Science Together

Sweet sweeter stevia - The entire story from analytical method development to a robust and effective online SPE purification of steviol glycosides with preparative HPLC

Kristin Folmert, Yannick Krauke, Juliane Böttcher, Johannes Menke, 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 with up to 400 times higher sweetening effect compared to sucrose or glucose. To enable a commercial usage in quality assurance and for the production of ultrapure reference compounds, the plant extract needs to be purified. Sample cleanup via solid phase extraction (SPE) and similar methods are time-consuming and cost-intensive procedures and the purification of complex plant extracts requires special care. We herewith introduce a robust and sensitive online SPE sample preparation method for the purification of rebaudioside A, stevioside, rebaudioside C, dulcoside A, rebaudioside B, and steviolbioside using reversed phase HPLC.

ANALYTICAL HPLC

In **Fig. 1** (red) the separation of the mixed standard of the six steviol glycosides rebaudioside A, stevioside, rebaudioside C, dulcoside A, rebaudioside B, and steviolbioside used for calibration at a level of 0.1 mg/mL for each compound is depicted. All determined steviol glycosides were baseline separated from each other within 12 min using a step gradient method with water and acetonitrile as mobile phase. The results of the sample measurement are included in **Fig. 1** (blue and red chromatograms). The quantification of the compounds was achieved with high accuracy and precision. As shown in **Tab. 1**, several of the calibrated compounds could be determined in both samples. According to the manufacturer of the stevia sweetener it contains rebaudioside A with a mass percentage of 3 %. The measurement shows that this is clearly not the case. Rebaudioside A was determined to be the main component with about 2.4 %, but also stevioside, rebaudioside C, and rebaudioside B were measured. The calculated amounts of steviol glycosides in the analyzed samples are summarized in **Tab. 1**.

Tab. 1 Average amount mass fraction and yield for dried stevia leaves and stevia sweetener samples

		Dried stevia leaves			Stevia sweetener		
#	**Compound**	**Average amount (mg/mL)**	**Mass fraction w (mg/g)**	**Yield y (%)**	**Average amount (mg/mL)**	**Mass fraction w (mg/g)**	**Yield y (%)**
1	Rebaudioside A	0.099	9.900	0.99	0.724	24.133	2.413
2	Stevioside	0.265	26.500	2.65	0.114	3.789	0.379
3	Rebaudioside C	0.045	4.500	0.45	0.002	0.056	0.006
4	Dulcoside A	0.014	1.400	0.14	-	-	-
5	Rebaudioside B	0.003	0.300	0.03	0.009	0.289	0.029
6	Steviolbioside	0.008	0.800	0.08	-	-	-

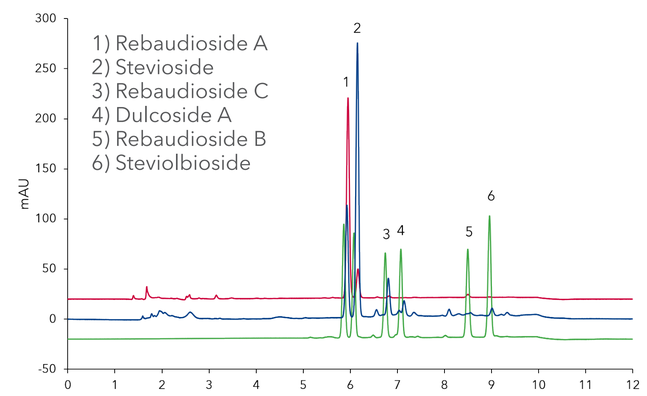

Fig. 1 Overlay of the chromatograms of calibration standard (0.1 mg/mL) (green), dried stevia leaves sample after SPE purification (blue), and stevia sweetener sample (red)

FAST ONLINE SPE PURIFICATION

To increase purification and analysis efficiency partly automated sample preparation and matrix reduction could be used for stevia food products. Here an extract of dried stevia leaves was used as sample. 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. 2 A**). 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 B**).

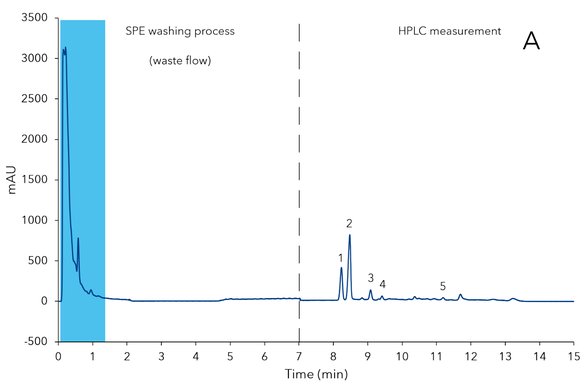

Fig. 2A Measurement of washing process; blue area: matrix; 20 µL injection of stevia extract; 0-7 min) measurement of SPE washing process, 7-15 min) HPLC measurement

