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Scale-Up of an analytical HPLC method for steviol glycosides to a preparative approach

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SUMMARY

Steviol glycosides are the main sweetening compounds in *Stevia rebaudiana* and can be used as natural sugar substitutes due to their far higher sweetening power than normal sucrose or glucose. The sweetness is estimated to be about 400 times higher. This application describes an easy transfer of an existing analytical HPLC method to a preparative HPLC using overload experiments together with the KNAUER Scale-Up converter. Furthermore, this approach of scale-up is also applicable for different analytes and thus provides a fast scale-up.

INTRODUCTION

For several years research has been undertaken to find sugar substitutes that are calorie free but have the same taste and properties as classic sugar, for diabetics and as part of a calorie-controlled diet. One popular substitute is the so-called "Stevia" which is a mixture of steviol glycosides isolated from the plant stevia rebaudiana [1]. The steviol glycoside rebaudioside A is the main compound of interest as it is the sweetest and less bitter compound of the extract. Often a mixture of rebaudioside A and stevioside is found in the "Stevia" products.

The development of a purification method with high yield of rebaudioside A, few stevioside impurities, and a high throughput would increase the economic output of stevia production.

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RESULTS

In analytical scale an isocratic method was developed for the purification of rebaudioside A and stevioside from stevia leaves. The previously described gradient method (application note **VFD0168**) was transferred to isocratic mode using the DryLab (Molnár-Institute, Germany) software. The isocratic method derived from the simulation was further developed for the gradient method. A concentration of 30:70 acetonitrile:water (v/v) showed the best performance (data not shown). A mix-standard of rebaudioside A, stevioside, rebaudioside C, dulcoside A, rebaudioside B, and steviolbioside with individual concentrations of 0.1 mg/mL was used as sample. Comparison of the gradient and isocratic method showed, that the two target peaks (rebaudioside A and stevioside) were nearly baseline separated but eluted later and were broader (**Fig. 1A**, 1 and 2). Hence, this isocratic method was transferred to an analytical column with 10 µm particles. This was done to ease the scale up to the preparative scale with a column with the same material.

Overload experiments in the analytical scale showed that 100 µL and 200 µL injection volume lead to overlapping of the two main peaks (**Fig. 1B**). Stevia extract obtained from dried stevia leaves was used for the experiments, both analytical and preparative scale.

The method was scaled up with the KNAUER ScaleUp Converter from the original ID 4.6 mm to an ID 20 mm column maintaining the length of 250 mm and thus keeping the HETP constant. The flow rate was increased from 1.2 mL/min to 22 mL/min.

Injections of up to 2 mL sample still showed a minimal separation of both peaks (**Fig. 2**). The matrix peak (1-5 min) increased significantly (**Fig. 2**, blue bars).

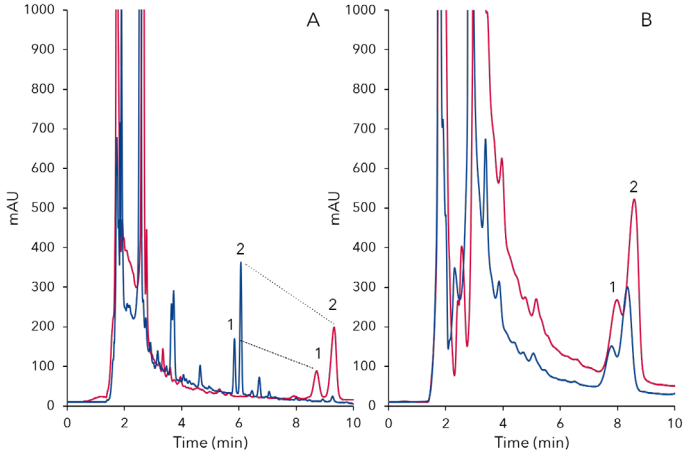


Fig. 1 A - Transfer gradient to isocratic method; 1) rebaudioside A, 2) stevioside; isocratic 30:70 acetonitrile/H2O, 20 µL injections, C18, 5 µm particle, 1.2 mL/min, 30°C B - Overlay chromatograms 100 µL (blue) and 200 µL (red) sample injection on analytical 10 µm particle column; 1) rebaudioside A, 2) stevioside; 30:70 acetonitrile/H2O, C18, 1.2 mL/min, 30°C

