CHAPTER 6

Ultra-High Performance Liquid Chromatography for the analysis of steviol glycosides

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ABSTRACT

When analyzing natural products, such as steviol glycosides, one must always be aware of the fact that the analytical method used might not reveal every compound that is actually present in the sample. For instance, a chromatogram representing the separation of steviol glycosides on two 20 cm C_{18} columns in series (d_p= 5.0 µm) indicates the presence of 12 compounds; four of them not being identified yet. Some compounds may be “invisible” to the instrument, because they are hidden under larger peaks, or because they elute very late and the band broadening renders them undetectable.

The van Deemter equation predicts a dramatic increase in column performance (and hence: resolution) by using much smaller particle diameters to pack the column. However, these columns will generate a substantially larger back pressure in the system, so a pump is needed that can deliver this pressure. Hence the name of the technique: Ultra-High Performance (or: Pressure) Liquid Chromatography (UHPLC). Moreover, a high inlet pressure allows the use of longer column lengths, further increasing the resolution of the separation.

An automatic column coupling device, installed on an Agilent Infinity UHPLC system with a maximum inlet pressure of 1200 bar, was used to couple several Acquity BEH C_{18} columns (particle size: 1.7 µm) in series. This set-up was used to evaluate the separation of a steviol glycosides sample on different column lengths, ranging between 5 cm and 25 cm. Various gradients of aqueous formic acid (0.1 %) – acetonitrile were first run on the shortest column length (5 cm), allowing selection of the best gradient conditions in the shortest possible time. The gradient conditions resulting in the best separation were subsequently repeated on a 10, 15, and 20 cm support. A total of 26 peaks could be separated on the longest column length (20 cm), obtained by coupling two 2.1 x 100 mm columns. None of the extra peaks could be identified at this moment, but it is hoped to identify them using LC-MS.

KEYWORDS
Steviol glycosides, Ultra High Performance Liquid Chromatography, UHPLC, unknown compounds.

Introduction

It is well known that the taste of steviol glycosides is strongly dependent on their structure. For example, rebaudioside C is about 50 to 120 times sweeter than sucrose, where rebaudioside A is 300 to 450 times sweeter (Geuns, 2010). Moreover, the taste of rebaudioside A is more similar to the taste of sucrose, whereas stevioside has a slight bitter aftertaste. As a consequence, it is very important that stevia extracts are very well characterized. This is even more vital, as a routine HPLC analysis of steviol glycosides usually quantifies only 7 or 8 different steviol glycosides out of the more than 10 that have been described to date (Geuns, 2010; MassBank, 2010). Moreover, we report elsewhere (Amery *et al.*, 2010) on the analysis of steviol glycosides in dairy products, and find at least one additional peak that is consistently present in our chromatograms. Hence, the fact that there are even more unidentified compounds present cannot be excluded. Furthermore we cannot exclude the possibility that at least part of the off-taste of some stevia

extracts could be due to the presence of compounds that have not yet been identified.

There are several recent publications on the analysis of steviol glycosides using various techniques, however, most of them deal with the optimization of the analysis of a limited number of steviol glycosides (Gardana *et al.*, 2010; Hoekstra *et al.*, 2009; Jaitak *et al.*, 2008; Kolb *et al.*, 2001; Liu and Li, 1995; Pol *et al.*, 2007; Rajasekaran *et al.*, 2008). Recent publications about detecting a larger number of steviol glycosides are relatively rare (Pol *et al.*, 2007). We will discuss some of these publications in brief.

The purpose of the work of Kolb *et al.* (2001)was to develop a relatively fast routine analysis. Hence, they targeted their analysis on the two main compounds: stevioside (Ste) and rebaudioside A (RebA) and made no attempt to quantify or identify any of the other steviol glycosides that were present. The separation was done on a NH_2 column, with detection at 210 nm.

Liu and Li (1995) developed a capillary electrophoresis analysis for steviol glycosides and compared the results with HPLC. They found good agreement between the results of both techniques. Another less common analysis technique was developed by Jaitak *et al* (2008). They validated an HPTLC thin layer analysis of three main glycosides. The recovery ranged between 93 and 101 % ; the LOD were 120 ; 180 and 80 ng/spot for SteB, Ste and RebA respectively.

Rajasekaran *et al.* (2008) used a C_{18} column to separate the steviol glycosides, and ESI-MS for identification. Their research was primarily aimed at identification of the main steviol glycosides that were present in stevia plants, grown in India.

Hoekstra *et al.* (2009) compared and optimized the separation of steviol glycosides on a NH_2 and a C_{18} column. They obtained baseline separation for 12 steviol glycosides with an aqueous – acetonitrile gradient. The best results were obtained with 0.1 % TFA as the aqueous solvent and an ELSD as detector. Total running time was about 50 min, including the wash and re-equilibration.

Attempts to characterize as much steviol glycosides as possible are quite rare in the recent literature. In an interesting publication, Pól *et al.* (2007) used comprehensive LC with Time Of Flight (TOF) mass spectrometry as detector. They were able to separate 17 compounds with a C_{18} column in the first dimension and a NH_2 column in the second using a modulation time of 60 s. Both eluents were acetonitrile – water; with a gradient in the first dimension, and isocratic elution in the second. Not all steviol glycosides could be unambiguously identified, even with the TOF.

