

Application Note

► Separation of steviol glycosides

| | |
|-------------|--|
| Category | Food analysis |
| Matrix | Plant |
| Method | HPLC |
| Keywords | Stevia, steviol glycosides, sweeteners |
| Analytes ID | Stevioside, rebaudioside A VFD11, 08/09(1) |



Summary

A robust, selective, and sensitive separation method for the determination of four steviol glycosides in stevia plant matrix is demonstrated. In addition to the two calibrated substances two more glycosides could be found in stevia plant extracts by LC-MS. These two substances were quantified by means of a stevioside calibration. Small HPLC columns and the use of a KNAUER Smartline HPLC system provide a fast and easy way for the separation of complex stevia extracts. Using a popular Eurospher NH₂ phase and applying moderate separation conditions results in an inexpensive and precise analysis method.

Introduction

Steviol glycosides are responsible for the sweet taste of stevia plant leaves. These compounds are 75 to 450 times sweeter than sucrose. The taste profile is pure sweet without the typical bitter aftertaste of some well-known artificial sweeteners. Steviol glycosides are almost free of calories and show no fermentation. In addition these glycosides inhibit the formation of caries and plaque.

The four main steviol glycosides of all glycosides in stevia leaf extracts are stevioside (5 – 10%), rebaudioside A (2 – 4%), rebaudioside C (1 – 2%) and dulcoside A (0.5 – 1%).

In the course of the accreditation of these four steviol glycosides by the public health authority a stable and sensitive analytical quantitative separation method is highly recommendable.

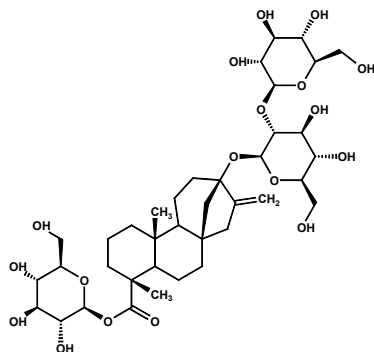
Experimental sample preparation

2 g of dried and pulverized stevia plant leaves are dissolved in 20 ml water at 70°C. 1 ml of the extract was injected on a C2 SPE column, washed with water and 20% methanol and rinsed with 80% methanol on 62 ml volume.

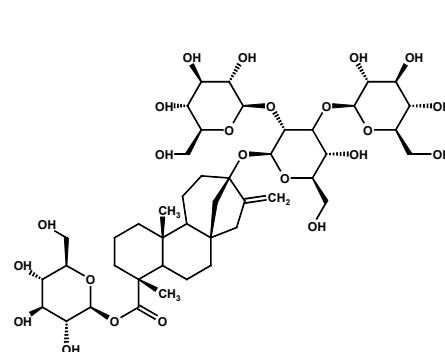
Experimental preparation of standard solution

2.5 mg of stevioside and rebaudioside A were dissolved in 10 ml 80% methanol. From this stock solution (250 µg/ml), the concentrations 100 µg/ml, 50 µg/ml, 10 µg/ml and 1 µg/ml are prepared.

