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Extraction of steviol glycosides from fresh Stevia using acidified water; clarification followed by ultrafiltration and nanofiltration

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Summary

As part of the PPS Kleinschalige bioraffinage project (WP1b), fresh Stevia material was used in the extraction of steviol glycosides using water acidified through conversion of sugar by microorganisms naturally present on the plant. Two successive harvests from the same plot were used. Previous experiments had resulted in high steviol glycoside extraction rates of 80 % to 90 % but the purity of the final extract was low (15 % to 20 % of steviol glycosides in the dry matter). The first batch of plants was used to test a clarification step by filtration on a small scale. A second batch of plants was used to perform clarification, purification using ultrafiltration, and concentration by nanofiltration on a larger scale.

The clarification step performed as desired, reducing the load applied to the ultrafiltration membrane. The final concentration step by nanofiltration also performed well, as it concentrated the ultrafiltration permeate while hardly losing any glycosides to the nanofiltration permeate. The purification by ultrafiltration itself did not perform as desired, as nearly all of the material still dissolved or suspended in the clarified extract passed through the membrane to the permeate. This resulted in 9 % to 10 % glycosides in the dry matter of both the ultrafiltration permeate, as well as the nanofiltration retentate, the final product.

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 this study, fresh Stevia plant material is extracted in water at room temperature. In order to facilitate the extraction of steviol glycosides through the cell wall, the water is acidified in order to increase cell wall hydrolysis. The acidification is achieved by letting the microorganisms present on the plant material convert added sugar to organic acids. In the previous study (Kootstra, 2015) Stevia extracts thus obtained from three successive harvests were purified by ultrafiltration and concentrated by nanofiltration. The Stevia material was found to be quite variable in steviol glycoside content, and apart from an increasing dry matter content –seemingly due to thicker stems-, no major difference was found between the three successive harvests. It was concluded that the extraction of steviol glycosides from fresh Stevia material is very effective (80 % to 90 % of all present glycosides were extracted), but that the purity in the final product –the nanofiltration retentate- was too low: 14 % to 19 % steviol glycosides in the dry matter. It was concluded that, following the extraction, a more selective downstream process is needed in order to result in a product with higher purity. A limit market study/discussion lead to the goal of producing a semi-finished product of 30 % to 50 % steviol glycosides in the dry matter. In a final full scale process, this product could then be further purified, if desired, at a central location, or it may be used for application for which the lower purity does not pose a problem.

Therefore, for the current study, three major process adjustments have been made: 1) a clarification step is added, and 2) the ultrafiltration step is performed using a membrane with a smaller pore size. It is expected that the smaller pore size leads to increased purity, while the clarification step reduces the load on the ultrafiltration membrane. 3) The nanofiltration is performed using membranes of two different pore sizes, to see whether additional purification (loss of molecules smaller than the steviol glycosides) can be achieved here. Unrelated to the three abovementioned points, only leaves were used for the experiments, instead of the whole plant, as was done in the experiments of 2014.

Two sets of experiments are performed. The first of two sets of experiments is performed to evaluate the efficiency of a clarification by filtration on a small scale. The second experiment is performed on a larger scale and included the full sequence of clarification by microfiltration, purification by ultrafiltration, and concentration by nanofiltration.

2 Materials and Methods

2.1 Stevia plants

Cultivation took place in Lehliu Gara, in south-east Rumania. Seed had been acquired from Everstevia, Canada. The plants the first sown in pots in spring 2015, and planted in soil in the beginning of May 2015. Cultivation was done organically, so without use of artificial fertiliser. The used soil can be described as fertile heavy clay with an organic matter content of 7 % and could be well dewatered. Drip irrigation was applied. Field edges and areas used for turning farming equipment were avoided. Material was harvested two times from the same area of land: early August, and early October. Harvesting was done manually and ideally consists of cutting of the plant just above the bottom pair of leaves. However, due to an unforeseen drought prior to harvesting, the lower part of the plants had withered and most of the harvest therefore consisted of the top part of the plants. Both harvests, the October harvest more so than the one of August, contained a relatively large amount of flowering material. Also, both harvests contained relatively many withered and browned leaves and both harvests contained a noticeably larger amount of sand on the plants, compared to the material of the trials in 2014. Harvest was timed just before the arrival of a cooled truck, by which the harvested material was transported at 2 °C to ACRRES in Lelystad, the Netherlands. Transport typically took two days, after which the material was kept at ACRRES at 4 °C for two days until the start of the extraction.

2.2 Methods

2.2.1 Processing

2.2.1.1 Leaf picking

Only leaves were used for the experiments, instead of the whole plant as was done in the experiments of 2014. The reason for this change is the final process design as envisaged by NewFoss, in which the stems are seen as unnecessarily increasing the needed extraction volume. Therefore only the leaves were used, to keep processing volume and associated costs down. A total of 23.2 kg leaves was handpicked from 33.3 kg of harvest for the August experiments, and 13.8 kg of leaves from 54.6 kg of plants for the October experiments.

2.2.1.2 Acidification and extraction

In a 0.5 m^3 (1.0 m^2 surface and 0.5 m height) vessel 15.5 and 14.9 L of demineralised water were added per kg of fresh leaves, in August and October respectively. In both experiments, 1.0 kg of sucrose (Kristalsuiker; Van Gilse, The Netherlands) was added to the mixture. A level of mixing was achieved by, several times per day, pushing under the plant material, which tended to float. The mixture was left to stand at room temperature. Acidification was monitored by regular pH measurements, and acidification/extraction was considered complete when pH 4 was achieved. After total acidification/extraction times (starting from when water was added to the plant material) of 67 and 44 hours respectively for the experiments in August and October, the extraction liquids were considered ready for further processing.

2.2.1.3 Clarification by filtration: small-scale experiment

During transfer of the extraction liquid to the ultrafiltration vessel, a meshed bag was placed over the pump inlet, so that very large particles (leaves, pieces of leaf, twigs, etc.) were discarded before the clarification. The mesh size of the bag was about 2 mm. Filtration through a sequence of three filters was performed on the extraction liquid (see Table 1 and Appendix 1 for specifications), in order to filter out larger particles and possibly micro-organisms present. The final product of this experiment was a clarified extract.

2.2.1.4 Clarification, ultra- and nanofiltration: large-scale experiment

Also in this experiment, the meshed bag was applied. Micro- and ultrafiltration were performed on the extraction liquid, in order to filter out larger particles and micro-organisms present. The ultrafiltration permeate of the 30 kDa membrane, which contains most of the extracted glycosides was then concentrated by nanofiltration. In this step, mostly water but also some smaller molecules such as minerals pass the membrane into the permeate, resulting in a concentration of the ultrafiltration permeate to the nanofiltration retentate. The nanofiltration retentate is the final product of this experiment.

