most of these diseases are induced by oxidative stress which means that the amount of reactive oxygen species (ROS) in the body increases. The positive effects of SVGlys have been attributed to the ROS scavenging activity of SVGlys (J.M.C. Geuns & Struyf, 2010; Shukla, Mehta, Bajpai, & Shukla, 2009).

A plausible hypothesis could refer to the double bond of the SV scaffold that might react with the hydroxyl radicals (Lee, Koo, & Min, 2004).

To verify the hypothesis of the scavenging capabilities, the scavenging experiment with SVGlys and sugars (Glc, Suc) was repeated. Furthermore, we tried to measure the sugar-free compound, SV, also containing the double bond with the same assay. Prior to that, a solvent for SV had to be chosen which did not influence the experiment.

Finally, leaf extracts and the hydrogenated compound, that means without the double bond, dihydro-stevioside (DH-Stev) shown in Figure 1, was also investigated in this experiment to confirm the reactive unit.

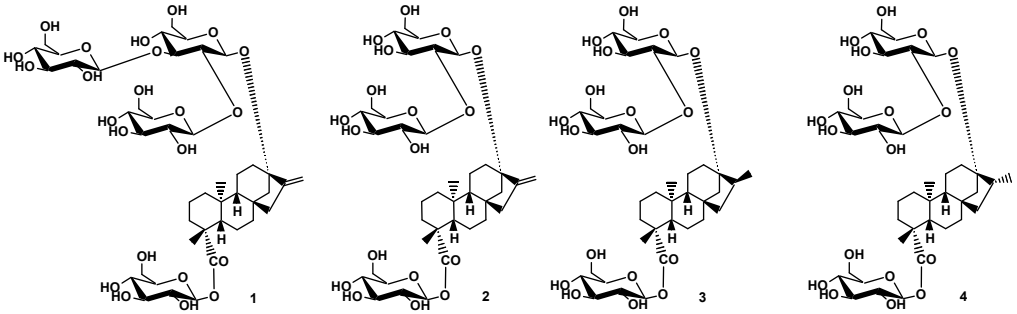

Figure 1: Structures of SVGlys with 1 RebA, 2 Stev, 3 and 4 DH-Stev

However, it should be considered that sugars are also able to scavenge radicals and that SVGlys contain sugars. To check if there is a relationship between the scavenging activities of SVGlys and sugars, some hypotheses were tested.

First, an interaction between the scavenging activity and the number of sugar units is possible. With regard to glucose it means: one glucose molecule equals one sugar unit, and sucrose (consisting of glucose and fructose) corresponds to two sugar units, Stev contains three glucose molecules and thus corresponds to three sugar units and finally RebA which corresponds to four sugar units. The hypothesis was that with increasing numbers of glucose units, the IC_{50} would be reduced by multiples of the IC_{50} of glucose, according to the number of sugar units. The measured IC_{50} values were compared with the calculated theoretical IC_{50} values.

Second, a correlation between scavenging activity and the number of OH-groups was examined. The different types of OH-groups (primary, secondary) and their total number were investigated.

MATERIALS AND METHODS

SVGlys samples as scavengers

The scavenging capabilities of four different SVGlys were tested. As the major compound of SVGlys, and also used as sweeteners, RebA and Stev were analyzed. Further, steviol, as the derivate containing no sugars, and DH-Stev, containing sugars but without the double bond, were investigated.

The samples of RebA and Stev were a donation by Prof. Geuns (KU Leuven). Prior to their use, the samples were dried overnight at 105°C. After measuring the exact amount of sample before and after drying, the dry weight or water content could be calculated. The composition of these samples is shown in Table 1:

Table 1: Composition of the SVGly([a]) samples

Sample	RebA	Stev	RebC	RebG	Rub	RebB	SB	Total
RebA	93.27%	0.51%	0.49%	0.05%	0.00%	0.11%	0.69%	95.11%
Stev	6.16%	84.72%	0.00%	0.00%	0.86%	0.00%	1.77%	93.51%

(a) RebF ; DulcA ; DulcB and SV were absent in both samples

Steviol was available as such; the preparation of it was performed at the laboratory by microbial degradation of SVGlys (Smedts, Amery, Moons, Jooken, & Meesschaert, 2008; Smedts, Ceunen, Amery, Geuns, & Meesschaert, 2009).

The sample of dihydro-stevioside (DH-Stev) was obtained from KU Leuven where it was prepared according to the method of Prakash et al. (2012).

In addition to the use of the pure SVGlys standards of RebA and Stev, it was also examined if other SVGlys have ROS scavenging capabilities. For that purposes, a SVGlys mixture was extracted twice from Stevia leaves. 2.5 g of homogenized, dried Stevia leaf powder (Durabilis, Gent, Belgium) was suspended in 20 mL ultrapure water and boiled for 30 min in closed centrifuge tubes. The extracts were centrifuged (5000 rpm, 15 min; Biofuge Stratos from Heraeus) and the combined supernatants were tranfered to a 25 mL volumetric flask and brought to volume. Before being used in the further experiments, the extract was filtered through a 0.20 µm syringe membrane filter.

Other chemicals

All chemicals, unless otherwise noted, were purchased from Acros Organics (Geel, Belgium) and were used without further purification.

