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Extraction of steviol glycosides from fresh Stevia using acidified water; comparison to hot water extraction, including purification

Authors: A.M.J. Kootstra, S. Huurman

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Summary

This report describes a practical comparison of an acidified water extraction of freshly harvested Stevia plants (the NewFoss method) to the hot water extraction of dried Stevia plants, the industry standard. Both extracts are subsequently purified using lab-/bench scale standard industrial technology: coagulation, filtration, adsorption, and ion exchange.
The reason for this comparison is the low purity of steviol glycosides reached in tests where an acidified water extract was purified using mainly ultrafiltration in 2014 and 2015. Therefore, the purification potential of the acidified water extract while using standard industrial technology needed to be assessed, in turn in order to show the potential for the acidified water extraction as a viable extraction technology for steviol glycosides from Stevia.

The combination of coagulation, filtration, adsorption, and ion exchange results in a 80.6 wt% steviol glycosides in the dry matter of the purified acidified water extraction. This is very close to the 85.4 wt% reached when the same purification processes are performed on a hot water extract. Acidified water extract of fresh Stevia can be purified using the same standard industrial technology, to a similarly high level as a hot water extract of dried Stevia. This strengthens the potential for the acidified water extraction of steviol glycosides from fresh Stevia.

1 Introduction

Stevia rebaudiana is a plant that originates from Paraguay, South America, and it produces high potency low-calorie sweeteners in its leaves, mainly stevioside and rebaudioside A, both steviol glycosides. Locally, the plant leaves have been used for their sweetening capacity since long ago, but not until the 1960's was commercial cultivation started in Paraguay and Japan, and later in other countries as well. In the late 1990's most of the Stevia cultivation was taking place in China, with Japan being the major market. Stevioside and rebaudioside A extracted from Stevia leaves are now more or less widely used in Japan, South Korea, China, South-East Asia and South America, as a sweetener in a wide variety of foods. Since the approval of Stevia sweeteners in the US by the FDA in 2008, and by the European Union in 2011, industrial interest has risen accordingly (Stoyanova et al, 2011; González et al, 2014).

For the extraction and purification of the steviol glycosides from the plant material, several possibilities exist. A commonly used extraction method consists of extracting dried and powdered leaves with hot water, after which a primary clarification is reached by filtration and centrifugation. Another common method for the extraction of leaves uses an ethanol-water mixture, followed by an evaporation of the extract. Other techniques include clarification using hexane, or solvent extraction followed by purification using selective adsorption by ion exchange, or addition of chelating agents followed by crystallisation, or extraction followed by adsorption using zeolites (Chhaya et al, 2012; González et al, 2014). For purification purposes, ultra- and nanofiltration are also suggested, including a centrifugation step for clarification of the extract, in a study using dried and powdered Stevia leaves (Chhaya et al, 2012).

To reduce process costs related to drying, it may be preferable to process fresh Stevia, possibly at relatively small scale –for instance close to the area of cultivation. In two studies performed previously to the one described in this report , fresh Stevia plant material is extracted in water at room temperature, as proposed by the company Newfoss (Kootstra, 2015; Kootstra et al, 2016). In order to facilitate the extraction of steviol glycosides through the cell wall, the water is acidified in order to promote cell wall permeability; hence the term 'acidified water extraction'. The acidification is achieved by letting the microorganisms present on the plant material convert added sugar to organic acids. In these previous studies, Stevia extracts thus obtained were purified by ultrafiltration and concentrated by nanofiltration. Although the extraction of steviol glycosides was found to be 80 % to 90 % effective, the purity of the final product was too low: 10 % to 19 % glycosides in the dry matter.

Therefore, in the current study, an acidified water extract from fresh Stevia is purified using technology that is standard in the Stevia-extraction industry: coagulation, filtration, adsorption, and ion exchange. For further comparison with industrial standard processing, a hot water extract of dried Stevia material is also produced and subjected to the same purification procedure.

