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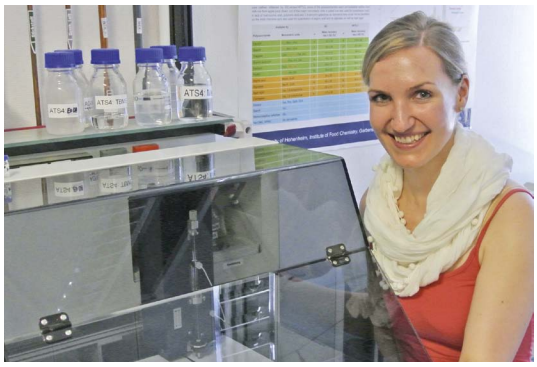
In Food Analysis HPTLC is a most cost effective and robust method – examples in this issue

CAMAG 109

Quantitative determination of steviol glycosides (Stevia sweetener)



Stephanie Meyer

The following HPTLC method for rapid analysis of the herbal sweetener in *Stevia rebaudiana* was developed by Prof. Dr. Morlock, Justus Liebig University of Giessen, and validated during the master thesis of Ms. Meyer, also in cooperation with Dr. Jean-Marc Roussel, consultant in analytical method development and validation, Aix-en-Provence.

Introduction

For centuries, diterpene sweeteners, i.e. steviol glycosides, of the plant *Stevia rebaudiana* have been used due to their attribute of exceptionally strong sweetness (up to 450 fold if compared to sucrose). In dried leaves, stevioside (ca. 10 %) and rebaudioside A (2–4 %) are present. Since December 2011, steviol glycosides have been permitted for use as food additive and sweetener (E 960) in the EU. For steviol glycosides a daily intake of 4 mg/kg body weight, expressed as steviol equivalents, was defined as acceptable.

The analysis of steviol glycosides is normally performed by HPLC using detection at 210 nm or by mass spectrometry. However, evaluation at 210 nm is difficult for complex food matrices, whereas the routine use of mass spectrometers is cost intensive. As the food industry increasingly develops products sweetened with steviol glycosides or Stevia formulations, a robust HPTLC method was developed for food matrices using a selective derivatization of the steviol glycosides. The performance data of the rapid and cost-effective HPTLC method proved its suitability for routine use in the control of tinctures/fluids, granulates and tablets as well as tea, yoghurt and candies.

Chromatogram layer

HPTLC plates silica gel 60 F_{254} (Merck), 20 × 10 cm, if required, prewashed with methanol and dried at 100 °C for 15 min

Standard solution

Steviol glycosides were dissolved in methanol as mixtures or individually (30 ng/µL each). For limit of detection, the standard mixtures were diluted 1:3 with methanol. For accuracy, a stevioside solution of 5 µg/µL was prepared for spiking.

Sample preparation

20 mg granulate were dissolved in 20 mL water and diluted with methanol 1:5. 3 g tea were extracted with 200 mL boiling water and filtered after 5 min. One tablet (60 mg) was dissolved in 10 mL water and diluted 1:10 with methanol. 200 and 50 µL of fluids I and II, respectively, were filled up to the 2-mL mark with methanol and diluted 1:10 with methanol. Sea buckthorn candies were pestled in a mortar and 1 g was dissolved with 50 mL methanol. After ultrasonication for 15 min, the extract was centrifuged at 3500 U for 3 min and the supernatant was used. For method validation, 100 mg natural yoghurt each were spiked with stevioside at 3 different concentrations of 0.02, 0.13 and 0.2 % (additions of 4, 24 and 40 µL of the 5-µg/µL solution), homogenized and dissolved in 2 mL using methanol. Thereof 5 µL were applied (50, 300 and 500 ng/band).

Sample application

Bandwise with Automatic TLC Sampler 4, 22 tracks, band length 7 mm, track distance 8 mm, distance from lower edge 8 mm and side edge 16 mm, application volume 1–5 µL (samples) and 1–20 µL (standards)

Chromatography

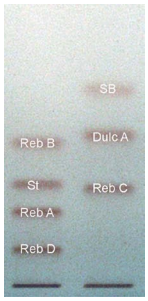
In Automatic Developing Chamber (ADC 2) with 10 mL ethyl acetate – methanol – acetic acid 3:1:1 (v/v/v), migration distance 60 mm, drying time 30 s before and 2 min after development

Post chromatographic derivatization

The HPTLC plate was immersed in the ß-naphthol reagent (2 g ß-naphthol were dissolved in 180 mL ethanol and 12 mL sulfuric acid 50 %) using the TLC Immersion Device (immersion time 2 s, speed 3.5 cm/s) and heated on the TLC Plate Heater at 120 °C for 5 min. The reagent stored in the refrigerator is stable for months.

Documentation

The chromatograms were documented under white light illumination (transmission and reflection mode) using the TLC Visualizer.



