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

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1 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. The extraction was followed by clarification using ultrafiltration and concentration by nanofiltration. Three successive harvests from the same plot were used. A mass balance was set up for more insight in the process. The Stevia material was found to be quite variable in steviol glycoside content, and apart from an increasing dry matter content, no major difference was found between the three successive harvests. Two smaller experiments are performed with direct acidification of the plant material with lactic or citric acid, of which the results are similar to the main experiment.
It is concluded that the extraction of steviol glycosides from fresh Stevia material is very effective (80 % to 90 % of all present glycosides are extracted), but that the purity in the final product –the nanofiltration retentate- is too low: 15 % to 20 % steviol glycosides in the dry matter. Following the extraction, a more selective downstream process is needed in order to result in a product with high purity.

2 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 [1, 2].

For the extraction and purification of the steviol glycosides from the plant material, several possibilities exist. A commonly used extraction method consists mixing 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 a evaporation of the extract. Other techniques include pretreatment 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 [2, 3]. For purification purposes, ultra- and nanofiltration is also suggested, including a centrifugation step as pretreatment of the extract, in a study using dried and powdered stevia leaves [3].

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 a limited preliminary study, promising results were observed (results not shown), but these were based on literature values on steviol glycoside concentration in the plant, and no mass balance was included.

The main goal of this study is to focus on the efficacy of the extraction of steviol glycosides from fresh Stevia plant material, including subsequent ultra- and nanofiltration steps as a downstream process. In order to reach the desired acidity of the water used for extraction, sugar is added which is to be converted to organic acid by microorganisms present on the plant material, effectively making the first stage a combined extraction/acidification. Mass balances are set up over all process steps to be able to calculate process yields and clarify any losses that may occur. Also, as Stevia can be harvested three times per growth season, three subsequential harvests form the same area of land were used for the experiments, in order to see whether this has an effect on the envisaged process. Finally, organic acid is also measured in the extraction liquid.

3 Materials and Methods

3.1 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 2014, and planted in soil in the beginning of May 2014. 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. Harvesting was done manually and consisted of cutting of the plant just above the bottom pair of leaves. Field edges and areas used for turning farming equipment were avoided. Material was harvested three times from the same area of land: beginning of July, mid-August (the first regrowth), and late September (the second regrowth). The 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 until the start of the extraction, which was usually two more days. It should be noted that with the material from harvest 3 (the second regrowth), the harvested material was stored for two days without cooling before delivery to ACRRES, which resulted in a decreased quality of the material, i.e. a notable amount of dried and browned leaves. With the material of the first two harvests this issue did not occur.

3.2 Methods

3.2.1 Processing

3.2.1.1 Acidification and extraction

In a 1 m^3 (1m x 1m x 1m) vessel, ~380 L of demineralised water was added to ~50 kg of fresh plant material. 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. The following day, as natural acidification did not readily occur (typically the pH decreased from 7 to 6 overnight) 0.5 kg of gelling sugar was added to induce acidification. The following day, at a pH of around 5, another 1.5 kg of gelling sugar was added. After a total acidification/extraction time (starting from when water was added to the plant material) of about 72 hours, the extraction liquid was considered ready for filtration.

The gelling sugar used for the material of harvest 1 and 3 was ‘Geleisuiker Speciaal (Van Gilse, the Netherlands)’ and for the experiment with material from harvest 2, ‘Geleisuiker (Van Gilse, the Netherlands)’ was used. Both kinds consist mainly (97 % to 98 %) of sucrose, the disaccharide of glucose and fructose, with added pectin and citric acid. ‘Geleisuiker Speciaal’ contains more pectin than ‘Geleisuiker’. Gelling sugar was used in these experiments only because this was also used in preliminary experiments earlier in the project (results not shown). No effect of the pectin is assumed.

In the experiment with plant material from harvest 3, acidification occurred much faster than in the other two, resulting in that pH 4 was reached after 48 hours, after only adding 0.5 kg of gelling sugar. No addition gelling sugar was added and filtration was started at that point.

3.2.1.2 Ultra- and nanofiltration

During transfer of the extraction liquid to the ultrafiltration vessel, a meshed bag was placed over the pump inlet, so that very large particles (large parts of leaf, twigs, etc.) would not end up in the ultrafiltration vessel. The mesh size of the bag was several millimetres. Ultrafiltration was performed on the extraction liquid, in order to filter out larger particles and micro-organisms present. The ultrafiltration permeate, 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 passes the membrane into the permeate, resulting in a concentration of the ultrafiltration permeate to the nanofiltration retentate. The nanofiltration retentate in the final product of these experiments.

For the ultrafiltration, an inlet pressure of 200 to 300 kPa was applied, and the nanofiltration pressure was 3 to 4 MPa.

The membranes used for the ultra- and nanofiltration are described in Table 1, with more detailed information in Appendix 1 to Appendix 3.

Table 1. Types of membranes used for ultra- and nanofiltration

Harvest	Ultrafiltration	Nanofiltration
1	Romicon PM 50; MW cut off 50 kD, 1.9 m^2	
2	IMT Sevenbore UF MW cut off 100-150 kD, 5.8 m^2	Dow Filmtec NF 270-4040 MW cut off 200-400 D, 7.6 m^2
3	Romicon PM 50; MW cut off 50 kD, 1.9 m^2	

3.2.2 Analysis

3.2.2.1 Dry matter of plant material

The dry matter content of the fresh plant material was determined after keeping the material at 105 °C, until no change of weight. For the material from harvest 1, 100 g of fresh material was used, while for harvests 2 and 3 samples of around 1 kg were used. For each of the three tests, 10 samples were dried.

3.2.2.2 pH and conductivity

Conductivity and pH were measured using a Hanna Instruments HI 98129 Combo-apparatus.

3.2.2.3 Dry matter of processing samples and steviol glycoside levels

Levels of steviol glycosides were determined by the external laboratory Prisna 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. In short (translated from Appendix 4), for the dried plant material: sample were ground in their entirety, after which sub-samples were taken for dry matter determination by freeze drying. The HPLC analysis was performed after extraction of the ground freeze dried sub-samples.
Wet samples were first freeze dried as a whole to determine the dry matter content and were consequently ground, also as a whole. The HPLC analysis was performed after extraction of this ground freeze dried material.
Of the liquid samples, the dry matter content was determined using 50 mL liquid. The HPLC analysis was performed using the liquid directly.
The samples were prepared in duplicate following the preparation protocol and analysed by HPLC using UV-detection. The levels of stevioside, rebaudioside A (Reb A), rebaudioside C (Reb C), and 'others' (among which rebaudiosides D, E, F, and dulcoside A) have been determined. Because no sufficiently pure reference material was available for rebaudioside C and 'other', these levels are expressed using rebaudioside A as reference.

3.2.2.4 Organic acids

Organic acid analysis was performed at Wageningen University and Research Centre - Food & Biobased Research, using Dionex RSLC equipment (Dionex Corporation, Sunnyvale, CA, USA), consisting of a Ultimate 3000 RS (Rapid Separation) pump and a Ultimate 3000 autosampler, a Ultimate 3000 column compartment with a thermostable column area, and a Ultimate 3000 variable wavelength detector, operating with the Dionex ChromeleonTM 7.1 software. The organic acids where separated using a Bio-Rad Aminex HPX-87H column (7.8 mm x 300 mm) and a Bio-Rad IG Cation H guard column. The LC-analysis is performed by using a isocratic run of 45 minutes with an eluent flow rate of 0.6 mL/min (250 µL of 85 % phosphoric acid 85 % added to 1 L ultrapure water). Column temperature was maintained at 30 °C. Detection was done at a wavelength of 210 nm. Sample preparation consisted of mixing of 1.00 mL of appropriately diluted sample (with demineralised water) and 1.00 mL internal standard solution (phthalic acid, 0.2 g/L), after which the mixture was filtered with a Sartorius 0.45 µm filter and put into a vial. All vials were placed in the cooled autosampler of the LC apparatus. Calibration curves were used of DL-malic, lactic, oxalic, citric, glycolic, formic, acetic, and levulinic acid.

3.3 Experimental setup

In order to draw conclusions from the data from these experiments, a major goal was to set up a mass balance over all the processing steps from the raw material to the final product: the nanofiltration retentate (concentrate). The mass balance was set up for the overall mass, but also specifically for the steviol glycosides. This facilitates not only the calculation of process yields, but also offers the possibility of identifying specific points of process improvement. For example, it may be that a specific yield is lower than expected, but without a mass balance it may not be possible to say whether this is due to, for example, a poor extraction, less-than-ideal selectivity of a filtration step, specific losses, or possible breakdown of the glycosides.

In order to set up the mass balance, all ingoing and outgoing material from all process steps are weighed and sampled, and these samples are analysed for dry matter content and steviol glycoside content.

As raw material, three harvests of Stevia plant from the same plot of land are used: the first harvest, the first regrowth, and the second regrowth. The idea is to see to what extent any differences in the three harvested crops influence the results from extraction and filtration steps.

Another point of interest is the acidification of the extraction liquid. In past preliminary experiments (results not shown), it was assumed that acidification was due to the formation of lactic acid. This was however not checked by analyses, which is why in these experiments an organic acid analysis by HPLC is included. Lastly, some attention will be directed towards the quality of the end product of these tests; the nanofiltration retentate. This means that the amount of steviol glycosides, but also the specific glycoside composition will be focused upon.

In order to see what is the effect of direct acidification, smaller experiments are performed using lactic or citric acid. Differences in set up and execution between these and the main experiment are explained in the ‘Additional experiments’ section.

4 Results

4.1 Main experiment

4.1.1 Raw material properties

4.1.1.1 Dry matter and variability in steviol glycoside levels

The dry matter content of the harvested Stevia increases with the time of harvest (Table 2). Between harvest 1 and 2, there seems to be little difference, if any. The material of the second regrowth (harvest 3) contains 22.5 % of dry matter, almost 10 % more than the first growth. The stems of the third harvest were clearly more pronounced and more woody compared to the earlier two harvests. Possibly, the fact that the harvest 3 material was stored for two days without cooling before delivery to ACRRES, has also contributed to a higher dry matter content.

Looking at the standard deviation, it is striking how much the concentration of steviol glycosides varies between samples in all three harvests. When the harvest 1 results became available, it was thought that the size of the individual samples –around 100 g wet weight- might have been the cause of the observed variability. For this reason, the size of the samples for harvest 2 and 3 was increased to around 1 kg. This increased sample size did not have a notable effect on the observed variability, as can be seen in Figure 1. Ignoring the possibility of an analytical error, the variability may be caused by the fact that the Stevia used for this study is a 'wild type', and not a 'cloned line' of plants (Roel Koers, personal communication). Although this variation may be problematic for constructing the mass balance of steviol glycosides for this study –as it increases uncertainty on the amounts of steviol glycosides originally present for the extraction-, it can be said that the observed variability points towards possibilities for increasing the steviol glycoside content –general and/or specific- of the plant in breeding programs.

Regarding the steviol glycoside levels, there seems to be little difference between harvest 1 and 2. Any difference in average stevioside and rebaudioside A level is lost in the large variability. Possibly, the rebaudioside C and 'other' steviol glycoside level are a little higher in harvest 2, compared to harvest 1. Overall, the steviol glycoside content in the dry matter of harvest 3 seems lower than in the other two. This was as expected, as the stems in harvest 3 were thicker and more pronounced than in the earlier two harvests. Still, the large variability that occurs in the material from all three harvests should be taken into account.

Table 2. Stevia raw material: dry matter and steviol glycoside concentration

	Harvest 1	Harvest 2	Harvest 3
Dry matter (% of mass)	13.3 (0.8)	15.0 (0.5)	22.5 (1.6)
Stevioside (mg/g dm)	32.9 (7.1)	30.7 (6.6)	25.1 (7.0)
Rebaudioside A (mg/g dm)	15.7 (6.3)	17.6 (5.0)	14.0 (5.2)
Rebaudioside C (mg/g dm)	3.8 (1.0)	5.0 (1.4)	3.2 (0.9)
Other steviol glycosides (mg/g dm)	5.9 (1.3)	8.2 (1.4)	6.4 (1.8)

Average values from 10 samples, standard deviation (stdev.s) between brackets. Harvest 1: 48 h drying; harvests 2 and 3: 72 h drying. No notable decrease in sample wait occurred between 48 h and 72 h drying for harvests 2 and 3.

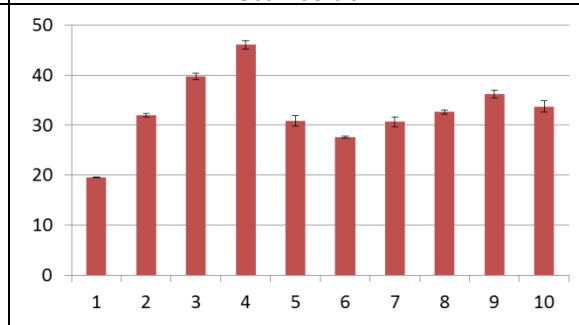
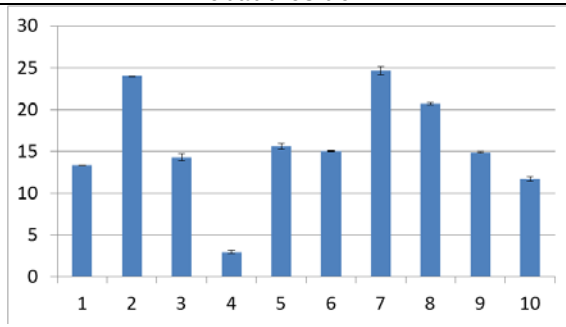
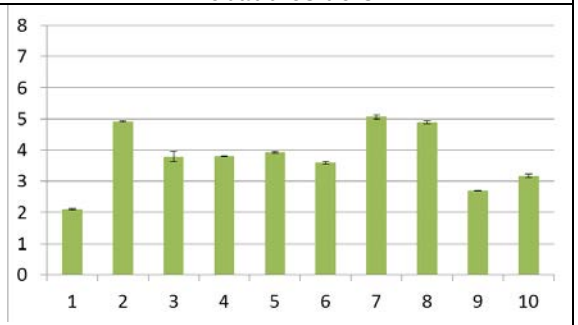
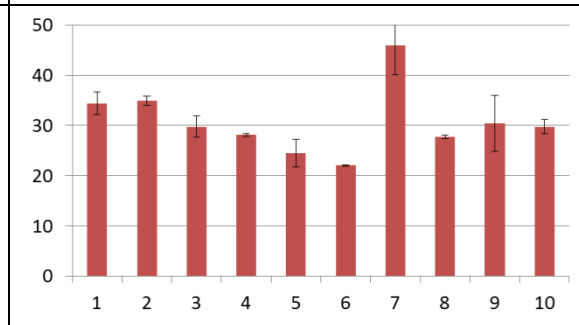
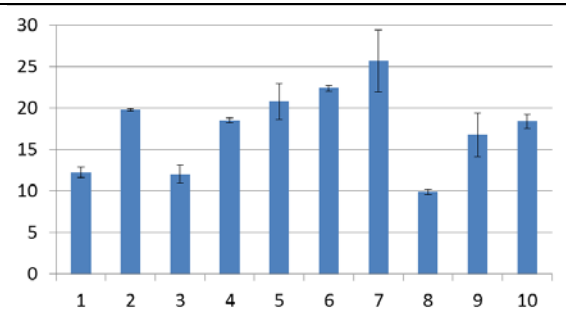
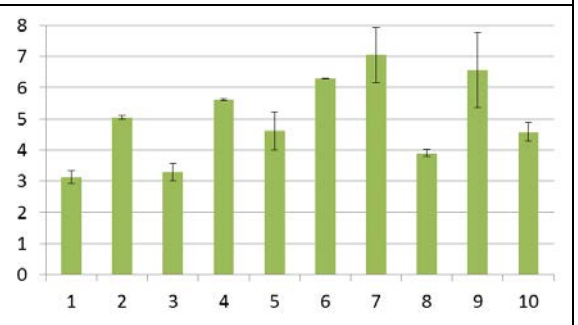
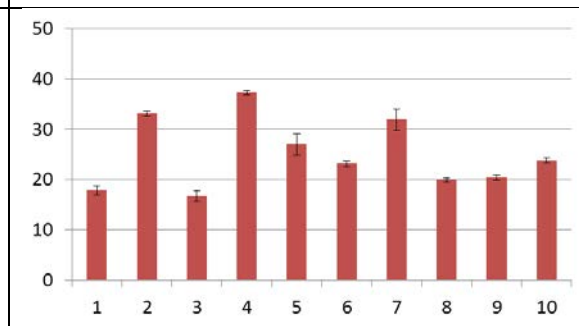
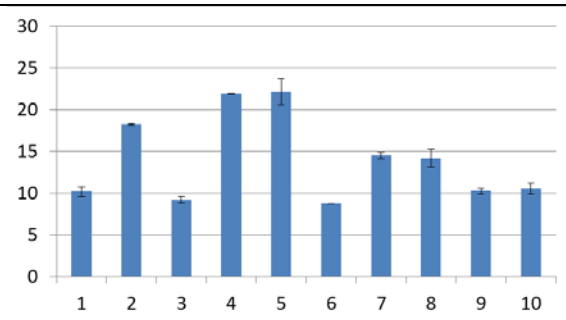
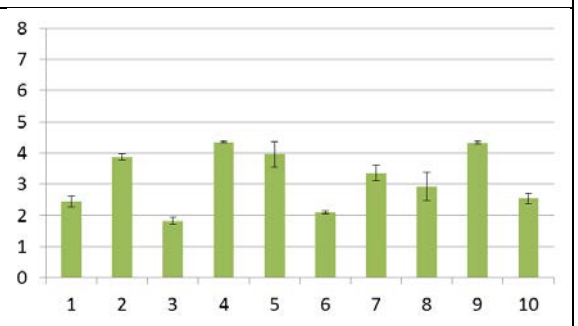
Harvest	Stevioside	Rebaudioside A	Rebaudioside C
1			
2			
3			

Figure 1. Steviol glycosides in three consequential harvests of Stevia (mg/g dry matter). Average values of two samples, error bar = standard deviation.