2 Materials and Methods

2.1 Stevia plants

Cultivation took place in a greenhouse on location at ACRRES, Lelystad, the Netherlands, using *Stevia rebaudiana* 153 rooted cuttings acquired via Globe Plant, the Netherlands, from Hishtil, Israel. The cuttings were planted two or three per pot (20 cm diameter) containing press potting soil and 5 gram Osmocote exact fertilizer pellet (N-P-K-Mg: 14-8-11-2) per pot, on 10 August 2016. The plants received artificial light for 18 h per day, and were watered regularly. Harvest took place on 19 September 2016. Plants were cut at ~5 cm above the soil. Half of the plants were dried (48 h, 80 °C) to be used in the hot water extraction of dried material, and the other half was used immediately in the extraction of fresh material.

2.2 Methods

2.2.1 Processing

2.2.1.1 Acidified water extraction of fresh Stevia

In a 150 L barrel, a total of 77.0 L of demineralised water was added to 11.8 kg of plant material –leaves and stems–, after which 478 g sucrose (crystalline food grade sugar, Sundale, the Netherlands) was added. Mixing was achieved by closing the vessel and rolling and turning it. After re-opening, the vessel was left to stand at room temperature (20.6 to 21.8 °C). After 24 h, pH had dropped to 5.5-5.7. The following morning, so after 48 h, pH 4.0 was measured. The extract was filtered using a 150 µm nylon filter bag (NMO-150-P1S, Industrial Filter Manufacturing Ltd, Canada). The extract was relatively free of large particles, and somewhat light green.

2.2.1.2 Hot water extraction of dried Stevia

The dried plant material –leaves and stems– was crushed manually so that the stems pieces were ~3-7 cm in size; leaves fell apart in smaller pieces quite readily. In a 30 L barrel, a total of 16.9 L of demineralised water at 80 °C was added to 1.23 kg of dried plant material. After stirring, the vessel was closed and placed in a 80 °C incubator for 1 hour. The extract was then filtered using a 150 µm nylon filter bag (NMO-150-P1S, Industrial Filter Manufacturing Ltd, Canada). The filtered extract was cooled to ~40 °C by pouring it back and forth using two vessels. The extract was relatively free of large particles, and dark brown - comparable to very strong tea, or weak coffee.

2.2.1.3 Clarification by coagulation

To induce coagulation, $CaOH_2$ (>96%, Sigma Aldrich) was dissolved in the extract to a final pH 10. The initial pH of the hot water extract was 5.7 (at 38.1 °C), with final pH at 10.1. For the fresh-material extract, these values are 4.0 and 10.2, respectively.

After approximately 30 minutes, the coagulated fresh material extract was filtered through a series of filters. First a 150 µm nylon filter bag (NMO-150-P1S, Industrial Filter Manufacturing Ltd, Canada), followed by three separate polypropylene meltblown cartridge filters (50 µm, 5 µm, and 0.5 µm; BMBN series, 10", double open end, Fuhr Filtertechnik GmbH, Germany). As hardly any material was retained in the >0.5 µm filters, it was decided to only use the 0.5 µm pore size cartridge filter for the coagulated hot water extract. This caused no observed clogging, or other issues.

2.2.1.4 Adsorption resin

In preparation, a volume of 1 L resin (Diaion HP20, Mitsubishi Chemical, Japan) was mixed with 1 L of 50 vol% ethanol, and the mixture was transferred to a glass column (4.5 cm internal diameter). After settling,

the column was washed with 5 L (5 column volumes) demineralised water, making sure the resin stayed under the water surface.

For loading the resin, 18 L of fresh material extract was used, and 7 L of demineralised water for the subsequent wash step. For the hot water extract, 7 L was used for loading, and also 7 L demineralised water for the wash step.

Desorption was performed with 70 vol% ethanol -made from pure ethanol (ethanol absolute, Emsure, 100983, Merck) and demineralised water. Approximately 10 fractions were collected, of 250 mL to 350 mL each. All fractions were boiled and evaporated down to a mass of 40 g to 90 g, in order to remove the ethanol. All fractions were then normalised to their original volume by adding demineralised water. After sampling (results of separate fractions not shown), all fractions were pooled together in their original ratio by volume.

