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

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SUMMARY

Steviol glycosides are the main sweetening compounds in *Stevia rebaudiana*. Due to their up to 400 times higher sweetening power compared to sucrose or glucose they are often used as natural sugar substitutes. To enable a commercial usage, the plant extracts need to be purified. In this work preparative online SPE (solid phase extraction) was investigated for improvement of overall purity due to reduction of matrix contamination.

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. Such subtitutes are important especially in diets necessary for diabetics and increasingly as part of the so-called "low-carb" movement. One popular substitute is “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 Stevia products contain a mixture of rebaudioside A and stevioside. The development of a purification method with high yield of rebaudioside A, only few stevioside impurities, and high throughput would increase the economic output of Stevia production.

RESULTS

For purification of rebaudioside A and stevioside from stevia leaves a gradient method for analysis of stevio glycosides was transferred to an isocratic method (VFD0170). The final method was up-scaled with the KNAUER up-scale converter [2] to an ID 20 mm column of same length as the analytical column, increasing the flow rate from 1.2 mL/min to 22 mL/min. Sample injections of up to 2 mL still showed a slightly separation of the rebaudioside A and stevioside peaks (**Fig. 1**). The matrix peak (1-5 min) increased significantly (**Fig. 1**, blue). Large sample matrix can negatively affect the separation abilities and wear off the main column therefore elimination of matrix prior to the purification is desirable. An online-SPE method was developed with a short preparative column in front of the main column. 10 mL of sample were loaded, the matrix washed away and then the target compounds were injected on the main column (**Fig. 2**). Comparison of the chromatograms of the classical batch process (**Fig. 1**) and the online-SPE process (**Fig. 2**) showed that the automated SPE process significantly decreased the matrix. The fraction analysis revealed that only a small part of the overlapping peak contained nearly pure rebaudioside A; fractions 3-5 approx. 15 mL with >90 % rebaudioiside A and <10 % stevioside (**Fig. 3**, B). The later fractions contained high amounts of stevioside but also still rebaudioside A (**Fig. 3**, C). The results showed that purification of highly pure rebaudioside A is possible by introducing an additional online-SPE step, however yield is sacrificed.

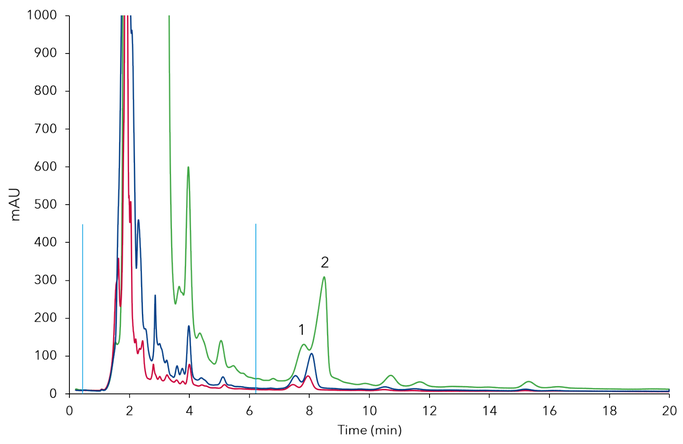


Fig. 1 Overload experiments on preparative column, 200 µL (red), 500 µL (blue), 2000µL (green); 1) rebaudioside A, 2) stevioside, blue bars – matrix, 25°C, 22 ml/min

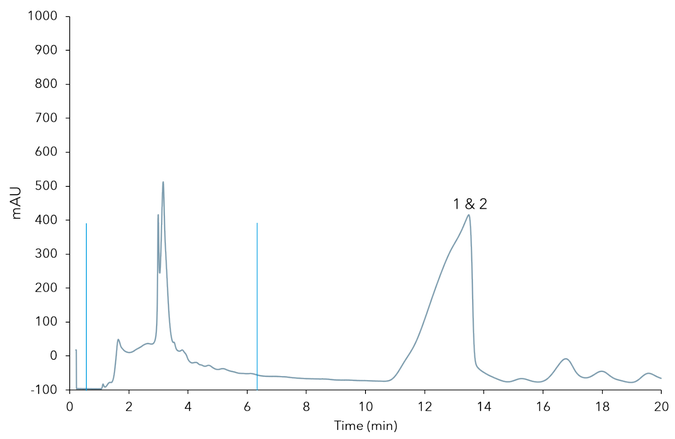


Fig. 2 Preparative online SPE, 10 mL loading; 1) rebaudioside A, 2) stevioside, blue bars – matrix, 25°C, 22 mL/min