For clarification pressures applied were maximum 0.6 and 1.5 bar for 12-40 and 0.6 µm filters respectively. For the ultrafiltration, an inlet pressure of 150 kPa was applied, and the nanofiltration pressure was 3,05 MPa. The filters and membranes used for the micro-, ultra- and nanofiltration are described in Table 1, with more detailed information in Appendices 1, 2 and 3.

Table 1 Types of filters and membranes used for micro-, ultra- and nanofiltration

Experiment	Microfiltration	Ultrafiltration	Nanofiltration
1 (small scale)	StrassBurger Filter 12-40 (SK 0), 0.6 (SS1) and SteriliTech PES 0.45 µm Diameter 142 mm	-	-
2 (large scale)	StrassBurger Filter 12-40 (SK 0), 0.6 (SS1) 2.1 m^2 of each filter	Romicon PM30 and PM50 MW cut off 30 and 50 kDa, 0.09 m^2 of each filter	SR3D MW cut off 200 Da Selro MPF36 MW cut off 1000 Da, 28 cm^2 of each filter

2.2.2 Analysis

2.2.2.1 pH, temperature and conductivity

pH, temperature and conductivity were measured using a Hanna Instruments HI 98129 Combo-apparatus.

2.2.2.2 Dry matter of processing samples and steviol glycoside levels

Levels of steviol glycosides were determined by the external laboratory ExPlant Technologies in Leiden, the Netherlands, as well as the dry matter determination and the extractions needed for those analyses. A protocol is included in Appendix 4. 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 analyzed 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. Results of both analyses are shown in Appendices 5 and 6 of this report.

2.3 Experimental setup

In these experiments, the goal is to improve the quality of the end product of steviol glycosides extraction from fresh Stevia plants: the nanofiltration retentate. This means that the amount of steviol glycosides as percentage of the dry matter, the absolute amount of steviol glycosides, but also the specific glycoside composition will be focused upon. The effect of the inclusion of a clarification step, in combination with smaller pore size of the ultrafiltration membrane will be identified, as well as the effect of the two different pore sizes of the nanofiltration membrane.

3 Results and Discussion

3.1 Clarification experiment

3.1.1 pH, dry matter and steviol glycoside levels

The pH of the primary extract was 4.03. In the first filtration, using a 12-40 µm filter, a large part of the suspended solids are retained, resulting in a visible clarification of the liquid and a decrease in dry matter content from 12.0 (±0.3) to 11.2 (±0.1). Visibly, the liquid also clarified after the 0.6 µm filtration, but the removed material was not enough to noticeably reduce the dry matter content. 0.45 µm filtration did not result in further visible clarification, nor did it retain enough material to reduce the dry matter content. The concentration of steviol glycosides in the filtrates did not noticeably change, meaning that the glycosides are not retained in the filtrations, and/or that the glycosides are dissolved in the liquid –as expected– and not present specifically in the removed suspended particles. The purity, expressed as glycoside fraction of the dry matter did not increase as a result of the clarification, remaining constant at 14 % to 15 %. All data are shown in Appendix 5. On the basis of these experiments, it was decided to apply consecutive filtration using the 12-40 µm and the 0.6 µm filters in the larger scale experiment described below.

3.2 Main experiment

3.2.1 Visual clarification

As in the clarification experiment, the primary extract was visually clarified after the 12-40 µm and 0.6 µm filtration steps (Figure 1).

Figure 1 The fluids after extraction (left), after 12-40 µm (middle) and after 0.6 µm (right) clarification steps

3.2.2 pH, temperature, conductivity, and concentration factor

The pH of the primary extract was 3.85 and did not vary with further processing, except in the nanofiltration permeate where pH decreased to 3.43 and 3.76, for the 200 Da and 1000 Da membranes, respectively (Table 2). Ultrafiltration lead to a slight increase in temperature (Figures 2 & 3), probably partly due to the filter unit and collected permeate being at room temperature, while the retentate was replenished with somewhat cooler unfiltered material. The nanofiltration at 1000 Da (Figure 5) ran quite long due to a lower flow compared to the 200 Da filtration (Figure 4), leading to a larger increase in temperature. Conductivity of the liquid did not vary in downstream processing, except in the nanofiltration, as is to be expected when the liquid is concentrated. The concentration factor using the 200 Da membrane was 3.39, leading to a 2.46 times increased conductivity, while nanofiltration with the 1000 Da membrane lead to a concentration factor of 2.73 and a 1.76 times increased conductivity. The achieved concentration factor was mostly determined by the flow and time available for the experiment (paragraph 3.2.3.2).

Table 2 pH, temperature and conductivity after the different extraction and filtration steps

Product	pH (-)	T (°C)	EC (µS/cm)
Leaves in water at start	5.88	19.5	
Primary extract	3.85	16.8	
Filtrate microfiltration	3.88	nd	1576 *
Permeate UF 30 kDa	3.91	14.9	1568 *
Retentate UF 30 kDa	3.92	16.3	1636 *
Permeate UF 50 kDa	3.91	14.5	785
Retentate UF 50 kDa	3.91	16.5	1602 *
Permeate NF 200 Da	3.43	22.0	360
Retentate NF 200 Da	3.95	21.6	3852
Permeate NF 1000 Da	3.76	20.6	634
Retentate NF 1000 Da	3.93	24.3	2756

* calculated from measured ppm/0.5

3.2.3 Flow, pressure and temperature data during ultra- and nanofiltration

3.2.3.1 Permeate flow, inlet pressure and temperature data during ultrafiltration

The permeate flow during 50 kDa ultrafiltration decreases more steeply and starts at a higher flow rate than during 30 kDa ultrafiltration (Figures 2 and 3). The fact that a larger pore size results in a higher initial flow rate may be expected. But, flow rate decreases more steeply during the 50 kDa ultrafiltration, while less fouling could expected for larger pore size. Apparently, other factors play a role here, possibly having to do with the design of the two membrane units.

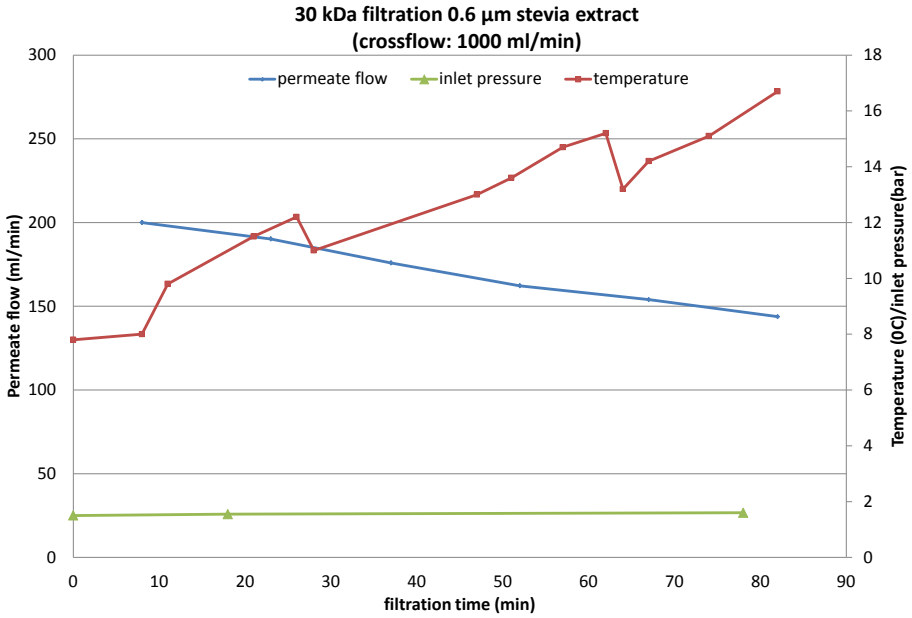

Figure 2 Permeate flow, inlet pressure and temperature during ultrafiltration 30 kDa