2.2.1.1 Ion exchange resins

From both anion (Relite RAM2, Mitsubishi Chemical, Japan) and cation (Relite RPS, Mitsubishi Chemical, Japan) exchange resins 1 L was mixed separately with 1 L demineralised water and transferred to separate glass columns (4.5 cm internal diameter, 85 cm length). After settling each column was washed with 3 L demineralised water making sure the resin stayed under the water surface. In preparation for anion exchange, the anion exchange resin was rinsed with 2 wt% NaOH solution, followed by an additional wash step with 5 L demineralised water to remove remaining NaOH. The cation exchange resin was rinsed with 5 wt% HCl, in order to load the resin with H^+ in preparation for cation exchange, followed by an additional wash step with 5 L demineralised water remove remaining HCl.

To both pooled extracts (~500 mL used; pH of acidified water extract: 7.03; pH of hot water extract: 7.12) an excess of anion exchange resin was added (~130 gram charged resin material) and gently mixed manually for 1 minute, after which the extract and the resin were separated using a 150 micron mesh sieve. The same procedure is repeated on the collected extract using the cation exchange resin. The finally collected product is used for analysis.

2.2.2 Analysis

2.2.2.1 pH and temperature

pH and temperature were measured using a calibrated pH electrode with built-in thermometer.

2.2.2.2 Dry matter and steviol glycoside levels

Levels of steviol glycosides were determined by the external laboratory ExPlant Technologies in Leiden, the Netherlands. The dry matter determinations and the extractions needed for these analyses were also performed by this laboratory. A protocol is included in Appendix 1. Dry matter content of the homogeneous liquids were determined by freeze-drying 50 mL of liquid. The samples were processed according to a fixed protocol in duplicate and analysed by HPLC with UV detection. Concentrations of stevioside, rebaudioside A (‘reb A’) and ‘sum other’ (a.o. rebaudiosides C, D, E, F, and dulcoside A) are determined. In this sample series the concentration of rebaudioside C (‘reb C’) was high enough to quantify separately. However, since there was no pure reference material for reb C, its concentration and that of the other glycosides lumped together under ‘sum other’ were expressed using a calibration curve based on rebaudioside A. For all samples an independent duplicate HPLC analysis was performed. In addition to the processed results in the Results section, raw analysis results can be found in Appendices 2, 3, and 4.

2.3 Experimental setup

In these experiments, the goal is to assess the purification potential of the acidified water extract, using standard industrial technology: coagulation, filtration, adsorption, and ion exchange. For further comparison with industrial standard processing, a hot water extract of dried Stevia material is also produced and

subjected to the same purification procedure (Figure 1). If the purity levels of the two final products are similar, this strengthens the potential of the acidified water extraction.

