Quantification of steviol glycosides and steviol/isosteviol

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The analysis of steviol glycosides in food and *Stevia* products presented in CBS 109 has been expanded not only to carbohydrate-rich food, but also to the breakdown products steviol/isosteviol, confirmation by HPTLC-MS and proof of product falsification [1]. This research was performed by Julian Wald and Prof. Dr. Gertrud Morlock at the Justus Liebig University Giessen, Germany.

Introduction

In the EU, the sweetener steviol glycosides (E 960) isolated from the plant *Stevia rebaudiana* has been permitted for use as food additive since December 2011, and food products containing E 960 are increasingly launched on the European market. The broad variety of matrices requires a streamlined, but robust method to screen marketed samples that may be adulterated with cheaper synthetic sweeteners. These guidelines were paramount in developing the method presented here.

This HPTLC method proved to be robust with regard to varying sample matrices and resolution between steviol glycosides was improved. Up to 23 different samples can be separated on one plate, which took 1 h (2.6 min/sample) with solvent consumption of only 0.4 mL/sample. Through derivatization with the 2-naphthol reagent, the detection was selective and inexpensive. Additionally, steviol and isosteviol were detected on the same plate using the primuline reagent. The latter reagent was superior for sugar-containing samples, though detectability was not as good for steviol glycosides. HPTLC-ESI-MS spectra were only recorded from the zones of interest, not from matrix or background, which had prolonged the runtime between MS cleaning cycles. Among the samples analyzed a falsified marketed sample was discovered, qualifying this method for food control.

Chromatogram layer

HPTLC plates silica gel 60 F_{254} (Merck), 20 x 10 cm, if required, prewashed with methanol and dried (100 °C, 30 min)

Standard solution

Steviol glycosides (33 ng/µL) and steviol/isosteviol (333 ng/µL) dissolved as methanolic mixture; further solutions in [1]

Sample preparation

Table-top sweetener powders (25–50 mg) and tablets (75 mg) were dissolved in methanol (5 mL) via 5-min ultrasonication, whereas tinctures and liquids were diluted with water (0.5 mL/10 mL). Tea formulations (0.3–3.0 g) and dried/pulverized *Stevia* leaves were boiled for 10 min (0.5 g/30 mL). These suspensions were filtered into a volumetric flask to be filled up to the 50-mL mark with water and diluted 1:10 with methanol. Chocolates were dissolved with ethanol set at 50 °C for 15 min, treated with 0.5 mL Carrez 1/2, filled up to be 4 g/25 mL, centrifuged (3 min, 3000 x g), defatted by freezing (–18 °C, 5 h) and filtered; further preparations in [1].

Sample application

Bandwise with Automatic TLC Sampler 4, 23 tracks, band length 6 mm, track distance 7 mm, distance from lower edge 8 mm and side edge 24 mm, application volumes 1–20 µL for standards and samples

Chromatography

In Automatic Developing Chamber (ADC 2) with 10 mL ethyl acetate – methanol – formic acid 93:40:1 after adjustment of the plate activity for 5 min with lithium chloride (830 g/L) or magnesium chloride (540 g/L), migration distance 60 mm (15 min), drying times 0.5 min before and 3.0 min after development

Baseline separation of steviol and isosteviol on the cut upper plate part at 52 mm in the Twin-Trough Chamber with 7 mL *n*-hexane – acetic acid 19:1 up to 45 mm (10 min)

Mass spectrometry

Underivatized zones were marked and directly eluted with the oval elution head (4 mm × 2 mm) of the TLC-MS Interface using methanol (0.2 mL/min) into the electrospray ionization mass spectrometer (ESI-MS, Agilent Technologies, Waldbronn, Germany).