Scavenging experiment

Investigating the scavenging capabilities requires radicals. These radicals are produced by the Fenton reaction, and then entered a competitive reaction between terephthalic acid (TPA) and the scavenger. The radicals that did not react with the scavenger are detected by measuring the fluorescent reaction product between TPA and hydroxyl radical using a fluorescence detector. Depending on the scavenging capacity of SVGlys, less radicals can react with TPA so that less TPA-reaction product can be formed. This means that SVGlys quench the reaction with TPA. The quenching, also known as scavenging capability, is expressed by the half maximal inhibitory concentration (IC_{50}). The lower IC_{50} value the better the scavenger.

To observe the $^{\bullet}OH$ scavenging activity, the methods of Linxiang et al. (2004) and Hajihashemi and Geuns (2013) using the Fenton reaction were modified. The reaction mixture consisted of 500 µM TPA, 10 µM ethylenediaminetetraacetic acid (EDTA), 10 µM $FeSO_4$, 100 µM sodium ascorbate, scavengers solution in a range of 0.25 – 10 mM and 100 µM H_2O_2. All solutions were prepared in a 50 mM potassium phosphate buffer (pH 7.2; measured with SevenMulti™ pH-meter from Mettler Toledo) and filtered over a membrane filter AO-20/25 from Chromafil®. The fluorescence of HTPA formed was measured by HPLC.

The method to determine the scavenging activity of SVGlys is already published (J.M.C. Geuns, Hajihashemi, & Claes, 2012; J.M.C. Geuns & Struyf, 2010; Hajihashemi & Geuns, 2013; Stoyanova, Geuns, Hideg, & Van den Ende, 2011) but not yet optimized. To guarantee reproducibility, different measurements were performed to check the influence of the parameters ascorbate, time, volume and temperature on the reaction.

Time monitoring: For observing the formation of the end product HTPA, the fluorescence of a blank sample (1.5 mL) was measured every hour in a time window of 30 hours (excluding the hours 8 - 15) at 37°C (Loading Model 100-800 from Memmert). A further 1.5 mL-blank sample at 70°C was analyzed from hours 1 - 6 and also after 23 hours.

Volume: Blank samples with a volume of 40 mL were determined hourly over 25 hours at 37°C to notice if the surface-to-volume ratio had an influence on the formation of HTPA.

Temperature: To compare the temperature of 37°C and 70°C 1.5 mL-blank samples were incubated for 16 hours.

Finally, the different scavengers in a 1.5 mL-reaction mixture were measured after 1 h at 37°C, 16 h at 37°C and also after 16 h at 70°C. Leaf extract and DH-Stev were only measured at 70°C.

The concentration of hydrogen peroxide was verified every week by titration with potassium permanganate solution according to Vogel (1961). To determine the titer of the potassium permanganate solution, Vogel's method (Vogel, 1961) using sodium oxalate was used.

Experiments with steviol

Steviol is not soluble in water, and hence to study the behavior of steviol, an organic and water-miscible solvent was needed which dissolves the sample and – most important – does not quench the fluorescence in its own right. First, the following solvents were tested as a blank (50% organic solvent) and treated as described in scavenging experiment. The following solvents were tested: methanol (MeOH), acetic acid (HAc), aceton, acetonitrile (ACN), N,N-dimethylformamide (DMF), dimethylsulfoxid (DMSO), 1,4-dioxan, tetrahydrofuran (THF). Particular attention was paid to the quenching of the fluorescence signal induced by the organic solvent. Because of the aqueous conditions of the scavenging experiment, the next step was to reduce the amount of the organic solvent without losing the solubility of steviol.

Analysis of SVGlys

The concentration of the SVGlys was measured by HPLC (Thermo Scientific, Waltman): the device is supplied with a SCM1000 vacuum degasser, a P1000XR pump, an AS1000 autosampler with an injection volume of 20 µL and a

UV6000LP diode array detector with a flow cell of 10 µL and a path length of 5 cm. The method was modified from Jooken et al. (2012). All samples were separated on a KINETEX-column (C18; 2.6 µm; 150 x 4.6 mm), (Phenomenex, Utrecht, The Netherlands) using a gradient with 25 mM H_3PO_4 (solvent A) and ACN (solvent B) as follows: 0 min: 30 % B ; 2 min: 30 % B ; 6 min: 40% B; 10 min, 48% B; 14 min, 82% B. (Flow: 1 mL/min.) The analytes were quantified at 200 nm.

Samples were diluted immediately in the HPLC-vial (Verex vial, 8 mm screw cap, 2 mL from Phenomenex) that was closed with the appropriate cap (Verex cap, 8-425 from Phenomenex). Dilutions were done with ultra-pure water produced by Simplicity® 0.22 µm from Merck Millipore.

The calibration line was prepared based on RebA as the only standard and calculated over the molar mass of the different SVGlys according to Jooken et al. (2012). Finally, the purity and SVGlys-composition was evaluated.

Fluorescence detection

Fluorescence detections were performed with the HPLC device as mentioned above, now provided with a (single beam) FL3000 fluorescence detector (Thermo Scientific, Waltman).

The eluent consisted of ultra-pure water with a flow of 1 mL/min. The HPLC column was replaced by an empty tube (length: 1 m). Excitation and emission wavelength were 316 nm and 420 nm respectively. Each run took about 0.3 min, allowing an injection every 2 min.