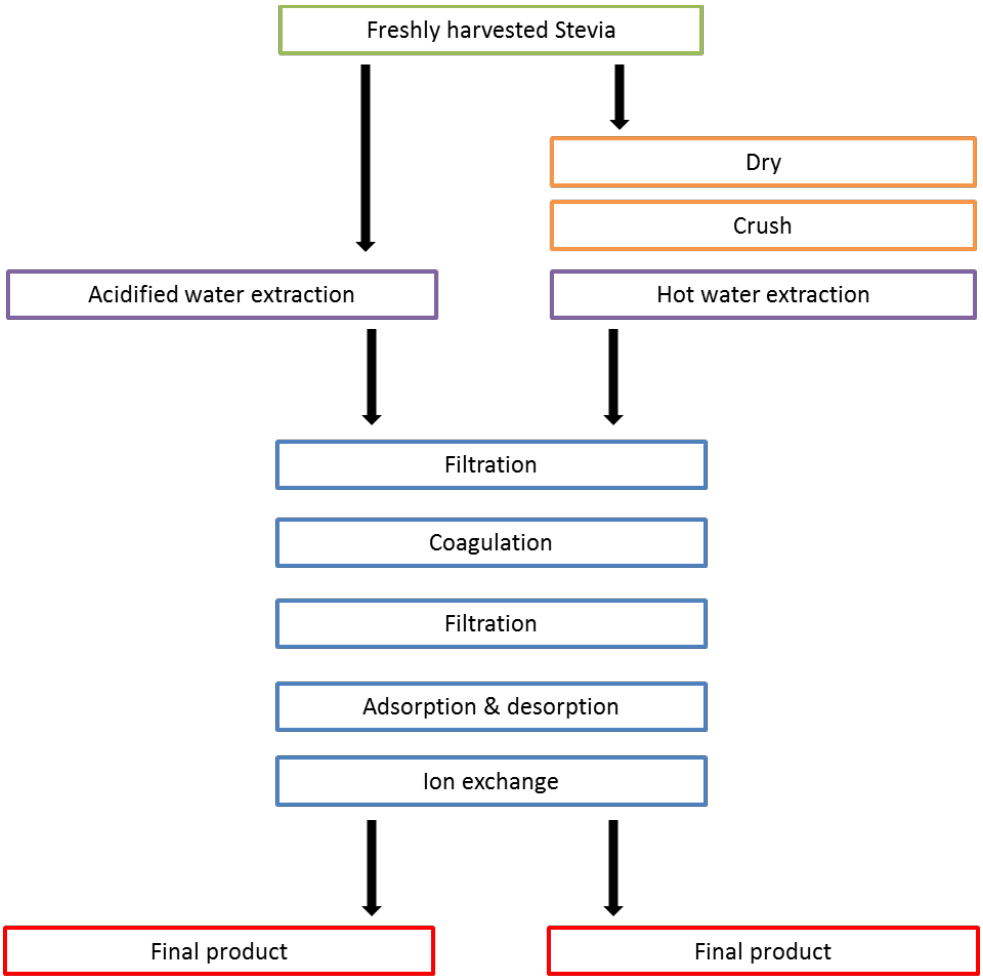

Figure 1. Process scheme: two different extractions with identical purification steps (in blue)

3 Results and Discussion

3.1 General results

The Stevia material used in this study was grown in an onsite greenhouse and contained 4.8 wt% steviol glycosides in the dry matter (Table 1), which is in the same range as the three commercially produced harvests used for the experiments in 2014. The relative concentration of each steviol glycosides is also similar [Kootstra, 2015]. Compared to the 2014 study, the small standard deviation between the three samples analysed for the current study indicate that the rooted cuttings are more consistent in their glycoside content. This was expected, as the 2014 material originated from more 'wild type' plant seed.

Table 1. Dry matter content and steviol glycoside content of the dry matter of the dried Stevia. Dry matter: value is average of 9 samples. Steviol glycosides: values are average of three samples. Standard deviation shown between brackets.

Product	Dry matter (wt%)	Stevioside in dm (%)	Reb A in dm (%)	Reb C in dm (%)	other in dm (%)	Sum in dm (%)
Fresh Stevia	11.6 (0.3)	3.19 (0.05)	0.92 (0.01)	0.37 (0.01)	0.35 (0.02)	4.83 (0.07)

3.2 Main results on extraction and purification

The dry matter content of the primary extract after hot water extraction contains more than twice the dry matter than that after acidified water extraction. This is to be expected, as in the hot water extraction 16 L water is used for 1.23 kg dry Stevia, versus 77 L for 11.8 kg fresh Stevia in the acidified water extraction; 5.5 times the amount of water as extractive. In fact, when taking into account the added 478 g of saccharose and assuming all of it remains as dry matter either as sugar or as organic acid, it would account for ~5.5 g/L of dry matter in the acidified water extract. The remaining 4.5 g/L would actually have been extracted from the plant material: a little over 5x less than the 22.9 g/L dry matter in the hot water extract, corresponding to the relative difference in volume. All in all, it can be reasonably stated that both methods extract a similar amount of material from the plant. Focussing on the purity of the extract, it is clear that the fraction of steviol glycosides in the dry matter of the hot water extract is almost twice that present in the dry matter of the acidified water extract. Again, this may largely be explained by the added sugar in the latter.

After coagulation at pH 11, the dry matter content and glycoside content of the dry matter in the filtrate do not change much, compared to the primary extract. The coagulation clearly mostly serves to decrease fouling of the following step, involving the adsorption resin.

In the final product, after the adsorption and ion exchange resins, the purity of the acidified water extract exceeds 80 wt% of steviol glycosides in the dry matter. This is very close to the 85 wt% that is reached in the final product resulting from hot water extraction. This obviously is much higher than the 10 to 20 % purity reached in the two studies preceding the current one [Kootstra, 2015; Kootstra et al, 2016], showing that acidified water extraction certainly does have the potential of resulting in a high purity product, just like hot water extraction. In fact, even higher purity does not seem out of reach, as the execution of processes as performed for this study had not been optimised. Another reason to think higher purity can be reached, is that decolouration was not included in this study. Not only would decolouration improve the appearance of the final product, it would also increase purity even more.