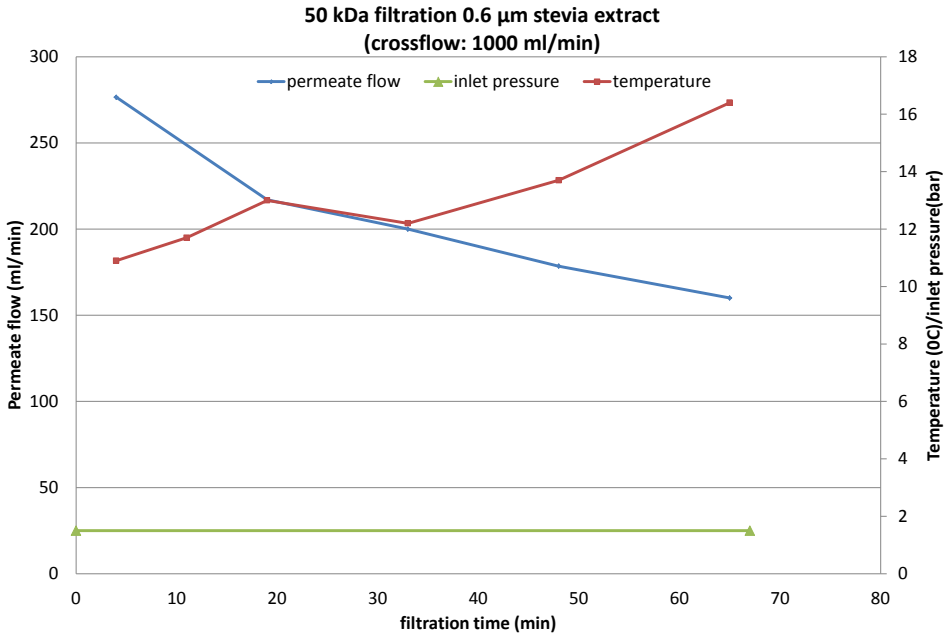

Figure 3 Permeate flow, inlet pressure and temperature during ultrafiltration 50 kDa

3.2.3.2 Permeate flow and transmembrane pressure data during nanofiltration

During nanofiltration, the permeate flow of the 1000 Da membrane decreased, while that of the 200 Da membrane stayed more or less constant at a higher flow rate (Figures 4 and 5). It might be expected that larger pore size leads to a higher initial flow and a less steep decrease of the flow rate in time, but this was not found in the experiments described here. No apparent fouling of the 200 Da membrane occurred.

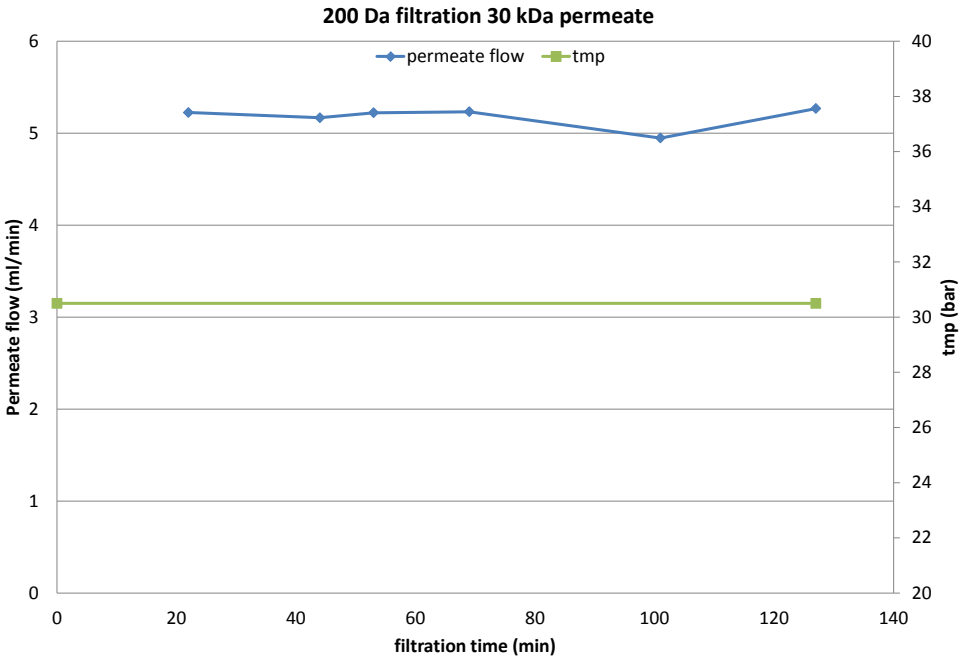

Figure 4 Permeate flow and transmembrane pressure (tmp; bar) during nanofiltration 200 Da