In the analysis of real leaf extracts, the authors also noted a lot of matrix compounds which could not be identified.

Gardana *et al.* (2010) used UHPLC – MS to quantify steviol and three steviol glycosides (Ste, RebA and SteB) in stevia leave extracts, grown in southern Italy, and a commercial stevia sweetener. However, this study was primarily aimed at a fast analysis, suitable for routine control.

In this contribution, we report on the analysis of steviol glycosides with UHPLC. Contrary to most other publications, we tried to discover as many compounds in our samples as possible. Because the results are still quite preliminary, the unknowns could not yet be identified at the time of writing.

Methods and materials

UHPLC experiments were done on two different instruments from Dionex and Agilent. In order to couple several columns in series, both systems were equipped with a custom build column coupler. This allowed up to four columns to easily be coupled. The experimental setup has been described elsewhere (Cabooter *et al.*, 2010a; 2010b). This column coupler is schematically represented in Figure 1. For details, see below.

Columns can be coupled by connecting the appropriate ports through rotating the rotors. In Figure 1 respectively one, two, three and all four columns are connected to the system. Of course it is also possible to connect only one column, other than the first to the system, e.g., in order to use only column 3 the ports R3 and L5 are used.

The Dionex UHPLC system was an Ultimate 3000 system (Dionex Corporation, Germering, Germany) equipped with a dual binary pump, an autosampler, a thermostatted forced-air oven with a maximum temperature of 110 °C and a variable wavelength detector with a flow cell of 45 nL (1 cm path length). The dwell volume of the system was 600 µL and the maximum pressure 800 bar. The system was operated with Chromeleon software (Dionex). The UHPLC system was equipped with high-pressure switching valves with a pressure limit of 1000 bar (TitanHT, HT715-000) from Rheodyne (Rhonert Park, CA, USA). The rotors consisted of 6 peripheral ports with one central port and had a port-to-port volume of 300 nL. The stator was custom made in order to allow the valves to be used in 6 different positions. The valves were operated with the Chromeleon software.

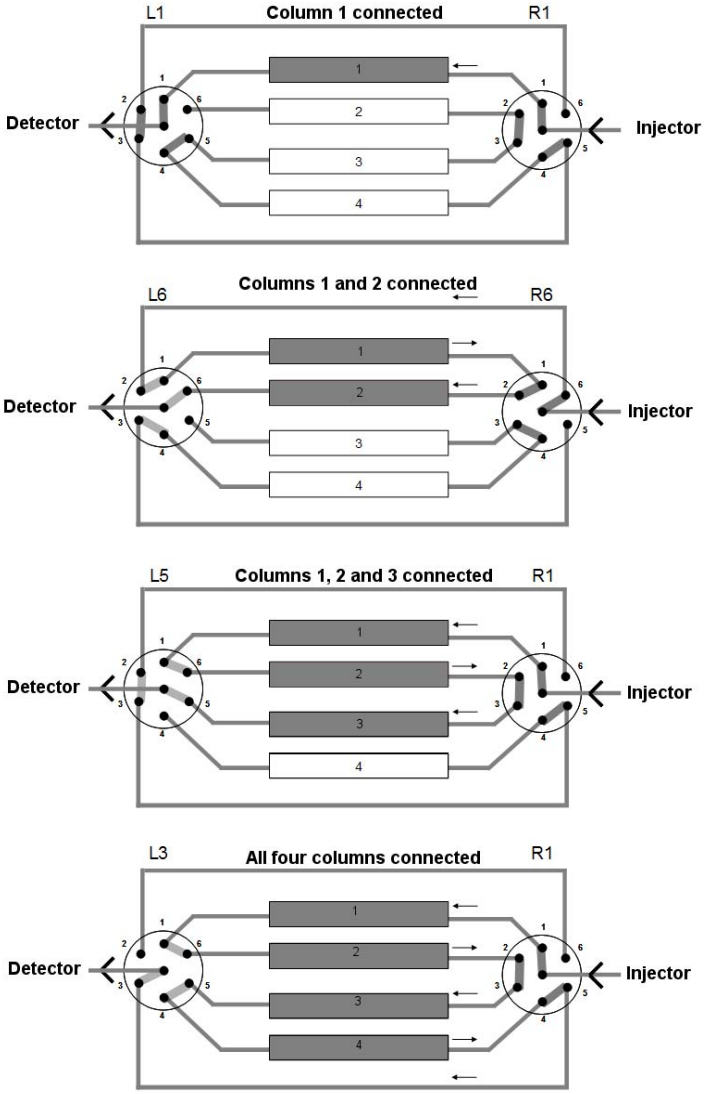

Figure 1: Schematic representation of the column coupler

The other instrument was an Agilent 1290 Infinity system (Agilent Technologies, Waldbronn, Germany) equipped with a dual binary pump, an auto-sampler, two thermostatted column compartments with a maximum temperature of 100°C and a diode-array detector with a flow cell of 1 µL (1 cm path length). The dwell volume of the system was 112 µL and the maximum pressure 1200 bar. The system was operated with Chemstation software (Agilent). Each thermostatted column com-

partment was equipped with a high-pressure switching valve with a pressure limit of 1200 bar (TitanHT, HT715-000) from Rheodyne (Rhonert Park, CA, USA). The rotors consisted of 8 peripheral ports with one central port and had a port-to-port volume of 300 nL. The stator was custom made in order to allow the valves to be used in 8 different positions. The valves were operated with the Chemstation software. For details about the columns and eluents that were used, the reader is referred to the next section.