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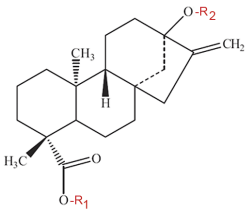
Steviol glycosides:
Stevioside (St)
Rebaudioside (Reb)
Dulcoside A (Dulc A)
Steviolbioside (SB)



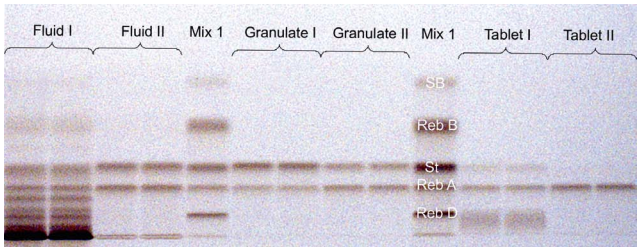
Structure of steviol glycosides (R1 and R2: different sugars)

Densitometry

TLC Scanner 3 with winCATS software, absorption measurement at 500 nm, slit dimension 5 x 0.3 mm, scanning speed 20 mm/s, evaluation by polynomial regression

Results and discussion

After a reduced sample preparation, steviol glycosides in Stevia products on the market (fluids, granulates and tablets) as well as in food (tea, yoghurt and sweets) were separated in only 20 min. After derivatization with the ß-naphthol reagent, the plates were documented and then evaluated quantitatively after absorbance measurement at 500 nm.



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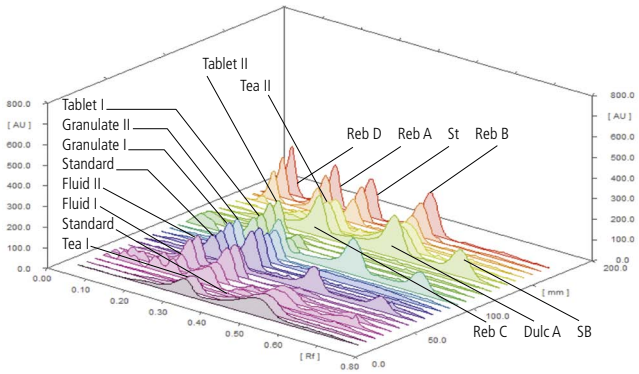

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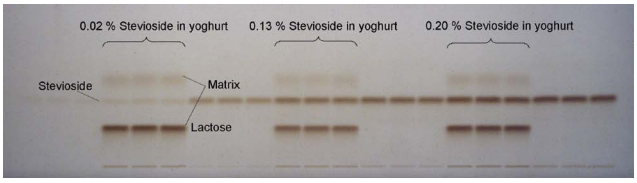
Chromatogram (excerpt) and analog curves of samples and standard mixtures:

For method validation, natural yoghurt was spiked with stevioside. The chromatograms clearly showed the stevioside zones without any interfering matrix. The specificity of the method was sufficient for the different sample matrices depicted. The limit of detection and quantitation (S/N 3 and 10) was determined to be 10 and 30 ng/band (peak height or area), respectively. Using the calibration curve method, the LOQ was even reduced to 12 ng/band (peak height) and 20 ng/band (peak area). The precision of the method was proven using repeatability (*%RSD*; *n*=3; additionally at 3 concentration levels per plate) and intermediate laboratory precision (*%RSD*; *n*=5; fresh sample preparation and spiking each time, quantitation on different days). The calculated expected tolerance range over the whole procedure inclusive sample preparation considered recovery rates (Rec) at 3 different concentration levels.

Accuracy of the whole procedure inclusive sample preparation (exemplarily for peak height)

Stevioside concentration in natural yoghurt (%)	Stevioside concentration (ng/band)	Lower tolerance limit of the Rec (%)	Upper tolerance limit of the Rec (%)	Repeatability (*%RSD*, *n*=3)	Laboratory precision (*%RSD*, *n*=5)
0.02	50	92	120	4.2	8.4
0.13	300	96	108	3.1	4.0
0.20	500	95	110	5.4	5.4



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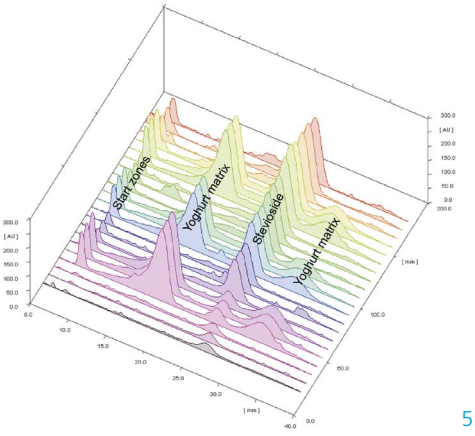

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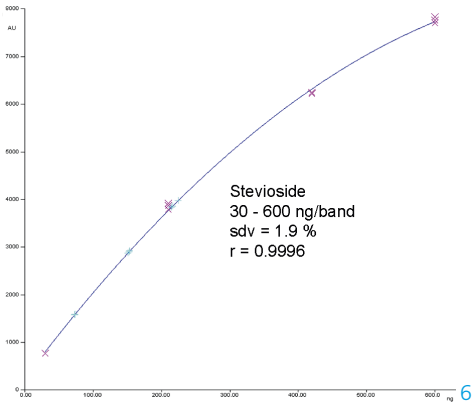

