Development of a Comprehensive 2D-LC-MS Method for the Analysis and Identification of New Steviol Glycosides in *Stevia Rebaudiana*

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ABSTRACT

Objective: *Stevia Rebaudiana* extracts and plant materials are increasingly being used as natural sweeteners, because of their low-caloric characteristics. Steviol glycosides are responsible for the sweet taste of stevia plant material. Up to date, more than fifteen steviol glycosides have been described and identified. It has, however, been reported that Stevia extracts can have a bitter, metallic aftertaste. It is therefore crucial to detect and characterize as many steviol glycosides as possible to identify the compound(s) responsible for the bad aftertaste and eliminate them in the production process (Gardana et al., 2010, Zimmerman et al., 2011 and Chaturvedula et al., 2011). In this study, a comprehensive two-dimensional liquid chromatography set-up was used to obtain a high efficiency separation of a *Stevia Rebaudiana* extract.

Materials and Methods: A capillary RP-C18 column (Acclaim PepMap RSLC RP-C18; 300 μm x 150 mm; 2 μm) was used for the first dimension separation in gradient mode at a flow rate of 1.0 μl/min. Mobile phase was gradually increased from 22% ACN to 60% ACN in 120 min. The second dimension consisted of a HILIC-Amide column (Acquity UPLC BEH Amide; 2.1 mm x 100 mm; 1.7 μm) operated isocratically at a flow rate of 0.55 ml/min. This allowed a 2nd dimension separation/cycling time of less than two minutes.
The comprehensive 2D-LC was subsequently connected to an electrospray ionisation micro quadrupole time-of-flight mass spectrometer (ESI-Micro-Q-TOF) for the detection of the steviol glycosides.

Results: A highly efficient 2D-LC-MS separation was obtained and 34 steviol glycosides were tentatively identified based on their MS fragmentation patterns.

Conclusion and Broader Impacts: A previous study (Cabooter et al., 2010) demonstrated the existence of a number of unknown steviol glycosides that could not be identified at that time. In this study, these unknown steviol glycosides were identified based on their MS fragmentation pattern at different fragmentation voltages. The identification of new steviol glycosides and elucidation of their structure is crucial to improve the taste of Stevia extracts.

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Introduction

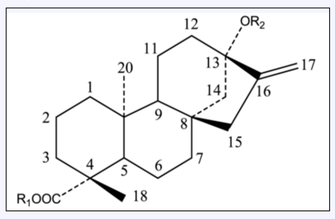
Stevia rebaudiana is a plant originating from Brazil and Paraguay and is often referred to as "the sweet herb of Paraguay". *Stevia rebaudiana* plant materials and extracts are increasingly being used as natural sweeteners, because of their low-caloric characteristics. It has been used for many years in Japan, Korea, China, Brazil and Paraguay and has also recently been approved in the US and the European Union (2008 and 2011, respectively).

The plant contains high concentrations of steviol glycosides which are responsible for the sweet taste. Steviol glycosides are ent-kaurene-type diterpene glycosides, composed of an aglycon backbone with a various number of sugars attached to it. Up to date, more than twenty steviol glycosides have been described and identified. The structures of the main known steviol glycosides are shown in Figure 1 [1,2,3].

It has, however, been reported that Stevia extracts can have a bitter, metallic aftertaste. It is therefore crucial to detect and characterize as many steviol glycosides as possible to identify the compound(s) responsible for the bad aftertaste and eliminate them in the production process.

A previous study of Cabooter et al. [4] demonstrated the existence of a number of unknown steviol glycosides based on their *m/z* ratio, that could not be identified at the time. The study also indicated the presence of co-eluting compounds which were impossible to separate through conventional one-dimensional high-performance liquid chromatography.

In the present study, a capillary liquid chromatography (cap-LC) set-up was designed to obtain a high efficiency separation of a *Stevia rebaudiana Bertoni* extract. The LC set-up was subsequently coupled to an electrospray ionisation micro quadrupole time of flight mass spectrometer (ESI-micro-q-TOF MS) to identify as many new steviol glycosides as possible based on their fragmentation patterns at different fragmentation voltages. Due to the large number of (overlapping) peaks, a 2D-LC-MS method was developed as well.