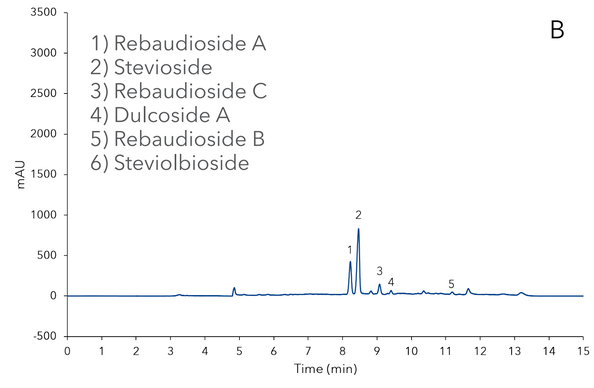

Fig. 2B Measurement of sample only; 20 µL injection of stevia extract

PREPARATIVE HPLC WITH ONLINE SPE

Sample injections of up to 2 mL with common extraction still showed a weak separation of the rebaudioside A and stevioside peaks (**Fig. 3**). 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. Therefore, an online SPE method was developed with a short preparative column in front of the main column. 10 mL of sample was loaded, the matrix washed away and then the target compounds were injected on the main column (**Fig. 4**). Comparison of the chromatograms of the classical batch process (**Fig. 3**) and the online SPE process (**Fig. 4**) 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. 5, B**). The later fractions contained high amounts of stevioside but also still rebaudioside A (**Fig. 5, C**). The results showed that purification of highly pure rebaudioside A is possible by introducing an additional online SPE step.

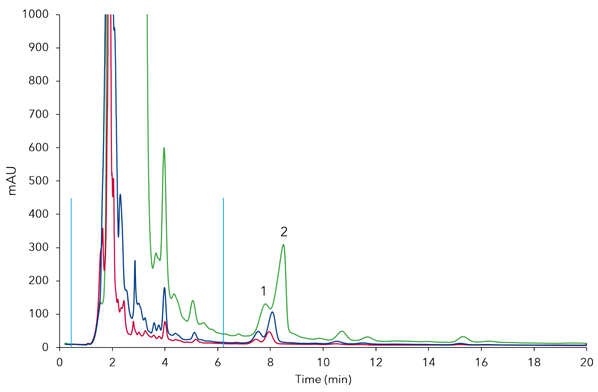

Fig. 3 Overload experiments on preparative column, 200 µL (red), 500 µL (blue), 2000 µL (green); 1) rebaudioside A, 2) stevioside, blue bars – matrix, 25°C, 22 ml/min

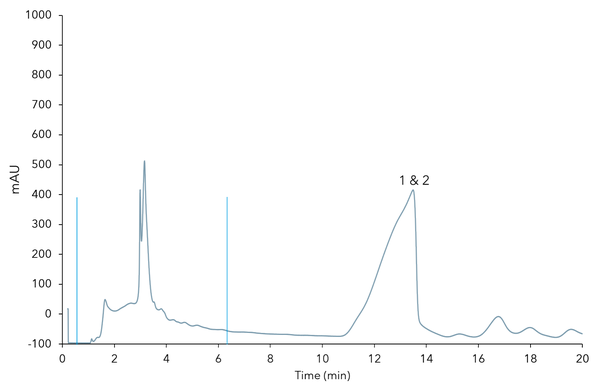

Fig. 4 Preparative online SPE, 10 mL loading; 1) rebaudioside A, 2) stevioside, blue bars – matrix, 25°C, 22 mL/min

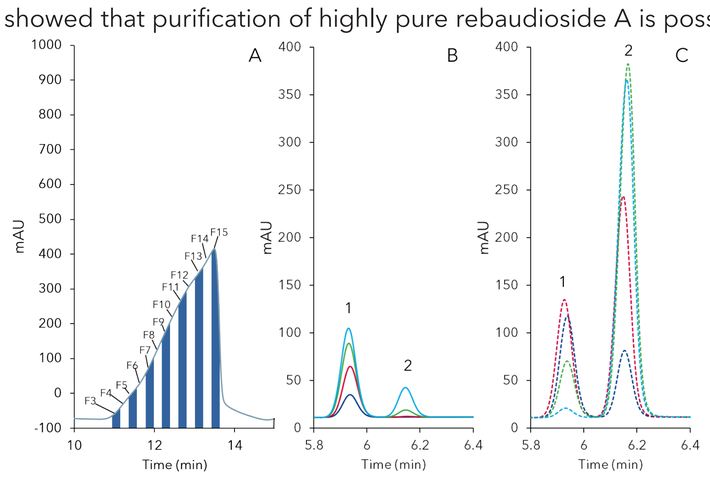

Fig. 5 Fraction analysis of preparative online SPE purification (Fig.2) of rebaudioside A (1) and stevioside (2); A) fractionation of target peak, 5 mL fractions B) F3 (blue), F4 (red), F5 (green), F6 (light blue); C) F7 (red dashed), F10 (blue dashed), F12 (green dashed), F15 (light blue dashed)

Find the full applications notes, more information and details on the systems used here: