Science Together

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

Johannes Menke, Yannick Krauke, Juliane Böttcher, Kate Monks; applications@knauer.net
KNAUER Wissenschaftliche Geräte GmbH, Hegauer Weg 38, 14163 Berlin; www.knauer.net

SUMMARY

Steviol glycosides are the main sweetening compounds in Stevia rebaudiana and can be used as natural sugar substitutes, because they have a far higher sweetening power than normal sucrose or glucose. The sweetness is estimated to be about 400 times higher. This gradient method provides a fast determination of six steviol glycosides using reversed phase HPLC and UV detection. Furthermore, an automated matrix reduction is achieved by online SPE (solid phase extraction), speeding up sample preparation and guaranteeing a high sample throughput.

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 (REF1). The steviol glycoside rebaudioside A is the main compound of interest as it is the sweetest and less bitter compound of the extract but often a mixture of rebaudioside A and stevioside is found in the "Stevia" products. In addition, also other steviol glycosides like rebaudioside B, rebaudioside C, dulcoside A and steviolbioside are commonly present in stevia mixtures and were therefore also analytes of interest in the developed method, as they are not desired in finished products. A determination method for steviol glycosides in stevia samples, with partly automated sample preparation and matrix reduction could thus be used for an easy quality control of stevia food products.

RESULTS

A gradient method for six steviol glycosides was developed beforehand (application note VFD0168). For this method a manual SPE protocol was used which was then transferred to the online SPE approach. The online SPE method was previously applied in preparative HPLC (application note VFD0171). For the analytical method valve switching sequences, as well as washing and conditioning solutions, were adopted from the preparative method. An extract of dried stevia leaves was used as sample. The extraction was performed as described in the application note for the original analytical method (VFD0168) as well as the 5-point calibration with mix-standard solutions of rebaudioside A, stevioside, rebaudioside C, dulcoside A, rebaudioside B and steviolbioside. The calibration was set for a range from 0.01 mg/mL to 0.15 mg/mL of each individual compound. The injection volume was 20 µL in full loop mode. The flow passing the SPE column was monitored to see the effect of the washing procedure (**Fig. 1**). After 7 min the main column flow was directed to the detector by switching the stand-alone 6-port 2-position valve. In a second run the same sample was injected again with the same amount, this time measuring the main column flow only (**Fig. 2**).

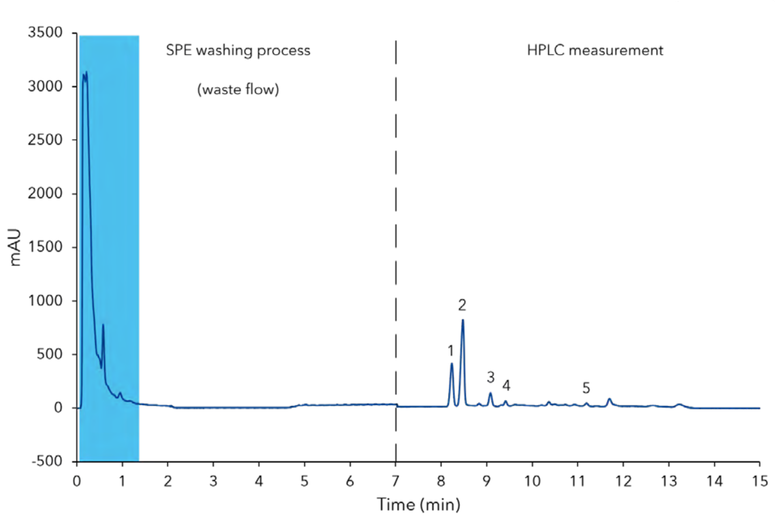


Fig. 1 Measurement of washing process; blue area: matrix; 1) rebaudioside A, 2) stevioside, 3) rebaudioside C, 4) dulcoside A, 5) steviolbioside; 20 µL injection of Stevia extract; 0-7 min) measuring of SPE Washing Process, 7-15 min) measuring of HPLC