To control the stability of the lamp output, two Bisphenol A (BPA) samples with a concentration of 5 and 10 ppm respectively were measured once a day with an excitation wavelength of 226 nm and an emission wavelength of 310 nm (Maimbourg, 2013).

RESULTS AND DISCUSSION

Scavenging capabilities of SVGlys and sugars

Time monitoring

After incubation at 37°C, the measured fluorescence values did not increase anymore after a reaction time of 16 hours as shown in Figure 2. Every sample was measured ten times, and the values were reproducible. The stable values indicate that the formation of the end product is finished after 16 hours.

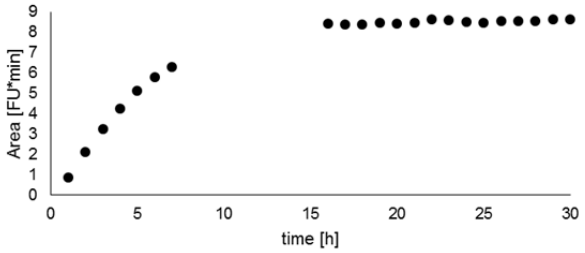

Figure 2: Formation of HTPA depending on time at 37°C

When the samples were incubated at 70°C, it turned out that the value after six hours could be reproduced also after 23 hours (Figure 3).

This proved that after six hours the fluorescence value, and thus the end product formation, was stable.

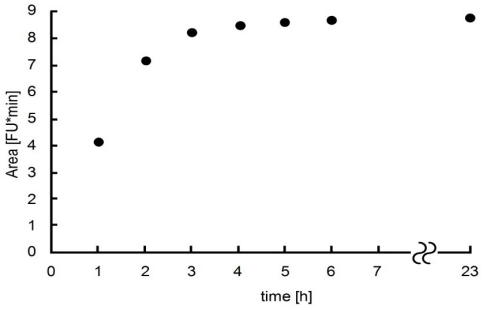

Figure 3: Formation of HTPA depending on time at 70°C

Comparing these results of the different temperatures during the reaction, it was proven that with a higher temperature, the formation of the end product HTPA was

finished faster. For simpler handling of overnight reactions, the reaction time for 70°C was also set to 16 hours.

Volume

The possibility that $^{\bullet}OH$ radicals react with the glass wall cannot be ruled out, and by consequence will be less available for reaction with the scavenger or TPA if the surface over volume ratio is high. Therefor an experiment was set up to investigate if this ratio of the reaction vessel influences the measured fluorescence intensity.

The monitoring of 40 mL blank samples revealed that after 16 hours a stable value (Area around 3 FU*min) was obtained (ref. Figure 4). Two different 40 mL blank samples were prepared. To keep the time window without monitoring results due to the overnight reaction as low as possible, the reactions of the two samples were started at different times.

Comparing these areas with those of the time monitoring of 1.5 mL samples, which had a value of 8 FU*min, it became clear that the results of the bigger volume were lower although the surface-to-volume ratio of a 1.5 mL vial was higher than in a 40 mL flask. If radicals react with the glass wall, it means that in a volume of 1.5 mL more radicals escape due to the higher surface-to-volume ratio. Our test results did not confirm this assumption. Up till now no explanation has been found for the higher values of the 1.5 mL samples. The different volumes were measured on different days. The results indicate that there is a further parameter that influences the reaction we do not know yet. For calculating the IC_{50} the single series was measured on the same day with the same reaction mixture, so that it would not influence the calculation.

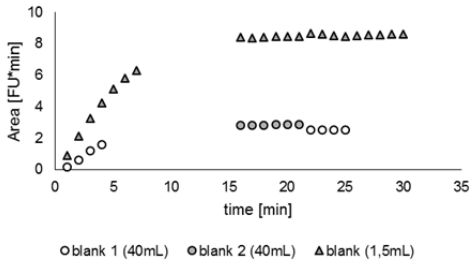

Figure 4: Formation of HTPA depending on the volume

Temperature

After a reaction time of 16 hours, the fluorescence signal at 70°C was 54% higher than at 37°C (Figure 5), indicating a formation of larger amounts of HTPA under higher temperatures.

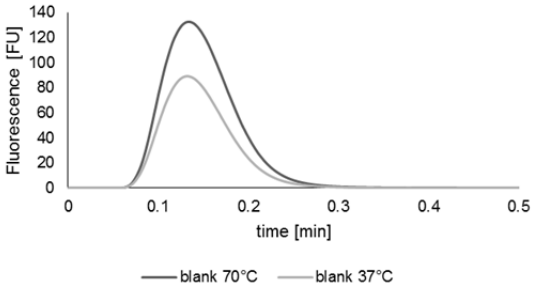

Figure 5: Chromatogram of blank samples at 37°C and 70°C after 16h

However, a temperature dependent formation of end product is in contrast to the result of the time monitoring which showed that a stable end product was formed faster at a higher temperature. The actual amount of the formed end product was similar.

It seems that there is another parameter which influences the reaction. Within the framework of this project, this parameter could not be controlled and identified. The fact that there is an unknown parameter, which might influence the measurement, makes the results not completely reproducible.

Experiments with a reaction time of 16h at 37°C

A first experiment was performed at a temperature of 37°C. This temperature was chosen for ease of thermostatization, yet it was not too high above room temperature, used by other authors. To ensure a complete reaction, the reaction time was set to 16 h.