Acetonitrile, phosphoric acid and formic acid (Biosolve, Valkenswaard, The Netherlands) were all HPLC quality and were used without further purification. Water was prepared "in-house" using a Milli-Q apparatus (Millipore, Billerica, MA). The stevia sample was "sample 1" that was used for the round-robin testing (Geuns and Struyf, 2009). The advantage of using this sample is that it is well characterized, both qualitatively and quantitatively.

Results and discussion

Background

There are two very important and well known equations in chromatography that must be used as a guide whenever one wants to increase the resolution in a chromatogram. These equations are, of course, the general resolution equation and the van Deemter equation. The resolution equation (See e.g. Snyder *et al.*, 1997) takes the form of (Eq. 1).

$$R_s = \frac{1}{4}\left(\frac{k}{1+k}\right)\left(\frac{\alpha-1}{\alpha}\right)N^{1/2} \qquad \text{(Eq. 1)}$$

In this equation, k is the retention factor, defined as:

$$k = \frac{t_R - t_M}{t_M}$$

t_R is the retention time and t_M the mobile phase time, i.e. the retention time of an unretained compound.

α is the separation factor:

$$\alpha = \frac{k_2}{k_1}$$

Finally, N is the plate count of the column.

This equation reveals the three important parameters influencing the resolution: the retention: $\left(\frac{k}{k+1}\right)$; the selectivity: $\left(\frac{\alpha-1}{\alpha}\right)$ and the column efficiency: $\sqrt{N}$

The second important equation is the well-known van Deemter equation, (Eq. 2), (see e.g. Scott, 1992)

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{1+6k+11k^2}{24(1+k)^2}\frac{{d_p}^2}{D_m}u + \frac{8}{\pi^2}\frac{k}{(1+k^2)}\frac{{d_f}^2}{D_s}u \qquad \text{(Eq. 2)}$$

In this equation, H is the height equivalent of a theoretical plate; λ and γ are the packing and obstruction factors of the column; D_m and D_s are the diffusion constants of the analyte in the mobile and stationary phases respectively; d_f is the film thickness of the stationary phase on the inert support; d_p is the particle diameter of the column packing; and u is the linear velocity of the mobile phase. H and N are interrelated through the column length L:

$$L = H \cdot N \qquad \text{(Eq. 3)}$$

The van Deemter equation is a hyperbolic function with a minimum, so there will be an optimum velocity u_{opt} at which H is minimal and hence the column has the greatest efficiency.

The easiest way to increase the resolution for a given chromatographic system (which fixes k and α) is to increase the column length. Doubling the column length L will double the plate count N, but of course from (Eq. 1) it follows that the resolution R_s will only increase by a factor $2^{1/2}$. However, the total retention time is proportional to the column length, and will increase by a factor 2.

Another way of increasing the resolution through a bigger plate count, is by decreasing the particle diameter d_p. In (Eq. 2) it can be seen that d_p has a major influence on H. In Figure 2 the van Deemter curve is plotted for three different particle diameters, the other parameters are kept constant, and have a realistic value (Scott, 1992).

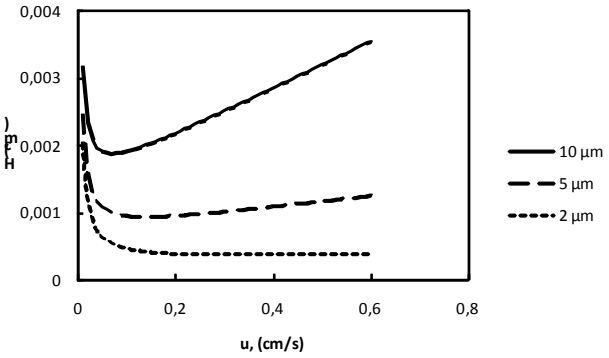

Figure 2: van Deemter curve for three different particle diameters

We see that H decreases drastically as d_p becomes smaller, and there is an added benefit because the right side of the curve becomes more flat, thus enabling higher speeds without loss of column efficiency.

There is of course a catch: if we increase the column efficiency, either by increasing L or by decreasing d_p, then the column back-pressure will also increase. An approximate equation for the pressure drop over a column is given in (Eq. 4) (Snyder *et al.*, 1997)

$$\Delta p = \frac{207 L \eta}{t_M d_p^{\,2}} \qquad \text{(Eq. 4)}$$

In this equation, η is the viscosity of the eluent in cP, L is expressed in cm, Δp in bar, d_p in µm and t_M in min. Of course the pressure drop will also increase when the flow is increased, through the parameter t_M. An "ordinary" HPLC pump can cope with a maximum pressure of about 400 bar, which is certainly not enough, especially when columns packed with small particles are to be used. Hence, it is necessary to use dedicated instruments which can deliver these high pressures (UHPLC instruments).