Of course, other processing differences between acidified water extraction and hot water extraction exist which certainly would influence further downstream processing. For example: in the case of the acidified water extraction, the extract volume is 5.5 times larger. Also, acidified water extraction as performed here was allowed to last 48 hours, while the hot water extraction was performed in 1 hour. Another issue is the sugar addition in the acidified water extraction. Here, it should be realised that in the original Newfoss process, acidification is to be separated from the extraction. Doing so and assessing the effects needs to be studied further, as well as further optimisation and upscaling. Finally, it should also be taken into account that the hot water extraction requires both drying of the plant material, as well as heating of the water used as the extraction solvent, both of which come at a cost.

Table 2. Dry matter content and steviol glycoside content of the dry matter in the primary extract, after coagulation and in the final purified product. Values are average of three samples, standard deviation shown between brackets.

Extraction	Product	Dry matter (g/L)	Stevioside in dm (%)	Reb A in dm (%)	Reb C in dm (%)	other in dm (%)	Sum in dm (%)
Acidified water	Primary extract	10.0 (0.0)	5.9 (0.0)	1.8 (0.0)	0.9 (0.0)	1.0 (0.0)	9.7 (0.0)
	After coagulation	10.9 (0.1)	5.6 (0.0)	1.6 (0.0)	0.9 (0.0)	0.9 (0.0)	9.0 (0.0)
	Final product	6.2 (0.2)	56.1 (1.3)	13.7 (0.3)	5.8 (0.1)	4.9 (0.2)	**80.6** (1.8)
Hot water	Primary extract	22.9 (0.1)	12.2 (0.2)	3.2 (0.1)	1.2 (0.0)	1.1 (0.0)	17.7 (0.2)
	After coagulation	21.2 (0.0)	13.8 (0.3)	3.6 (0.1)	1.5 (0.0)	1.1 (0.0)	20.0 (0.4)
	Final product	7.4 (0.0)	57.8 (2.0)	15.5 (0.5)	6.6 (0.1)	5.5 (0.3)	**85.4** (2.9)

Regarding the composition of the extracted mixture steviol glycosides, results show that relatively more rebaudioside C and 'other' are extracted in the acidified water extraction, and a correspondingly smaller fraction of stevioside compared to the hot water extraction (Table 3). In the final product however, the composition of the steviol glycoside mixture of both extracts is close to identical, with 69 % to 70% stevioside, and 17 % to 18 % rebaudioside A. Together these represent 86 % to 87 % of all steviol glycosides, which exceeds the former requirement of JECFA from 2006 that stated that no less than 70 % of all present steviol glycosides should consist of the sum of stevioside and rebaudioside A (JECFA, 2006). In its later publications JECFA no longer stated this requirement.

The fact that the purification results in very similar mixtures of steviol glycosides is not highly surprising, as purification was successful for both extracts and the purification process was the same in both occasions, as well as the original stevia material. Apparently, the differences upstream, mainly the drying and extraction steps, do not cause a hugely different feed as far as the purification by adsorption and ion exchange is concerned. Of course, it should also be realised that the performed purification processes were not optimised, and possibly somewhat over-scaled. If a cost-oriented optimisation is performed it is quite possible that differences between the extraction methods become apparent. But as shown in this study: in principle, the acidified water extract can be purified to a similarly high level as the hot water extract.

Table 3. Relative steviol glycoside content of the dry matter in the primary extract, after coagulation and in the final purified product. Sum of all steviol glycosides set at 100 %. Values are average of three samples, standard deviation shown between brackets.