The results of the fluorescence quenching of the different scavengers are presented in Figure 6. SVGlys needed a lower concentration for the same effect of quenching than sugar. The values of different measurements of one day were similar.

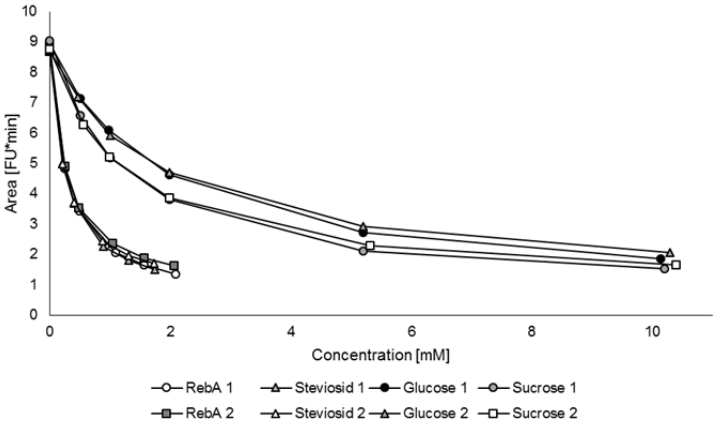

Figure 6: Area of the fluorescence signal plotted against the scavenger concentration [mM]; 16h at 37°C

Table 2: Results of scavenging experiment 16h at 37°C

Scavenger	IC_{50} [mM]	Standard deviation [mM]	RSD [%]	Number of measurements	Sugar units
RebA	0.20	0.06	32	10	4
Stev	0.18	0.07	36	10	3
Suc	1.01	0.38	38	10	2
Glc	1.64	0.46	28	10	1

The calculated results are shown in Table 2.

As the Stev sample also contained an amount of 6.59% RebA (see Table 1), the value of IC_{50} for Stev needed to be corrected. This was done, assuming that IC_{50} values are summative, with the formula:

$$IC_{50}^{Stev\ measured} = \frac{100}{91}\left(0.06 \cdot IC_{50}^{RebA} + 0.85 \cdot IC_{50}^{Stev}\right)$$

$$IC_{50}^{Stev} = \frac{\frac{91}{100} \cdot IC_{50}^{Stev\ measured} - 0.06 \cdot IC_{50}^{RebA}}{0.85}$$

The coefficients being used were based on the analysis for purity determination. As RebA and Stev accounted for only 91% of SVGlys, a correction factor of 100/91 was introduced. After rewriting the second equation was received.

The corrected value of IC_{50} for Stev based on the measurement was: 0.18 ± 0.06 mM. As shown in this experiment, there was no significant difference between the scavenging capabilities of Stev and RebA.

The results were comparable with the literature values of Hajihashemi and Geuns (2013).

In comparison with the determined values of this assay, several differences with the literature values need to be mentioned: The IC_{50} values in the literature show a high range of variation. In general, SVGlys have a lower IC_{50} than sugars. Further, the results of Geuns and Struyf (2010) and Hajihashemi and Geuns (2013) were measured under the same conditions (room temperature, 16 hours) whereas Stoyanova et al. (2011) received their results at room temperature with an unknown reaction time. Of particular note is also that the values of Hajihashemi and Geuns (2013) are lower than the values of Geuns and Struyf (2010). The diversity between these values is an indication for the difficulty to reproduce this assay. Our results correlate with Hajihashemi and Geuns (2013) and with Stoyanova et al. (2011) for Stev. Incidentally, the two research teams worked at room temperature during the reaction. In this experiment a temperature of 37°C was chosen to assure a stable temperature during the whole project. In spite of the difference in temperature, a good correlation is recognizable among the measured values of this project and those in the literature.

Experiments with a reaction time of 16h at 70°C

The possibility that a higher temperature would yield an even more complete reaction could not be ruled out. Therefor a substantially higher temperature of 70°C was chosen for a second experiment.

Plotting the received fluorescence values of SVGlys against their concentration, it was evident that RebA, Stev and DH-Stev had a similar curve shape. Leaf extract indicated the same behavior but less concentration was needed. The curves that were obtained were similar to Figure 6.

The value for the IC_{50} of Stev was corrected as mentioned above. The IC_{50} calculated with the measured value for Stev was: 0.487 ± 0.064 mM.

In this experiment the leaf extract needed the smallest concentration to scavenge 50% of the radicals: 0.012 ± 0.007 mM followed by Stev (0.483 ± 0.068 mM), DH-Stev (0.512 ± 0.063 mM), RebA (0.541 ± 0.080 mM), Suc (3.961 ± 0.283 mM) and Glc (7.040 ± 0.448 mM). For the sugars a RSD value around 6-7% was achieved. For SVGlys the value was around 14% and finally for the leaf extract the RSD reached 57% (cf. Table 3). Compared with the experiments at 37°C, the range of the RSD was lower.

Table 3: Results of scavenging experiment 16h at 70°C

Scavenger	IC_{50} [mM]	Standard deviation [mM]	RSD [%]	Number of measurements	Sugar units
RebA	0.54	0.08	15	15	4
Stev	0.48	0.07	14	15	3
DH-Stev	0.51	0.06	12	12	3
Suc	3.96	0.28	7	14	2
Glc	7.04	0.45	6	14	1
Leaf extract	0.01	0.01	57	11	-

The values for the half-inhibitory concentration were higher than in the experiments at a lower temperature and also than the values of the mentioned literature. The same rank order for the scavenger RebA, Stev, Glc and Suc was found compared to the experiment with a reaction time of 1 h at 37°C and also the one with 16 h at 37°C. To sort DH-Stev into this rank order, it was between Stev and RebA. The leaf extract had the lowest IC_{50}.

The results for R^2 were higher than 0.99 for each test series. Only for the leaf extract a lower determination coefficient of 0.94 was found. For the sugars, the value was also 0.99 within the whole data series. During the whole experiment at 70°C, the fluorescence values were in case of intra-daily and inter-daily measurements repeatable. Hence, the better values for the reproducibility, which were expressed by the values of R^2. It was possible to guarantee reproducibility at 70°C in contrast to a reaction temperature of 37°C. But for the IC_{50} of leaf extract a very high RSD

value was reached. This was also due to the very small value of the IC_{50} about 0.012 mM.

The overlayed chromatograms of RebA, DH-Stev and leaf extract are presented in Figure 7 - Figure 8. It is obvious that the fluorescence signal decreased with an increasing concentration of scavenger. The quenching induced by RebA and DH-Stev was similar. The leaf extract had a stronger inhibition with a smaller concentration compared to RebA, Stev and DH-Stev. With the leaf extract at concentrations of 1.5 mM and 2.0 mM, the fluorescence signal showed a split peak, cf. Figure 8. This was interpreted as an influence of the colored sample.

The leaf extract was not colorless. The color changed from yellow-green to green-brown during incubation. It shows that further ingredients were dissolved e.g. phenolic compounds (Hajihashemi & Geuns, 2013) and also the coloring agent chlorophyll. It was assumed that the split peak was probably induced by self-quenching of these further compounds. This effect on the fluorescence was only visible in higher concentrations. To study the behavior of leaf extract in more detail, one possibility is the treatment with e.g. active coal or polyvinylpyrrolidone to remove the polyphenols and colored pigments (Hajihashemi & Geuns, 2013) and thereby the effect of self-quenching.

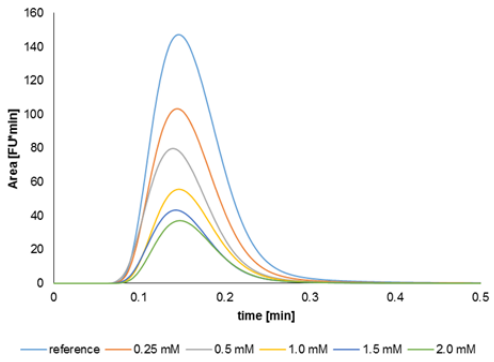

Figure 7: Fluorescence quenching induced by RebA after 16h at 70°C

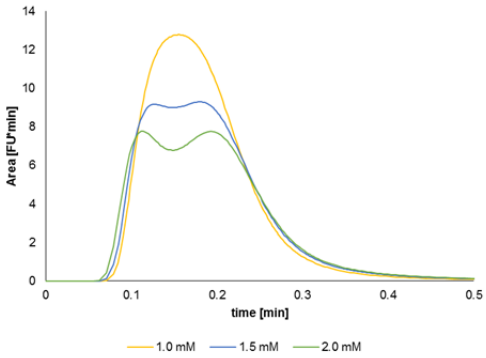

Figure 8: Fluorescence signal quenched by leaf extract showing the split peak

Another scavenger, added to this assay at 70°C, was DH-Stev. In contrast to the other SVGlys, DH-Stev has no double bond. Our expectations concerning the double bond were not confirmed. DH-Stev, containing the same number of sugar units as Stev, reached similar IC_{50} values that RebA and Stev have. Based on this fact, we conclude that the double bond is not the main reason for the scavenging activity of SVGlys.

It has already been noted that sugars are also able to act as scavengers even though not with the same intense capabilities as SVGlys. The first hypothesis of scavenging activity associated with the number of sugar units could not be proven. The reason is that with increasing numbers of glucose units, the IC_{50} would have been reduced by multiples of the IC_{50} of glucose, according to the number of sugar units. This was calculated using the following equation:

$$IC_{50}^{expected} = \frac{IC_{50}^{Glucose}}{number\ of\ glucose\ units}$$

The results of this calculation are shown in Table 4.

The measured IC_{50} values for RebA, Stev and DH-Stev were always lower than the expected value. Only the calculated value for Suc was smaller than the measured value. Summing up, the measured and calculated values are completely different.

Thus, a correlation between the scavenging activity and the number of sugars is refuted. This result was also found by Hajihashemi and Geuns (2013).

Table 4: Comparison of IC_{50} [mM] measured and the calculated IC_{50} [mM] based on Glc

	Glc-units	IC_{50} (16h, 37°C)	IC_{50} expected	IC_{50} (16h, 70 °C)	IC_{50} expected
Glc	1	1.64		7.04	
Suc	2	1.01	0.82	3.96	3.52
Stev	3	0.18	0.55	0.48	2.35
DH-Stev	3			0.51	2.35
RebA	4	0.20	0.41	0.54	1.76

The second hypothesis was based on a correlation between scavenging activities and the number of OH-groups. Calculations were done for the total number of OH-groups as well as for the primary and secondary OH-groups. An overview of the number of sugar units and OH-groups of the scavengers is given in Table 5.