4.1.2 Monitoring during acidification extraction, ultra-, and nanofiltration

4.1.2.1 Acidification, conductivity and temperature during extraction

The acidification and increase in conduction in the extraction liquid were monitored, as was temperature.

In all three experiments, pH decreased at a more or less constant rate, as expected; although when extra saccharose was added an extra rate of decrease was notable. The acidification of the harvest 3 material was faster than that of harvest 1 and 2. This probably is because of the fact that the harvest 3 material was stored for two days without cooling before delivery to ACRRES, which resulted in a decreased quality of the material, i.e. a notable amount of dried and browned leaves. Temperature during the acidification extraction stage remained close to constant, again as expected.

Conductivity increased from close to zero –as expected for demineralised water- to close to 2500 µS/cm during the ~70 hours of acidification and extraction, for all three experiments (Figure 2).

For more detailed information, see Appendix 5, Appendix 6, and Appendix 7 .

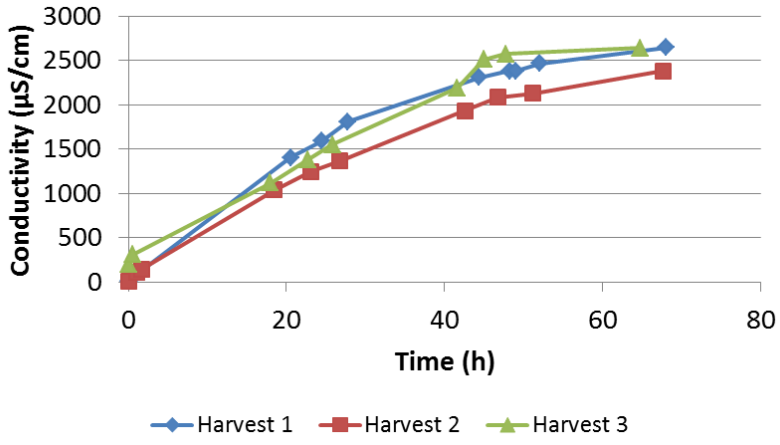


Figure 2. Conductivity (µS/cm) during the acidification & extraction stage.

4.1.2.2 pH, conductivity, temperature, and flow during ultrafiltration

The pH remained constant in both the permeate and the retentate during ultrafiltration in the experiments with harvest 1 and 2. During the experiment with material from harvest 3, the pH seemed to show a minor increase, from pH 4.3 to 4.4, in the retentate as well as in the permeate. As expected, the ultrafiltration membrane did not pose a barrier for the acid present, resulting in no pH difference between permeate and retentate.

The conductivity remained more or less constant in both the permeate and the retentate in alle three experiments, but the permeate was a little less conductive than the retentate; ~100 µS/cm difference on average.

The retentate temperature increased during ultrafiltration: from 23 °C to 33 °C for harvest 1, from 14 °C to 21 °C for harvest 2, and from 14 °C to 25 °C for harvest 3. This increase is caused by the heat added to the liquid by the recirculation pump. The reason that harvest 2 and 3 filtration started at a lower temperature is that the filtration was performed the day after the acidification and extraction, and the extraction liquid had been in cooled storage overnight (4 °C room). The temperature of the permeate also rose somewhat, caused by the heating up of the retentate.

The flow during ultrafiltration decreased during ultrafiltration for harvest 1 and 3 (Figure 3). Harvest 3 filtration started at a lower flow, probably due to a lower pressure, although pressure was not logged for the harvest 1 filtration. During ultrafiltration of the harvest 3 extraction liquid, flow increased over time, likely because the inlet pressure was increased from 200 to 300 kPa, although the flow increase lagged the pressure increase somewhat. It may be interesting to mention here that the harvest 2 ultrafiltration was performed using a different membrane than that used for harvest 1 and 3.

For more detailed information, see Appendix 5, Appendix 6, and Appendix 7 .

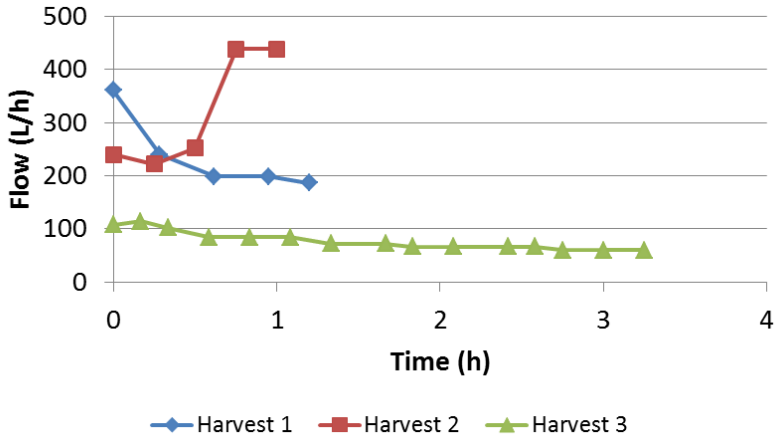


Figure 3. Flow (L/h) during ultrafiltration.

4.1.2.3 Conductivity, temperature, pH, and flow during nanofiltration

The conductivity of the retentate increased during nanofiltration, but much less so in the nanofiltration of harvest 1 liquid, compared to that of harvest 2 and 3 possibly due to a shorter filtration time (Figure 4) . The conductivity of all three nanofiltration permeates rose as well, likely due to the increase in temperature caused by the recirculation of the retentate –the feed for the nanofiltration. Clearly, some acid passed the membrane, as demonstrated by the conductivity of the permeate and the fact that the pH of the permeate was only 0.1 to 0.3 higher than that of the retentate. All in all, the pH remained relatively constant in both the retentate as well as the permeate, in all three nanofiltrations. The flow in all three nanofiltrations decreased in time, and increasing the inlet pressure from 3 to 4 and 8 MPa in the end stage of the harvest 2 nanofiltration did not result in a flow increase.

For more detailed information, see Appendix 5, Appendix 6, and Appendix 7.

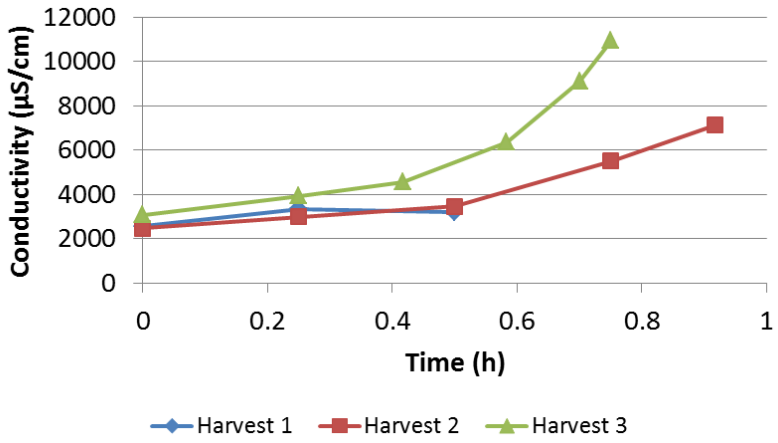


Figure 4. Conductivity (µS/cm) in the retentate during nanofiltration.

4.1.3 Organic acid formation

After the extraction, mostly citric acid and some lactic acid are present in the extraction liquid of the test done with material from harvests 1 and 2 (Table 3). This was somewhat surprising as only lactic acid formation was assumed in previous tests (results not shown). In the extraction liquid in the test using material from harvest 3, more lactic acid was found. This material also acidified more quickly -to pH 4.2 within 44 hours, instead of to pH 4.8 in 68 hours as was the case with harvest 1 and 2. It may be that a speedy acidification is connected to more lactic acid formation, but this cannot be concluded from these sparse results.

Table 3. Organic acids in extraction liquid (g/L).

Organic acid	Harvest 1	Harvest 2	Harvest 3
Citric acid	2.8 (0.02)	4.3 (0.02)	2.4 (0.04)
Lactic acid	1.6 (0.05)	1.4 (0.04)	3.0 (0.02)
Formic acid	0.4 (0.00)	0.3 (0.00)	n.i.
Acetic acid	0.2 (0.01)	0.1 (0.00)	n.i.

Average values from 3 samples, standard deviation between brackets. N.i. = Not identified. (too little to identify, or too many artefacts)

Interestingly, the amount of organic acids found suggest that not all were formed during an acidification consuming the added saccharose. Using about 400 L of demineralised water in the extraction, an organic acid formation of around 5 to 6 g/L suggests nearly all the 2.5 kg of saccharose added in the harvest 1 and 2 tests was converted into organic acid, assuming very little sugar was used for microbial biomass formation, cell maintenance, or other processes. In fact, only 0.5 kg saccharose was added in the harvest 3 test, which displayed the fastest and most pronounced acidification. This makes it evident that, at least in the test with harvest 3, not all organic acids present could have resulted from conversion of the added saccharose, and it seems

likely that the same goes for the tests with harvest 1 and 2.
This leaves other options for consideration. Firstly, some acids could have been already present in the plant material and were extracted. Secondly, some acids could have been formed from plant compounds. And thirdly, the HPLC data may not have been fully dependable. For example, it is important to note that a large peak that appeared in all HPLC chromatograms was not identified. Its retention time did not correspond to: oxalic, citric, malic, glycolic, lactic, formic, acetic, and levulinic acid that were used in these analyses. Its retention time was between those of glycolic and lactic acid, and closer to the latter. Assuming comparability to the concentration-response of citric, glycolic, and lactic acid, the concentration in the extraction liquid of this unknown compound is in the same order of magnitude: grams per litre. Furthermore, the presence of another compound in the g/L level in the extraction liquid strengthens the idea that not all these compounds can have been formed by conversion of the added saccharose.
All in all, the HPLC results of Table 3 are indicative only. HPLC is a method that compares retention times of compounds in samples to those of known compounds. In principle, it is possible that identification is erroneous. For more certainty, mass spectrometry may be an option. Furthermore, it should also be determined which acids are formed by micro-organisms present and which are extracted directly from the plant material, or are formed otherwise. For example, in the added gelling sugar, some citric acid is present. As the gelling sugar used was supermarket-bought, it is not certain how much citric acid (or pectin) they contained. Assuming a citric acid content of 1 to 3 % of the gelling sugar mass, using 2.0 kg of gelling sugar, or 20 g to 60 g of citric acid, in 400 L to 450 L of extraction liquid would result in around 0.05 g/L to 0.15 g/L citric acid. Far from the 2 g/L to 4 g/L citric acid that was determined by HPLC in the extraction liquid. Still, for future experiments in which a carbon source is to be added to induce acidification, it would be better to use glucose instead of saccharose mixed with a not precisely known amount of pectin, citric acid, and other components.

4.1.4 Mass balance

4.1.4.1 General mass balance

The amounts used in the experiments, respectively for harvest 1, 2, and 3, were: 56.4 kg, 52.3 kg, and 54.1 kg of fresh Stevia; added demineralised water: 379 kg, 366 kg, and 382 kg.

The overall mass balance closure was over 99 % in the tests after harvests 2 and 3 (Table 4). In the harvest 1 test, some material was lost during the ultra- and nanofiltration steps -respectively, 7.4 kg and 2.6 kg-, resulting in lower mass balance closure. These losses are probably due to material being left in the equipment. In the Harvest 2 and 3 tests, more care was taken to collect also this material and the mass balance closure over these process steps improved accordingly. The loss during extraction can most likely be attributed to evaporation of water, as this process step took place in an open vessel and lasted 68 hours for the test with material from harvest 1 and 2, and 48 hours in the test with harvest 3 material.

Table 4. Total mass balance (%)

	Harvest 1	Harvest 2	Harvest 3
Extraction	99.7	99.5	99.7
Ultrafiltration	97.9	99.8	100.0
Nanofiltration	99.1	99.9	100.0
Total	96.8	99.2	99.8

4.1.4.2 Steviol glycoside mass balance

The mass balance concerning the steviol glycosides was calculated independently from the overall mass balance, and contains several points of interest (Table 5). First of all, it is striking that in all three tests, more steviol glycosides are determined in the extraction liquid than in the original material. Possibly, this is caused by the variability of the material as shown in Figure 1, but this would mean that by chance, the extra boxes or crates of Stevia added to reach ~50 kg all contained higher levels of steviol glycosides than was on average present in the samples taken from the 10 sampled crates, for all three tests using material from three different harvests. This may not be very likely. More likely is that this points towards an underestimation of the steviol glycoside level in the original Stevia. It appears that the extraction as performed in the analysis lab –from dried and ground up material; see protocol in Appendix 4- is less effective than the extraction with acidic water as a solvent as performed in this study. Even when ignoring the result for rebaudioside C and for 'Other' steviol glycosides, as the results of these compounds are based on calibration curves made using rebaudioside A, it remains that about 43 % 'too much' stevioside and 84 % 'too much' rebaudioside A is found in the combined extraction liquid and rest fraction from harvest 1. Using material from harvest 2, these numbers are 31 % and 43 %, respectively. And with harvest 3 material, these numbers are 24 % and 31 %, respectively. Clearly, this is problematic for setting up the mass balance. One thing that can be concluded however, is that most of the steviol glycosides are extracted from the plant material, as around 20 % of the glycosides remain in the rest fraction after extraction. It should be noted that this 'Rest' number is also based on the amount present in the original plant material, meaning that this percentage is likely somewhat inflated, although the 'rest' fraction also required extraction in the analysis lab, similar to the original material. If the fraction of steviol glycosides that remains in the 'rest' material were to be based on the 'Total out' of the extraction, the percentage 'Rest' would be between 9 % and 15 %. It is probably a safe estimation that between 80 % and 90 % of the steviol glycosides is extracted from the plant material in the performed tests.

Table 5. Steviol glycoside mass balance

Harvest 1			Mass (g)				Percentage (%)			
			Stev.	Reb A	Reb C	Other	Stev.	Reb A	Reb C	Other
Extraction	In	Stevia	247	118	28	44	100	100	100	100
	Out	Extraction liq.	302	185	43	47	125	161	156	110
		Rest	43	26	5	10	18	23	19	22
		Total out	345	212	49	57	143	184	175	132
Ultrafiltration	In	Extraction liq.	293	180	42	46	100	100	100	100
	Out	Filtrate	192	118	27	24	66	66	65	53
		Retentate	75	52	11	19	26	29	26	41
		Total out	267	170	38	43	91	95	92	94
Nanofiltration	In	UF-filtrate	190	117	27	24	100	100	100	100
	Out	NF-filtrate	0	0	0	0	0	0	0	0
		NF-retentate	170	107	22	46	90	92	81	191
		Total out	170	107	22	46	90	92	81	191
Harvest 2			Mass (g)				Percentage (%)			
			Stev.	Reb A	Reb C	Other	Stev.	Reb A	Reb C	Other
Extraction	In	Stevia	242	139	39	64	100	100	100	100
	Out	Extraction liq.	274	173	59	45	113	124	149	70
		Rest	43	27	6	0	18	19	16	0
		Total out	320	203	66	43	131	143	165	70
Ultrafiltration	In	Extraction liq.	274	174	59	43	100	100	100	100
	Out	Filtrate	226	143	49	36	83	82	84	86
		Retentate	40	26	8	6	14	15	14	14
		Total out	266	169	57	42	97	97	98	100
Nanofiltration	In	UF-filtrate	225	142	49	36	100	100	100	100
	Out	NF-filtrate	0	0	0	0	0	0	0	0
		NF-retentate	222	139	50	38	99	97	102	105
		Total out	222	139	50	38	99	97	102	105
Harvest 3			Mass (g)				Percentage (%)			
			Stev.	Reb A	Reb C	Other	Stev.	Reb A	Reb C	Other
Extraction	In	Stevia	305	170	39	78	100	100	100	100
	Out	Extraction liq.	327	190	39	22	107	112	101	29
		Rest	52	34	6	4	17	20	15	5
		Total out	379	224	45	26	124	131	115	33
Ultrafiltration	In	Extraction liq.	319	185	39	22	100	100	100	100
	Out	Filtrate	248	142	29	15	78	76	75	69
		Retentate	78	46	10	7	24	25	27	30
		Total out	326	188	39	22	102	101	101	99
Nanofiltration	In	UF-filtrate	246	141	29	15	100	100	100	100
	Out	NF-filtrate	0	0	0	0	0	0	0	0
		NF-retentate	178	96	22	27	72	68	76	181
		Total out	178	96	22	27	72	68	76	181

Average values from 3 samples; standard deviation is estimated to be < 5/100 of the average value.