Compound	R1	R2	M_r
Steviol	H	H	318
Steviolbioside	H	β-Glc-β-Glc(2-1)	642
Rubusoside	β-Glc	β-Glc	642
Dulcoside A	β-Glc	β-Glc-α-Rha(2-1)	788
Stevioside	β-Glc	β-Glc-β-Glc(2-1)	804
Rebaudioside B	H	β-Glc-β-Glc(2-1)β-Glc(3-1)	804
Rebaudioside F	β-Glc	β-Glc-β-Xyl(2-1)β-Glc(3-1)	936
Rebaudioside C	β-Glc	β-Glc-α-Rha(2-1)β-Glc(3-1)	950
Rebaudioside A	β -Glc	β-Glc-β-Glc(2-1)β-Glc(3-1)	966
Rebaudioside E	β-Glc-β-Glc(2-1)	β-Glc-β-Glc(2-1)	966
Rebaudioside D	β-Glc-β-Glc(2-1)	β-Glc-β-Glc(2-1)β-Glc(3-1)	1128

Figure 1 : *Structures of the main known steviol glycosides from Stevia rebaudiana. (Glc= Glucose, Rha= Rhamnose and Xyl= Xylose)*

Experimental

Apparatus

Dionex Ultimate 3000 RSLC system with capillary NC pump and UHPLC loading pump.

Sample

Commercial Stevia rebaudiana extract (Medherbs, Germany).

1000 ppm, 10 000 and 20 000 ppm solution in H_2O (m/v)

Samples were filtered prior injection.

1st dimension separation parameters

- Capillary Columns:
  - Acclaim PepMap RSLC RP-C18 (**300 µm** x 150 mm; dp=1.9 µm)
  - Hypersil Gold (**125 µm** x 150 mm, d_p= 2.0 µm)
- Mobile phases:
  A: H_2O, 0,1% formic acid (FA) (pH= 2.8)
  B: ACN 0,1% formic acid
- Gradient mode
- Temperature: 40 °C
- Flow rate: 1 µl / min
- Injection Volume: 1 µl
- UV detection: 200 nm

Min	% A	% B
0	22	78
36	35	65
50	40	60
62	46	54
70	54	46
85	62	38
115	95	5
120	95	5

Table 1 : *Gradient 1st dimension separation*

2nd dimension separation parameters

- Columns:
  - Acquity BEH Amide column (**2.1 mm** x 100 mm, d_p= 1.7 µm)
  - Acquity BEH Silica column (**2.1 mm** x 100 mm, d_p= 1.7 µm)
- Mobile phase:
  75/25 ACN/H_2O 10 mM ammonium acetate pH 6.0
- Isocratic mode
- Temperature: 40 °C
- Flow rate: 0.5 ml / min

Mass Spectrometer parameters

- ESI-Micro-Q-TOF in negative ionisation mode
- Source Temperature: 160 °C
- Capillary voltage: 4500 V
- Mass spectrometer analytical software: Brüker Compass v 1.3
- Data processing: Progenesis & Microsoft Excel

2D-LC parameters

- Sample loops: 5 µl loops
- Sampling time: 2 minutes

Results: Chromatographic separation

Optimization of 1st dimension separation: Capillary RP-LC

- Two different capillary columns were evaluated (Acclaim Pepmap and Hypersil Gold). Both columns showed similar separation efficiency, but Acclaim Pepmap was chosen because of better sample loadability (data not shown).
- The effect of organic modifier (ACN and MeOH) and mobile phase pH (pH 2 and pH 7) on the resulting separation was evaluated
- Gradient settings were optimized for maximal spreading of the peaks

- **1st dimension separation:**
  - **Acclaim Pepmap RP-18 column (300 µm x 150 mm; 1.9 µm)**
- **A total of 82 peaks were separated in 120 min (Fig 2)**
- **A large number of (overlapping) peaks were present**

➔ **Development of a comprehensive 2D-LC method**

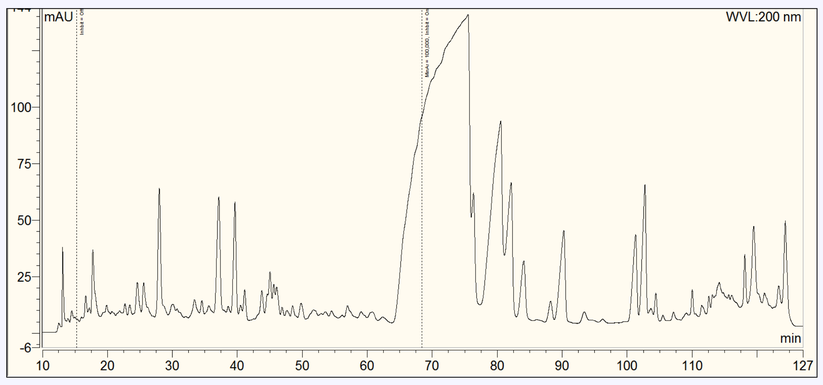


Figure 2 : *UV chromatogram for the 1D-separation of Stevia rebaudiana sample 20 000 ppm. Gradient: 22% ACN 0.1% FA (pH 2.8) to 95% ACN 0.1% FA (pH 2.8) in 120 min. UV detection 200 nm. Flow rate: 1 µl / min. Injection Volume 1 µl. Temperature: 40 °C.*