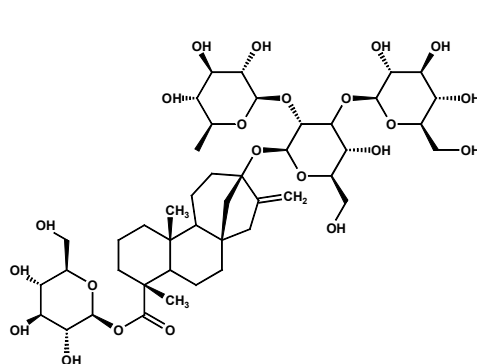
Chemical structures



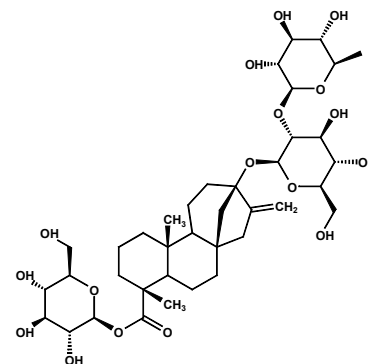
Stevioside



rebaudioside A



rebaudioside C



dulcoside A

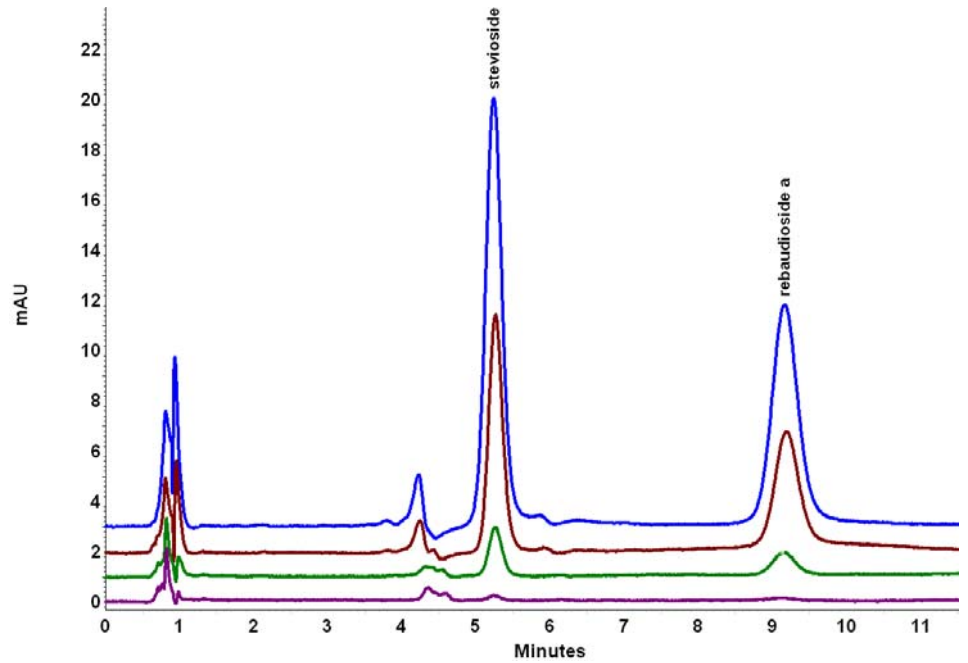
Method parameters

| | |
|--------------------|--|
| Column | Eurospher 100-5 NH ₂ , 150 x 3 mm |
| Mobile phase | acetonitrile/water 80:20 (v/v) |
| Flow rate | 1.0 ml/min |
| Injection volume | 10 µl |
| Column temperature | 35°C |
| System pressure | 50 bar |
| Detection | UV at 210 nm |
| Run time | 10 min |

Results

Fig. 1

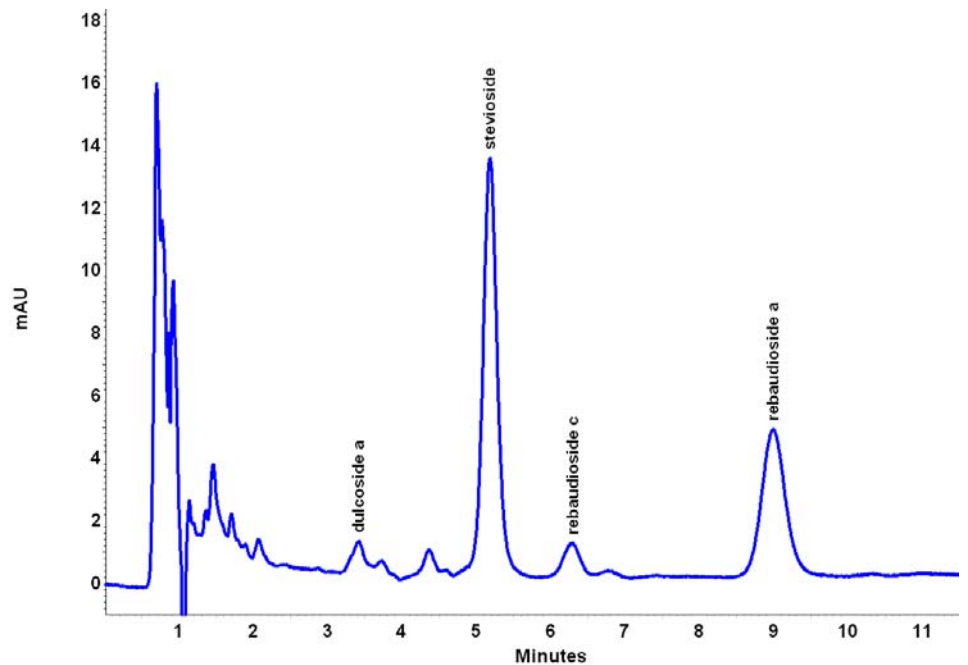
Overlay chromatogram of different sample concentrations