Table 5: Overview of the number of sugar units and OH-groups

Scavenger	glucose	fructose	primary OH	secondary OH	total OH
RebA	4	0	4	10	14
Stev	3	0	3	8	11
DH-Stev	3	0	3	8	11
Glc	1	0	1	4	5
Suc	1	1	3	5	8

Plotting the IC_{50} against the respective OH-number, the coefficient of determination was calculated for a linear correlation and a quadratic function. The results are presented in Table 6.

Table 6: Coefficient of determination for the respective correlation between IC_{50} and the number of OH-groups for a linear and quadratic function

	R^2 (linear)			R^2 (quadratic)		
IC_{50} [mM]	**Total OH**	**1° OH**	**2° OH**	**Total OH**	**1° OH**	**2° OH**
37°C, 1h	0.891	0.763	0.863	0.962	0.767	0.998
37°C, 16h	0.893	0.763	0.864	0.963	0.768	0.998
70°C, 16 h	0.891	0.788	0.85	0.974	0.796	0.993

The first striking point is that the numbers are very similar for each OH-group category, despite of the different temperatures and reaction times. The lowest values are achieved with the primary OH-groups in case of linear and quadratic functions. The differences between the obtained values for the total number of OH-groups and these of the secondary OH-groups are small, in both the linear and the quadratic correlation. For a linear relationship the highest values are reached for the total OH-number. Concerning the quadratic function, the highest values are obtained for the secondary OH-groups, with coefficients of determination lager than 0.990. The quadratic function reaches better values for the coefficient of determination compared with the linear correlation.

The majority of OH-groups in sugars are secondary OH-groups. Therefore, we expected a similarity in the correlation between IC_{50} values and the total OH-groups as well as in the correlation between IC_{50} values and the secondary OH-groups. In conclusion, the coefficients of determination of the quadratic function give a better correlation than those of the linear function. However, it does not prove a quadratic relationship. This correlation suggests that OH-groups are responsible for radical scavenging activity. On the one hand, this information is contrary to the results of Hajihashemi and Geuns (2013). They did not find a correlation for the scavenging capacity of SVGlys with neither the sugar units nor the number of alcoholic hydroxyl groups. On the other hand Morelli et al. (2003) showed a strong interaction between the $^{\bullet}OH$ scavenging activity and the number of OH-residuals of sugars, e.g. glucose, fructose and sucrose. They demonstrated that sucrose had a higher activity than glucose. This fact was confirmed in our assay.

It seems that sugars play a role in the scavenging activity of hydroxyl radicals although it is not the final reason. To confirm the relationship of sugar and scavenging capabilities, experiments with different alcohols, e.g. ethanol and 2-propanol, could be done.

In this context of a correlation between the scavenging activity of SVGlys and the OH-groups of the sugars, the question arises what happened with the sugars and

their alcoholic groups after an attack of hydroxyl radicals. Stranic et al. (2014) showed the possible reaction between ethanol and hydroxyl radicals. They measured the overall and non-β rate constants for the reaction between ethanol and hydroxyl radicals behind reflected shock waves in a shock tube.

The hydroxyl radical can attack ethanol via three possible reaction sites: α, β, and O (Stranic, et al., 2014):

$CH_3\text{-}CH_2\text{-}OH + {}^{\bullet}OH \rightarrow CH_3\text{-}C^{\bullet}H\text{-}OH + H_2O$ (reaction at α position)

$\rightarrow {}^{\bullet}CH_2\text{-}CH_2\text{-}OH + H_2O$ (reaction at β position)

$\rightarrow CH_3\text{-}CH_2\text{-}O^{\bullet} + H_2O$ (reaction at O position).

With their result it is possible to conclude that sugars are damaged by hydroxyl radicals on various positions. As reaction product sugar radicals and water are achieved.

The question rises as to why the blank samples had a different IC_{50} value at 37°C and 70°C. The gap of the fluorescence value indicates a different amount of formed HTPA, as already discussed above. At a higher temperature the rate of the reaction is higher (Pérez, Torrades, Domènech, & Peral, 2002). That explains why the end product formation was already finished after 6h at 70°C. However, it does not explain the formation of different HTPA amounts. A possible explanation is that at 37°C the reaction is not completely finished, even in spite of the long reaction time. The various educts were available in surplus, and Fe(II) was generated by ascorbate. Thus, the limiting factor to produce radicals was hydrogen peroxide. However, its concentration was checked regularly, and the preparation of the hydrogen peroxide solution was adapted to the loss of concentration. Thereby the same concentration in the reaction mixture was ensured for every measurement. Another factor, the lamp output, was checked every day measuring two BPA samples. The achieved fluorescence values were stable so that we concluded a stable lamp output. Moreover, SVGlys and sugars do not have a significant influence (<10%) on the hydrogen peroxide concentration (Stoyanova, et al., 2011). Furthermore, with

no inter-daily reproducibility measured at 37°C, there was the option of an unfinished reaction. Most probably another parameter exists that influences the reaction. The higher quantity of HTPA at a higher temperature was induced by more radicals. This also means that more scavengers were needed to inhibit 50% of them. Therefore, a higher value for the IC_{50} was achieved.