In order to facilitate discussion on the mass balance of the filtration steps, the input of these steps are set at 100 %. Furthermore, the underestimation as mentioned above is assumed not to occur when steviol glycosides are determined directly in solutions. Looking at the results from harvest 1, it is clear that the mass balance of both filtration steps close only for 90 % to 95 %. Just to clarify, this does not say anything about the efficacy of the filtration. It does mean that in each separate filtration step about 10 % the stevioside does not appear in the filtrate, nor in the retentate, and is therefore considered lost. For rebaudioside A, about 5 % is unaccounted for in the ultrafiltration step, and 8 % is lost in the nanofiltration. The glycoside loss in the ultrafiltration may be partly explained by the 97.9 % mass balance closure of this step in the Harvest 1 test. Most of the lost 2.1 % or 7.4 kg was probably still present in the equipment, and if this is considered to be retentate which has a glycoside concentration 80 % higher than the filtrate, this means that about 3.5 % or 4 % of the loss could possibly be attributed to the material left behind in the equipment. When looking at the harvest 2 test, this displayed a much smaller loss of material in the general mass balance, and also a smaller loss of steviol glycosides in the ultrafiltration step, closing the glycoside mass balance for this step for 97 %. That said, the nanofiltration step of the harvest 1 test displayed a similar part of the glycosides unaccounted for, while the general mass balance is better than for the ultrafiltration. And in the test with harvest 3, the mass balance of the ultrafiltration step closes nicely, while in the nanofiltration step, around 30 % of the stevioside and rebaudioside A is lost.
This leaves three possibilities: 1) some of the steviol glycosides has been trapped in a fouling layer on the membrane, and/or 2) some glycosides broke down during processing via an unknown reaction, and/ or 3) the analytical error by chance resulted in lower average numbers. Still, it remains unexplained why this would most strongly occur in the test with harvest 3, less so with harvest 1, and almost not in the tests with harvest 2.

4.1.4.3 Steviol glycosides: efficacy of extraction and filtration

Using the data in Table 5, the efficacy of the total process can be calculated, taking into account that only the filtrate of the ultrafiltration, and the retentate of the nanofiltration is used to obtain the final product (Table 6). It should be taken into account that the extraction yield is calculated as 100 % minus the amount of glycosides determined in the rest material after extraction. Because of the uncertainty of this number as explained above, the total efficacy is calculated over the entire process (as Ex * UF * NF), as well as only over the combined filtration steps (as UF * NF).

It is clear that in the test with harvest 1, the ultrafiltration is the most limiting factor, with only about two thirds of the glycosides ending up in the filtrate. For harvest 1, the end result was that a little less than half of the original steviol glycosides stevioside, rebaudioside A and rebaudioside C end up in the final product, with about 50 % to 60 % passing through the two filtration steps.

In the test with harvest 2, the ultrafiltration was run using a different membrane and at similar pressure, resulting in over 80 % of the glycosides ending up in the filtrate. Combined with a nanofiltration step which was equally selective as with harvest 1 but with a much better mass balance closure, this resulted in 80 % to 85 % of the glycosides passing the two filtration steps. Taking into account the extraction as well, the resulting end product contained about 65 % to 71 % of the original steviol glycosides (again keeping in mind the uncertainty of the extraction results).

In the test with harvest 3, the ultrafiltration was run with the same membrane as in the test with harvest 1. Results are similar to those with harvest 2, except that the yield in the nanofiltration is a lot lower, resulting in a total yield of 42 % to 48 %. As mentioned above, no glycosides were lost to the permeate, and the nanofiltration overall mass balance closed for 100 %. Also as mentioned above, this leaves three possibilities: 1) a substantial amount of the glycosides somehow was lost in the nanofiltration unit, possibly in a fouling layer of the membrane, and/or 2) some glycosides

broke down during processing via an unknown reaction, and/ or 3) the analytical error by chance resulted in lower average numbers (although all of the triplicate samples showed the same result).

Table 6. Steviol glycoside yields (%)

Harvest 1	Percentage (%)		
	Stev.	Reb A	Reb C
Extraction	82	77	81
Ultrafiltration	66	66	65
Nanofiltration	90	92	81
Ex * UF * NF	48	46	43
UF * NF	59	60	53
Harvest 2			
	Stev.	Reb A	Reb C
Extraction	82	81	83
Ultrafiltration	83	82	84
Nanofiltration	99	97	102
Ex * UF * NF	67	65	71
UF * NF	82	80	85
Harvest 3			
	Stev.	Reb A	Reb C
Extraction	83	80	85
Ultrafiltration	78	76	75
Nanofiltration	72	68	76
Ex * UF * NF	46	42	48
UF * NF	56	52	57

Average values from 3 samples; standard deviation is estimated to be < 5/100 of the average value.

4.1.5 End product composition and -quality

In the final product of these tests, the retentate of the nanofiltration, about 14 % to 19 % of the dry matter consists of steviol glycosides, with about 7 % to 10 % stevioside and 4.5 % to 5.5 % rebaudioside A (Table 7). This steviol glycoside content in the dry matter reached in these tests is much less than desired, with JECFA requiring at least 95 % of the dry matter consisting of steviol glycosides [4, 5]. When only focussing on stevioside in the permeate after ultrafiltration, the 6 % to 11 % reached in this study is very much less than the 43 % to 70 % that was achieved in literature, in a somewhat similar process, but which using dried powdered leaved instead of the fresh whole plant, hot water extraction, and a primary clarification by centrifugation [3].

It is clear that after the extraction step, about 85 % to 90 % of the extracted dry matter consists of other material than steviol glycosides and, contrary to expectation, the ultrafiltration step does not improve this. In fact, it seems in the tests with harvests 1 and 2 that the ultrafiltration retains steviol glycosides more than other dissolved and dispersed compounds. In the test with harvest 3, a small improvement is reached, but in the retentate as well as in the filtrate, which is probably due to an artefact, as in principle this is not possible. While it is likely that an ultrafiltration would retain larger (un-)dissolved compounds from the extraction liquid, it seems that a lot of compounds are not removed, while steviol glycosides are partly retained. Clearly, the ultrafiltration step is not having the desired effect.

Table 7. Steviol glycosides in the dry matter.

Harvest 1	Total mass (kg)	Dry matter (kg)	Stev. (%wt)	Reb A (%wt)	Reb C (%wt)	Other (%wt)	Total (%wt)	Stev +Reb A (%wt)
Stevia	56	7.3	3.3	1.6	0.4	0.6	5.8	4.9
Extraction liquid	373	4.5	6.7	4.1	1.0	1.0	12.8	10.8
Extraction rest	63	5.4	0.8	0.5	0.1	0.2	1.6	1.3
UF filtrate	289	3.2	6.0	3.7	0.9	0.8	11.3	9.7
UF retentate	65	1.0	7.5	5.2	1.1	1.9	15.7	12.7
NF filtrate	264	0.6	0.0	0.0	0.0	0.0	0.0	0.0
NF retentate	20	2.4	7.2	4.5	0.9	2.0	14.6	11.8
Harvest 2	Total mass (kg)	Dry matter (kg)	Stev. (%wt)	Reb A (%wt)	Reb C (%wt)	Other (%wt)	Total (%wt)	Stev +Reb A (%wt)
Stevia	52	7.8	3.1	1.8	0.5	0.8	6.2	4.8
Extraction liquid	357	4.5	6.1	3.9	1.3	1.0	12.3	10.0
Extraction rest	62	5.8	0.7	0.5	0.1	0.0	1.3	1.2
UF filtrate	307	3.8	5.9	3.7	1.3	1.0	11.9	9.7
UF retentate	44	0.6	6.4	4.2	1.3	1.0	12.8	10.5
NF filtrate	276	0.5	0.0	0.0	0.0	0.0	0.0	0.0
NF retentate	29	3.2	6.9	4.3	1.5	1.2	13.9	11.2
Harvest 3	Total mass (kg)	Dry matter (kg)	Stev. (%wt)	Reb A (%wt)	Reb C (%wt)	Other (%wt)	Total (%wt)	Stev +Reb A (%wt)
Stevia	54	12.1	2.5	1.4	0.3	0.6	4.9	3.9
Extraction liquid	360	3.6 (3.2)	9.1	5.3	1.1	0.6	16.1	14.4
Extraction rest	75	9.8	0.5	0.3	0.1	0.0	1.0	0.9
UF filtrate	271	2.3	10.8	6.2	1.3	0.7	18.9	17.0
UF retentate	83	0.8	10.2	6.0	1.3	0.9	18.4	16.2
NF filtrate	245	0.3	0.0	0.0	0.0	0.0	0.0	0.0
NF retentate	25	1.7	10.2	5.5	1.2	1.6	18.5	15.7

Stevia: Average values from 10 samples; standard deviation for dry matter is < 1.6/100 of average ; standard deviation for glycosides is around 20/100 to 30/100 of the average value.
Other: Average values from 3 samples; standard deviation is estimated to be < 5/100 of the average value.
Extraction liquid dry matter value harvest 3 : second set of samples (after overnight storage) just before UF resulted in lower number: between brackets.

The low purity results in these experiments do not compare well with literature, where higher purity of up to 70 % is reached [3]. It seems that the extraction process used in literature (hot water extraction of dried and ground Stevia), followed by a primary clarification by centrifugation, results in an extraction liquid that is more easily clarified by ultrafiltration than the combined acidification and extraction of fresh Stevia material as was done in the current study. Possibly, using fresh material results in more compounds present in the extraction fluid that are smaller or similar of size compared to steviol glycosides, enabling to pass through the ultrafiltration membrane to the permeate. Seeing as the application of extraction of fresh material is desired by the project partners, it is recommended to investigate other, more selective processes to apply than ultrafiltration; resin adsorption, for example.

In Table 7, the sum of the dry mass of 'extraction liquid' and 'extraction rest' is more than that of the Stevia material. This can partly be explained by the added gelling sugar (2 kg for harvest 1 and 2, 0.5 kg for harvest 3), partly by analysis inaccuracy, possibly caused by the inhomogeneity of the 'extraction rest'.

In these tests, the sum of stevioside and rebaudioside content of the Stevia plant material as well as of the final product –the nanofiltration retentate– account for around 80 % of the total amount of steviol glycosides (Table 8). 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 [6]. 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.

Table 8. Composition of steviol glycoside mixture (wt%)

Harvest 1	Stev.	Reb A	Reb C	Other	Stev+Reb A
Stevia	56	27	7	10	83
Extraction liquid	52	32	7	8	84
UF filtrate	53	33	8	7	86
UF retentate	48	33	7	12	81
NF retentate	49	31	6	13	80
Harvest 2	Stev.	Reb A	Reb C	Other	Stev+Reb A
Stevia	50	29	8	13	79
Extraction liquid	50	32	11	8	82
UF filtrate	50	31	11	8	81
UF retentate	50	33	10	7	83
NF retentate	50	31	11	8	80
Harvest 3	Stev.	Reb A	Reb C	Other	Stev+Reb A
Stevia	52	29	7	13	80
Extraction liquid	57	33	7	4	89
UF filtrate	57	33	7	4	90
UF retentate	55	33	7	5	88
NF retentate	55	30	7	8	85

Stevia: Average values from 10 samples; standard deviation for dry matter is < 1.6/100 of average ; standard deviation for glycosides is around 20/100 to 30/100 of the average value.
Other: Average values from 3 samples; standard deviation is estimated to be < 5/100 of the average value.

In regard to the colour of the final product, no objective measurements were performed. However, it can be said that the nanofiltration retentate was very dark brown for all three tests; between dark tea and coffee, so to speak. It seems likely that this is related to the large amount of components other than steviol glycosides in the dry matter, or that oxidation and/or enzymatic reactions take place during the filtration of the only slightly coloured extraction liquid, although the time needed –more or less one hour for each filtration– seems a little short. Furthermore, a ten- or twentyfold concentration of the extraction liquid may also account for a darkening effect. It may be that if more of the non-steviol glycosides could be filtered out, the dark colour of the nanofiltration retentate can be at least partly avoided. In short, no solid conclusion can be stated on this subject, but it seems clear that the colour of the final product in these tests is not acceptable, except for applications for which the dark brown colour of the product is unimportant.

4.1.6 Comparison of the three harvests

The dry matter content of the Stevia used in these tests increased with harvest time, meaning that compared to the first harvest, the dry matter content increased in the regrowth, and again in the second regrowth. This was as expected, due to the observed more pronounced presence of stems in the plant material. Another factor that may have contributed to the higher dry matter content of

the material of harvest 3 is the fact that this material was stored for two days without cooling before delivery to ACRRES.

The acidification in the extractions of harvests 1 and 2 was similar, reaching a final pH 4.95 and 4.80, respectively, after about 68 hours of extraction. The mass balance closure for the harvest 2 test was better, but this seems largely related to processing, not so much to differences in harvest. Apart from the dry matter content and the mass balance closure, the results of the tests performed with material from harvest 1 and 2 are similar in relation to the extraction and filtration of steviol glycosides. The total efficacy of the process was higher in the test with material from harvest 2. This can mostly be attributed to a more effective separation in filtrate and retentate during the ultrafiltration in the second test.

The acidification of the harvest 3 material was a lot faster and more pronounced. A pH of 4.20 was reached after less than 44 hours, with the extraction being allowed to run for 4 hour longer. Apart from the much faster acidification in the test with material from harvest 3, extraction and filtration results were similar for the tests with material from all three harvests. See also the section on the organic acid analysis.

4.2 Additional experiments

4.2.1 Lactic acid addition

Note: The experiment described in this section was not well documented, and the author was not supplied with laboratory notes of the execution of this experiment. Methods and results are therefore to be taken as indicative only.

50.3 kg of original stevia plant material from harvest 3 was washed. Leaving behind an unknown amount of washed-off material, the washed material was transferred to a 1 m^3 (1m x 1m x 1m) vessel and 489 L of tap water was added. After this, 250 mL of lactic acid (90 % pure, unknown origin) was added. The resulting acidity after 15 minutes was pH 3.25, and pH 3.50 after 1.5 hours. The mixture was left to stand for overnight (20 hours and 15 minutes starting from the acid addition), after which the pH was measured to be 4.44.

Steviol glycoside levels determined in the washed and dried harvest 3 Stevia material seem to be lower than in the original unwashed harvest 3 material (Table 9 and Figure 5). This cannot be stated with much certainty, as only three washed samples were taken, and the variation of the results is quite high (20 % to 30 % standard deviation). No samples were taken from the wash water, so it could not be checked what was lost during the wash step. In principle, it is possible that some glycoside containing leaves were washed off, resulting in a lower glycoside content of the remaining material which would then contain relatively more stems. On the other hand, if mostly sand was washed off, one could expect to see an increase in steviol glycoside levels per amount of dry matter in the washed material.

The ultrafiltration of the extraction liquid was performed with the IMT Sevenbore UF membrane (the same as used for the harvest 2 experiment, leaving the Romicon PM 50 to be used for the ultrafiltration in the main experiment of harvest 3), and the nanofiltration was performed using the Dow Filmtec NF 270-4040. No data is available on the mass of different fractions before and after filtration steps.

The composition of the final product –the nanofiltration retentate- of the experiment with washed Stevia and lactic acid addition is quite comparable to that of the experiment without washing and acidification by conversion of saccharose (Table 9 and Table 10) . Similarly low purity, similar composition of the steviol glycoside content. For this experiment, no yield could be calculated, as no data on mass of raction before and after filtration steps were available. It may be concluded that washing Stevia combined with acidification by direct addition of lactic acid results in a similar steviol glycoside purity and composition of the final product, compared to using unwashed Stevia and acidification by saccharose conversion.

The concentration of organic acids in the extraction liquid of this experiment was also determined, by the same HPLC method as described earlier. Although artefacts made it difficult to determine exact amounts of lactic acid, around 2 g/L was found. A somewhat high result, taking into account the 250 mL of 90 % pure lactic acid that was added to a total of around 550 kg of material (stevia and water), which may have been caused by said artefacts. More surprisingly, a similar concentration of around 2 g/L of citric acid was also determined. As microbial production of this high citric acid concentration seems unlikely, partly because the pH of the extraction liquid was lowered instantaneously by lactic acid addition, it seems apparent that the citric acid found was present in the Stevia material. Other possibilities may be that the HPLC-based identification was wrong (as explained above), but this would still leave a rather large amount of acid unidentified. Also, the added lactic acid may have been a source, but this cannot be checked, as the lactic acid itself was of unknown origin and not analysed. Lastly, a relatively small amount of acetic acid (~0.3 g/L) was found was found as well. As with the citric acid, it seems apparent that this originates from the Stevia material. Clearly, the situation regarding presence of organic acids in the extraction liquid still needs more clarification.

Due to the lack of information concerning this experiment, no solid conclusions can be stated.

Table 9. Steviol glycosides in the dry matter; washed and unwashed harvest 3, and nanofiltration retentate.