| Substance | t_r (min) | Limit of detection, LOD (ng) |
|----------------|-------------|------------------------------|
| stevioside | 5.197 | 7.5 |
| rebaudioside A | 9.017 | 16.0 |

Fig. 2

Separation of stevia plant extract



| Substance | t_r (min) | C_{absolute} | C_{leaves} |
|------------------|-------------|--------------------------|---------------------|
| stevioside | 5.197 | 69.248 $\mu\text{g/ml}$ | 42.93 mg/g leave |
| rebaudioside A | 9.017 | 50.211 $\mu\text{g/ml}$ | 31.13 mg/g leave |
| rebaudioside C * | 6.300 | 6.591 $\mu\text{g/ml}^*$ | 4.09 mg/g leave |
| dulcoside A * | 3.417 | 5.773 $\mu\text{g/ml}^*$ | 3.58 mg/g leave |

* calculated with calibration of stevioside

Method performance

| | |
|----------------------------------|--|
| Limit of detection | ng range (S/N = 3) |
| Linearity (r²) | 0.9996-0.9998 |
| Linearity range | 1 to 100 ng stevioside 5 to 100 ng rebaudioside A |
| Retention time precision* | < 0.2% RSD stevioside < 0.2% RSD rebaudioside A |
| Peak area precision* | < 2.7% RSD stevioside < 1.4% RSD rebaudioside A |

* calculated for 9 replicate runs

Conclusion

A fast and very good separation of four steviol glycosides in real samples with excellent peak symmetry is easily accomplished by reversed-phase HPLC using the Eurospher NH₂ column in combination with a Smartline HPLC system. The simple isocratic method with moderate parameters guarantees robust and sensitive results over a long time period. The demonstrated method provides "enough space" between the peaks of the target substances and matrix peaks for safe quantitation.

Physical properties of recommended column

The Eurospher NH₂ packing material is an excellent choice for very polar substances in reversed-phase mode and can also be used as a weak anion exchanger. Furthermore this stationary phase is also suitable for normal phase (NP) and polar organic (PO) mode separations.



| | | |
|-------------------------|---------------------------------|---------|
| Stationary phase | Eurospher 100-5 NH ₂ | |
| USP code | L8 | |
| Pore size | 100 Å | |
| Particle size | 5 µm | |
| Form | spherical | |
| Surface area | 350 m ² /g | |
| % C | 3 | |
| Endcapping | no | |
| Dimensions | 150 x 3 mm | |
| Parameter limits | maximum temperature | 60°C |
| | maximum pressure | 400 bar |
| | pH range | 2 – 8 |
| Order number | 15CE190ESJ | |

Recommended instrumentation



This application requires an isocratic HPLC system equipped with degasser, autosampler, column oven, and PDA detector. Other configurations are also available. Please contact KNAUER to configure a system that's perfect for your needs.

| Description | Order No. |
|--|-----------|
| Smartline Pump 1000, incl. 10 ml pump head | A50303 |
| Smartline Manager 5000 with degasser | A5316 |
| SmartMix static mixer | A5351 |
| Autosampler 3950 | A5005-1 |
| Smartline Oven 4050 | A5300 |
| Smartline PDA Detector 2800 | A5251 |
| 10 mm flow cell | A4074 |
| ChromGate software | A1493 |

Author

René Borstel, Columns and Applications Department, KNAUER

Contact information

Wissenschaftliche Gerätebau
Dr. Ing. Herbert Knauer GmbH
Hegauer Weg 38
14163 Berlin, Germany

Tel: +49 (0)30 / 809727-0
Fax: +49 (0)30 / 8015010
E-Mail: info@knauer.net
Internet: www.knauer.net