Since SVGlys are stable up to 80°C for 4 h and also in the used pH range (Kroyer, 2010), it is possible to measure with a temperature of 70°C. However, with a reaction time of 16h we were above the time frame of stability of SVGlys found by Kroyer. Several authors (Jooken, et al., 2012; Wölwer-Rieck, Tomberg, & Wawrzun, 2010) observed the stability of different SVGlys in several foodstuffs. They noticed decomposition over various time periods. For example, in soft drinks the amount of RebA and Stev decreased by 15% and 32% respectively after a treatment with 80°C for 24 hours (Wölwer-Rieck, et al., 2010). In this case the low pH of soft drinks must be considered, which is lower from the one used in our assay. Jooken et al. (2012) tested the SVGlys stability under different conditions. Concerning the heat stability, RebA is stable up to 180°C for 2 hours. For Stev decomposition was observed. In general RebA is more stable than Stev. In terms of the test circumstances with a pH of 7, RebA is stable during the reaction. For Stev decomposition is expected. Through decomposition induced by e.g. splitting of sugars, the amount of the minor SVGlys (i.e. RebB, SB) increases. These compounds can also act as scavenger.

Furthermore, SVGlys prevent the degradation of ascorbate (Kroyer, 2010), so it has also a positive influence to the Fenton reaction.

Finally, the IC_{50} value of Stev was lower than the value of RebA. This result is in contrast to the literature values of Geuns and Struyf (2010) and Hajihashemi and Geuns (2013). Both research teams found a higher scavenging activity for RebA. This correlates with the fact, that RebA is more stable and contains more OH-groups due to four glucose molecules instead of three molecules (Stev). In our sample of Stev an amount of 6% RebA was present. The measured IC_{50} values of Stev were recalculated to the true IC_{50} values. The calculation was based on the

assumption that these values are summative. Based on this calculation, the IC_{50} values for Stev were lower than those of RebA, resulting in a different ranking order of scavenging activity as mentioned in literature.

If the IC_{50} values were not summative, they could be calculated based on following equation:

$$IC_{50}^{Stev\ measured} = \frac{100}{91}\left(0.85 \cdot IC_{50}^{Stev} - 0.06 \cdot IC_{50}^{RebA}\right)$$

$$IC_{50}^{Stev} = \frac{\frac{91}{100} \cdot IC_{50}^{Stev\ measured} + 0.06 \cdot IC_{50}^{RebA}}{0.85}$$

Recalculating the IC_{50} values of Stev with this formula resulted in the ranking order that RebA had a higher scavenging activity and with this a lower IC_{50} value than Stev (cf. Table 7). This would correspond with the values mentioned in literature. Further research concerning interaction of SVGlys is suggested, because the behavior of SVGlys mixtures, and whether it is summative or not, is unknown, as well as the existence of synergetic effects.

Table 7: Overview of IC_{50} concentrations in mM recalculated with the new formula for Stev, compared with the available literature

Scavenger	Geuns and Struyf, 2010	Stoyanova et al., 2011	Hajihashemi and Geuns, 2013	this work, 16h at 37°C	this work, 16h at 70°C
RebA	0.360	-	0.196	0.200	0.541
Stev	0.383	0.188	0.219	0.209	0.560
Glc	8.913	0.514	1.562	1.642	7.040
Suc	5.303	0.615	1.119	1.014	3.961

Huang et al. (2005) pointed out that a standard assay to determine the antioxidant or scavenging capacity is missing. Thus, it is difficult to compare the results of the different research teams. Furthermore, Huang et al. comment critique to the hydroxyl radical scavenging assay. The formation of hydroxyl radicals based on the Fenton reaction has disadvantages. One disadvantage is the fact that many antioxidants also act as a metal chelator (Huang, et al., 2005; Karadag, Ozcelik, & Saner,

2009). Through the chelation of the metal ions, it is inert toward hydrogen peroxide and no radicals will be produced. Therefore, it is impossible to identify if the scavenger is just a good metal chelator or a radical scavenger (Karadag, et al., 2009). In the present assay, EDTA was used in the reaction mixture. Chelating agent, e.g. EDTA, is added to protect the metal ion against the chelating activity of scavengers (Karadag, et al., 2009). However, in this assay the influence of EDTA was not checked. Further, the influence of SVGlys as chelator is not known. It is also unknown if a competitive reaction between EDTA and SVGlys exists.

An alternative assay is a metal-independent Fenton reaction. Zhu et al. (2000) reported an organic Fenton reaction without using a metal compound. The generation of hydroxyl radicals is induced by tetrachlorohydrochinone (TCHQ) with H_2O_2 dissolved in 4-(2-hydroxy-ethyl)-1-piperazinethane-sulfonic acid buffer. The formation of the radical takes place via a trichlorohydroperoxyl-1,4-benzoquinone intermediate. Its decomposition by homolytic splitting generates hydroxyl radicals (Zhu, Kalyanaraman, & Jiang, 2007). These radicals are caught by salicylate forming 2,3- and 2,5-dihydroxybenzoic acid; separated by HPLC and detected via electrochemical detection (Zhu, et al., 2000). Avoiding the use of metals and chelating agents, this assay can be an alternative to the general Fenton reaction.