	Stev. (%wt)	Reb A (%wt)	Reb C (%wt)	Other (%wt)	Total (%wt)	Stev+Reb A (%wt)
Washed harvest 3	1.7	1.0	0.2	0.4	3.3	2.7
Original harvest 3	2.5	1.4	0.3	0.6	4.9	3.9
NF retentate (lac. ac. exp.)	10.7	5.1	1.4	1.7	18.9	15.9

Washed harvest 3: average values from 3 samples; standard deviation is around 20/100 to 30/100 of the average value. Original harvest 3: Average values from 10 samples; standard deviation is around 20/100 to 30/100 of the average value. NF retentate: Average values from 2 samples, standard deviation is <10/100 of the average value

Table 10. Composition of steviol glycoside mixture (%)

	Stev.	Reb A	Reb C	Other	Stev+Reb A
Washed harvest 3	53	30	6	11	83
Original harvest 3	52	29	7	13	80
NF retentate (lac. ac. exp.)	57	27	7	9	84

Washed harvest 3: average values from 3 samples; standard deviation is around 20/100 to 30/100 of the average value. Original harvest 3: Average values from 10 samples; standard deviation is around 20/100 to 30/100 of the average value. NF retentate: Average values from 2 samples, standard deviation is <10/100 of the average value

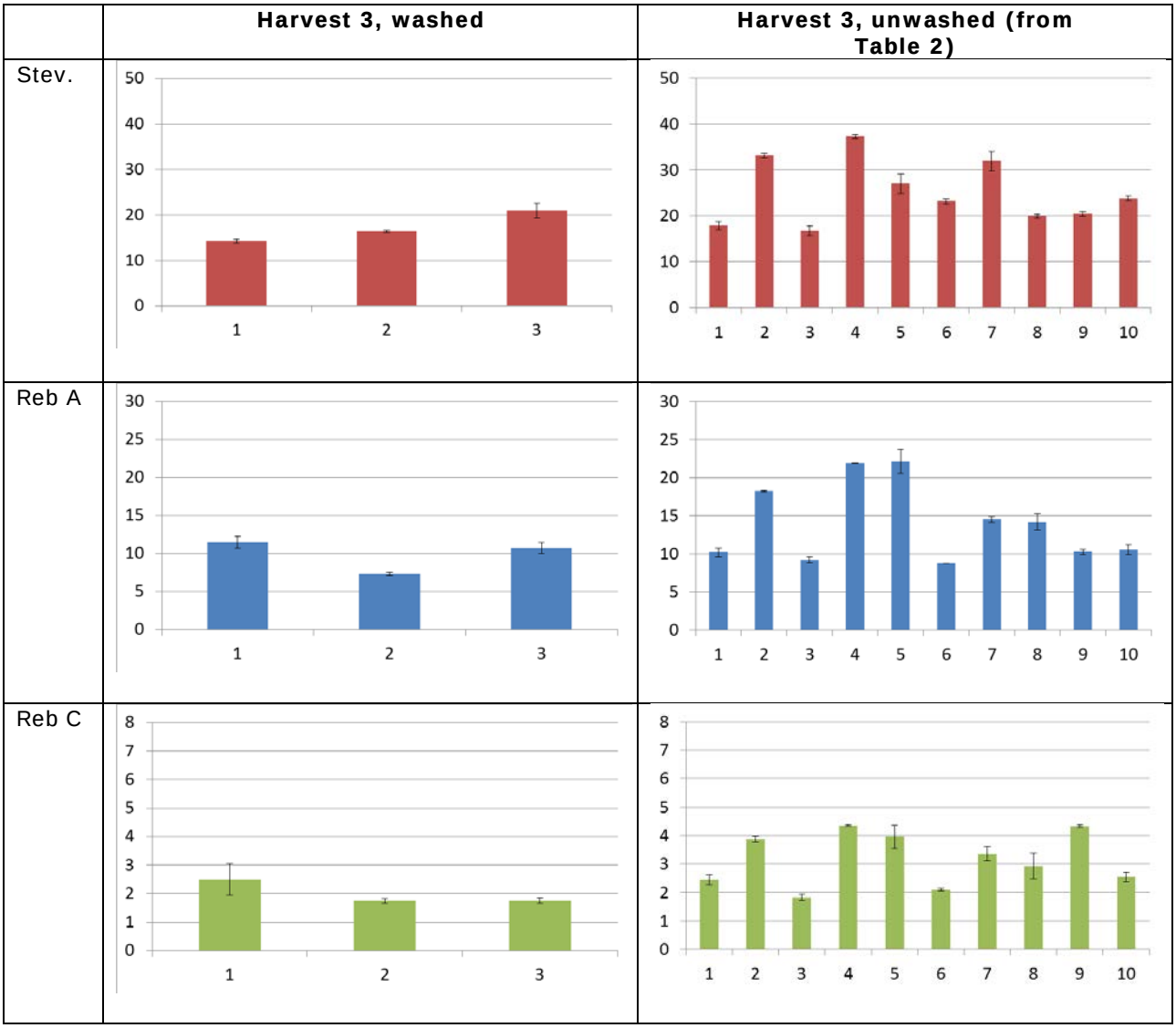


Figure 5. Steviol glycosides washed Stevia from harvest 3 (mg/g dry matter).
Average values of two samples, error bar = standard deviation.

4.2.2 Citric acid addition

A small scale test was performed to see whether a extraction in which pH 4 was maintained using addition of citric acid would yield similar results as the larger scale main experiments in which acidification was reached by micro-organisms present on the plant material converting added saccharose to organic acids. Triplicate experiments were run in which ~1 kg fresh Stevia from harvest 3 was kept at 20 °C for 24 hours in ~9 litres of a pH 4 solution consisting of citric acid in demineralised water (see Table 11). During the experiment, solid citric acid was added when the acidity rose to over pH 4. After 24 hours, samples of the liquid were taken, and the rest fraction of each experiment was divided over three samples. Dry matter content and steviol glycoside content were determined. Regardless of the extraction results, it is expected that a certain variability occurs, due to the smaller scale of the experiments, regarding the variability of the glycoside content in smaller samples, as shown for all three Stevia harvests in Table 2 and Figure 1.

Table 11. Amounts used for extraction with added citric acid

Experiment	Fresh Stevia (g)	Added water (g)	Citric acid (g)
1	903	8592	8.8
2	817	8001	7.3
3	832	8170	8.0

In terms of glycoside yields the results of the extraction with citric acid are very similar to those in the main experiments where the acidification is achieved by conversion of added saccharose (Table 12). Taking into account the same underestimation of the glycoside levels in the plant material, 80 % to 90 % of all steviol glycosides are extracted. In experiment 3, a lower yield seems to be achieved, but this was as expected (see above), meaning that the variability may be due to the smaller size of the experiment, compared to the ~50 kg of the main tests.

Table 12. Citric acid experiments: Mass balance for dry matter and steviol glycosides

Experiment 1		Dry matter	Mass (g)				Percentage (%)			
		(g)	Stev.	Reb A	Reb C	Other	Stev.	Reb A	Reb C	Other
In	Stevia	203	5.1	2.8	0.6	1.3	100	100	100	100
Out	Liquid	54	5.6	3.4	0.6	0.3	109	120	100	26
	Rest	166	0.6	0.4	0.1	0.0	12	15	10	3
	Total	220	6.2	3.8	0.7	0.4	122	135	110	29
Experiment 2		Dry matter	Mass (g)				Percentage (%)			
		(g)	Stev.	Reb A	Reb C	Other	Stev.	Reb A	Reb C	Other
In	Stevia	183	4.6	2.6	0.6	1.2	100	100	100	100
Out	Liquid	43	4.5	3.1	0.6	0.3	98	123	98	24
	Rest	142	0.7	0.5	0.1	0.0	14	20	15	3
	Total	185	5.2	3.7	0.7	0.3	112	143	113	27
Experiment 3		Dry matter	Mass (g)				Percentage (%)			
		(g)	Stev.	Reb A	Reb C	Other	Stev.	Reb A	Reb C	Other
In	Stevia	187	4.7	2.6	0.6	1.2	100	100	100	100
Out	Liquid	46	3.7	3.2	0.6	0.3	79	124	93	21
	Rest	139	0.6	0.6	0.1	0.0	12	21	13	3
	Total	186	4.3	3.8	0.6	0.3	91	146	106	24

Stevia: Average values from 10 samples; standard deviation for dry matter is < 1.6/100 of average ; standard deviation for glycosides is around 20/100 to 30/100 of the average value.
Other: Average values from 2 samples; standard deviation is estimated to be < 5/100 of the average value.
Dry matter content assumed 22.4 wt% as in main experiment with harvest 3.

In the extraction liquid of the citric acid experiments, about 17 % to 20 % of the dry matter consists of steviol glycosides, with about 8 % to 10 % stevioside and 6.3 % to 7.3 % rebaudioside A (Table 13). This is similar to or possibly somewhat more than achieved in the main tests. As the extraction liquid of the experiments with citric acid addition has not been processed with ultra- and nanofiltration, it can only be stated that, assuming similar filtration effects, the resulting product would probably contain around 20 % to 22 % steviol glycosides in the dry matter. This may be somewhat better than in the main tests, but still far from the desired 95 % purity [4, 5]. The composition of the extracted steviol glycoside mixture, with the sum of stevioside and rebaudioside accounting for 77 % to 81 % of all glycosides (Table 14), is very comparable to that of the

extraction liquids of the main tests, and therefore also comparable to the glycoside composition in the fresh Stevia material from harvest 3.

Table 13. Steviol glycosides in the dry matter

	Stev. (%wt)	Reb A (%wt)	Reb C (%wt)	Other (%wt)	Total (%wt)	Stev+Reb A (%wt)
Stevia, harvest 3	2.5	1.4	0.3	0.6	4.9	3.9
Extraction liquid 1	10.3	6.3	1.2	0.6	18.3	16.5
Extraction liquid 2	10.4	7.3	1.3	0.7	19.6	17.7
Extraction liquid 3	8.0	7.0	1.2	0.5	16.8	15.0

Stevia: Average values from 10 samples; standard deviation is around 20/100 to 30/100 of the average value.
Extraction liquid: Average values from 2 samples; standard deviation is estimated to be < 5/100 of the average value.

Table 14. Steviol glycoside composition (wt%)

Experiment	Stev.	Reb A	Reb C	Other	Stev+Reb A
Stevia, harvest 3	52	29	7	13	80
Extraction liquid 1	50	31	6	3	81
Extraction liquid 2	46	32	6	3	78
Extraction liquid 3	41	36	6	3	77

Stevia: Average values from 10 samples; standard deviation for glycosides is around 20/100 to 30/100 of the average value.
Extraction liquid: Average values from 2 samples; standard deviation is estimated to be < 5/100 of the average value.

5 Conclusions

- The extraction of steviol glycosides from Stevia plant material performed in these tests was very effective. In fact, more so than those performed in the lab in order to determine the steviol glycoside content of the original plant material, which means that the steviol glycoside content of the plant material was somewhat underestimated. When also taking into account the un-extracted steviol glycosides left in the material after extraction, it is estimated that 80 % to 90 % of all glycosides present are extracted in these tests.
- The ultra- and nanofiltration steps as applied in these tests were not sufficient as a means of selectively concentrating the extracted steviol glycosides. Only 13.9 % to 18.5 % steviol glycosides in the dry matter in the end product is very low, compared to the desired 95 %. 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.
- There is a large variability of steviol glycoside levels in the Stevia plant material used in these tests. This was clearly noticeable between samples of the same harvest. Steviol glycoside levels in the material from harvest 3 seem somewhat lower compared to harvest 1 and 2. A likely explanation is that this is because of the more pronounced presence of stems in the second regrowth.
- In these tests, no influence of the harvest time on the efficacy of the extraction and filtration was apparent.
- The overall mass balance closure of the described experiments was close to 100 %. The more specific mass balance for steviol glycosides over the filtration steps showed that varying amounts of glycosides are lost, without a concrete explanation (~10 % with harvest 1, no large losses with harvest 2 and a loss with harvest 3 of 30 % of in the nanofiltration step).
- The best overall yield of steviol glycosides from plant material to end product was around 65 % to 67 % and occurred with harvest 2, as no large unexplained losses seemed to occur here, which did occur in the filtration steps of the experiments using harvest 1 and 3.
- More organic acid was found than could have resulted from conversion of the added sugar during acidification. It is not known whether organic acid was already present in the fresh plant material, or that the organic acids were formed using plant components during acidification, as acidification and extraction were performed simultaneously.
- The additional experiment with citric acid showed that direct addition of citric acid to pH 4 and overnight extraction leads to similar extraction results as did the acidification of the added sugar, while the latter also necessitates a longer for acidification/extraction.

6 Acknowledgements

The author wishes 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 using in these experiments. Ivo Kretzers and Rob Kwinten (both from Newfoss) for their help during execution of the main experiments, and for conducting the measurements of conductivity and pH. Ton Franken (MACT) for doing the ultra- and nanofiltrations, and the execution of the additional experiment using lactic acid together with Paul a'Campo (ACConsult). Alniek van Zeeland (WUR-FBR) for doing the organic acid analyses. Leen Verhagen and Teus Luijendijk (both from Prisna) for performing the steviol glycoside analyses. The author also acknowledges 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), Paul a'Campo (ACConsult), Ton Franken (MACT), and Roel and Remco Koers (both from Koers).

7 References

1. Stoyanova, S., et al., *The food additives inulin and stevioside counteract oxidative stress.* International Journal of Food Sciences and Nutrition, 2011. **62**(3): p. 207-214.
2. González, C., et al., *Main properties of steviol glycosides and their potential in the food industry: a review.* Fruits, 2014. **69**(02): p. 127-141.
3. Chhaya, et al., *Clarifications of stevia extract using cross flow ultrafiltration and concentration by nanofiltration.* Separation and Purification Technology, 2012. **89**(0): p. 125-134.
4. JECFA *Compendium of Food Additive Specifications. Monograph 5. Steviol glycosides.* 2008.
5. JECFA *Compendium of Food Additive Specifications. Monograph 10. Steviol glycosides.* 2010.
6. JECFA *Compendium of Food Additive Specifications. Monograph 1. Steviol glycosides.* 2006.

8 Appendices

Appendix 1. Information on ultrafiltration membrane Romicon PM50

KOCH MEMBRANE SYSTEMS

ROMICON™ 3" HOLLOW FIBER CARTRIDGES

3" 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 PM5, PM10, PM50, PM100 and PM500 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

Special Construction for Specific Applications:
- **Vinegar Filtration:** Selected PM50 and PM500 cartridges are available for vinegar filtration.
- **Citrus Filtration:** Selected PM10, PM50 and PM500 cartridges are available for citrus filtration.
- **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

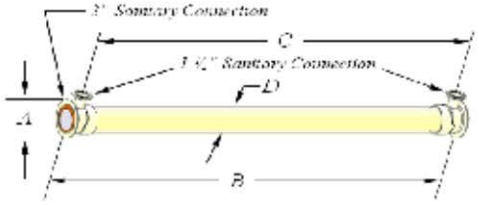
Membrane Type	MWCO (Dalton) or Pore size (µm)	Fiber Diameter [mil (mm)]				
		20 (0.5)	43 (1.1)	60 (1.5)	75 (1.9)	106 (2.7)
PM5	5,000		•			
PM10	10,000	•	•	•		
PM30	30,000		•			
PM50	50,000		•	•	•	
PM100	100,000	•	•			
PM500	500,000		•		•	•
Membrane Area [ft² (m²)]		53 (4.9)	25 (2.3)	25 (2.3)	21 (1.9)	16 (1.5)

OPERATING AND DESIGN INFORMATION*

Maximum Inlet Pressure: 40 psi (2.8 bar)
Maximum Transmembrane Pressure: 35 psi (2.4 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	A inches (mm)	B inches (mm)	C inches (mm)	D inches (mm)	Permeate Connection	Process Connection
3043	4.0 (102)	43 (1092)	40 15/16 (1040)	3 (76)	1½" T/C	3" T/C

Appendix 2. Information on ultrafiltration membrane IMT Sevenbore

Ultrafiltration element 4100 UF/MB/TAP

Specifications 4100 UF/MB/TAP		SI		US	
Element data					
Material housing			PVC		PVC
Material connector			PVC		PVC
Housing length		mm	1000 +/- 2,0	inch	39
Element length**		mm	1142 +/- 3,0	inch	45
Element length		mm	1200 +/- 3,0	inch	47
Distance permeate connector-feed connector**		mm	236 +/- 2,0	inch	9
Distance permeate connector-feed connector		mm	265 +/- 2,0	inch	10
Distance permeate connector-element center		mm	90 +/- 2,0	inch	4
Permeate connection male OD			1"		1"
Feed connection male OD			1"		1"
Housing OD		mm	110	inch	4
Element OD		mm	125	inch	5
Endcaps ID**		mm	32	inch	1
Weight		kg	4,5	lbs	10
Membrane Type					
Material			PES		PES
Type			SevenBore®		SevenBore®
Diameter bores ID		mm	0,9	inch	0,04
Diameter fiber OD		mm	4,0	inch	0,16
MWCO		kD	100-150	kD	100-150
Area		m^2	5,8	ft^2	62
Typical Process Conditions					
Maximum operating temperature		°C	40	°F	104
Maximum system pressure		bar	10	psi	145
Trans membrane pressure	operation	bar	< 1,0	psi	< 14,5
	maximum	bar	2,5	psi	36
Productivity	clean water at 25° C (77°F)	l/m^2barh	1300-1600	gfd/psi	53-65
	process	l/m^2h	100 to 350	gfd	60 to 210
	backwash/forward flush	l/m^2h	250	gfd	150
Permeate flow at 100 l/m^2 h		l/h	580	gpm	3
pH range during operation			3-10		3-10
Cleaning					
Soaking time during cleaning			min 5		
pH range during cleaning			1-13		
Disinfecting chemicals	hypochlorite (NaOCl)		ppm 50-200		
	hydrogenperoxide (H_2O_2)		ppm 100-200		

** without screw ends (provided separately)

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IMT

Appendix 3. Information on nanofiltration membrane Dow Filmtec NF 270-4040

Product Information

DOW

FILMTEC™ Membranes

FILMTEC NF270 Nanofiltration Elements for Commercial Systems

Features

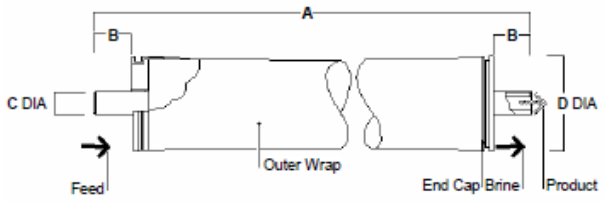
The FILMTEC™ NF270 membrane elements are ideal for removing a high percentage of TOC and THM precursors with medium to high salt passage and medium hardness passage. The FILMTEC NF270 membrane is an ideal choice for surface water and ground water where good organic removal is desired with partial softening.