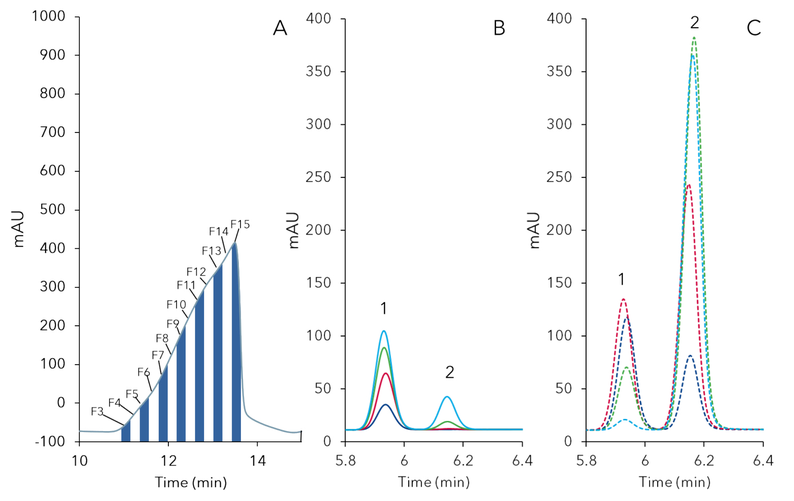


Fig. 3 Fraction analysis of preparative online-SPE purification (Fig.2) of rebaudioside A (1) and stevioside (2); A) fractionation of target peak, 5 mL fractions B) F3 (blue), F4 (red), F5 (green), F6 (light blue); C) F7 (red dashed), F10 (blue dashed), F12 (green sashed), F15 (light blue dashed)

MATERIALS AND METHODS

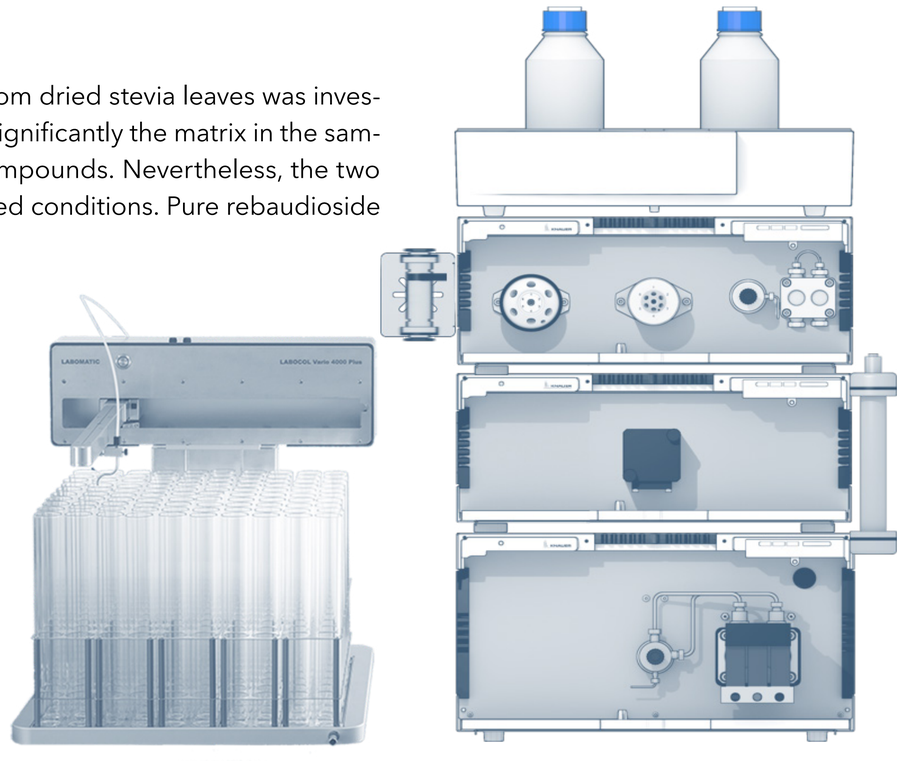
The AZURA Preparative HPLC system consisted of AZURA P 2.1L 100 mL sst pump with ternary LPG module, AZURA UVD2.1L detector with 3 mm, 2 µl flow cell, an AZURA assistant module with a 6 port multi position 1/8” sst valve (solvent selection), a 6 port 2 position 1/16” sst injection valve, a P 4.1S 50 ml sst feed pump and a Labocol vario-4000 fraction collector. Final purification method was divided into two phases: SPE loading and target purification. SPE loading: 1) Conditioning 1.5 min with 20 mL/min 100 % ACN; 2) Re-equilibration 2.5 min with 20 mL/min 20/80 ACN/H_20; 3) sample loading 1 min 5 mL/min 4) Washing 6.5 min with 20 mL/min; target purification: 20 min with 22 mL/min 30/70 ACN/H_2O; at 210 nm and 25°C. Fraction analysis was performed with AZURA analytical HPLC system as described in application note VFD0168.