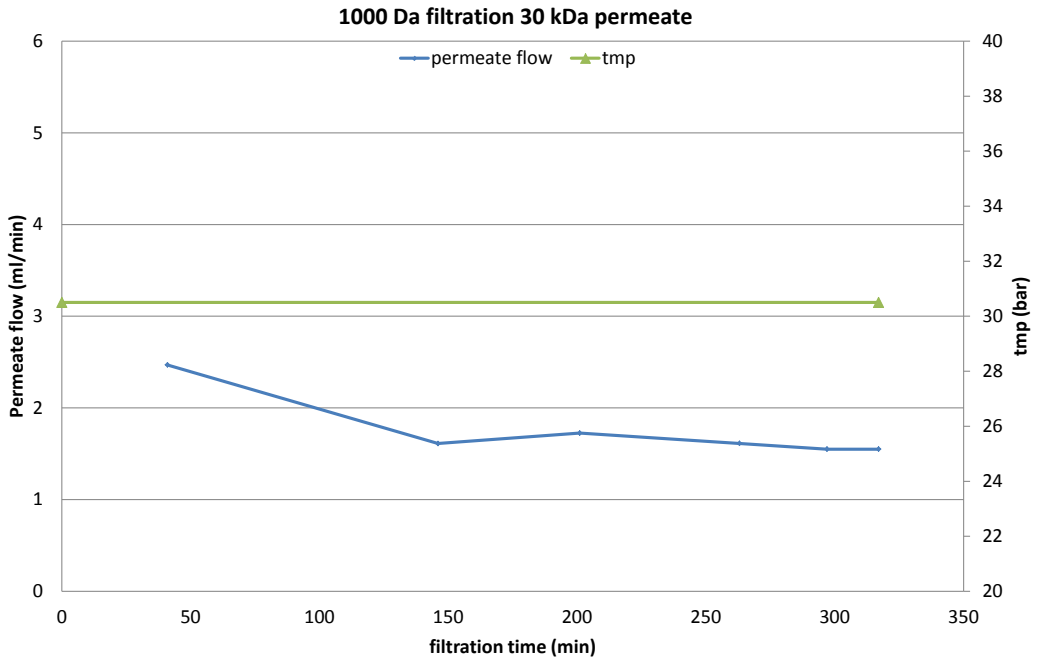

Figure 5 Permeate flow and transmembrane pressure (tmp; bar) during nanofiltration 1000 Da

3.2.4 Mass balance of the process

3.2.4.1 General mass balance

In the four treatment steps (30 kDa, 50 kDa, 200 Da and 1000 Da) respectively 0.1, 0.1, 6.8 and 24.4 % of the input liquids were lost (Table 3). The highest loss (after 1000 Da nanofiltration) was due to the fact that some of the permeate was not weighed.

Table 3 Mass balance of ultra- and nanofiltration steps

Membrane	Input (g)	Permeate (g)	Retentate (g)	Difference (g)
30 kDa	16375.9	13946.7	2420.1	9.1
50 kDa	16066.5	13102.4	2941.5	22.6
200 Da	944.9	602.2	278.2	64.4
1000 Da	939.7	366.9	343.9	228.9*

*Total mass of the permeate was not measured

3.2.4.2 Steviol glycoside mass balance

For the steviol glycosides mass balance (Table 4) during ultra- and nanofiltration, it was assumed that the loss of 1000 Da permeate was negligible when focussing on the glycosides.

Table 4 Mass balance (dry matter), amount of extracted steviol glycosides, and relative contribution of each glycoside to the extracted total (%)

Step	Fraction	Dry matter (g)	Stev. (mg)	RebA (mg)	RebC (mg)	Other (mg)	Stev. (%)	RebA (%)	RebC (%)	Other (%)
Ultrafiltration 30 kDa	**Filtrate MF**	**139**	**7610**	**4111**	**677**	**1611**	54	29	5	12
	Permeate	115	6148	3316	570	1360	54	29	5	12
	Retentate	21	1160	630	104	211	55	30	5	12
	Total out	**137**	**7308**	**3946**	**673**	**1572**				
	Loss %	**1.7**	**4.0**	**4.0**	**0.5**	**2.5**				
Ultrafiltration 50 kDa	**Filtrate MF**	**136**	**7466**	**4033**	**664**	**1581**	54	29	5	12
	Permeate	110	5960	3193	535	1153	55	29	5	11
	Retentate	25	1373	748	124	275	55	30	5	11
	Total out	**135**	**7333**	**3941**	**659**	**1427**				
	Loss %	**0.8**	**1.8**	**2.3**	**0.8**	**9.7**				
Nanofiltration 200 Da	**Permeate 30 kDa**	**8**	**417**	**225**	**39**	**92**	54	29	5	12
	Permeate	0	13	6	8	0	47	22	31	0
	Retentate	7	359	196	34	63	55	30	5	10
	Total out	**7**	**372**	**202**	**42**	**63**				
	Loss %	**10.9**	**10.7**	**10.1**	**-9.2**	**31.8**				
Nanofiltration 1000 Da	**Permeate 30 kDa**	**8**	**414**	**223**	**38**	**92**	54	29	5	12
	Permeate	1	2	1	4	0	25	12	64	0
	Retentate	7	385	207	36	74	55	30	5	11
	Total out	**8**	**386**	**208**	**41**	**74**				
	Loss %	**7.6**	**6.7**	**6.8**	**-5.8**	**18.9**				

Average values from 3 samples

During nanofiltration higher losses of dry matter and steviol glycosides occurred than during ultrafiltration. This could be due to the application of smaller volumes during nanofiltration (Table 3) which increases analytical errors. During nanofiltration, a small amount of stevioside and Reb A seems to be lost, while also a small amount of Reb C seems to appear. This probably is due to experimental error due to small volumes

and/or analytical error, as permeate concentrations are very low and the structures of Reb A and Reb C are very similar. Another possible explanation is that a small amount of Reb A somehow got converted to Reb C, and/or some stevioside was broken down in an unknown reaction, but this was not studied further. All in all, while some material might be lost to fouling of the membranes, no substantial losses were observed.

In all other fractions, the sum of stevioside and rebaudioside content of the extracts always account for around 80 % to 85 % of the total amount of steviol glycosides (Table 4). Regarding the final product, this meets a former requirement of JECFA from 2006, which stated that no less than 70 % of all present steviol glycosides should consist of the sum of these two components (JECFA, 2006). In later JECFA publications, this requirement was no longer present. It is clear that the different process steps do not have a large effect on the relative concentration of the different steviol glycosides.

3.2.4.3 Steviol glycosides: efficacy of filtration

Using the data in Table 4, the efficacy of the total process can be calculated, taking into account that only the filtrate of the 30 kDa ultrafiltration, and the retentates of the 200 Da and 1000 Da nanofiltration steps are used to obtain the final product (Table 5). Like in the previous experiments, ultrafiltration is more limiting than nanofiltration and the results for the two different pore sizes within the ultrafiltration and the nanofiltration steps are similar, with a somewhat higher yield for the 1000 Da membrane (72 % yield of total steviol glycosides for the 1000 Da membrane as compared to 69 % yield for the 200 Da membrane).

Table 5 Steviol glycoside yields (%).The primary extract is regarded as 100 %.

	Stev.	RebA	RebC	Sum other	Total
Extraction	100	100	100	100	100
Ultrafiltration 30 kDa	81	81	84	84	81
Ultrafiltration 50 kDa	80	79	81	73	79
Nanofiltration 200 Da	86	87	88	68	84
Nanofiltration 1000 Da	93	93	95	81	92
UF 30 kDa*NF 200Da	70	70	74	58	69
UF 30 kDa*NF 1000Da	74	73	76	59	72

Average values from 3 samples

3.2.4.4 End product composition and quality

In comparison to the 2014 experiments, the colour of the final product (Figure 6) was much less dark, and somewhat greenish brown.