Optimizing the resolution

In a first series of experiments, the resolution between the critical pair in the chromatogram (Ste and RebA) was optimized. Using a C_{18} column a rather long column (and hence a long analysis time) is needed for a baseline separation (Geuns and Struyf, 2009). For a baseline separation between two peaks an R_s = 1.5 is required. The optimization experiments discussed here were done on the Dionex

instrument, with up to four Dionex Rapid Resolution columns, each 100 x 2.1 mm and d_p = 2.2 µm. The dwell volume V_D of this system was 0.6 mL. The mobile phase consisted of 25 mmol.L^{-1} aqueous H_3PO_4 and acetonitrile, ACN. In order to obtain a sufficiently wide elution window ($2 < k < 10$) for the previously characterized compounds (Amery *et al.*, 2010), a shallow gradient of 32 % B to 34 % B was used. The gradient time, t_G, was increased proportionally to the number of coupled columns. The ratio t_G/t_M should be kept constant in order to be able to evaluate the peak width (see below) (Cabooter *et al.*, 2010a). The flow F used was 0.2 mL.min^{-1}. The column(s) were kept at 30 °C and 2 µL of a sample of 1 mg.mL^{-1} was injected. Detection wavelength was 200 nm. The resulting chromatograms, with 1 to 4 columns coupled to the system are shown in Figure 3, a – d.

Upon inspection of Figure 3 a – d is it clear that the resolution of the critical pair (the first two peaks) increases, as it should be theoretically. A total of 13 peaks is visible in the chromatograms. Corresponding peaks in the four chromatograms are numbered 1 to 13; the identification is made by comparing with chromatograms of a similar column known from the literature (for a review: see Geuns, 2010).

There are a few peculiar features in these chromatograms. First, the absence of peak 4 in the chromatograms a and b is apparent. Next, peaks 3 and 4 invert in the chromatograms c and d. This indicates a certain change in selectivity of the stationary phase upon changing from three to four columns. This is probably related to the fact that the four coupled columns are operated at a pressure of nearly 750 bar, whereas the three columns are only operated at some 480 bar. It has been demonstrated previously that the retention factor of analytes increases linearly with increasing pressure (for a pressure increase of 1000 bar, an increase in retention of some 10 % is expected for neutral compounds, whereas this can be up to 50 % for charged analytes) (Fallas *et al.*, 2008). The observation that peak 4 changes its relative position upon increasing the pressure from 480 to 750 bar, indicates that its retention behaviour is less prone to the pressure increase than that of compounds 3 and 5, resulting in a selectivity shift.

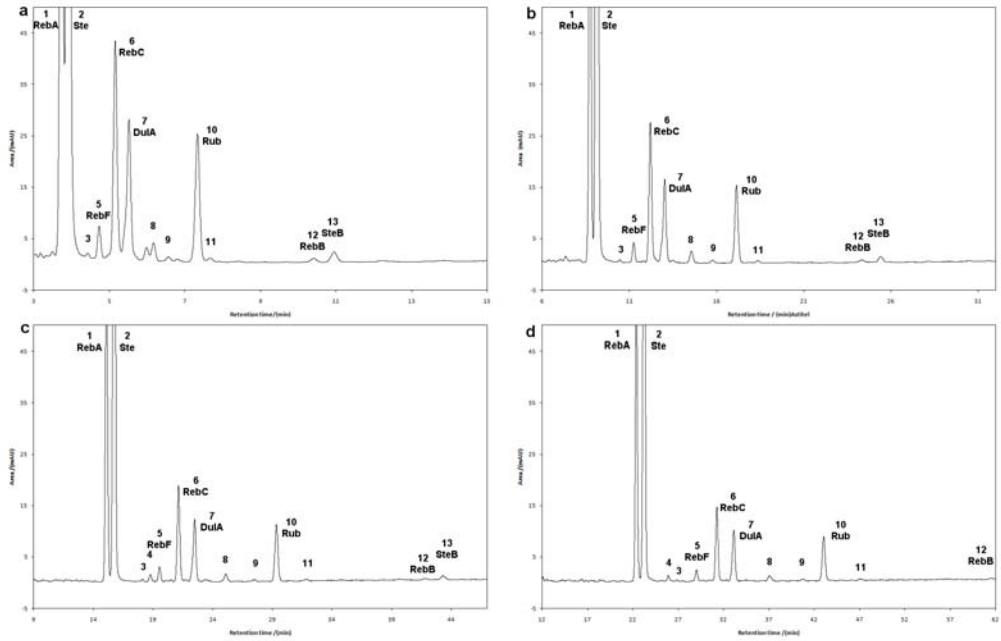

Figure 3: Resolution optimization with 1 to 4 columns:
a: 1 column ; L = 100 mm ; t_G = 12 min + 0 min isocratic hold ; R_s RebA – Ste = 1,3
b: 2 columns ; L = 200 mm : t_G = 24 min + 3 min isocratic hold ; R_s RebA – Ste = 1,8
c: 3 columns ; L = 300 mm ; t_G = 36 min + 6 min isocratic hold ; R_s RebA – Ste = 2,1
d: 4 columns ; L = 400 mm ; t_G = 48 min + 9 min isocratic hold ; R_s RebA – Ste = 2,2
Other conditions: see text.