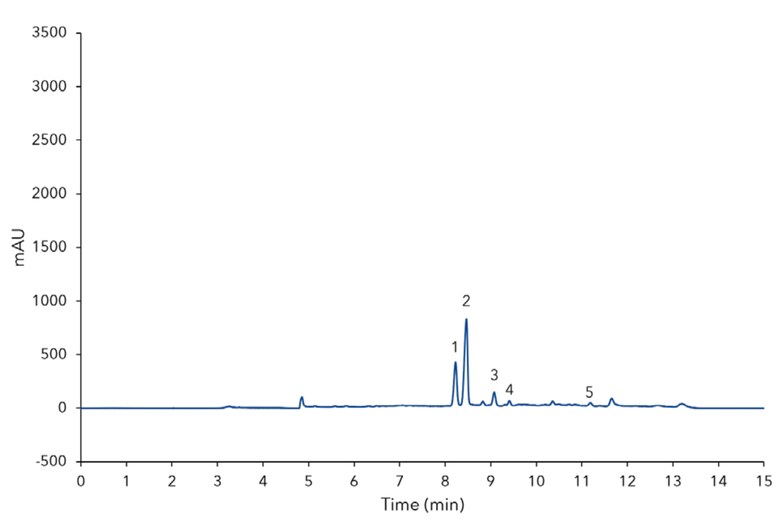
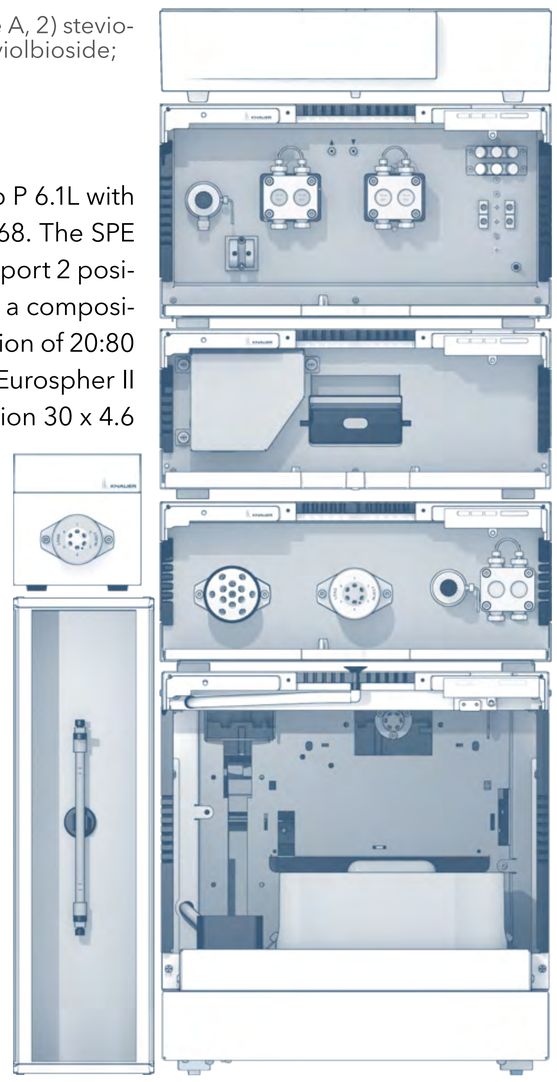


Fig. 2 Measurement of sample only; 1) rebaudioside A, 2) stevioside, 3) rebaudioside C, 4) dulcoside A, 5) steviolbioside; 20 µL injection of Stevia extract

MATERIALS AND METHODS

As analytical system a KNAUER AZURA Online SPE System equipped with an autosampler AS 6.1L, a binary high-pressure gradient pump P 6.1L with 10 mL pump head, a CT 2.1L column thermostat and a diode array detector DAD 2.1L was used as described in application note VFD0168. The SPE module of the system consists of an AZURA assistant ASM 2.1L equipped 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. Injection was automated using an autosampler AZURA AS 6.1L. The eluent was a composition of A: water and B: acetonitrile and was also used to elute the analytes from the SPE column. As washing solution a premixed composition of 20:80 acetonitrile:water (v/v) was used. Conditioning of the column was performed with acetonitrile. A KNAUER Vertex Plus column filled with Eurospher II 100-5 C18 silica in a dimension 250 x 4.6 mm ID with precolumn was used. For the SPE a column with the same material, but with dimension 30 x 4.6 mm ID was chosen. The sample was applied to an already conditioned and equilibrated SPE column, followed by a washing procedure. Next, elution on the SPE column is started by introduction of the water:acetonitrile gradient in reverse flow. Subsequently, the flow is then directed to the main column. Meanwhile the SPE column is conditioned and reequilibrated with washing solution using the feed pump.