Figure 6 Nanofiltration retentate (200 Da)

3.2.4.5 Dry matter and steviol glycosides

In the final product of the tests, the retentate of the nanofiltration, about 9 % to 10 % of the dry matter consists of steviol glycosides, with about 5 % to 6 % stevioside and 3 % rebaudioside A (Table 6). The steviol glycoside content in the dry matter reached in these tests is much less than desired, as 30 % to 50 % steviol glycosides in the dry matter would be desirable for an intermediate product. Dry matter content and glycoside concentration (as percentage of dry matter) were both higher in the clarification experiment (paragraph 3.1) than in the main experiment.

Table 6 Dry matter concentrations and steviol glycosides concentrations (mass % of dry matter) in the different fractions

Sample	Dry matter (g/L)	RebA (%)	Stevio (%)	RebC* (%)	sum other* (%)	Total (%)
Primary extract	9.7 (0.04)	3.0	5.4	0.5	1.0	9.8
12-40 µm	9.3 (0.03)	2.8	5.3	0.5	0.9	9.6
0.6 µm 1	8.6 (0.02)	2.9	5.4	0.4	0.9	9.7
0.6 µm 2	8.5 (0.01)	3.0	5.5	0.5	1.2	10.1
Retentate UF 30 kDa	8.8 (0.05)	2.9	5.4	0.5	1.0	9.9
Permeate UF 30 kDa	8.3 (0.09)	2.9	5.3	0.5	1.2	9.9
Retentate UF 50 kDa	8.6 (0.03)	3.0	5.5	0.5	1.1	10.0
Permeate UF 50 kDa	8.4 (0.01)	2.9	5.4	0.5	1.0	9.8
Retentate NF 200 Da	25.1 (0.36)	2.8	5.1	0.5	0.9	9.3
Permeate NF 200 Da	0.7 (0.13)	1.3	2.8	1.8	0.0	6.0
Retentate NF 1000 Da	20.0 (0.22)	3.0	5.6	0.5	1.1	10.2
Permeate NF 1000 Da	1.3 (0.03)	0.1	0.2	0.5	0.0	0.8

average from 3 samples, standard deviation between brackets

It is clear that after the extraction step, about 90 % to 91 % of the extracted dry matter consists of other material than steviol glycosides and, contrary to expectation, the added clarification step and the smaller pore size of the ultrafiltration do not improve this.

Some discussion points on this subject:

1. The concentration of glycosides in the dry matter of the extract for the clarification experiment was similar to that of the 2014 experiments. In the extract of the main experiment, the glycoside concentration of the dry matter was lower than in 2014. This may be due to two causes. Firstly, the acidification for the main experiment was faster than for the clarification experiment, leading to a shorter extraction time (44 h compared to 67 h). As the concentration in the original plant leaves was not determined, it is unknown what the influence of extraction time was. Secondly, and likely more importantly: the sugar addition. The extraction for the main experiment was performed with less liquid and leaves (but in the same ratio), compared to the clarification experiment, but the same amount of sugar was added. This would lead to more dry matter, and therefore a lower glycoside concentration in the total dry matter.
2. Remaining on the subject of added sugar, 1000 g was added to 360 and 200 L, or 2.8 g/L and 4.9 g/L, for the clarification and the main experiment, respectively. Due to their size -smaller than glycosides-, sugar molecules and the organic acids resulting from microbial conversion pass through the UF membrane. The dry matter of the resulting product will therefore consist of a considerable amount of sugar and/or organic acid. In the case of the main experiment, this accounts for about 50% of the dry matter, hereby making it difficult to reach the desirable purity of glycosides in the dry matter.
3. The quality of the Stevia plants used for the experiments was quite low. Quite a lot of the material was wilted, browned, and very sandy. It is unknown to what extent breakdown products already present in the material negatively influence the extraction focusing on the purity of glycosides in the dry matter but it would likely be preferable to use fresher material.
4. Lastly, the results of the current trial and those of 2014 indicate that combination of the envisaged extraction of fresh material and purification by ultrafiltration may be something to reconsider. The choice for UF was based on literature in which Stevia was first dried, then ground, and then extracted with hot water. It is possible that the envisaged extraction of fresh material leads to a primary extract in need of a different DSP. It is recommended to keep this in mind for planning future experiments. For comparison, the current DSP system could be used on hotwater-extracted dried & ground material, and for the currently used extraction of fresh material, alternative DSP options to improve purification should be looked in to.

4 Conclusions

- Clarification of the primary extraction liquid was successful, and the clarified liquid caused no fouling issues in the ultrafiltration that followed.
- 69 % and 72 % of all glycosides present in the primary extract end up in the retentate of the 200 Da and 1000 Da nanofiltration steps respectively.
- 9.3 % to 10.2 % steviol glycosides in the dry matter in the end product is low. The ultrafiltration step as applied in these tests is not sufficient as a means of selectively concentrating steviol glycosides from the extract from fresh Stevia plants. The lack of selectivity for glycosides makes it clear that the downstream process of selectively concentrating the extracted steviol glycosides needs to be improved, to increase the quality and value of the envisaged product.

5 Acknowledgements

The authors wish to acknowledge the following people and organisations for their contribution to the work described in this report: Roel Koers for supplying the Stevia plant material used in these experiments. Ivo Kretzers (Newfoss) for his help during execution of the experiments. Leen Verhagen (ExPlant) for performing the steviol glycoside analyses. The authors also acknowledge all project partners who were consulted for the experimental set up and involved in discussions on the results: Chris de Visser (WUR-ACRRES), Ivo Kretzers and Geert van Boekel (both from Newfoss), and Roel and Remco Koers (both from Koers).

6 References

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7 Appendices

Appendix 1 Information on clarification membranes Strassburger filter SS 1 and SK 0

STRASSBURGER FILTER

Technisches Datenblatt SF - Tiefenfilter

Type: SK 0

SF Hochleistungs-Tiefenfilterschicht für den groben bis mittleren Klärschärfenbereich, deren Zusammensetzung im Wesentlichen aus einer reinen Zellstoffmatrix In der Regel wird diese Filterschicht für industrielle Filtrationsaufgaben eingesetzt.

Anwendungsgebiete:
Zur Vor- und Grobfiltration von Flüssigkeiten; im Lebensmittelbereich zur Vorklärung von Zuckersirup, Gelatine, Kakaobutter, verschiedene pflanzliche Öle, Suppe; im Labor sowie in der chemisch-pharmazeutischen Industrie für Harze, Gelatine usw. Diese Filterschicht besteht nur aus Zellulose und kann nahezu vollständig verascht werden.