Optimization of 2nd dimension separation: UHPLC HILIC

- Orthogonal separation mechanism compared to 1st dimension
- Larger internal diameter (2.1 mm) for second dimension column
  → Dilute sample composition eluting from 1st dimension separation
  → Large flow rate (0.5 mL/min) to allow for short analysis time (1-2 min)
  → Injections of 1-2 µl on HILIC column
- Isocratic elution omits re-equilibration ⇒ faster analysis
- 2 columns were compared: Acquity BEH Amide and Acquity BEH Silica → Amide column was chosen because of better robustness of separation with different sample compositions (data not shown)
- Mobile phase was adjusted for maximal spreading of peaks of interest
- 2D-Hyphenation:
  - sampling time: 2 min
  - Sampling loops: 5 µl

- **2nd dimension separation:**
  - **Acquity BEH Amide column (2.1 mm x 100 mm; 1.7 µm)**
- **Large dilution effect 2nd dimension → insufficient detection sensitivity for smaller peaks (no detection problems for most abundant peaks)**

➔ **Future prospect: heart-cut 2D-LC-MS**

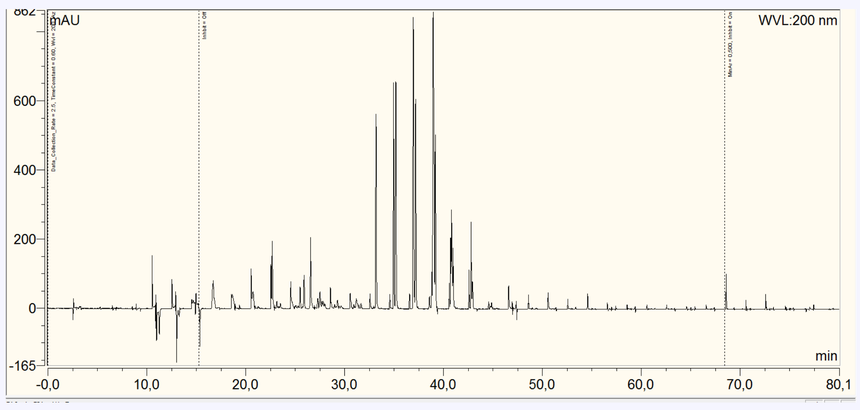


Figure 3 : *UV chromatogram of 2D-LC separation of Stevia rebaudiana sample 100 000 ppm (after blank run subtraction). 1st dimension: gradient mode, 2nd dimension: isocratic mode. UV detection 200 nm. Flow rate 1st dimension: 1 µl / min. Flow rate 2nd dimension: 0.5 ml / min. Injection Volume 1 µl. T= 40 °C.*

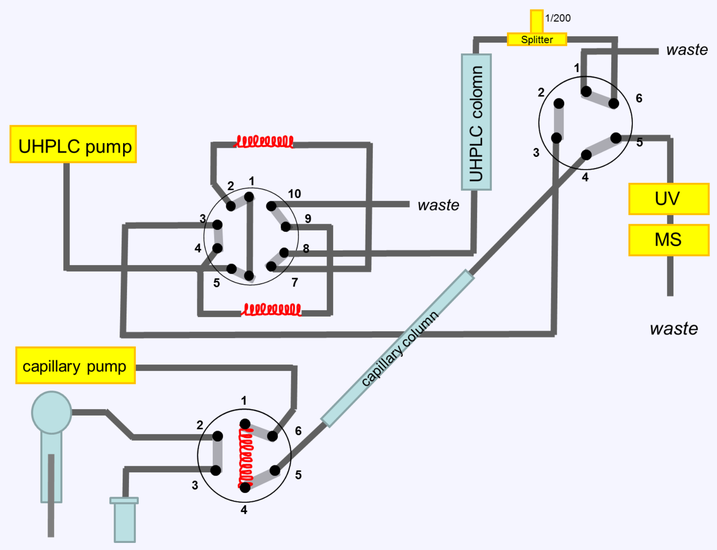


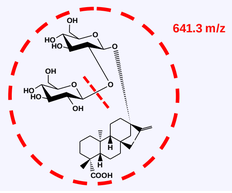
Figure 3 : *Scheme heartcut-2D-LC setup*