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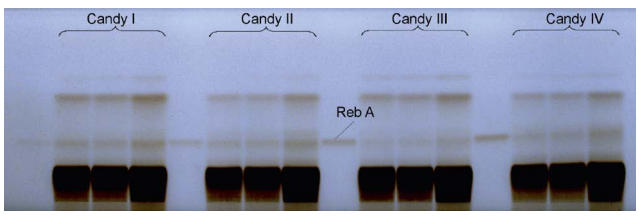
Chromatogram and analog curves of the spiked natural yoghurt samples and stevioside standard (30–600 ng/band)

For the analysis of different batches of candies (not labeled), which could not be analyzed by HPLC-UV due to their heavy matrix, a second development with an increased ethyl acetate content was required (6 instead of 3 parts). With regard to the pronounced matrix zone in the lower hR_F range, isomalt was assumed to be the sugar alcohol basis of the candies.



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Chromatogram for quantitation of rebaudioside A (30–210 ng/band) in candy batches (2 × 5 µL and 10 µL applied each)

The results obtained for the sample matrices were realistic, and the calculated sugar content correlated with the sensory test.

Sample	Tea		Candy		Yoghurt		Fluid		Granulate		Tablet	
Mean [%]	I	II	I	II	I	II	I	II	I	II	I	II
Stevioside	0.23	0.08			0.02	0.20	2.5	1.4	44.8	47.4	5.3	2.1
Rebaudioside A	0.02		0.08	0.09			2.1	1.3	19.8	48.7	14.9	22.9
Rebaudioside D	0.03						0.80	0.11				
Dulcoside A	0.20	0.10					1.7	0.03				
Steviolbioside							0.42					
Rebaudioside B							0.44					
Sum of steviol glycosides	0.48	0.18	0.08	0.09	0.02	0.20	7.9	2.8	64.6	96.0	20.2	25.0
Sugar content correlated [g]	4.2	1.4	30.4 %	37.6 %	6.0 %	60.0 %	6.9	5.0	2.3	3.3	5.9	6.0
Sample amount	3 g						10 / 5 drops		10 mg granulate		1 tablet	
per volume [mL]	150						100		100		100	
Target value [%]			0.09	0.09	0.02	0.20						
Recovery rate [%]			84	94	106	102						

Conclusion

The performance data of the validation showed that the HPTLC method is highly suited for quantitation of steviol glycosides. The developed and validated HPTLC method is effective with regard to sample throughput, matrix robustness, costs and analysis time.

In a following study, HPTLC results were verified by comparison with HPLC, alternative derivatization reagents were shown for food containing sugars, the separation of critical pairs was improved and the test for the absence of (iso)steviol was integrated.

Thanks to Dr. Reif and Dr. Schwarz, PhytoLab, Vestenbergsgreuth, Germany, for standard compounds.

Further information is available from the author on request.

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Chromatogram development under standardized conditions

CAMAG Automatic Developing Chamber ADC 2

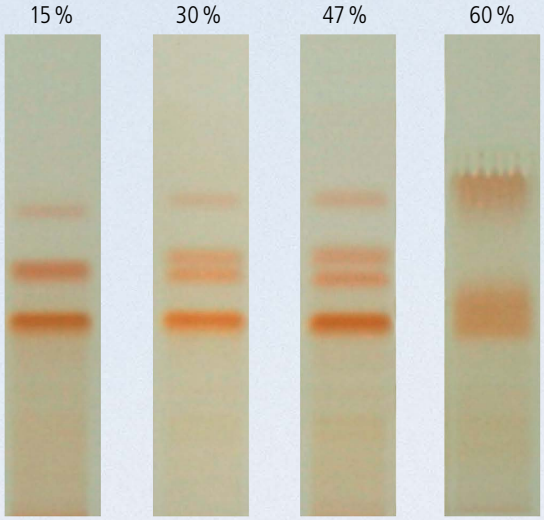
The Automatic Developing Chamber offers convenience, safety and reproducibility for the isocratic development of TLC/HPTLC plates and foils with the format 20 x 10 and 10 x 10 cm.

- Due to the chamber geometry and homogenity of the gas phase, identical developing distances from plate to plate are secured and thus reproducible results. Chromatography occurs in a closed system and is therefore independent of environmental conditions.
- The actual developing chamber is identical with a regular CAMAG Twin Trough Chamber, so that analytical procedures can be readily transferred in both directions.
- Pre-conditioning of the layer, the chamber saturation as well as final drying is fully automatic with pre-set parameters.
- The user is freed from all process monitoring responsibilities, a CCD sensor surveys the solvent migration distance.
- The option "Humidity Control" allows reproducible chromatography at a defined activity of the layer. It is advisable to always check the influence of relative humidity during method development.

Two modes of operation are possible: stand-alone with input of parameters via keypad, or software controlled operation with process monitoring, documentation of operating parameters, and reporting.

Further information in the special brochure "Automatic Developing Chamber ADC 2" or on **www.camag.com/adc2**

Also reference the application "Quantitative determination of steviol glycosides (sweeteners)" described in this CBS (p. 10–12).



Effect of relative humidity on separation of polyphenoles in green tea

Mobile phase: toluene – acetone – formic acid 9:9:2

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