Product Specifications

Product	Part Number	Active Area ft² (m²)	Applied Pressure psig (bar)	Permeate Flow Rate gpd (m³/d)	Stabilized Salt Rejection (%)
NF270-2540	149986	28 (2.6)	70 (4.8)	850 (3.2)	>97.0
NF270-4040	149987	82 (7.6)	70 (4.8)	2,500 (9.5)	>97.0

1. Permeate flow and salt rejection based on the following test conditions: 2,000 ppm $MgSO_4$, 77°F (25°C) and 15% recovery at the pressure specified above.
2. Permeate flows for individual NF270-2540 elements may vary by -20% / +30%. NF270-4040 individual elements may vary -15% / +50%.
3. Developmental products available for sale.

Figure 1



Dimensions – Inches (mm)

Product	A	B	C	D
NF270-2540	40.0 (1,016)	1.19 (30)	0.75 (19)	2.4 (61)
NF270-4040	40.0 (1,016)	1.05 (27)	0.75 (19)	3.9 (99)

1. Refer to FilmTec Design Guidelines for multiple-element systems.
2. NF270-2540 has a tape outer wrap. NF270-4040 has a fiberglass outer wrap.

1 inch = 25.4 mm

Operating Limits

• Membrane Type	Polyamide Thin-Film Composite
• Maximum Operating Temperature	113°F (45°C)
• Maximum Operating Pressure	600 psi (41 bar)
• Maximum Feed Flow Rate - 4040 elements	16 gpm (3.6 m³/hr)
- 2540 elements	6 gpm (1.4 m³/hr)
• Maximum Pressure Drop - tape wrapped	13 psig (0.9 bar)
- fiberglassed	15 psig (1.0 bar)
• pH Range, Continuous Operation[a]	2 - 11
• pH Range, Short-Term Cleaning (30 min.)[b]	1 - 12
• Maximum Feed Silt Density Index	SDI 5
• Free Chlorine Tolerance[c]	< 0.1 ppm

[a] Maximum temperature for continuous operation above pH 10 is 95°F (35°C).
[b] Refer to Cleaning Guidelines in specification sheet 609-23010 for NF90.
[c] Under certain conditions, the presence of free chlorine and other oxidizing agents will cause premature membrane failure. Since oxidation damage is not covered under warranty, FilmTec recommends removing residual free chlorine by pretreatment prior to membrane exposure. Please refer to technical bulletin 609-22010 for more information.

Appendix 4. Short description of sample preparation and analysis from Prisna analysis report (in Dutch)

PRISNA

ANALYSERAPPORT 20140926LV

Analyse van steviolglycosides in opwerkingsfracties en gedroogde plantenmaterialen

Op verzoek van ACRRES Lelystad is het droge stof gehalte en het gehalte aan steviolglycosiden bepaald in 23 aangeleverde opwerkingsfracties + 10 plantenmaterialen.

1. Materiaal en methoden

Ontvangen werden 33 monsters, gecodeerd 02FN 1 t/m 3, 02FU 1 t/m 3, 02LEVF 1 t/m 3, 02RN 1 t/m 3, 02RU 1 t/m 3, 02LENE 1 t/m3, 1-280814spoel en 2-290814spoel, 02RE 1 t/m 3 and plant monsters 02S 1 t/m 10.
Van de vloeistoffen is door middel van vriesdrogen het vaste stofgehalte bepaald. Hiervoor werd 50 ml gebruikt. Van de oplossingen is een HPLC analyse gedaan.

Van de vaste plantmonsters 02S1 t/m 02S10 werd het gehele monster gemalen, waarna van een deelmonster het drooggewicht werd bepaald door dit te vriesdrogen.
Van de gevriesdroogde deelmonsters is de HPLC analyse uitgevoerd.

Monsters 02RE1 t/m 02RE3 waren natte monsters en zijn eerst in zijn geheel gevriesdroogd om het droge stof gehalte bepalen en vervolgens in zijn geheel gemalen, waarna van het homogene poeder van de drie monsters de HPLC analyse uitgevoerd.

De monsters werden volgens een eerder opgesteld vast protocol in duplo opgewerkt en geanalyseerd met HPLC met UV-detectie. De serie RE en S moest eerst worden geëxtraheerd, de andere waren oplossingen en konden direct opgewerkt worden.
Normaal gesproken worden met de gebruikte HPLC methode de gehalten stevioside, rebaudioside A ('reb A') en 'overigen' (waaronder rebaudiosides 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 ook apart worden gekwantificeerd. Omdat er echter geen beschikking was over voldoende zuiver referentiemateriaal voor reb C, werd het gehalte hiervan (evenals dat van de 'overigen') uitgedrukt als reb A.

- 1 -

Appendix 5. Monitoring data for experiment with harvest 1

Acidification and extraction, harvest 1

Date	Time	Time (h)	Temp (°C)	pH	Conduc-tivity (µS/cm)	Sample code	Tube empty (g)	Tube full (g)
14-7-2014	12:50	0.00	22.6	7.63	10	1_140714_stap1A	11.3	57.6
	13:15	0.42	22.6	6.3	65			
	13:55	1.08	21.1	6.82	88	2_140714_stap1A	11.3	57.26
15-7-2014	9:24	20.57	20.7	5.8	1403	3_150714_stap1A	11.3	52.75
	13:19	24.48	20.5	5.53	1588			
	16:38	27.80	21.6	5.48	1810			
16-7-2014	9:00	44.43	21	5.04	2307	4_150714_stap1A	11.2	61.2
	12:50	48.27	21.9	4.79	2380			
	13:40	49.10	22.1	5.05	2380			
	16:40	52.10	21.9	4.98	2465			
17-7-2014	8:38	68.07	21.9	4.86	2647	5_140714_stap1A	11.16	65.14

Notes:
On 15/7/14 at 8:30 in the morning, 500 g of gelling sugar was added. pH measured around that time varied between 6.2 and 5.8.

On 16/7/14, at 13:40 another 1.5 kg gelling sugar added
Every day, in the morning, 1 h of recirculation in the fermentation broth

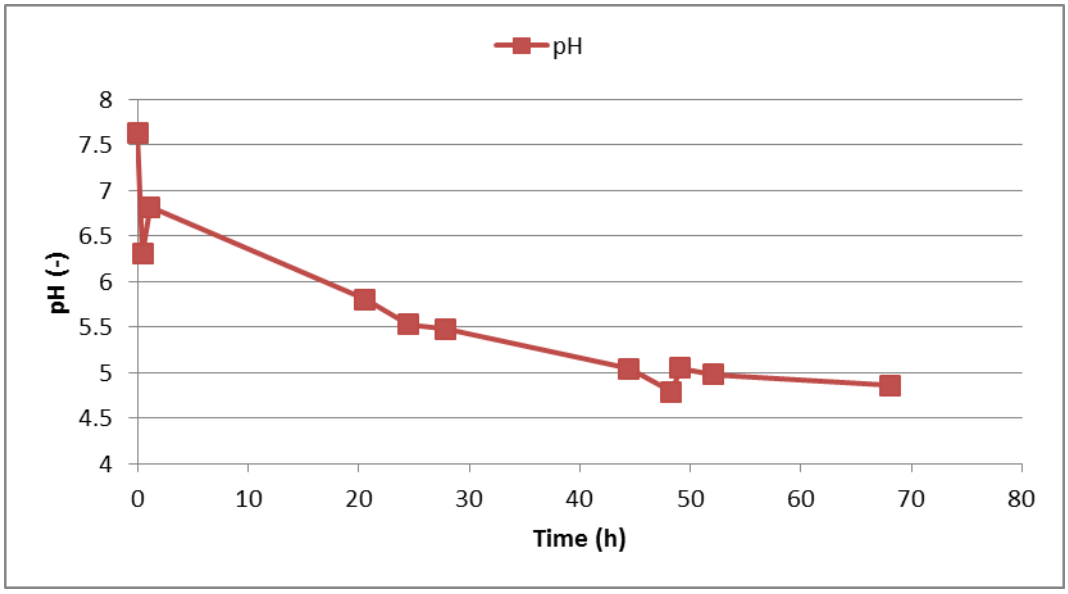


Figure 6. Harvest 1 material: pH during acidification and extraction

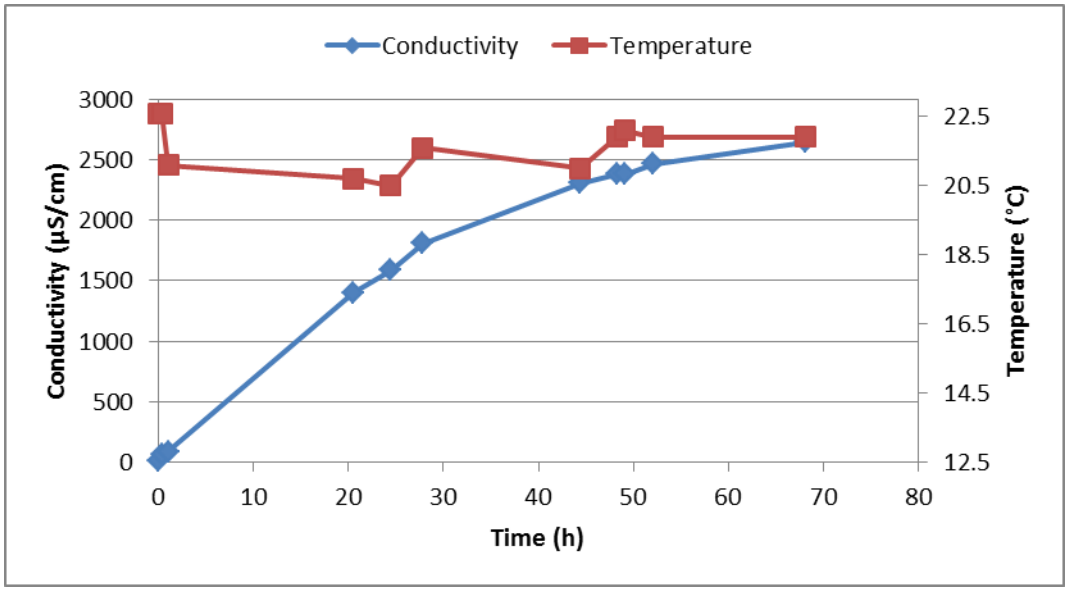


Figure 7. Harvest 1 material: conductivity (µS/cm) and temperature (°C) during acidification and extraction

Ultrafiltration, harvest 1

Data

Date	Time	Time (h)	pH Conc.	pH Perm.	Flow Perm. (L/h)	Conduc-tivity Conc. (µS/cm)	Conduc-tivity Perm. (µS/cm)	Temp. (°C) Conc.	Temp. (°C) Perm.
17-7-2014	10:43	0.00	4.8	4.85	360	2692	2636	23.4	23.3
	11:00	0.28	4.8	4.8	240	2714	2576	24.5	24.9
	11:20	0.62	4.78	4.76	198	2595	2640	25.6	26.7
	11:40	0.95	4.83	4.72	198	2830	2698	29.1	29.5
	11:55	1.20	4.81	4.8	186	2915	2710	32.7	31.1

Sample data

Date	Time	Sample code Conc.	Tube empty (g)	Tube full (g)
17-7-2014	10:43	1_170714_stap2C	11.16	60.82
	11:20	2_170714_stap2C	11.19	60.59
	11:40	3_170714_stap2C	11.15	61.1
Date	**Time**	**Sample code Perm.**	**Tube empty (g)**	**Tube full (g)**
17-7-2014	10:43	1_170714_stap2P	11.28	62.01
	11:20	2_170714_stap2P	11.29	59.91
	11:40	3_170714_stap2P	11.16	60.85

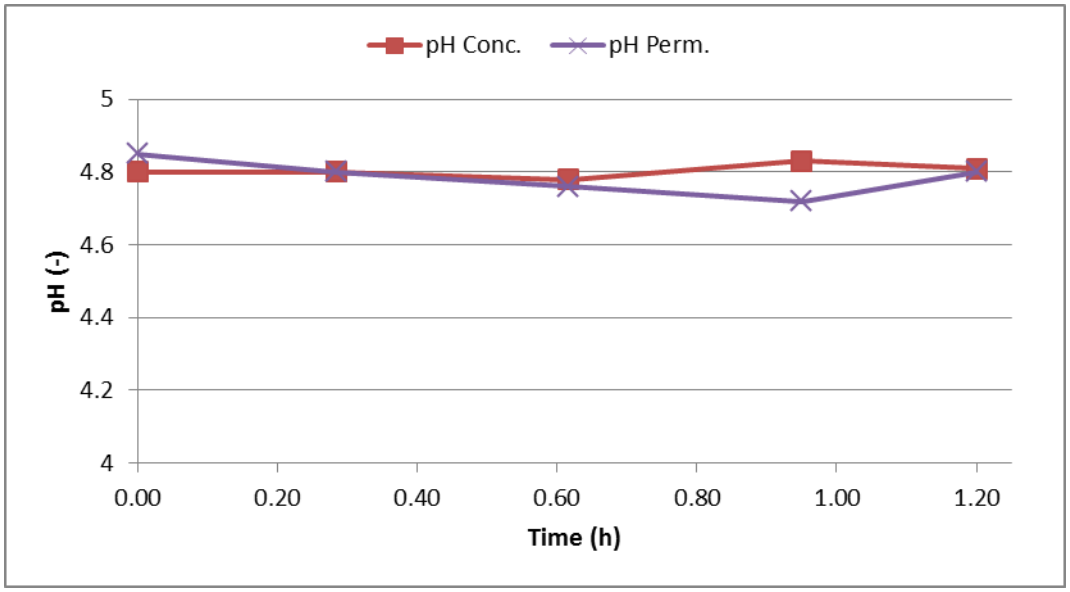


Figure 8. Harvest 1 material: pH during ultrafiltration.

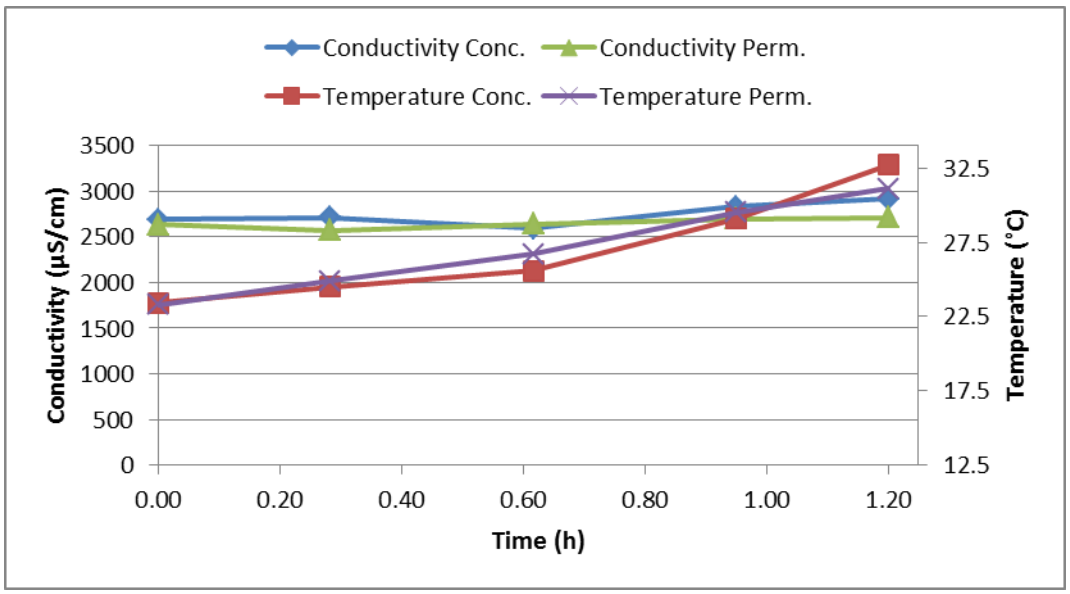


Figure 9. Harvest 1 material: conductivity (µS/cm) and temperature (°C) during ultrafiltration.

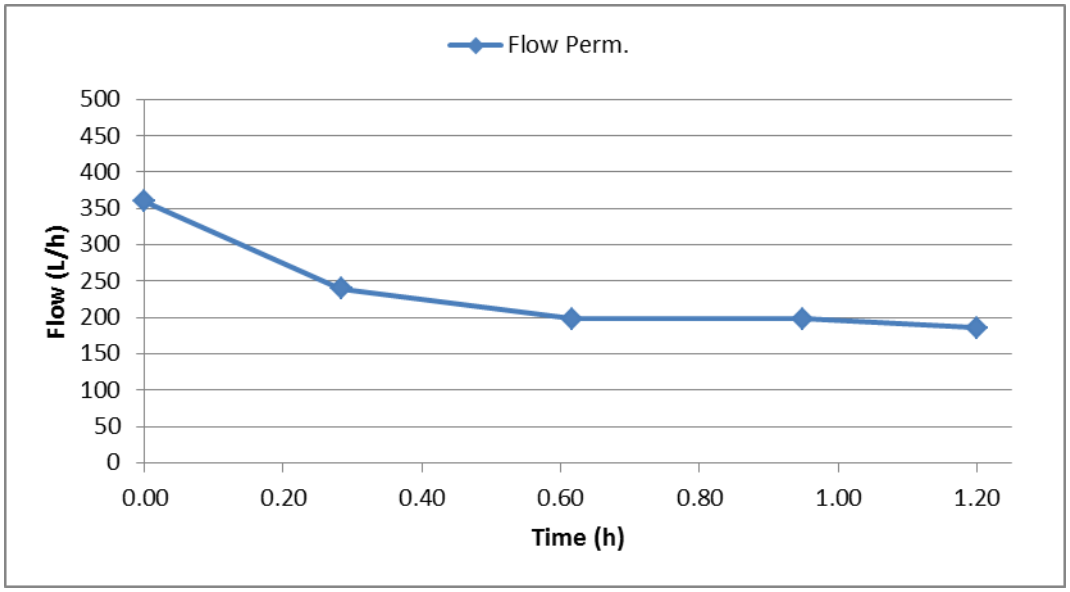


Figure 10. Harvest 1 material: permeate flow (L/h) during ultrafiltration.

