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 substitutes 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 stevioside 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 steviol 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 <10 % rebaudioside 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.

MATERIALS AND METHODS
The AZURA Preparative HPLC system consisted of AZURA P 2.1L 100 mL sip 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 1/16” sst valve (solvent selection), a 6 port 2 position 1/16” sip 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/H2O, 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/H2O 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 steviosid 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

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### ADDITIONAL RESULTS

![Graph](image)

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

### ADDITIONAL MATERIALS AND METHODS

#### Tab. A1 Method parameters (preparative online-SPE)

<table>
<thead>
<tr>
<th>Eluent A</th>
<th>100 % ACN</th>
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<tr>
<td>Eluent B</td>
<td>20 %/80 % ACN/H$_2$O</td>
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<td>Sample</td>
<td>Concentrated stevia extract</td>
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<tr>
<td>Step</td>
<td>Flow rate</td>
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<td>Re-equilibration</td>
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<td>Washing</td>
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<td>Injection volume</td>
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<td>Detection wavelength</td>
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#### Tab. A2 Method parameters (preparative method)

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#### Tab. A1 System configuration & data

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<td></td>
<td>Middle: 6Mpor2Po, 1/16**, SST</td>
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<td></td>
<td>Right: F4 15, 50 mL, Lut</td>
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### RELATED KNAUER APPLICATIONS

- **VFD0148**: Oh so sweet - Quantification of steviol glycosides in Stevia samples with RP-HPLC
- **VFD0155**: Sensitive online SPE determination of Bisphenol A in water samples