Permeabilität:	10.200 l/min m² bei 1 bar	
Flächengewicht:	700 g/m²	
Stärke:	2,9 mm	
Glührückstand:	<1 %	
Auswaschbare Ionen (Richtwerte)		
Löslich in Essigsäure (5%) z.B.	Ca	320 mg/m²
	Fe	1 mg/m²
	Al	6 mg/m²

Schwermetalle gem. Empfehlung XXXVI/1 im Rahmen des Lebensmittel- und Bestandegesetzes (LMBG) < 50 ppm

Abscheideraten: Filterschichten sind sogen. Tiefenfilter, bei denen die Größe der max. abzuscheidenden Partikel nicht wie bei einem Siebfilter festgelegt werden können. Tiefenfilter können wesentlich kleinere Teilchen zurückhalten, als die maximale Porenweite ausmacht. Die durch empirische Messungen ermittelten Werte liegen bei 12-40 µ.

Die Prüfungen erfolgen nach den Methoden des Arbeitskreises Technik/Analytik in der Europäischen Fachvereinigung Tiefenfiltration e.V. bzw. nach werksinternen Prüfvorschriften.

Das Produkt entspricht den Anforderungen der Empfehlung XXXVI/1 im Rahmen des Lebensmittel-und Bedarfsgegenständegesetzes (LMBG), insbesondere §§ 5, 30 und 31 und kann unbedenklich zur Kaltfiltration von Lebensmitteln eingesetzt werden-

Hauptbestandteile: Zellstoffe

Geringfügige Bestandteile: < 3% Harze entsprechend der Empf. XXXVI/1 und der 21 CFR

Alle Angaben beruhen auf unserem heutigen Kenntnisstand und erheben keinen Anspruch auf Vollständigkeit und entbinden nicht von der Pflicht zur Durchführung einer Wareneingangsprüfung und eigener Tests für den speziellen Anwendungsfall.

Das vorliegende Exemplar unterliegt nicht dem Änderungsdienst.

Westhofen, den 1.4.2011

STRASSBURGER Filter GmbH & Co. KG
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D 67593 Westhofen

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E-Mail: info@strassburger-filter.de
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STRASSBURGER FILTER

Technisches Datenblatt SF - Tiefenfilter

Type: SS 1

SF Hochleistungs-Tiefenfilterschicht für den Entkeimungsbereich, nassfest ausgerüstet. Die Zusammensetzung besteht aus hochaktivierter, feinfibrillierter Zellulose mit eingelagerter feinster Kieselgur. Polymere Kunstharze sowie synthetische Fasern bewirken die erhöhte Nassfestigkeit, auch bei Warmfiltration.

Anwendungsgebiete:
Entkeimungsfiltration von Flüssigkeiten: im Getränkebereich zur Sterilfiltration von Wein, Bier, Sekt, Fruchtsaft und anderen Lebensmitteln.

Das besondere Fasergefüge mit Feinstkieselgureinlagerungen, die eine weitere Schicht bilden, erzielt ein glanzhelles Filtrat. Durch besondere Faserverstärkung für Heißabfüllung geeignet.

Permeabilität:	65 l/min m² bei 1 bar	
Flächengewicht:	1.360 g/m²	
Stärke:	3,8 mm	
Glührückstand:	46 %	
Auswaschbare Ionen (Richtwerte)		
Löslich in Essigsäure (5%) z.B.	Ca	1950 mg/m²
	Fe	15 mg/m²
	Al	110 mg/m²
Schwermetalle gem. Empfehlung XXXVI/1 im Rahmen des Lebensmittel- und Beständegesetzes (LMBG)	< 50 ppm	
Bakterienrückhaltevermögen:	LRV 7	

Abscheideraten: Filterschichten sind sogen. Tiefenfilter, bei denen die Größe der max. abzuscheidenden Partikel nicht wie bei einem Siebfilter festgelegt werden können. Tiefenfilter können wesentlich kleinere Teilchen zurückhalten, als die maximale Porenweite ausmacht. Die durch empirische Messungen ermittelten Werte liegen bei 0,6 µ.

Die Prüfungen erfolgen nach den Methoden des Arbeitskreises Technik/Analytik in der Europäischen Fachvereinigung Tiefenfiltration e.V. bzw. nach werksinternen Prüfvorschriften.

Das Produkt entspricht den Anforderungen der Empfehlung XXXVI/1 im Rahmen des Lebensmittel-und Bedarfsgegenständegesetzes (LMBG), insbesondere §§ 5, 30 und 31 und kann unbedenklich zur Kaltfiltration von Lebensmitteln eingesetzt werden-

Hauptbestandteile: Zellstoffe
Natürliche Diatomeenerde

Geringfügige Bestandteile: < 3% Harze entsprechend der Empf. XXXVI/1 und der 21 CFR

Alle Angaben beruhen auf unserem heutigen Kenntnisstand und erheben keinen Anspruch auf Vollständigkeit und entbinden nicht von der Pflicht zur Durchführung einer Wareneingangsprüfung und eigener Tests für den speziellen Anwendungsfall.

Das vorliegende Exemplar unterliegt nicht dem Änderungsdienst.

Westhofen, den 1.4.2011

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Appendix 2 Information on ultrafiltration membranes Romicon PM30 and PM50

KOCH MEMBRANE SYSTEMS

ROMICON™ 1" HOLLOW FIBER CARTRIDGES

1" Diameter Hollow Fiber Ultrafiltration Cartridges

PRODUCT DESCRIPTION

Membrane Polymer: Polysulfone
Housing Construction: Polysulfone
Seal/Potting Material: Proprietary Epoxy Compound
Storage Solution: Glycerin
Regulatory Status: Selected PM50 and PM100 cartridges are compliant with US FDA CFR Title 21 and EC Reg. Nos. 1935/2004, and 10/2011.

Options:
Lumen size: 20 mil (0.5 mm), 43 mil (1.1 mm), 60 mil (1.5 mm), 75 mil (1.9 mm), 106 mil (2.7 mm)
Membrane Type: PM5, PM10, PM30, PM50, PM100, or PM500
ROMIPRO™ Cartridges: Selected cartridges of all membrane types are available with components that have passed USP Class VI test guidelines.

CARTRIDGE AVAILABILITY AND MEMBRANE AREA

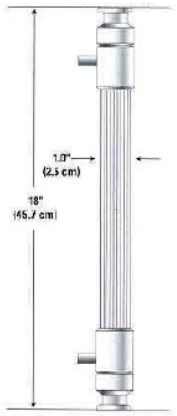
Membrane Type	MWCO Dalton) or Pore size (µm)	Fiber Diameter [mil (mm)]				
		20 (0.5)	43 (1.1)	60 (1.5)	75 (1.9)	106 (2.7)
PM5	5,000		•			
PM10	10,000	•	•	•		
PM30	30,000		•			
PM50	50,000		•	•	•	
PM100	100,000	•	•			
PM500	500,000		•		•	•
Membrane Area [ft² (m²)]		2.0 (0.18)	1.0 (0.09)	1.0 (0.09)	0.8 (0.07)	0.7 (0.06)

OPERATING AND DESIGN INFORMATION*

Maximum Inlet Pressure: 40 psi (2.8 bar)
Maximum Transmembrane Pressure: 30 psi (2.1 bar)
Maximum Operating Temperature (at pH 6.0): 140°F (60°C)
Maximum Permeate Side Back Pressure: 20 psi (1.4 bar)
Maximum Differential Pressure Feed Side: 30 psi (2.1 bar)
Allowable pH: 1.5 – 13.0 @ 130°F (54°C)
Maximum Total Chlorine (During Cleaning): 200 ppm @ pH 10-10.5, 130°F (54°C), 0 ppm @ pH < 9.5

* Consult KMS Process Technology Group for specific applications.