Inspecting the chromatograms c and d, one can see that the resolution does not increase much: from 2.1 to 2.2. On the other hand, the total time needed for the analysis increases from 45 to 62 min. Moreover, in chromatogram d peak 13, SteB, does not elute within this large timeframe, again most probably due to the dependency of retention on pressure. As 3 columns, with a total length of 300 mm give enough resolution, almost to the baseline, it can be concluded that the fourth column is not necessary and a lot of time can be saved by only using 3 columns.

Advantage can be taken from the fact that the van Deemter curve for these columns has a very flat slope beyond the optimum (see Figure 2). Thus, the flow can be increased, up to the practical pressure limit of the instrument. As the Dionex instrument has a pressure limit of 800 bar, one can safely increase the flow up to a back-pressure of 750 bar. The resulting chromatogram is shown in Figure 4. The total time of this chromatogram is 35 min, and the resolution of the critical pair is almost the same as in Figure 3 – c. The flow in this experiment was 0.3 mL.min^{-1}

and the back-pressure was 740 bar. The same shift in elution order is again observed for peak 4 upon increasing the pressure to 740 bar.

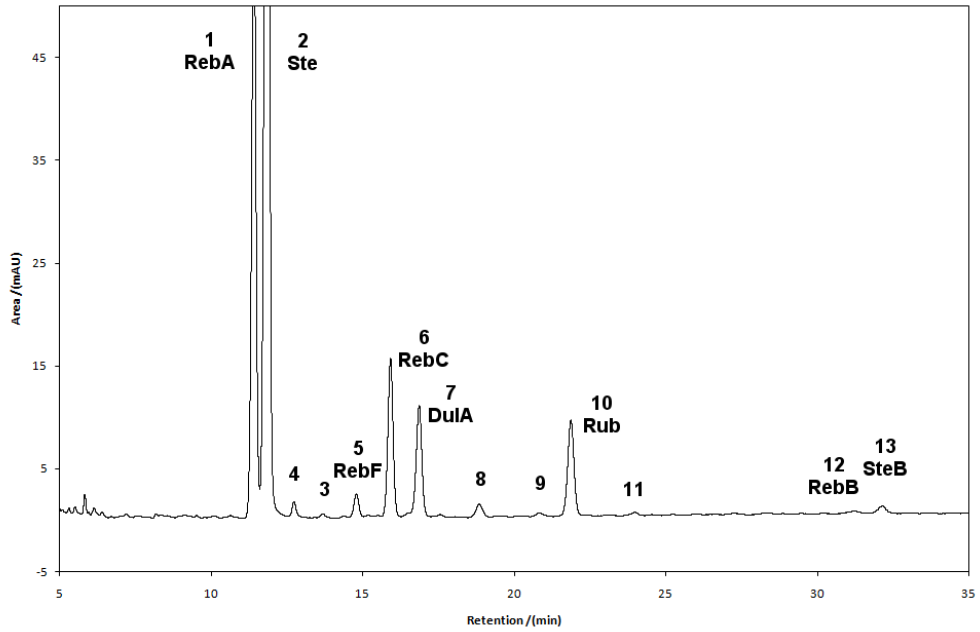

Figure 4: Resolution and speed optimized

There is of course more information in the chromatograms of Figure 3, a – d. The fact that peak 4 hides under another peak in chromatograms a and b raises the possibility that other peaks might also be co-eluting. The usual way to track co-eluting peaks is by assessing the "peak purity". This is a feature that is usually offered in the chromatography software. Each software vendor uses his own proprietary algorithm, but the basic principle is that the ratio of the absorbances at two different wavelengths is assessed across a peak, to a minimum of 3 different retention times, at the up slope, the apex and the down slope (Dolan, 2002). When two peaks co-elute, it is highly probable that the ratio of absorbances will change across the peak, allowing the possibility to trace these peaks.

However, in the analysis of steviol glycosides, this approach cannot be used, because all these compounds have the same chromophore, an ester function. Therefore, it is expected that the UV absorption spectra of the these compounds are all very similar. This is confirmed by experiment: all steviol glycosides have the same slope for their calibration curve, (Geuns, 2008). Consequently, an alternative approach is needed in this case.