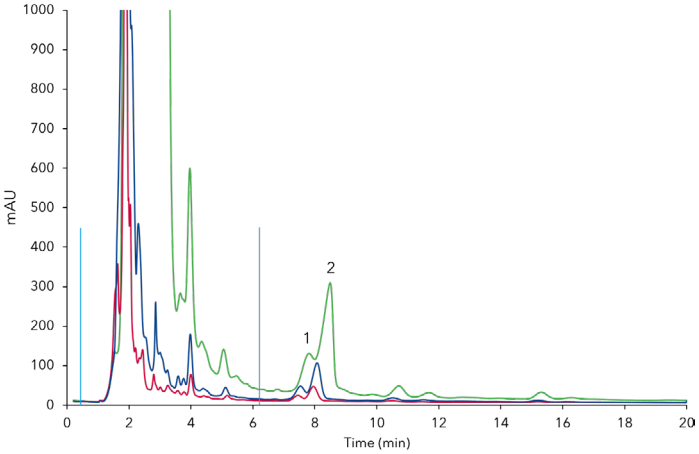
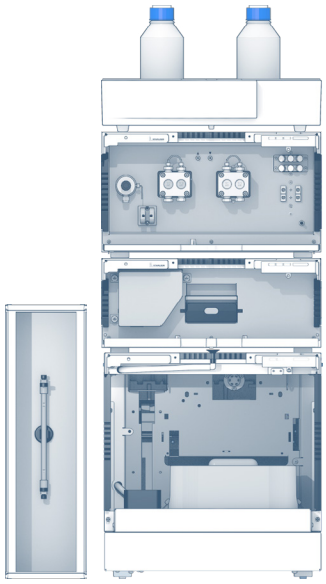


Fig. 2 Overload experiments on preparative column, 200 µL (red), 500 µL (blue), 2000µL (green); 1) rebaudioside A, 2) stevioside, blue bars – matrix, 25°C, 22 ml/min

MATERIALS AND METHOD

As analytical system a KNAUER AZURA HPLC Plus System was used as described in application note **VFD0168**. The eluent was a composition of A: water and B: acetonitrile. For the method transfer from gradient mode to isocratic a KNAUER Vertex Plus column filled with Eurospher II 100-10 C18 silica in a dimension 250 x 4.6 mm ID with precolumn was used. The flow was set to 1.2 mL/min. The scale-up was calculated using the KNAUER ScaleUp Converter [2] (**Fig A1**).

The AZURA Preparative HPLC system consisted of AZURA P 2.1L 100 mL sst pump with ternary LPG module, AZURA UVD2.1L detector with 3 mm, 2 µl flow cell, an AZURA assistant module with a 12 port multi position 1/8" sst valve (solvent selection), a 6 port 2 position 1/16" sst injection valve, a P4.1S 50 ml sst feed pump and a Labocol vario-4000 fraction collector. For the method transfer from analytical to preparative scale a KNAUER Vertex Plus AX column, Eurospher II 100-10 C18, 250 x 20 mm ID was used. The flow was scaled to a rate of 22 mL/min. Peaks were fractionated using 3 mL fractions and analyzed using an analytical HPLC as described in application note **VFD0168**.

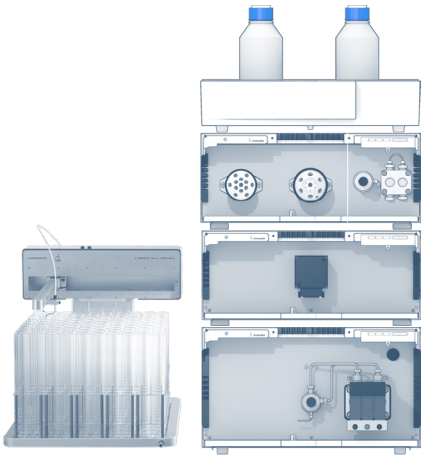


Analytical system configuration

CONCLUSION

Using simulation software provided a quick transfer from gradient elution to an isocratic method which could be established using the later preparative column material but in analytical column dimensions. In the following a fast method-transfer from an analytical to a preparative HPLC approach could be achieved using the KNAUER ScaleUp Converter. The isocratic HPLC method was successfully transferred to a preparative scale while keeping the elution characteristics of the target analytes rebaudioside A and stevioside. The overload experiments also showed a maximum column loading capacity both analytical and preparative. Thus, for following experiments an easy estimate concerning sample load is possible. In addition, the overload experiments depict the need for matrix reduction, as the matrix signal is close to overlay the target signal if not being decreased.