Results: Identification of steviol glycosides

Cap-LC-MS: Detection with ESI-micro-q-TOF MS

- Fragmentation tables were first established for known steviol glycosides
- Rules were deduced based on fragment intensity at different fragmentation voltages (10-100 eV) to identify sugar substituent types and positions (Figure 5)
- Fragmentation tables were constructed for commercial Stevia extract
- 37 steviol glycosides were detected and could tentatively be identified based on their retention time, m/z and fragmentation patterns at different voltages (10-100 eV)

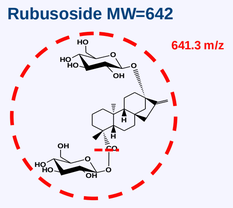
Steviolbioside MW=642



Fragment formed at intermediate voltage (30-40 eV): β-Glc-β-Glc (2-1)

m/z	317.2	479.3	641.3
10 eV			100%
20 eV			100%
30 eV	2%	13%	85%
40 eV	12%	47%	40%
50 eV	39%	53%	8%
60 eV	72%	28%	
70 eV	100%		

Rubusoside MW=642



Fragment formed at low voltage (10 eV): β-Glc (R2)

m/z	317.2	479.3	641.3
10 eV		57%	43%
20 eV		86%	14%
30 eV		93%	7%
40 eV	17%	83%	
50 eV	37%	63%	

Figure 5: *Example of fragmentation pattern of 2 steviol glycosides and elucidation of fragmentation behaviour which can be applied to identify unknown steviol glycosides*

Retention time	m/z	Compound	Retention time	m/z	Compound
45.8	965.4		92.6	949.4	
48.0	1127.5	Rebaudioside D	93.0	965.4	
50.2	1273.5		94.1	787.4	
53.6	1127.5		96.3	641.3	Rubusoside
53.9	949.4		100.5	787.4	
54.2	1111.5		100.6	965.4	
55.0	935.4		100.8	949.4	
55.2	1127.5		101.7	641.3	
57.9	803.4		101.8	479.3	
74.4	965.4		103.9	787.4	
76.6	965.4		105.0	787.4	
79.1	1127.5		107.5	803.4	
81.0	965.4	Rebaudioside A	108.4	641.3	Steviolbioside
83.5	641.3	Stevioside	109.9	787.4	
83.5	803.4		112.2	641.3	
84.8	965.4		113.3	317.2	
86.2	641.3		113.8	625.3	
91.0	935.4		119.5	317.2	Steviol
91.6	787.4				

Table 2: *Overview of detected steviol glycosides with their m/z and suggested structure*

Discussion & Conclusions

- **Steviol glycosides are responsible for the sweet taste of *Stevia Rebaudiana*. Up to date, over 20 steviol glycosides have been identified**
- **A cap-LC-MS method was succesfully developed for the separation of a large number of compounds in a commercial Stevia extract. Development of a 2D-LC-MS method to obtain an even higher efficiency is ongoing.**
- **First dimension: Capillary Pepmap column in gradient mode**
- **Second dimension: HILIC Amide column operated at high flow rate (0.5 ml/min)**
- **2D Hyphenation: - Sampling time: 2 min**
  - Sample loops: 5 µl
- **37 steviol glycosides were detected and could tentatively be identified based on their MS fragmentation pattern at different fragmentation voltages**
- **Future prospects:**
  - **Heartcut 2D-LC-MS**
  - **Further identification of these compounds**

References

(1) *Gardana, C.; Scaglianti M.; Simonetti, P. Evaluation of steviol and its glycosides in Stevia Rebaudiana leaves and commercial sweetener by ultra-high performance liquid chromatography-mass spectrometry. J.Chromatogr.A* ***2010****, 1217, 1463-1470.*

(2) *Zimmerman., B.; Tandem mass spectrometric fragmentation patterns of known and new steviol glycosides with structure proposals. Rapid Commun. Mass Spectrom.* ***2011****, 25, 1575-1582.*

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(4) Cabooter, D.; Amery, R.; Jooken, E.; Meesschaert, B.; Desmet, G. Ultra-High Performance Liquid Chromatography for the analysis of steviol glycosides. *Geuns, J. (Ed.), Proceedings of the 4th Stevia Symposium 2010 organized by EUSTAS: Stevia: Science, no fiction, Leuven* **2010,** pp.83

Acknowledgements

Remco Swart and his team of Thermo-Fisher Scientific (Amsterdam, Netherlands) is kindly thanked for the gift of the capillary column and their helpful suggestions.

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