For a single compound, eluting in a chromatogram, it is expected that the peak width will change proportionally to the increase in column length as the number of columns is increased; that is, if the ratio t_G/t_M is kept constant for the different column lengths (Cabooter *et al.*, 2010a). If two peaks are co-eluting on the other hand, the resolution will change at least a bit when more columns are connected to the system. This will cause peak broadening, even if the resolution is less than 0.5 ; the minimal resolution to discern two peaks. This peak broadening can be evaluated by plotting the ratio of the plate count *N* for two different column lengths, corrected for the length of the longest column. So, e.g. for plotting the ratio of 20 cm versus 10 cm, *N(2)/2*N(1)* is plotted against the peak number, with *N(2)* the plate count with two columns connected, and *N(1)* the plate count with one column connected. In Figure 5, this is done for the different chromatograms of Figure 3.

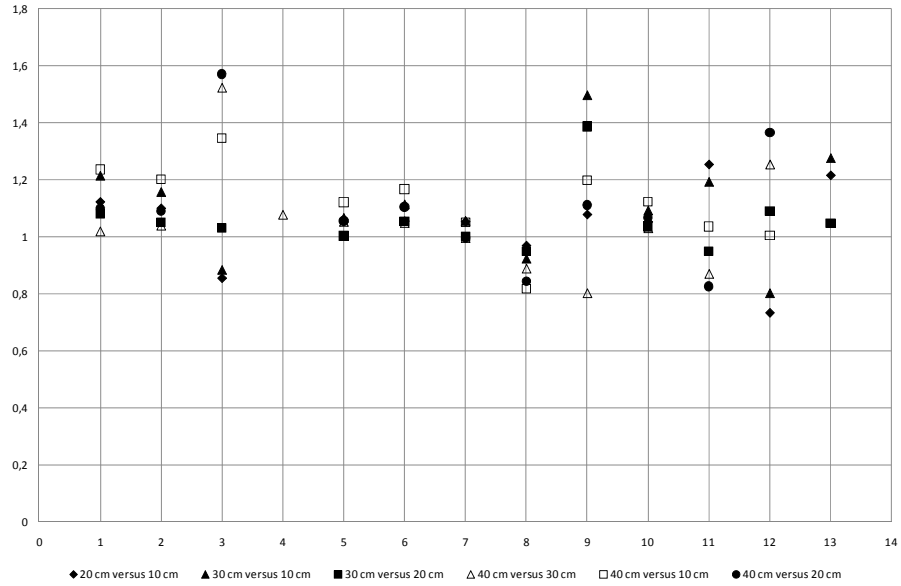

Figure 5: Ratio of peak counts for each of the peaks of steviol glycosides.

One can see that the ratio of plate counts is always greater than 1. This can easily be explained by the fact that a two column system is less prone to extra column band broadening than a one column system. For peaks one and two, the different points are nicely grouped, so probably these peaks originate from a single compound. The points for peak three on the other hand are more scattered, indicating that there is a problem with this peak. It could be a co-eluting pair, but as the peak area varies more than that of the other peaks on the different columns, it is not cer-

tain that it is the same compound in the different chromatograms. Other deviations are noted for peak 9 and to a lesser extent, peaks 11 and 12.

Of course, this approach has its limitations, just as the assessment of peak purity. It is impossible to prove that a peak originates from only a single compound. In other words: it can be proven that a peak comes from a co-eluting pair, and not the reverse. This means that a small peak hidden under a large peak cannot be detected, but that is eventually the limit of any chromatographic, or more generally any analytical system.

To conclude these experiments, it can be stated that there are enough hints to justify further experiments. There are almost certainly more compounds hidden under some of the peaks in the chromatograms of Figure 3 and Figure 4.

Optimizing the detection of peaks

As our previous experiments indicated that there might be several co-eluting compounds, a second series of experiments was done in order to detect as many compounds as possible. These experiments were done using the Agilent instrument (See Methods and Materials) using an Acquity BEH C_{18} column of 50 x 2.1 mm (d_p = 1.7 µm). The mobile phase consisted of 0.1 % aqueous formic acid (solvent A) and acetonitrile, ACN (solvent B). The injection volume was 0.5 µL and the detection wavelength 200 nm.

First, seven scouting runs were made using different gradients to find optimal conditions revealing the most peaks for the starting and end concentrations of ACN, and the gradient steepness. For each chromatogram, the number of peaks that was found by the integration software was recorded. The results are collected in Table 1. Runs 2 and 7 clearly show more peaks than any of the other runs.

Table 1: Results of the scouting gradient runs

	Gradient from … to …			number of
Run N°	Volume fraction B	Volume fraction B	t_G	peaks found
1	0.05	0.95	4	12
2	0.05	0.95	16	20
3	0.4	0.6	4	15
4	0.4	0.6	16	17
5	0.25	0.75	4	12
6	0.25	0.75	16	17
7	0.31	0.93	6	18

The chromatogram that was obtained from run 2 is depicted in Figure 6. The 20 peaks are indicated with bars. The first peak in the chromatogram has a leading shoulder, see Figure 6, inset.