CONCLUSION

This method enhances the already fast and robust analysis of steviol glycosides provided by the previous HPLC method keeping all its advantages but adding the capabilities of online SPE. Thus, an easy matrix reduction can be achieved very fast only adding 3 minutes to the original runtime of 12 minutes. This automated matrix reduction saves up time otherwise needed for manual solid phase extraction and allows a high sample throughput. In addition, the analytical main column is spared from being exposed to high concentrations of matrix, enhancing the columns longevity. The monitoring of the washing process also easily allows an optimization of the process. Although the method is now extended to a runtime of 15 min, the overall process of stevia analysis was shortened by a great deal. Additionally, since the SPE column is conditioned for the next run meanwhile sample analysis thus saving even more time.

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

Additional information

VFD0174

Science Together

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

ADDITIONAL MATERIALS AND METHODS

Tab. A1 Method parameters (analytical method)

Column temperature	40 °C
Injection volume	20 µL
Injection mode	Full loop
Detection	UV 210 nm
Data rate	20 Hz

Tab. A2 Pump parameters (main pump)

Eluent A	ddH_2O		
Eluent B	Acetonitrile		
Flow rate	1.2 mL/min		
Pump program			
Time (min)	A [%]	B [%]	Flow [mL/min]
0	70.0	30.0	1.2
3	70.0	30.0	1.2
10	55.0	45.0	1.2
11	55.0	45.0	1.2
11.02	70.0	30.0	1.2
15	70.0	30.0	1.2

Tab. A3 Pump parameters (SPE assistant feed pump)

Eluent A	20:80 ACN:Water (v/v)
Pump program	
Time (min)	flow [mL/min]
0	2
2	2
2.02	0.5
9.98	0.5
10	2
15	2

Tab. A4 SPE assistant valve program

Time (min)	**Valve left (Solvent selection)**	**Valve middle (Injection)**
0	Pos 2: 20:80 ACN:Water (v/v)	Load
2	Pos 1: ACN	Inject
10	Pos 1: ACN	Load
12	Pos 2: 20:80 ACN:Water (v/v)	Load
15	Pos 2: 20:80 ACN:Water (v/v)	Load

Tab. A5 System configuration & data

Instrument	**Description**	**Article No.**
Pump	AZURA P6.1L. (HPG) with 10 mL pump head sst	APH35EA
Autosampler	AZURA AS 6.1L	AAA00AA
Detector	AZURA DAD 2.1L	ADC01
Flow cell	Standard KNAUER LightGuide UV Flow Cell Cartridge 10mm, 2µL	AMC19XA
Thermostat	AZURA CT 2.1	A05852
Assistant	**AZURA ASM 2.1L** Left: 12 Mpos,1/8"", sst Middle:6 Port 2Pos,1/16", sst Right: P4.1S, 50 mL, sst	AYEKEABR
Valve Drive	AZURA Valve drive V 2.1S 6 Port 2Pos,1/16", sst	AWA10AA
Column	Vertex Plus Column, Eurospher II 100 5 C18, 250 x 4.6 mm ID with precolumn Vertex Plus Column, Eurospher II 100 5 C18, 30 x 4.6 mm ID	25VE181E2N 03EE181E2J
Software	ClarityChrom 7.4.2 – Workstation. autosampler control included ClarityChrom 7.4.2 – PDA extension	A1670 A1676

RELATED KNAUER APPLICATIONS

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

VFD0170 - Scale-Up of an analytical HPLC method for steviol glycosides to a preparative approach

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

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