Nanofiltration, harvest 1

Data

Date	Time	Time (h)	pH Conc	pH Perm	Flow Inlet (L/h)	Flow Perm. (L/h)	P Inlet (bar)	Conduc-tivity Conc. (µS/cm)	Conduc-tivity Perm. (µS/cm)	T (°C) Conc.	T(°C) Perm.
17-7-2014	13:30	0.00	4.79	4.88	900	400	33	2565	1448	26.9	26.8
	13:45	0.25	4.78	4.86	900	350	36	3347	1565	28.4	28.3
	14:00	0.50	4.73	4.88	900	250	36	3184	2062	31	29.4
	14:05	0.58					50				

Sample data

Date	Time	Sample code Conc.	Tube empty (g)	Tube full (g)
17-7-2014	13:30	1_170714_stap3C	11.26	62.34
	13:45	2_170714_stap2C	11.15	60.29
	14:00	3_170714_stap2C	11.2	58.47
Date	**Time**	**Sample code Perm.**	**Tube empty (g)**	**Tube full (g)**
17-7-2014	13:30	1_170714_stap2P	11.26	60.45
	13:45	2_170714_stap2P	11.25	63.14
	14:00	3_170714_stap2P	11.21	58.1

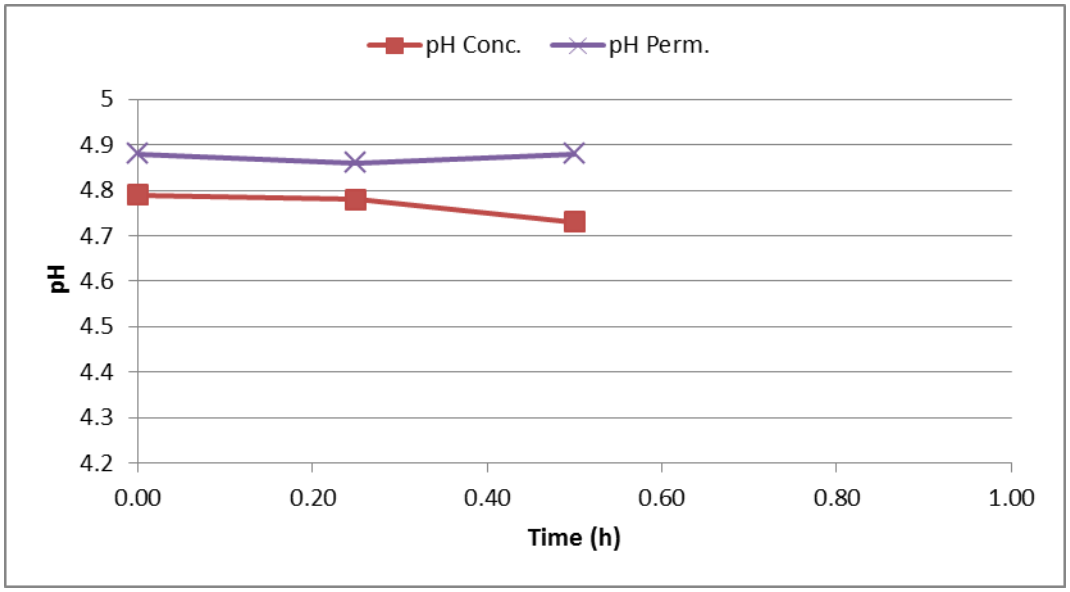


Figure 11. Harvest 1 material: pH during nanofiltration.

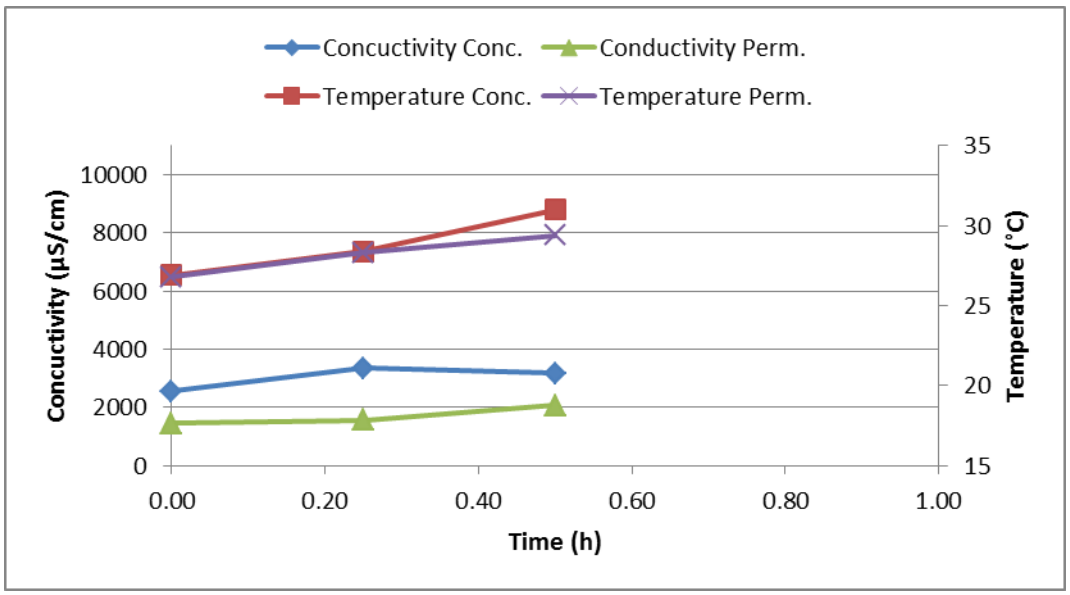


Figure 12. Harvest 1 material: conductivity (µS/cm) and temperature (°C) during nanofiltration.

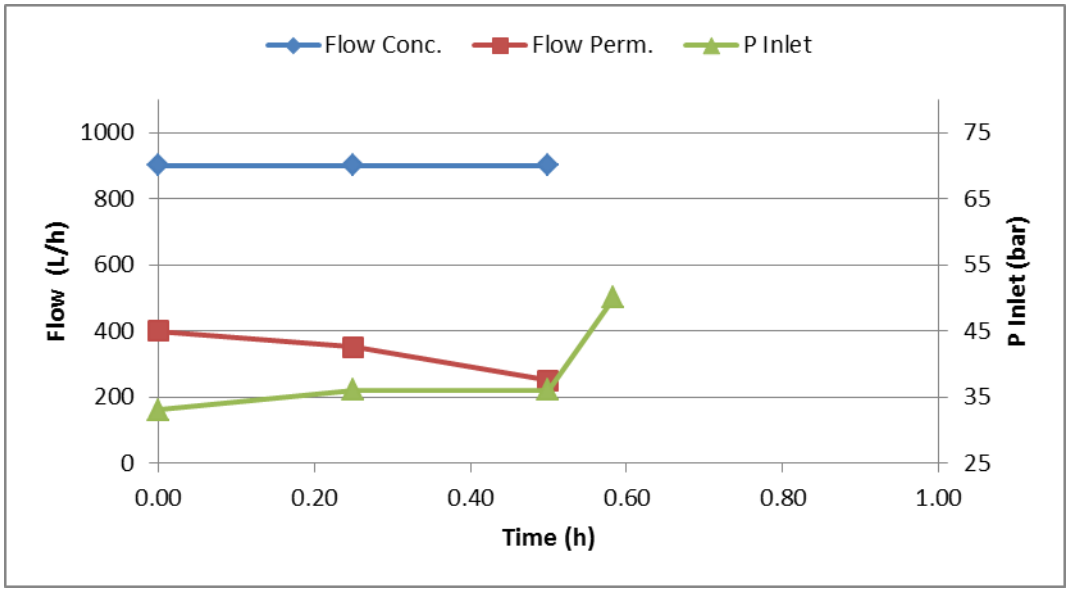


Figure 13. Harvest 1 material: flow (L/h) and pressure (bar) during nanofiltration.

Appendix 6. Monitoring data for experiment with harvest 2

Acidification and extraction, harvest 2

Date	Time	Time (h)	Temp (°C)	pH	Conductivity (µS/cm)	Sample code	Tube empty (g)	Tube full (g)
25-8-2014	14:00	0.00	19	5.4	9	1_250814_stap1	11.18	44.94
	15:05	1.08	18	6.22	107			
	15:45	1.75	18	6.2	143	2_250814_stap1	11.16	55.32
26-8-2014	8:30	18.50	18.1	5.87	1040	3_260814_stap1	11.39	59.45
	13:10	23.17	19.3	5.78	1247			
	16:45	26.75	19.2	5.45	1367			
27-8-2014	8:35	42.58	18.5	4.91	1930	4_270814_stap1	11.19	56.93
	12:45	46.75	19.4	4.81	2086			
	16:15	51.25	20.3	4.74	2128	5_270814_stap1	11.3	57.59
28-8-2014	8:45	67.75	19	4.75	2381	6_280814_stap1	11.2	59.59

Notes:

1. 26/08 recirculation switched on at 8.30
2. Gelling sugar is" van Gilse geleisuiker"
3. Extra components to saccharose: citric acid/pectin/vegetable oil
4. 502 g gelling sugar added at 9:15

1. 27/08 recirculation switched on from 9:30 to 10:15
2. 1472.4 g gelling sugar added at 13:00
3. recirculation switched on until 14:00

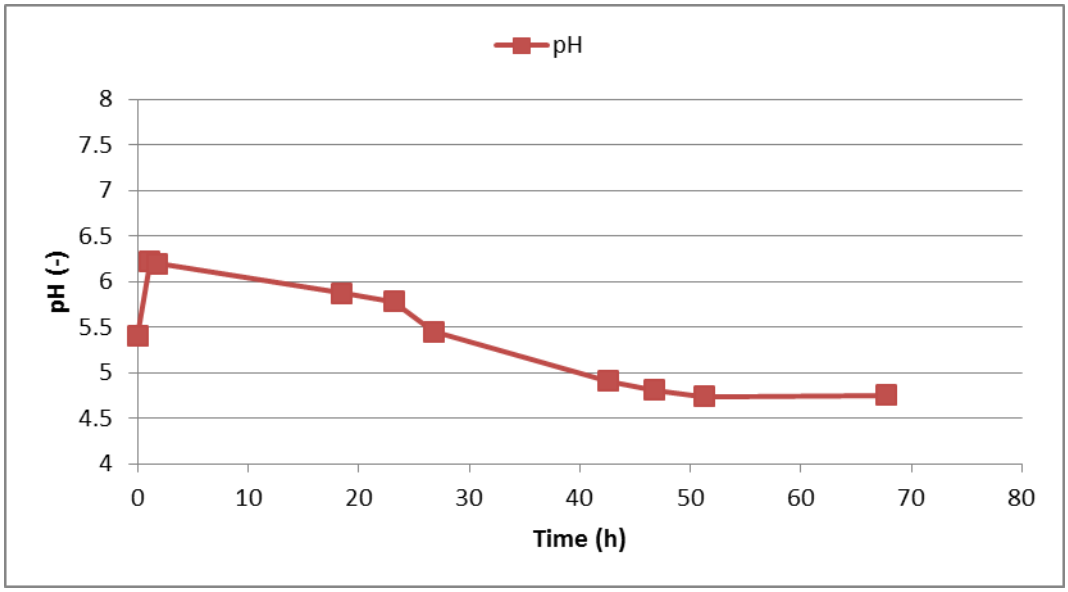


Figure 14. Harvest 2 material: pH during acidification and extraction

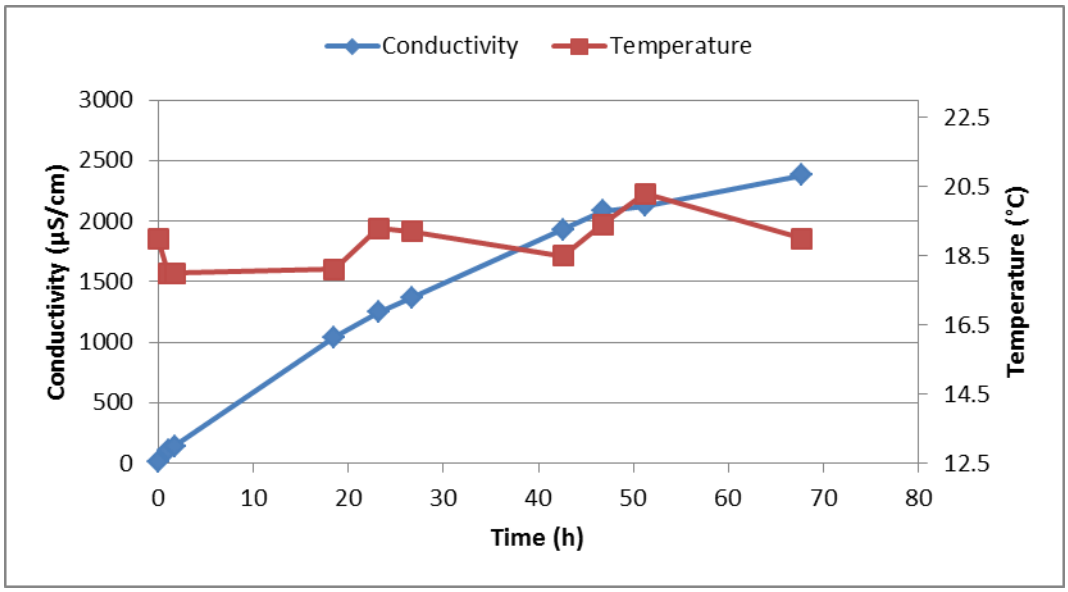


Figure 15. Harvest 2 material: conductivity (µS/cm) and temperature (°C) during acidification and extraction

Ultrafiltration, harvest 2

Data

Date	Time	Time (h)	pH Conc	pH Perm	P Inlet (bar)	P Outlet (bar)	Flow Perm (L/h)	Conduc-tivity Conc (µS/cm)	Conduc-tivity Perm (µS/cm)	T (°C) Conc	T (°C) Perm
29-8-2014	10:35	0.00	4.75	4.72	2	1	240	2424	2304	13.8	14
	10:50	0.25	4.74	4.75	2	1	222	2412	2330	14.8	14.9
	11:05	0.50	4.76	4.76	3	1.5	252	2415	2361	16	16.2
	11:20	0.75	4.75	4.74	3	1.5	438	2441	2399	17.6	17.9
	11:35	1.00	4.77	4.75	2.5	2	438	2498	2344	21.4	17.5

Sample data

Date	Time	Sample code Conc	Tube empty (g)	Tube full (g)
29-8-2014	10:35	1_290814_stap2C	11.36	57.77
	11:20	2_290814_stap2C	11.38	59.3
	11:35	3_290814_stap2C	11.47	61.25
Datum	**Tijd**	**Monstercode Perm**	**Buis leeg (g)**	**Buis vol (g)**
29-8-2014	10:35	1_290814_stap2P	11.35	58.1
	11:20	2_290814_stap2P	11.43	60.92
	11:35	3_290814_stap2P	11.47	57.13

Notes:

1. Measurements in liquid before UF: pH 4.73 / temperature 20.7 / conductivity 2363
2. Refilled concentrate from 10:37 to 10:55 and from 11:10 to 11:13
3. Filter was shut down at 11:21, flow was getting too high and could not be regulated, after this, a sample was taken

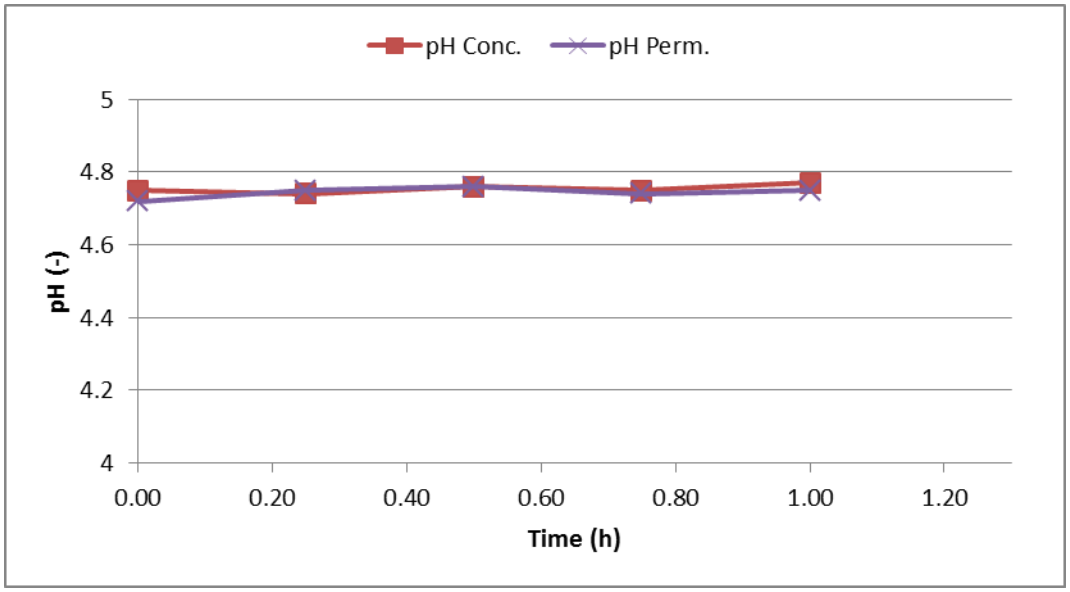


Figure 16. Harvest 2 material: pH during ultrafiltration.