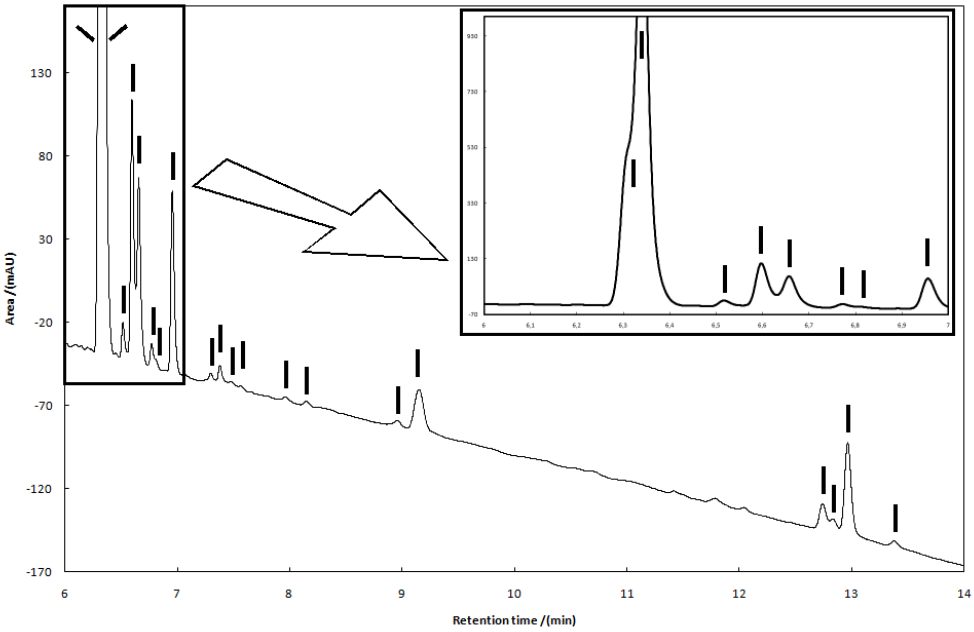

Figure 6: Chromatogram from stevia sample (Run 2), 0.05 - 0.95 B, t_G = 16 min. Inset: magnification of the first part of the chromatogram.

As some of the peaks in the chromatograms of Run 2 (Figure 6) and Run 7 (not shown) are very small, it is important to verify that these peaks are actual compounds, and not merely baseline disturbances. This can be done by applying a peak summation procedure. This summation is a very common procedure in spectroscopy (UV, IR, and especially NMR) where it is routinely used to enhance the signal-to-noise ratio (Skoog *et al.*, 2007). Run 2 and Run 7 were each repeated 7 times, and the resulting chromatograms summed up for each run. Because noise has a random nature, and actual signals (or peaks in this case) don't, summing up and averaging the resulting chromatograms will increase the signal – to – noise ratio by a factor $n^{1/2}$, with n the number of chromatograms. This technique makes it more straightforward to distinguish actual peaks from noise. This peak summing procedure confirmed that all peaks detected in Runs 2 and 7 were actual peaks.

In order to improve the resolution further, Runs 2 and 7 were repeated on a longer column. Run 2 was repeated on a 10 cm column (100 x 2.1 mm; d_p = 1.7 µm), and

t_G = 32 min. All other parameters were the same as above. This chromatogram yielded 25 peaks. Run 7 was also repeated on the same 10 cm column with t_G = 12 min. This chromatogram revealed 21 peaks.

In conclusion, Run 2 clearly gives the best result, and this chromatogram was repeated on a 20 cm column (200 x 2.1 mm; d_p = 1.7 µm) with t_G = 64 min. This chromatogram is shown in Figure 7.

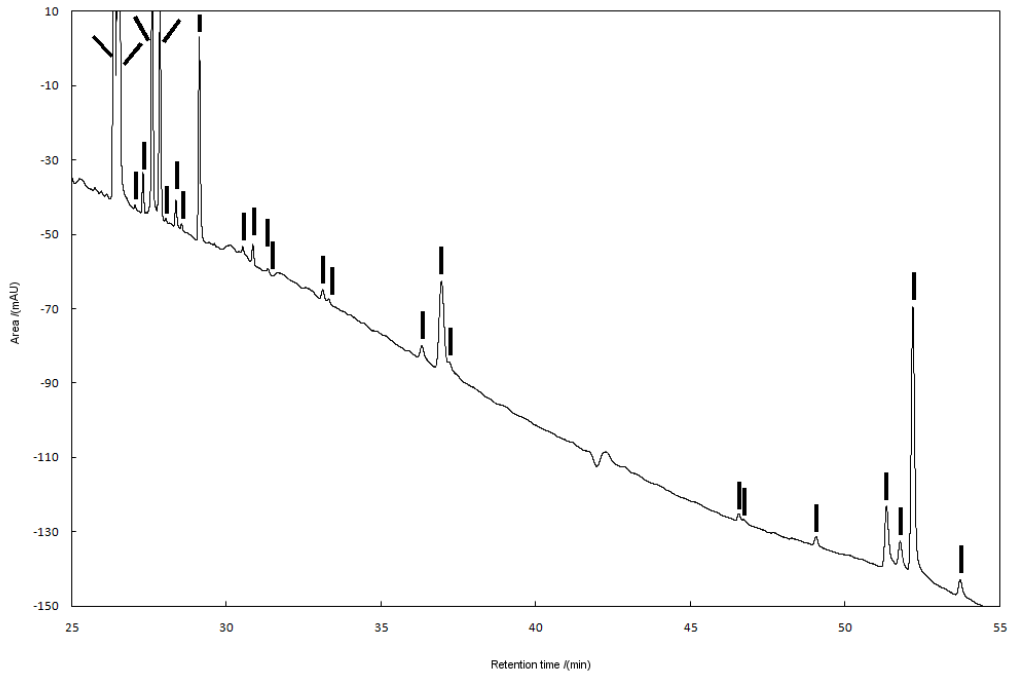

Figure 7: Chromatogram of stevia sample on 20 cm column.
Experimental conditions: see text.