Concluding the derived preparative method was well suited as a starting point for follow up experiments as described in application note **VFD0171**.



Preparative system configuration

REFERENCES

[1] "Stevia Leaf to Stevia Sweetener: Exploring Its Science, Benefits, and Future Potential" P. Samuel, K. T. Ayoob, B. A. Magnuson, et al. J Nutr, Volume 148, Issue 7, 1 July 2018, Pages 1186S–1205S.

[2] KNAUER HPLC Method Converter (link)

ADDITIONAL RESULTS

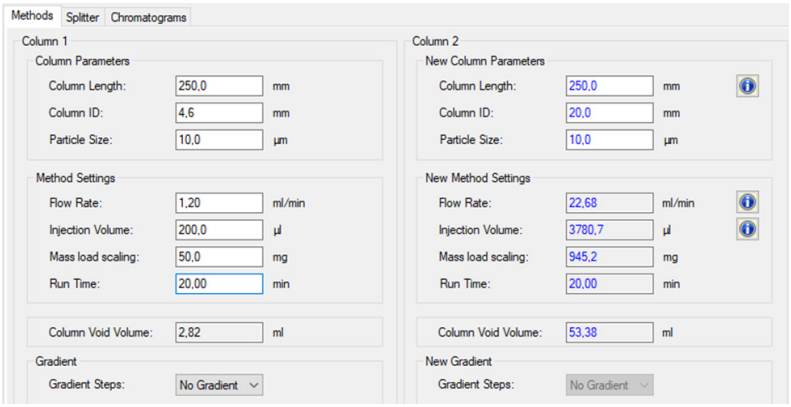


Fig. A 1 Linear scale up with KNAUER Scale-up converter

ADDITIONAL MATERIALS AND METHODS

Tab. A1 Sample preparation

For the analytical experiments 1 g of dried stevia leaves were extracted in water as described in application note VFD0168.
A highly concentrated sample of stevia extract was prepared for the preparative experiments. 15g of dried stevia leaves were extracted in 200 mL water, prepared as described in application note VFD0168 and adjusted to a final volume of 250 mL. Additional centrifugation steps were necessary to remove particles from the solution before and after filtration.

Tab. A2 Method parameters

	Analytical	Preparative
Column temperature	30 °C	RT
Injection volume	50 µL; 100 µL; 200 µL	500 µL; 1000 µL; 2000 µL
Injection mode	Full loop / Partial loop	Full loop
Detection wavelength	UV 210 nm	UV 210 nm
Data rate	20 Hz	2 Hz

Tab. A3 Pump parameters (analytical)

Eluent A	ACN:H_2O 30:70 (v/v)
Flow rate	1.2 mL/min
Pump program	isocratic 30% B

Tab. A4 Pump parameters (preparative)

Eluent A	ACN:H_2O 30:70 (v/v)
Flow rate	22 mL/min
Pump program	isocratic

Tab. A5 System configuration

Instrument	Description	Article No.
Pump 1	AZURA P 2.1L, 100 mL, sst	APE20KA
Pump 2	AZURA LPG ternary module for Pump P 2.1L	AZZ00AB
Detector	AZURA UVD 2.1L	APH30EA
Flow cell	3 µl; 1/16"	
Assistant	AZURA ASM 2.1L Left: 12 Mpos,1/8"", sst Middle:6 Port 2Pos,1/16", sst Right: P4.1S, 50 mL, sst	AYASM
Fraction collector	Labocol Vario-4000	A591024
Column	KNAUER Vertex Plus AX Eurospher II 100-10 C18, 250 x 20 mm ID	25PE181E2N
Software	PurityChrom5 Basic KNAUER HPLC Method Converter	A2650 Link

RELATED KNAUER APPLICATIONS

VFD0168 - Oh so sweet - Quantification of steviol glycosides in Stevia samples with RP-HPLC

VFD0171 - Advantages of preparative online SPE compared to batch LC for stevia purification

VFD0174 - Determination of six steviol glycosides using reversed phased HPLC and online SPE