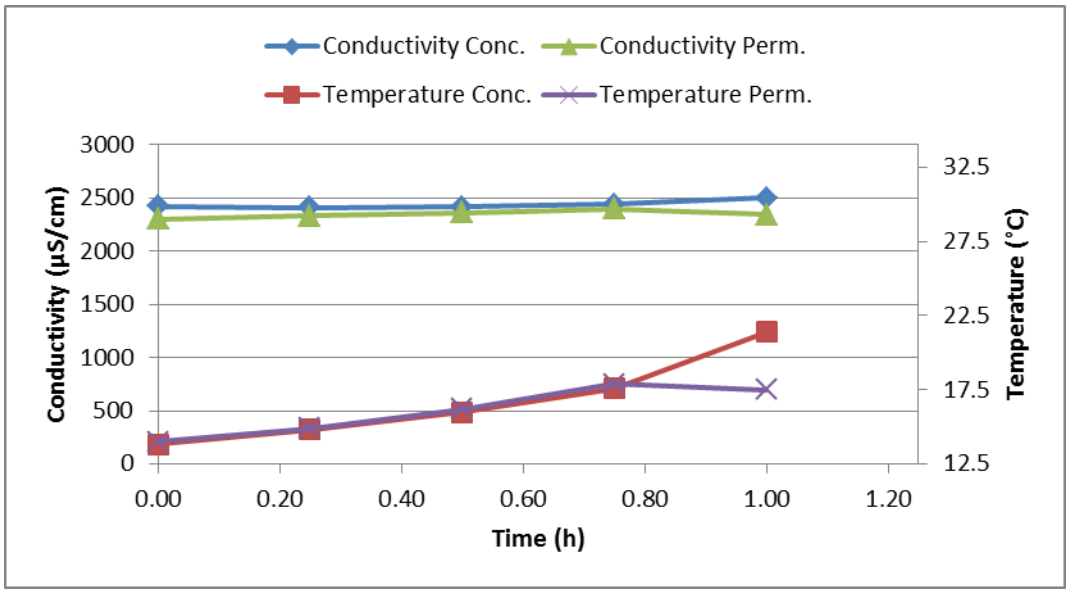


Figure 17. Harvest 2 material: conductivity (µS/cm) and temperature (°C) during ultrafiltration.

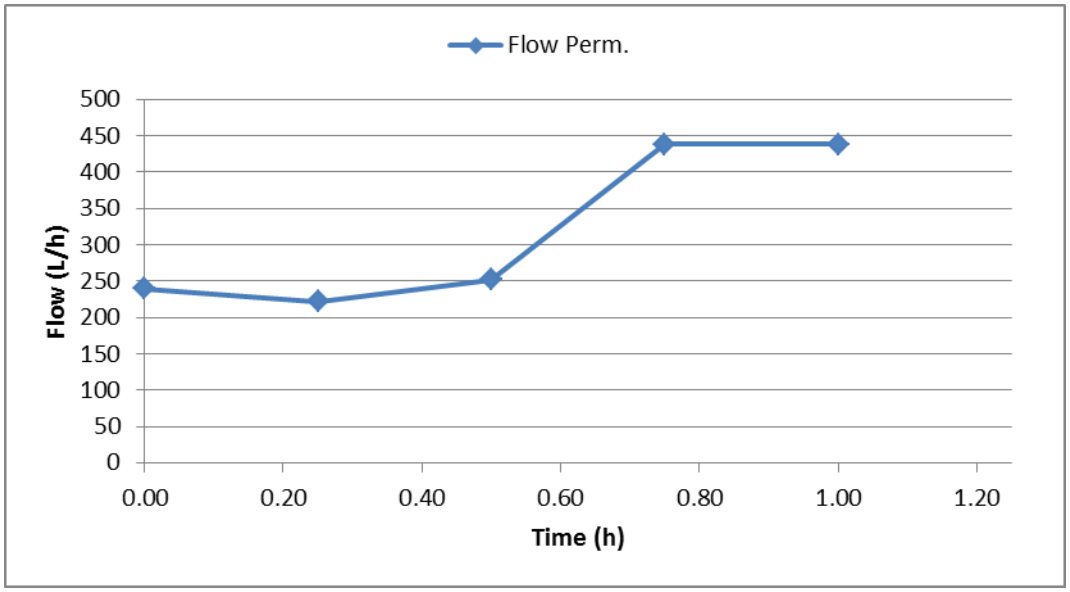


Figure 18. Harvest 2 material: permeate flow (L/h) during ultrafiltration.

Nanofiltration, harvest 2

Data

Date	Time	Time (h)	pH Conc.	pH Perm.	Flow Inlet (L/h)	Flow Perm. (L/h)	P Inlet (bar)	Conduc-tivity Conc. (mS/cm)	Conduc-tivity Perm. (mS/cm)	T (°C) Conc	T (°C) Perm
2-10-2014	14:25	0.00	4.33	4.62	1000	324	40	3.07	0.98	20.6	19.8
	14:40	0.25	4.32	4.67	1000	330	40	3.93	1.09	23	22.5
	14:50	0.42	4.31	4.65	1000	324	40	4.55	1.23	25	24.1
	15:00	0.58	4.28	4.63	1000	288	40	6.36	1.73	28.7	28.1
	15:07	0.70	4.27	4.55	1000		40	9.11	2.71	33.2	32
	15:10	0.75	4.23	4.43	1000		40	10.94	3.62	34.5	32.7

Sample data

Date	Time	Sample code Conc.	Tube empty (g)	Tube full (g)
2-10-2014	14:25	1_021014_stap3_concentraat	11.4	60.7
	15:10	2_021014_stap3_concentraat	11.3	61.6
Date	**Time**	**Sample code Perm.**	**Tube empty (g)**	**Tube full (g)**
2-10-2014	14:25	1_021014_stap3_permeaat	11.4	61.3
	15:10	2_021014_stap3_permeaat	11.3	60.2

Notes
:

1. Start filtration 12:39
2. At 13:22 raised pressure to 40 bar
3. Started with 360.5 L

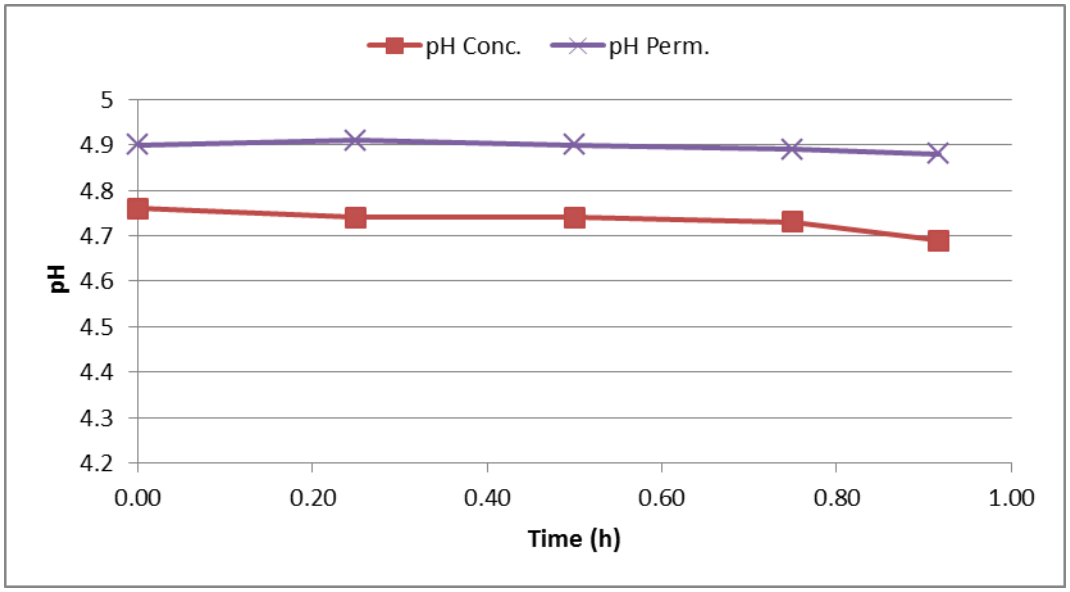


Figure 19. Harvest 2 material: pH during nanofiltration.

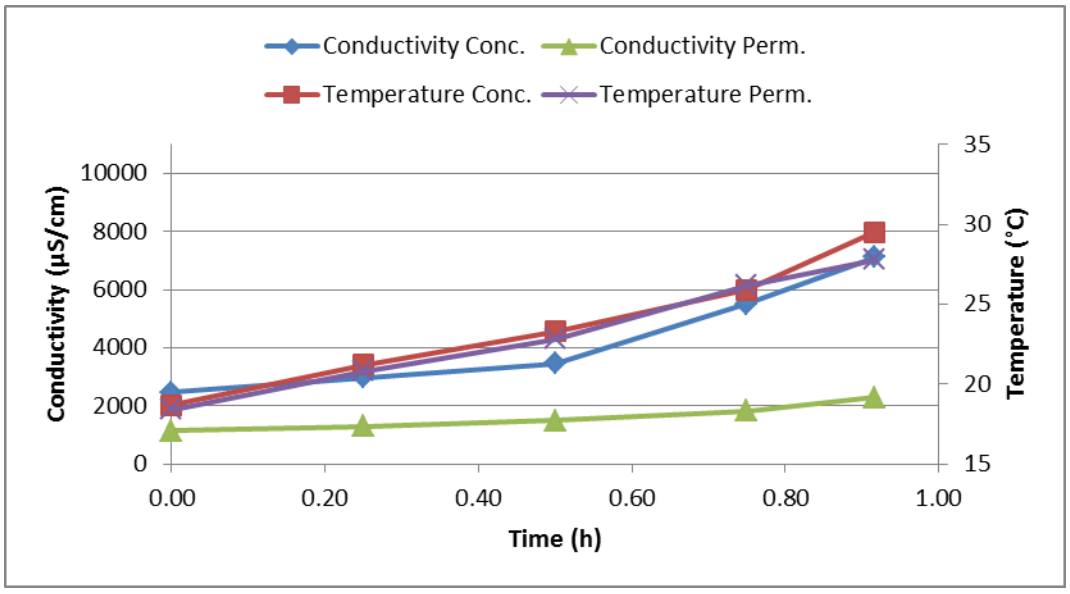


Figure 20. Harvest 2 material: conductivity (µS/cm) and temperature (°C) during nanofiltration.

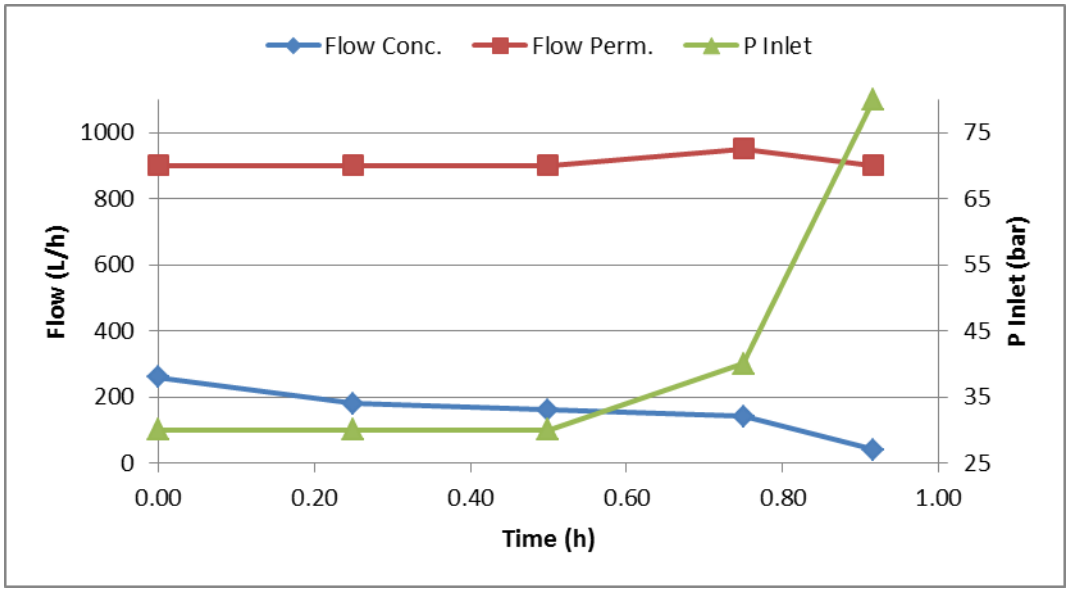


Figure 21. Harvest 2 material: flow (L/h) and pressure (bar) during nanofiltration.

Appendix 7. Monitoring data for experiment with harvest 3

Acidification and extraction, harvest 3

Date	Time	Time (h)	Temp (°C)	pH	Conduc-tivity (mS/cm)	Sample code	Tube empty (g)	Tube full (g)
29-9-2014	14:45	0.00	19.5	7.71	0.2			
	15:15	0.50	19.5	7.17	0.31	1_290914_stap1	11.2	57.5
30-9-2014	8:45	18.00	19	6.04	1.12	2_300914_stap1	11.5	61
	13:30	22.75	20.1	5.79	1.38			
	16:35	25.83	20.3	5.61	1.55			
1-10-2014	8:25	41.67	19.2	4.25	2.19	3_011014_stap1	11.5	59.2
	11:45	45.00	19.7	4.25	2.51			
	14:30	47.75	19.7	4.26	2.57	4_011014_stap1	11.4	60.6
2-10-2014	9:30	64.75	12.6	4.3	2.64	5_021014_stap1	11.4	56.6

Notes:
Indicated in red is compared to the pH meter of Maarten, which indicated 5.79
Gelling sugar added only once: on 30/09 at 9:10 at pH 6.12
Other type gelling sugar added than at experiments 1 and 2

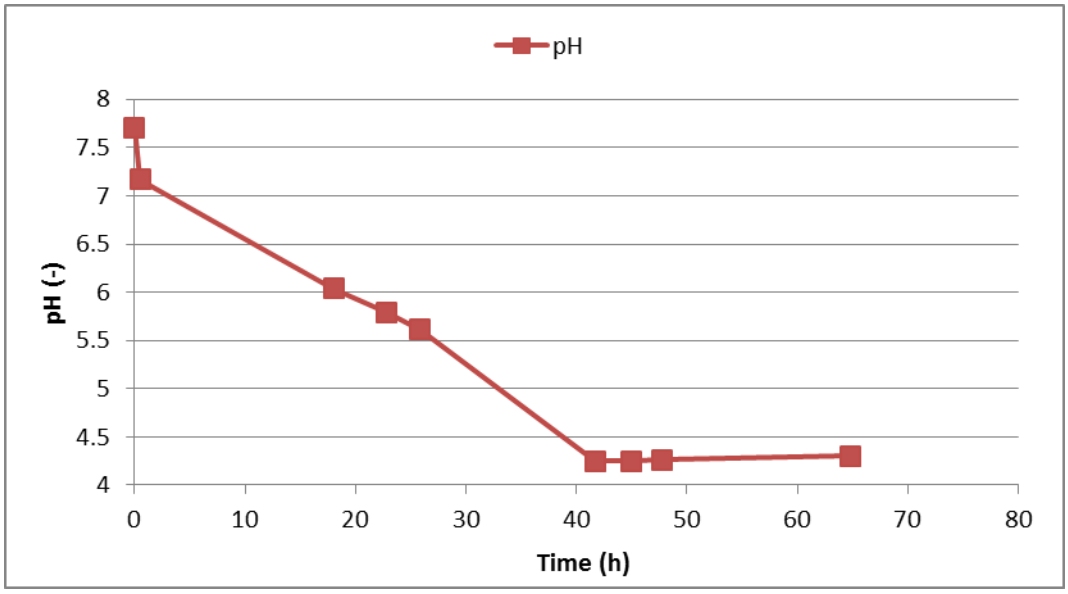


Figure 22. Harvest 3 material: pH during acidification and extraction

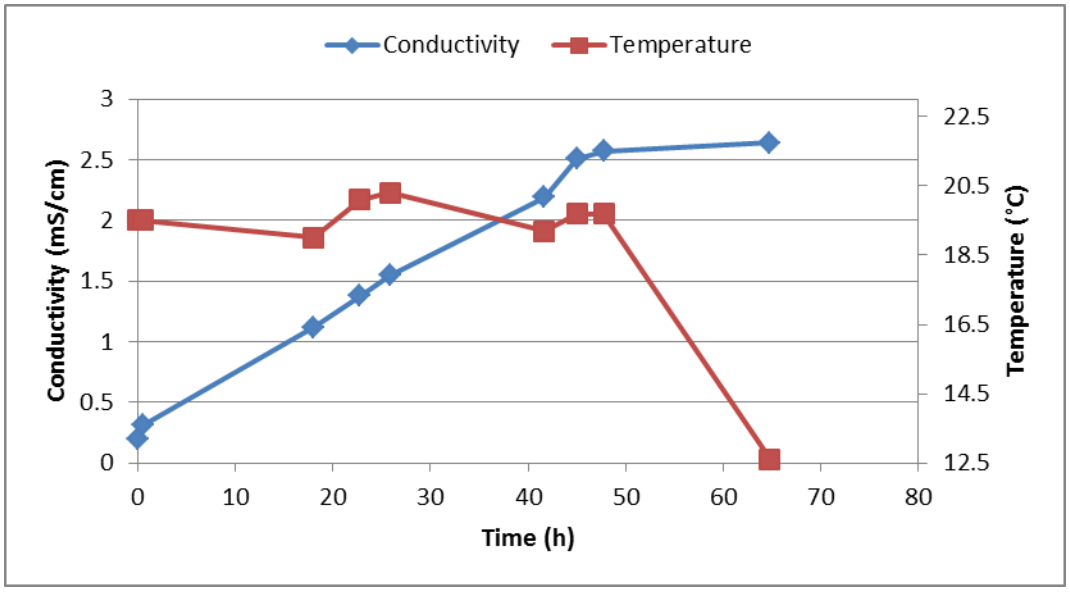


Figure 23. Harvest 3 material: conductivity (µS/cm) and temperature (°C) during acidification and extraction