A total of 26 peaks is visible, the majority of the unknowns elutes rather late in the chromatogram, indicating that these are fairly non polar compounds. This chromatogram was again repeated on a 25 cm column, but this did not yield any additional peaks, but confirmed the presence of the 26 already found. So, Figure 7 can be retained as the "best" chromatogram, i.e., it yields the most peaks in the shortest time.

Based on the known retention times of the 8 steviol glycosides that could be identified in Figure 4, the corresponding peaks in the chromatogram of Figure 7 can be assigned. This is shown in Figure 8, which is an enlargement of the first part of

Figure 7, up to a retention time of 40 min, and a slight enlargement of the area (Y) axis.

In the near future, the authors will certainly try to identify the unknown peaks by LC – MS. With this technique it will be possible to identify a number of additional steviol glycosides, although it is perhaps difficult – or impossible – to distinguish between steviol glycoside isomers. Moreover, it is perfectly possible that some other compounds that are co-extracted from the stevia leaves, such as triterpenes and flavonoids are present in the sample (Brandle *et al.*, 1998). It should be relatively easy to distinguish such compounds from steviol glycosides.

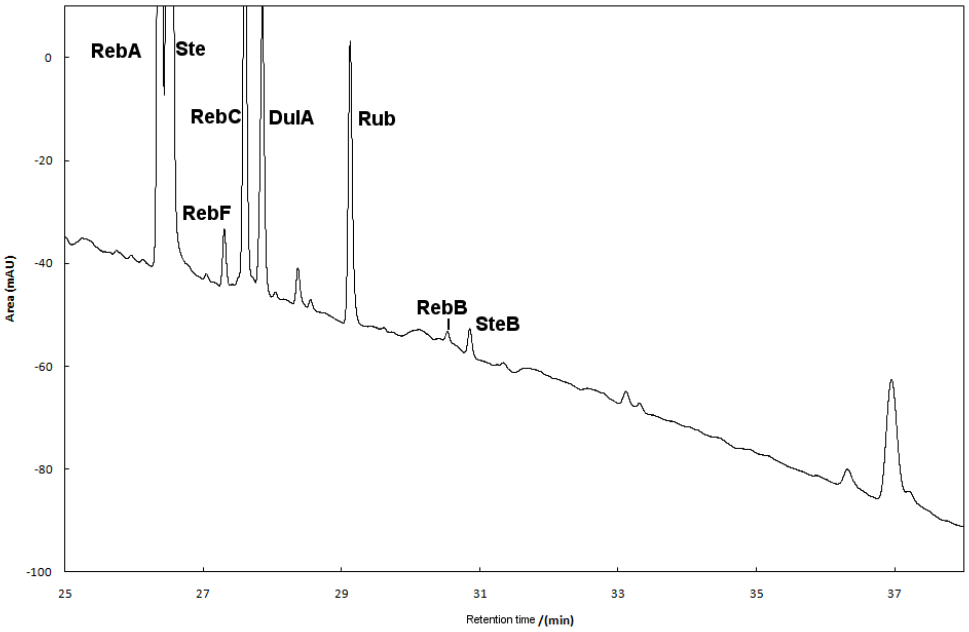

Figure 8: Enlargement of the previous chromatogram, with peaks assigned.

Conclusion

In this work it is shown by UHPLC analysis that samples of stevia are far more complex than initially assessed by normal HPLC. Some of these, yet unknown, compounds may be contributing to the off-taste of stevia, hence it is important that these compounds are fully characterized.

Using an automated column coupling system and the high resolution that is possible with an UHPLC instrument, and small particle columns, the analysis of a stevia sample could be optimized quite rapidly. The resolution of the critical pair in the chromatogram could be optimized in a total retention time of 35 min, using 300 x 2.1 mm columns with d_p = 2.2 µm. An added advantage of the column coupler is that co-eluting peaks can be tracked relatively easily by looking at their peak count on columns of different length. This method is in general more reliable than assessing peak purity, which is used commonly.

The column coupler was also very suitable for a rapid method development. Fast scouting runs could easily done on a short, 5 cm column, thus enabling optimal separation conditions to be found. After several scouting gradient runs, the stevia sample could be separated into 26 compounds in 55 min. Of these 26 peaks, only 8 could be assigned to known steviol glycosides.

It is hoped to identify the unknown peaks in these chromatograms by means of other techniques, such as LC-MS or LC-MS-MS. A full characterization might be possible by off-line NMR. A further increase in resolution is possible by LCxLC.

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