CONCLUSION

A preparative HPLC approach for the purification of the most preferred steviol glycoside rebaudioside A from dried stevia leaves was investigated. During the method development an automatic online-SPE method was established thus reducing significantly the matrix in the sample. That should protect the main column from contamination and increases the loading with the main compounds. Nevertheless, the two components rebaudioside A and stevioside are coeluting and a clean separation is not possible under tested conditions. Pure rebaudioside A can be purified but with low yield.

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] Scale up converter: https://www.knauer.net/en/knauer-scaleup-converter/p14082



Additional information

VFD0171

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ADDITIONAL RESULTS

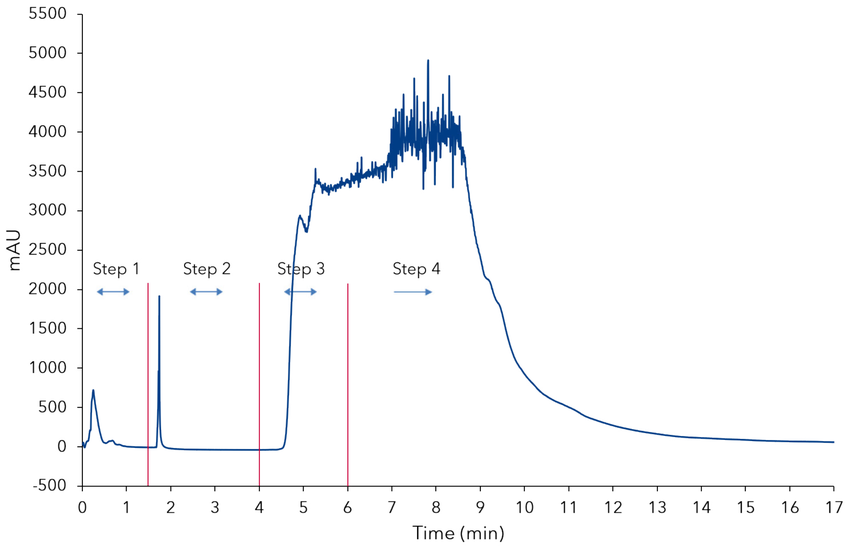

Fig. 1A Sample loading on SPE column, 10 mL sample, step 1 - conditioning, step 2 - re-equilibration, step 3 - sample loeading, step 4 - washing

ADDITIONAL MATERIALS AND METHODS

Tab. A1 Method parameters (preparative online-SPE)

Eluent A	100 % ACN				
Eluent B	20 %/80 % ACN/H_2O				
Sample	Concentrated stevia extract				
Step	Flow rate	Time (min)	% A	% B	Sample (%)
Conditioning	20 mL/min	1.5	100	0	0
Re-equilibration	20 mL/min	2.5	0	100	0
Sample loading	5 mL/min	2	0	0	100
Washing	20 mL/min	9.5	0	100	0
Run temperature	25°C	Run time	15.5 min		
Injection volume	10 mL	Injection mode	Feed pump		
Detection wavelength	210 nm	Data rate	2 Hz		
		Time constant	0.05 s		

Tab. A2 Method parameters (preparative method)

Eluent A	30 %/70 % ACN/H_20		
Eluent B	-		
Gradient	isocratic		
Flow rate	22 mL/min	System pressure	80 bar
Run temperature	25°C	Run time	20 min
Injection volume	From above	Injection mode	-
Detection wavelength	210 nm	Data rate	2 Hz
		Time constant	0.05 s

Tab. A2 System configuration & data

Instrument	**Description**	**Article No.**
Pump	AZURA P 2.1L, 100 mL, SST AZURA ternary module for P 2.1L	APE20KA AZZ00AB
Detector	AZURA UVD 2.1L	ADA01XA
Assistant	Left: 6 Mpos,1/8"",sst Middle:6Port2Pos,1/16",sst Right:P4.1S, 50ml,sst	AYASM
Flow cell	3 mm, 2 µL; 1/16"	A4069
Column	Eurospher II 100-10 C18 250x4.6 mm Eurospher II 100-10 C18 250x20 mm Eurospher II 100-5 C18 30x20 mm	25VE181E2N 25PE181E2N 03PE181E2J
Fraction collector	Labocol Vario-4000	A591022
Software	PurityChrom5 Basic	A2687

RELATED KNAUER APPLICATIONS

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

VFD0155 - Sensitive online SPE determination of Bisphenol A in water samples