Extraction	Product	Stevioside in dm (%)	Reb A in dm (%)	Reb C in dm (%)	other in dm (%)	Stev. + Reb A in dm (%)
Acidified water	Primary extract	61 (0.3)	18 (0.1)	10 (0.1)	11 (0.3)	80 (0.2)
	After coagulation	62 (0.3)	18 (0.1)	10 (0.2)	10 (0.2)	80 (0.4)
	Final product	70 (0.2)	17 (0.1)	7 (0.1)	6 (0.1)	**87** (0.1)
Hot water	Primary extract	69 (0.2)	18 (0.2)	7 (0.3)	6 (0.1)	87 (0.3)
	After coagulation	68 (0.1)	18 (0.1)	7 (0.1)	5 (0.0)	87 (0.1)
	Final product	69 (0.3)	18 (0.1)	8 (0.2)	6 (0.2)	**86** (0.2)

3.3 Indicative results on yield

The current study focusses on purification and mainly on the purity on the final product. It is important to realise that, as no mass balance was set up, the results concerning yields should be used as indicative only. Steviol glycoside yields for the two extractions are very similar. Both when using acidified water extraction of fresh material (120 % for stevioside; 125 % for rebaudioside), as well as when using hot water extraction of dried Stevia plants (121 % for stevioside; 110 % for rebaudioside), the extraction of glycosides from Stevia seem to be complete. Of course, apparent yields over 100 % are not realistic and probably caused by an sub-optimal extraction from the dried original material in the analysis lab. Still, it may be concluded that the two extraction methods as performed here do not differ much in the resulting yield.

Concerning the yield in the purification steps, the amount of steviol glycosides present in the final product can be compared with that present in the extract after coagulation and subsequent filtration, used to load the adsorption column (set at 100 %). For the acidified water extract, about 69 wt% of all glycosides (77 % for stevioside and 65 % for rebaudioside A) used to load the adsorption column end up in the final product after the ion exchange steps. For the hot water extraction, this is 49 wt% (48 % for stevioside and 49 % for rebaudioside A). Again, it should be clear that these numbers are indicative only. 69 % and 49 % may lead the reader into concluding that both yields are low, and/or that the yield for the acidified water extract is superior to that for the hot water extract. Neither conclusion should be drawn from these numbers, as they only serve to show that not only is the purity of the final products high (Table 2), also a substantial amount of steviol glycosides is present in the final product. Losses have clearly occurred during the adsorption and ion exchange steps: for example when loading/washing and especially in the elution of the adsorption column, or during evaporation of the ethanol, etc. However, minimisation of losses was not the focus on this study, and there obviously is room for optimisation.

4 Conclusions

- Purification of the acidified water extract of steviol glycosides from fresh Stevia is possible using standard industrial downstream processing technology.
- The final purity of the acidified water extract is 80.6 % steviol glycosides in the dry matter, very close to the 85.4 % purity reached for the hot water extract.
- Although no mass balance is constructed: steviol glycoside yields for the two types of extractions seem similar and high.
- The above shows potential for the acidified water extraction of steviol glycosides from fresh Stevia.

5 Suggestions for future study

As the main issue on the potential of purification of acidified water extract of Stevia has been resolved in this study, other questions have arisen. Some of these points have been mentioned above, and they include the following:

- Several questions remain on the subject of the acidified water extraction. For example: what is the effect of the separation on acidification and extraction, as is envisaged in the original process of Newfoss? And, what would be the minimal extraction time in the acidified water extraction? Clearly, reducing the 48 hours applied in this study would greatly improve the economics of the process.
- The purification should be optimised, with costs in mind. Obviously, optimisation of the adsorption and ion exchange steps should be performed. Also, it may be possible to reduce fouling of the resins even more my re-introducing ultrafiltration, or maybe microfiltration, before the adsorption resin.
- Larger scale application of the current study is needed as well. Possibly combined with the optimisation.
- The application of purification by adsorption opens the possibility for fractionation during purification. Being able to fractionate facilitates the production of more tailored products: single glycosides, or designed mixtures.
- The possible energetic advantage of not having to dry Stevia and not having to heat water for the hot water extraction needs to be calculated and compared to the larger volume applied for the acidified water extraction. Also, decreasing the needed solvent volume in the acidified water extraction should also be focussed upon.
- Several questions remain on the issue of the Stevia stems. Should the whole plant be used in the extraction, or would it be preferable to separate leaves and stems prior to extraction? This may not only affect the needed volume of the extraction vessel, but it may the intended application of the stems after extraction.