Ultrafiltration, harvest 3

Data

Date	Time	Time (h)	pH Conc.	pH Perm.	P Inlet (bar)	P Outlet (bar)	Flow Perm. (L/h)	Conduc-tivity Conc. (mS/cm)	Conduc-tivity Perm. (mS/cm)	Temp. (°C) Conc.	Temp. (°C) Perm.
2-10-2014	9:55	0.00	4.29	4.27	1	0.5	108	2.63	2.6	13.5	13.7
	10:05	0.17	4.27	4.29	1	1	114	2.62	2.6	14.1	14.1
	10:15	0.33	4.29	4.32	1	1	102	2.64	2.6	14.9	15.3
	10:30	0.58	4.31	4.31	1	1	84	2.61	2.6	15.4	15.6
	10:45	0.83	4.33	4.33	1	1	84	2.6	2.6	16.3	16.5
	11:00	1.08	4.33	4.31	1	1	84	2.66	2.61	16.8	16.8
	11:15	1.33	4.32	4.32	1	1	72	2.62	2.6	17.6	17.6
	11:35	1.67	4.33	4.32	1	1	72	2.65	2.61	18.3	18.4
	11:45	1.83	4.33	4.33	1	1	66	2.63	2.61	19.2	19.2
	12:00	2.08	4.35	4.35	1	1	66	2.64	2.61	19.7	19.7
	12:10	2.25	4.35	4.35	1	1		2.63	2.62	20.6	20.7
	12:20	2.42	4.37	4.37	1.2	1	66	2.63	2.61	21.5	21.5
	12:30	2.58	4.38	4.37	1.2	1	66	2.63	2.61	22.3	22.4
	12:40	2.75	4.38	4.38	1.2	1	60	2.64	2.6	23.4	23.3
	12:55	3.00	4.39	4.39	1.2	1	60	2.63	2.61	24.5	24.2
	13:10	3.25	4.42	4.35	1.2	1	60	2.63	2.64	25.1	25.1

Sample data

Date	Time	Sample code Conc.	Tube empty (g)	Tube full (g)
2-10-2014	9:55	1_021014_stap2_concentraat	11.4	56.6
	10:45	2_021014_stap2_concentraat	11.4	56.7
	11:35	3_021014_stap2_concentraat	11.4	60.5
	13:10	4_021014_stap2_concentraat	11.3	54.8
Date	**Time**	**Sample code Perm.**	**Tube empty (g)**	**Tube full (g)**
2-10-2014	9:55	1_021014_stap2_permeaat	11.5	56.4
	10:45	2_021014_stap2_permeaat	11.4	60
	11:35	3_021014_stap2_permeaat	11.4	57.4
	13:10	4_021014_stap2_permeaat	11.4	62.3

Notes:

UF performed with different membrane: same as at first experiment

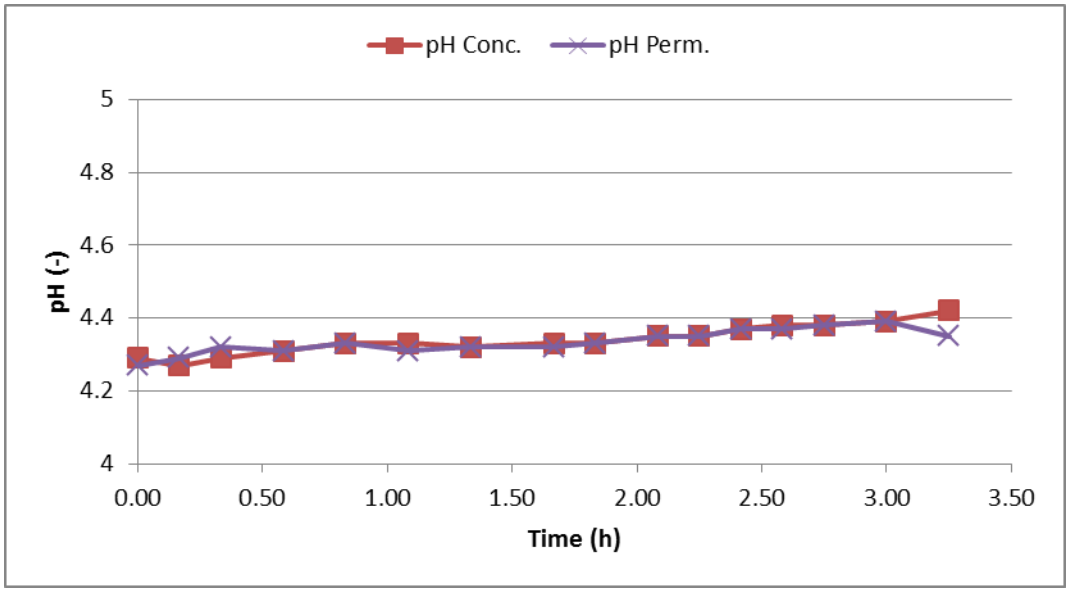


Figure 24. Harvest 3 material: pH during ultrafiltration.

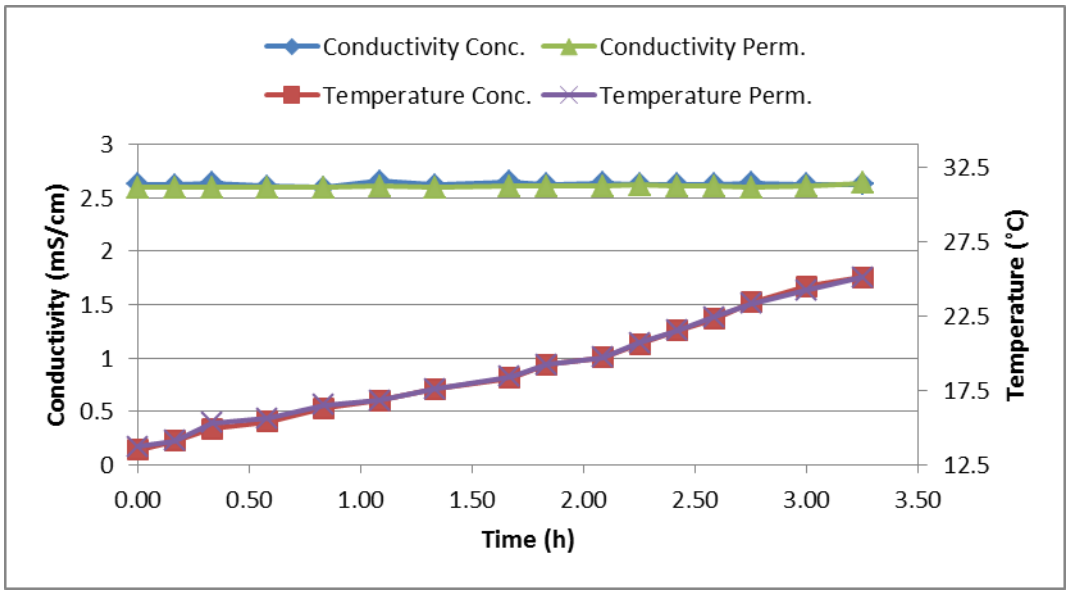


Figure 25. Harvest 3 material: conductivity (mS/cm) and temperature (°C) during ultrafiltration.

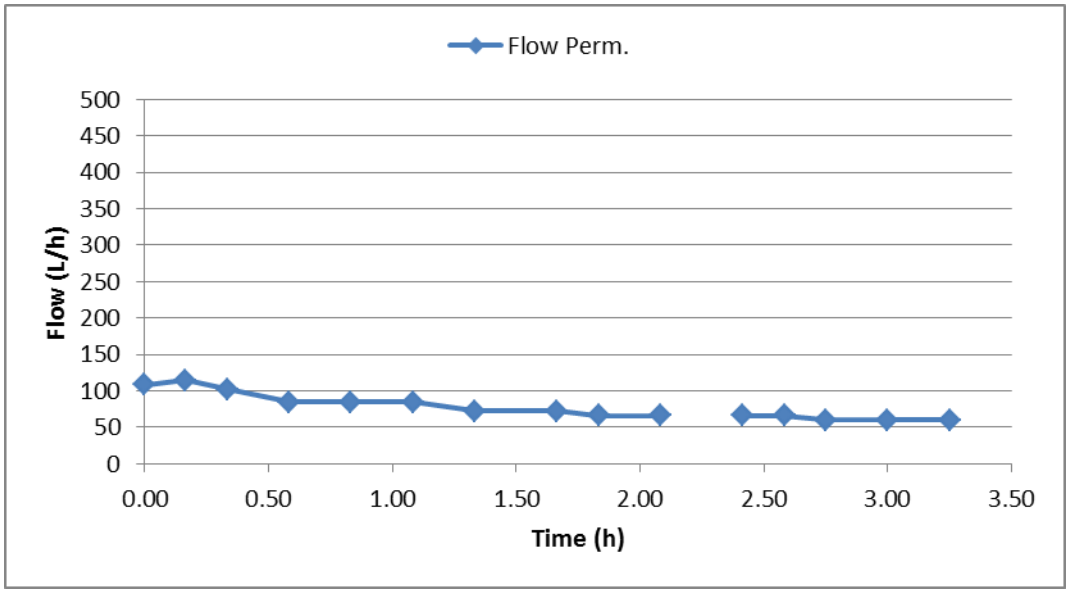


Figure 26. Harvest 3 material: permeate flow (L/h) during ultrafiltration.

Nanofiltration, harvest 3

Data

Date	Time	Time (h)	pH Conc	pH Perm	Flow Inlet (L/h)	Flow Perm (L/h)	P Inlet (bar)	Conduc-tivity Conc (mS/cm)	Conduc-tivity Perm (mS/cm)	T (°C) Conc	T (°C) Perm
2-10-2014	14:25	0.00	4.33	4.62	1000	324	40	3.07	0.98	20.6	19.8
	14:40	0.25	4.32	4.67	1000	330	40	3.93	1.09	23	22.5
	14:50	0.42	4.31	4.65	1000	324	40	4.55	1.23	25	24.1
	15:00	0.58	4.28	4.63	1000	288	40	6.36	1.73	28.7	28.1
	15:07	0.70	4.27	4.55	1000		40	9.11	2.71	33.2	32
	15:10	0.75	4.23	4.43	1000		40	10.94	3.62	34.5	32.7

Sample data

Date	Time	Sample code Conc.	Tube empty (g)	Tube full (g)
2-10-2014	14:25	1_021014_stap3_concentraat	11.4	60.7
	15:10	2_021014_stap3_concentraat	11.3	61.6
Date	**Time**	**Sample code Perm.**	**Tube empty (g)**	**Tube full (g)**
2-10-2014	14:25	1_021014_stap3_permeaat	11.4	61.3
	15:10	2_021014_stap3_permeaat	11.3	60.2

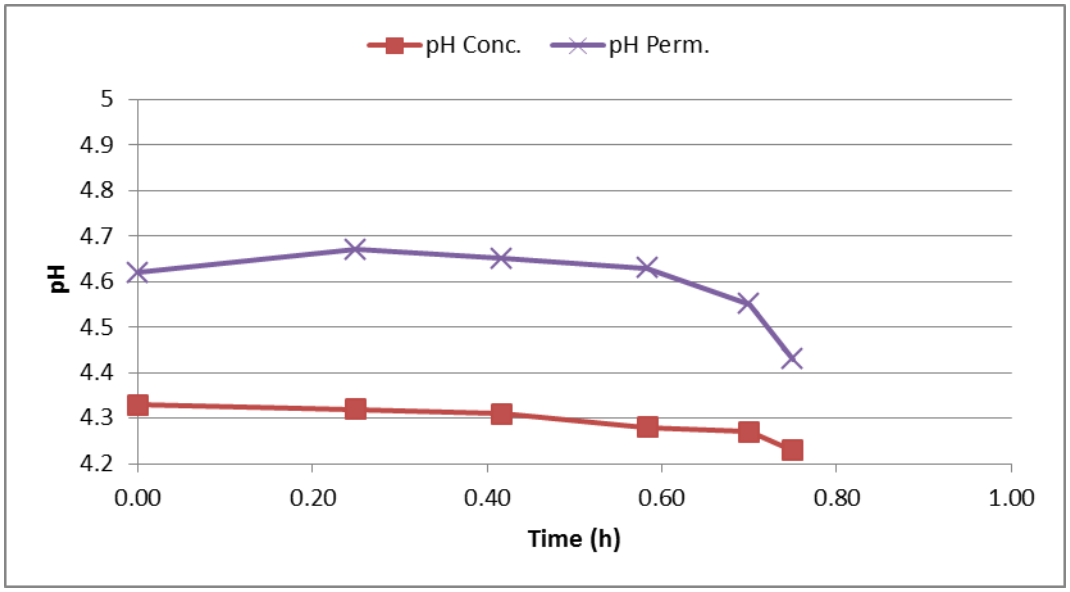


Figure 27. Harvest 3 material: pH during nanofiltration.

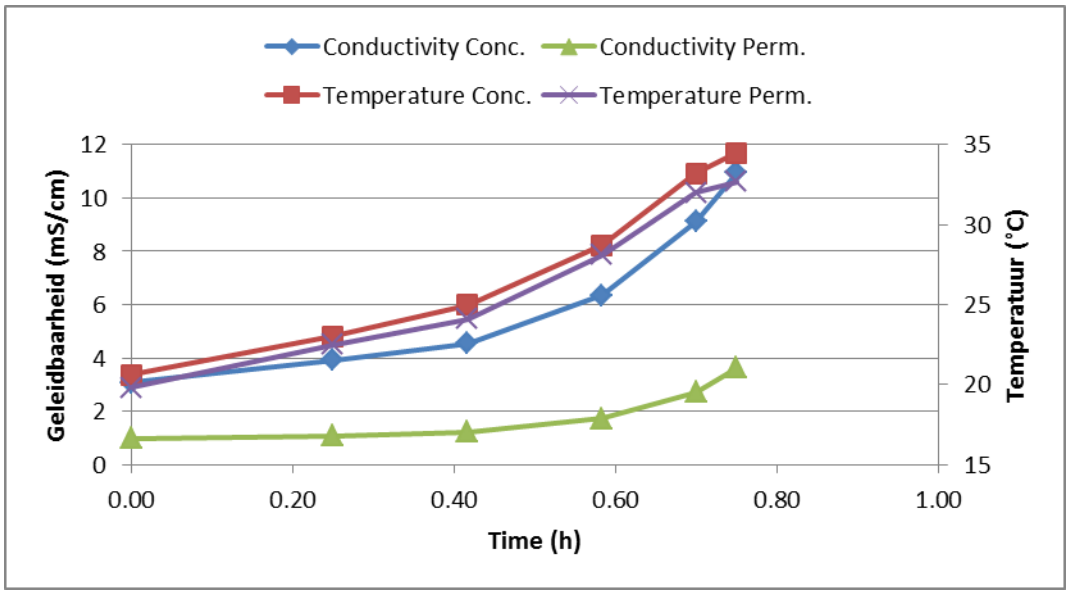


Figure 28. Harvest 3 material: conductivity (µS/cm) and temperature (°C) during nanofiltration.

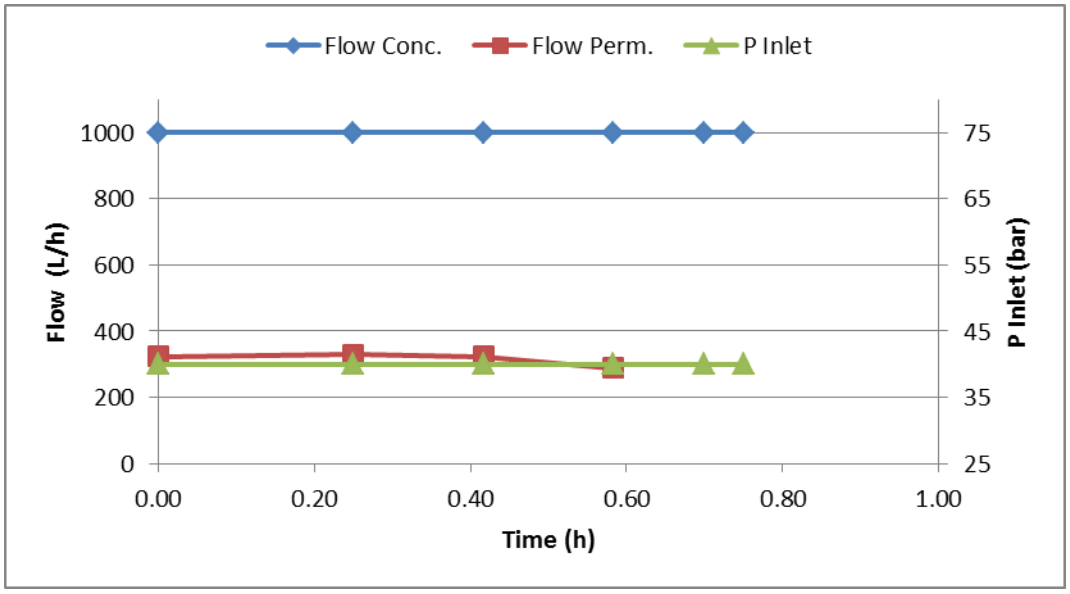


Figure 29. Harvest 3 material: flow (L/h) and pressure (bar) during nanofiltration.

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