NOMINAL DIMENSIONS



Model	Permeate Connection	Process Connection
1018	⅜" hose	¾" T/C

ROMICON™ 1" HOLLOW FIBER CARTRIDGES

Membrane Characteristics

- Koch Membrane Systems (KMS) ROMICON™ cartridges should be selected for filtration of process streams when the separation range is in the range of 5000 to 500,000 Daltons. They provide stable productivity, ease of cleaning and reliable operation.
- ROMICON cartridges should be selected for filtration of liquids based on the separation range needed. They provide stable productivity, ease of cleaning and reliable operation. KMS ROMICON cartridges are crossflow-type filters, in which the feed solution is pumped across the cartridge to minimize solids cake buildup on the membrane. Crossflow filters provide efficient filtration at low operating pressure, allowing long process runs while reducing cleaning time, cleaning frequency, and labor costs.

Product Nomenclature

	HF	,	Vinegar	50	43	-	40	-	106	-	PM	500
Field:	1		2	3	4		5		6			7

Field 1: HF – Hollow fiber cartridge

Field 2 (optional field): Market or application designation

Field 3: Cartridge diameter times 10 in inches

Field 4: Cartridge length in inches

Field 5: Active membrane area in ft^2

Field 6: Fiber diameter in mils (1000 mil = 1 inch)

Field 7: Molecular Weight Cutoff divided by 1000 in Daltons

The example shown above describes a 5-inch diameter by 43-inch long hollow fiber cartridge for vinegar filtration, utilizing fibers with diameter of 106 mil and 500,000 Dalton. The active membrane area of this cartridge is 40 ft^2.

Operating Limits

- **Operating Pressure:** Maximum operating pressure for a ROMICON® cartridge is 40 psi (2.8 bar) or 100 psi (if permeate side is pressurized). Actual operating pressure is dependent upon type of feed stream, recovery and temperature conditions.
- **Permeate Pressure:** Permeate pressure should not exceed 20 psi (1.4 bar) pressure at any time, including backflush.
- **Differential Pressure:** Maximum differential pressure limit is 30 psi (2.1 bar) per cartridge.
- **Temperature:** Maximum operating temperature is 140°F (60°C) and maximum cleaning temperature is 130°F (54°C).

Water Quality for Cleaning

- **pH:** Allowable range for cleaning is 1.5 to 13.0.
- **Guidelines:** Please refer to the "KMS Water Quality Guidelines" for more detailed information

Exposure to Chemical Oxidants

While not recommended for use on a daily basis, exposure to chemical oxidants for thorough cleaning and sanitization may prove necessary and useful.

Potassium metabisulfite (without catalyst such as cobalt) is the preferred chemical to eliminate residual chlorine or similar oxidizers prior to processing process liquid.

Lubricants

For cartridge installation, use only water or glycerin to lubricate seals. The use of petroleum or vegetable-based oils or solvents may damage the cartridge and will void the warranty

Service and Ongoing Technical Support

Koch Membrane Systems, Inc. has an experienced staff of professionals available to assist end-users and OEMs for optimization of existing systems and support the development of new applications. Along with the availability of supplemental technical bulletins, Koch Membrane Systems, Inc. also offers a complete line of KOCHKLEEN™ cleaning chemicals.

KMS Capability

KMS is the leader in crossflow membrane technology, manufacturing reverse osmosis, nanofiltration, microfiltration, and ultrafiltration membranes and membrane systems. The industries served include food, dairy and beverage, pharmaceutical, biotechnology, water and wastewater, semiconductors, automotive, chemical and general manufacturing. KMS adds value by providing top quality membrane products and by sharing its experience in the design and supply of thousands of crossflow membrane systems worldwide.

Koch Membrane Systems, Inc., www.kochmembrane.com

Corporate Headquarters: 850 Main Street, Wilmington, Massachusetts 01887-3388, US, Tel. Toll Free: 1-888-677-5624, Telephone: 1-978-694-7000, Fax: 1-978-657-5208
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Appendix 3 Information on ultrafiltration membranes SR3D (200D) and Selro MPF36 (1000D)

KOCH MEMBRANE SYSTEMS™

KMS FLAT SHEET MEMBRANE SAMPLES

Flat Sheet Membrane Samples for Feasibility Tests

MEMBRANE TYPE

HRX™:	High Rejection RO membrane
SR3D™:	NF membrane, 200 Dalton MWCO
SelRO™ MPF-34:	Acid/Base Stable NF membrane, 200 Dalton MWCO
SelRO™ MPF-36:	Acid/Base Stable NF membrane, 1,000 Dalton MWCO
HFK-328:	Polyethersulfone UF membrane, 5K Dalton MWCO
HFK-131:	Polyethersulfone UF membrane, 10K Dalton MWCO
HFM-180:	PVDF UF membrane, 100K Dalton MWCO
HFM-183:	PVDF Positive charge UF membrane, 100K Dalton MWCO
MFK-618:	Polyethersulfone MF membrane, 0.1 micron pore size
MFK-603:	Polyethersulfone MF membrane, 0.1 micron pore size, high temperature operation

PART NUMBERS AND DIMENSIONS

Part Number	Model	Membrane Dimensions
8150001	HRX™	40" x 12"
8150002	SR3D™	40" x 12"
0770002	SelRO™ MPF-34	18" x 18"
0770007	SelRO™ MPF-36	18" x 18"
0030896	HFK-328	18" x 18"
0030880	HFK-131	18" x 18"
0030887	HFM-180	18" x 18"
0030889	HFM-183	18" x 18"
0030898	MFK-618	18" x 18"
0030893	MFK-603	18" x 18"

OPERATING AND DESIGN INFORMATION*

Typical Operating Pressure:	NF/RO (SR3D™ / HRX™):	100 - 650 psi (7 - 45 bar)
	UF/MF:	30 - 120 psi (2 - 8 bar)
	SelRO™ NF:	200 - 510 psi (14 - 35 bar)
Typical Operating Temperature:	NF/RO (except SelRO™):	40 - 113°F (5 - 45°C)
	SelRO™ NF:	104 -158°F (40 - 70°C)
	UF/MF (except MFK-603):	40 - 130°F (5 - 55°C)
	MFK-603:	40 - 176°F (5 - 80°C)

* Consult KMS Process Engineering for specific applications and other operating parameters.