Despite the difficulties with the reproducibility, the IC_{50} values were calculated with the obtained fluorescence values which were considered in relation to the fluorescence values of the blank sample. This procedure ensured that the IC_{50} values were relative so that there is no problem to compare them.

Investigations with steviol

First, the test with different solvents (blank samples containing 50% organic solvent) with regard to the quenching of the fluorescence signal were done. The results are presented in Table 8. The measured values were very low (< 0,1 FU*min).

Table 8: Fluorescence values of blank samples containing 50% organic solvent

Solvent	Area [FU*min]
MeOH	0.031
HAc	0.027
Aceton	0.081
ACN	0.081
DMF	0.026
DMSO	0.090
Dioxan	0.030
Pyridine	0.071
THF	0.032

When trying to dissolve SV in a buffer with ACN, at least 50% of this organic solvent was needed to guarantee solubility. This scavenger solution was added to the reaction mixture, and the fluorescence was measured for a blank sample and a sample containing 2 mM SV after 16 hours at 37°C. The result of the blank sample measurement was an area of 0.90 FU*min and 0.87 FU*min for SV (2 mM). The difference in fluorescence intensity between these two results was very small, in spite of the highest difference in concentration used in this experiment for SVGlys. The area of this blank sample was higher than the area as shown in Table 8. These two samples were measured on different days. In all likelihood the difference in the area was induced by the unknown parameter influencing the reaction.

Blank samples have the highest fluorescence value because no scavenger is added, and the full amount of HTPA can be formed.

Using any organic solvents will be very difficult. The measured fluorescence values of the blank samples are already very small. Any added scavenger will quench the reaction, causing further decrease in fluorescence. The low difference of the quenching expressed by the fluorescence value was confirmed by the results with SV. Summing up, with an amount of 50% ACN, the quenching is too high.

It was not possible to find a solvent that does not quench the fluorescence and ensures the solubility of SV, so it was not feasible to continue the scavenging experiment and determine the half-inhibitory concentration of SV.

However, to observe the scavenging capabilities of SV and thus the influence of the double bond, the solubility in an aqueous system has to be enhanced. One possibility is to modify the carboxylic acid function (R_1) by esterification and the alcoholic hydroxyl group (R_2). Instead of sugars, such as are contained in SVGlys, alcohols can be used e.g. glycerol. The water solubility rises with the number of alcoholic hydroxyl groups. However, it must be considered that e.g. ethanol is also a radical scavenger (Buxton, Greenstock, Helman, & Ross, 1988) that might influence the scavenging activity of SV.

Conclusion

During the experiments to determine the scavenging activity of SVGlys a couple of difficulties were faced. These were particularly affecting reproducibility.

Summing up, ascorbate is necessary for the reaction to act as a pro-oxidant to generate Fe(II). The assumption of a reaction between radicals and the glass wall was not confirmed. After a reaction time of 16 hours the formation of the end product was finished and the end product reached a stable fluorescence value. Different scavenging capabilities were observed at 37°C and 70°C. Obviously, the temperature influences the amount of formed HTPA.

Notwithstanding the difficulties to reproduce this assay, we conclude that SVGlys have a remarkable scavenging activity compared with sugars as shown by the low IC_{50} values. The best scavenging activity after a reaction time of 16 hours was found at a temperature of 37°C. The IC_{50} values of several scavengers were: Stev: 0.181 ± 0.066 mM; RebA: 0.200 ± 0.063 mM; Suc: 1.014 ± 0.384 mM and Glc: 1.642 ± 0.456 mM.

The rank order found for the scavenging activity of SVGlys was Stev < RebA in opposite to the rank order found in literature with RebA < Stev. This means that the

behavior of SVGlys mixtures is unclear, and further investigations concerning the behavior of SVGlys in mixtures are suggested.

The expectation, that the double bond of the steviol scaffold is mainly responsible for the scavenging activity, was not confirmed. This was shown by the results of DH-Stev which had a similar scavenging activity as RebA and Stev, expressed by similar IC_{50} values.

However, a possible relationship between scavenging activity and number of OH-groups was found. To confirm this correlation, further scavenger experiments with sugars and also with alcohols, with different numbers and types of OH-groups, are suggested.

The investigations with steviol could not be finished. An organic solvent that does not quench the fluorescence signal has still not been found. An amount of 50% ACN was still too high concerning the quenching. For further research projects, the solubility of steviol is to be enhanced. This can be done by modifying the acid function by esterfication and modifying the alcoholic hydroxyl group with several alcohols or other polar functional groups.

Further investigation on the behavior of SVGlys should be done to analyze if SVGlys are able to act as a chelator. Also the role of EDTA should be checked to analyze competitive reactions between SVGlys and EDTA.

Due to the lack of a standard assay, the assay used within this project should be optimized. Another possibility is the use of an assay which guarantees a better reproducibility.

As mentioned in the beginning, SVGlys have positive effects on the human health. There is ample interest to see how SVGlys act as scavengers and to know which reaction takes place. To gain this knowledge structural analysis could be applied. One possibility would be the use of a Fenton reaction with e.g. RebA in a high concentration over a prolonged time. The reaction product would be separated by HPLC. Analysis and identification would be done by mass spectrometry. This project would need sufficient patience because of the unknown structure of the reaction product.

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