6 References

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7 Appendices

Appendix 1. Short description of sample preparation and analysis from ExPlant Technologies analysis report (in Dutch)

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Leiden, 05-10-2015

ANALYSERAPPORT 20151005LV

Analyse van steviolglycosides in vloeibare Stevia monsters.

Op verzoek van ACRRES Lelystad is het drogestof gehalte en het gehalte aan steviolglycosiden bepaald in 14 vloeibare monsters.

1. Methoden

Van de homogene vloeistoffen is door middel van vriesdrogen het vaste stof gehalte bepaald. Hiervoor werd 50 ml gebruikt.

De monsters zijn volgens een vast protocol in duplo opgewerkt en geanalyseerd met HPLC met UV-detectie. Met de gebruikte HPLC methode worden de gehalten Stevioside, Rebaudioside A ('reb A') en 'som overigen' (waaronder rebaudiosides C, D, E, F en dulcoside A) vastgesteld. Omdat in deze serie monsters sprake was van een voldoende meetbaar gehalte aan rebaudioside C ('reb C'), kon dit apart worden gekwantificeerd. Omdat er echter geen beschikking was over voldoende zuiver referentiemateriaal voor reb C, werd het gehalte hiervan (evenals dat van de ' som overigen') uitgedrukt als reb A.

2. Resultaten

Voor de vloeistoffen is het droge stof gehalte uitgedrukt in g/L (zie bijlage)

Van alle monsters is een onafhankelijke duplo analyse uitgevoerd. De resultaten van beide analyses (A/B) zijn vermeld in de bijlage bij dit rapport. Het gehalte van de verschillende componenten is vermeld in mg/L.

Bijlage: xls sheet met resultaten van de analyses

Appendix 2. Steviol glycoside content of dried Stevia material, stems and leaves together

Code	Concentration in mg/g			
	RebA	Stevioside	Reb C*	sum other*
Vast 1-1	9.0	31.4	3.5	3.4
Vast 1-2	9.3	31.8	3.7	4.1
Vast 2-1	9.1	31.8	3.7	3.1
Vast 2-2	9.1	31.3	3.8	3.4
Vast 3-1	9.2	32.6	3.7	3.4
Vast 3-2	9.3	32.4	3.8	3.7

Appendix 3. Steviol glycoside and dry matter content from acidified water extract; liquid samples

Code	Concentration in mg/L				Dry matter
	RebA	Stevioside	Reb C*	sum other*	g/L
Primary extract					
ZE1	176	582	91	99	9.850
ZE1d	178	591	97	105	
ZE2	182	599	98	114	9.976
ZE2d	173	579	87	100	
ZE3	178	598	95	106	9.920
ZE3d	173	588	94	94	
After coagulation					
ZNC1	179	609	100	91	10.760
ZNC1d	178	606	95	91	
ZNC2	176	601	96	95	10.976
ZNC2d	179	627	100	95	
ZNC3	183	604	101	99	10.950
ZNC3d	173	613	103	93	
Final product					
ZNR1	847	3458	366	295	6.106
ZNR1d	850	3445	354	309	
ZNR2	822	3430	353	307	6.062
ZNR2d	860	3510	357	296	
ZNR3	867	3470	367	290	6.416
ZNR3d	857	3545	375	312	

Appendix 4. Steviol glycoside and dry matter content from hot water extract; liquid samples

Code	Concentration in mg/L				Dry matter
	RebA	Stevioside	Reb C*	sum other*	g/L
Primary extract					
HE1	740	2800	297	231	22.930
HE1d	756	2852	264	255	
HE2	735	2792	272	261	22.892
HE2d	747	2809	296	241	
HE3	720	2785	293	244	23.094
HE3d	726	2759	300	242	
After coagulation					
HNC1	755	2901	316	248	21.132
HNC1d	807	3074	327	225	
HNC2	779	2967	323	238	21.198
HNC2d	746	2831	312	221	
HNC3	738	2820	303	228	21.068
HNC3d	776	2915	315	233	
Final product					
HNR1	1116	4279	478	418	7.306
HNR1d	1170	4367	477	408	
HNR2	1133	4189	466	419	7.228
HNR2d	1158	4312	503	418	
HNR3	1079	3997	468	368	7.380
HNR3d	1114	4197	483	398	

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