Koch Membrane Systems, Inc., www.kochmembrane.com

Corporate Headquarters: 850 Main Street, Wilmington, Massachusetts 01887-3388, US, Tel. Toll Free: 1-888-677-5624, Telephone: 1-978-694-7000, Fax: 1-978-657-5208
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06/14 Rev.14-2

Appendix 4 Short description of sample preparation and analysis from ExPlant Technologies analysis report (in Dutch)

ExPlant Technologies

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E-mail explant@kpnmail.nl

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 5 Data from clarification experiment: steviol gylcosides and dry matter analyses

Dry matter analyses

In extract	
Sample	DM g/L
1EX1	11.8
1EX2	12.4
1EX3	11.8
avg (g/L)	*12.0*
stdev.s (g/L)	*0.30*
After 12-40 µm	
Sample	DM g/L
12401	11.2
12402	14.3
12403	11.2
avg (g/L)	*11.2*
stdev.s (g/L)	*0.05*
In 12-40 µm pool	
Sample	DM g/L
1240P	11.2
After 0.6 µm	
Sample	DM g/L
061	11.2
062	11.4
063	11.6
avg (g/L)	*11.4*
stdev.s (g/L)	*0.18*
In 0.6 µm pool	
Sample	DM g/L
06P	11.4
After 0.45 µm	
Sample	DM g/L
0451	11.0
0452	11.5
0453	11.2
avg (g/L)	*11.2*
stdev.s (g/L)	*0.26*

Steviol glycosides analyses (mg/L)

Sample	RebA	Stevio	RebC*	sum other*
1EX1A	624	771	76	207
1EX1B	619	754	79	221
1EX2A	615	738	34	173
1EX2B	615	770	31	184
1EX3A	654	800	40	172
1EX3B	701	869	60	232
12401A	605	751	52	245
12401B	625	767	59	194
12402A	645	801	65	174
12402B	644	803	71	197
12403A	644	785	82	249
12403B	629	791	81	206
1240PA	594	726	75	125
1240PB	608	770	77	214
061A	623	767	75	203
061B	616	775	74	197
062A	585	709	33	151
062B	585	726	45	196
063A	618	750	54	210
063B	614	773	41	188
06PA	558	681	43	154
06PB	583	751	40	178
0451A	583	715	28	176
0451B	603	760	31	191
0452A	609	751	71	211
0452B	624	762	70	182
0453A	618	771	74	200
0453B	624	766	73	195

Not used

Appendix 6 Data from main experiment: steviol gylcosides and dry matter analyses

Dry matter analyses

In extract	
Sample	DM g/L
PE1	9.6
PE2	9.7
PE3	9.6
avg (g/L)	*9.7*
stdev.s (g/L)	*0.04*
After 0.6 µm 1*	
Sample	DM g/L
V1	8.6
V2	8.5
V3	8.6
avg (g/L)	*8.6*
stdev.s (g/L)	*0.02*
R30	
Sample	DM g/L
R301	8.8
R302	8.8
R303	8.9
avg (g/L)	*8.8*
stdev.s (g/L)	*0.05*
R50	
Sample	DM g/L
R501	8.5
R502	8.5
R503	8.6
avg (g/L)	*8.6*
stdev.s (g/L)	*0.03*
R200	
Sample	DM g/L
R2001	25.3
R2002	25.3
R2003	24.7
avg (g/L)	*25.1*
stdev.s (g/L)	*0.36*
R1000	
Sample	DM g/L
R10001	19.8
R10002	20.2
R10003	19.9
avg (g/L)	*20.0*
stdev.s (g/L)	*0.22*

After 12-40 µm	
Sample	DM g/L
12401	9.3
12402	9.2
12403	9.3
avg (g/L)	*9.3*
stdev.s (g/L)	*0.03*
After 0.6 µm 2*	
Sample	DM g/L
FIL119	8.5
FIL219	8.5
FIL319	8.5
avg (g/L)	*8.5*
stdev.s (g/L)	*0.01*
P30	
Sample	DM g/L
P301	8.2
P302	8.4
P303	8.2
avg (g/L)	*8.3*
stdev.s (g/L)	*0.09*
P50	
Sample	DM g/L
P501	8.4
P502	8.4
P503	8.4
avg (g/L)	*8.4*
stdev.s (g/L)	*0.01*
P200	
Sample	DM g/L
P2001	0.8
P2002	0.8
P2003	0.6
avg (g/L)	*0.7*
stdev.s (g/L)	*0.13*
P1000	
Sample	DM g/L
P10001	1.4
P10002	1.3
P10003	1.3
avg (g/L)	*1.3*
stdev.s (g/L)	*0.03*

Steviol glycosides analyses (mg/L)

Sample	**RebA**	**Stevio**	**RebC***	**sum other***
V1	257	479	37	71
V1D	257	480	37	66
V2	250	457	40	78
V2D	242	459	37	71
V3	243	457	39	74
V3D	245	459	38	95
R301	261	475	41	75
R301D	263	480	43	89
R302	256	473	44	93
R302D	272	500	45	83
R303	258	481	43	100
R303D	251	467	40	83
R501	259	453	42	91
R501D	242	457	40	89
R502	253	472	45	100
R502D	260	486	41	88
R503	258	460	45	94
R503D	252	474	41	97
P501	246	453	42	79
P501D	247	463	41	90
P502	243	453	41	88
P502D	251	466	40	86
P503	236	441	41	91
P503D	239	452	40	93
P2001	10	22	13	0
P2001D	10	21	14	0
P2002	10	22	14	0
P2002D	10	20	13	0
P2003	10	21	13	0
P2003D	10	22	14	0
R2001	711	1300	116	151
R2001D	673	1220	119	162
R2002	715	1320	124	265
R2002D	680	1245	123	258
R2003	725	1339	125	255
R2003D	721	1323	124	264
12401	270	493	43	90
12401D	272	501	44	79
12402	252	495	47	89
12402D	250	495	44	90
12403	272	497	45	91
12403D	269	496	44	89
P10001	1	3	7	0
P10001D	1	3	8	0
P10002	2	3	8	0
P10002D	1	3	7	0
P10003	1	3	7	0

P10003D	1	3	7	0
R10001	599	1116	105	203
R10001D	608	1127	107	220
R10002	599	1107	105	212
R10002D	608	1120	109	216
R10003	596	1104	103	203
R10003D	608	1137	104	244
FIL119	251	465	42	99
FIL119D	253	471	41	108
FIL219	254	467	42	97
FIL219D	246	458	43	100
FIL319	247	461	41	98
FIL319D	255	465	40	87
P301	236	442	41	99
P301D	236	435	41	99
P302	237	443	40	104
P302D	240	444	40	90
P303	234	434	43	100
P303D	244	448	40	93
PE1	280	508	48	101
PE1D	287	522	46	96
PE2	281	513	48	96
PE2D	285	520	49	105
PE3	283	512	45	87
PE3D	294	523	49	101

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