Postchromatographic derivatization

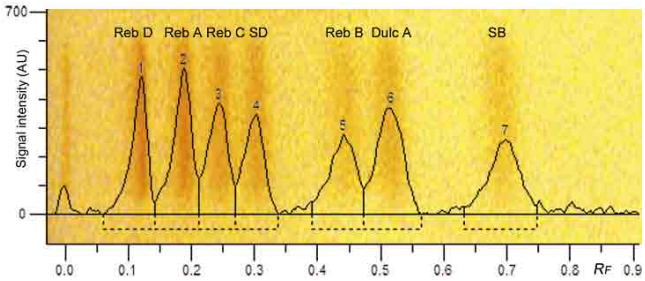
With Chromatogram Immersion Device (immersion speed 3 cm/s, immersion time 0 s), the HPTLC plate was first half-immersed in 135 mL primuline reagent (100 mg primuline in 200 mL acetone – water, 4:1; for isosteviol/steviol detection) from the opposite plate side and dried. Second, the plate was half-immersed in the direction of development in 135 mL 2-naphthol reagent (2 g 2-naphthol in 180 mL ethanol and 12 mL 50% sulfuric acid) and heated on the TLC Plate Heater (120 °C, 5 min). Alternatively, both reagents were used on separate plate halves after plate cut and baseline separation of steviol and isosteviol. Stored in the refrigerator, both reagents were stable for months.

Documentation

Chromatograms were documented at UV 366 nm (primuline reagent) and under white light illumination in transmission/reflection mode (2-naphthol reagent) using the TLC Visualizer.

Densitometry

TLC Scanner 3 with winCATS software, absorption measurement at 500 nm, slit dimension 4.0 mm × 0.3 mm, scanning speed 20 mm/s; VideoScan software was used as alternative.

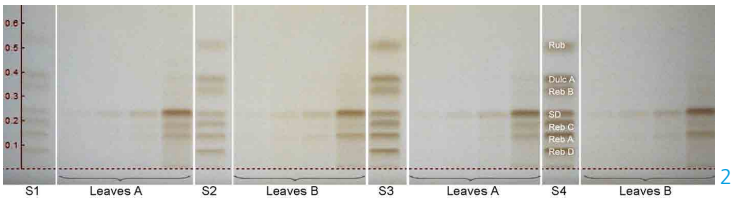


1

Videodensitogram of the underlaid steviol glycoside separation after derivatization with 2-naphthol reagent (60 ng/band each, Rebaudioside: Reb; Stevioside: SD; Dulcoside: Dulc; Steviolbioside: SB); reprinted with permission from [1]

Results and discussion

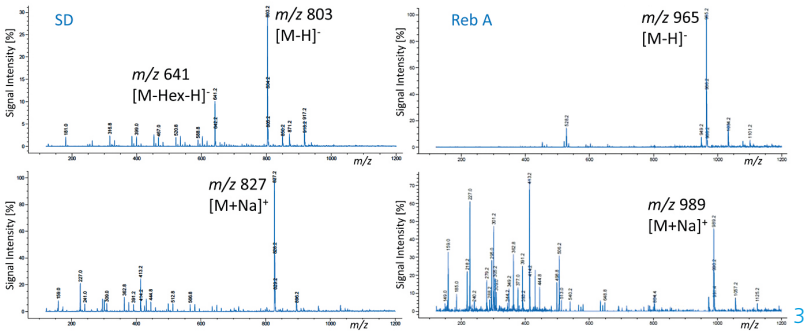
First, the resolution of the separation of the steviol glycosides was improved to separate seven on the same track. Up to 23 samples were separated in parallel within 15 min. The steviol glycosides were detectable down to 2–5 ng/band by absorbance measurement at 500 nm after derivatization with the 2-naphthol reagent. Calibration curves showed correlation coefficients between 0.9983 and 0.9995 and relative standard deviations between 2.7% and 4.8%. Sample results were confirmed by HPTLC-ESI-MS. Also various products were analyzed, resulting in the discovery of a product falsification, containing no steviol glycosides, but instead containing the inexpensive synthetic sweeteners sodium cyclamate and saccharine [1].



2

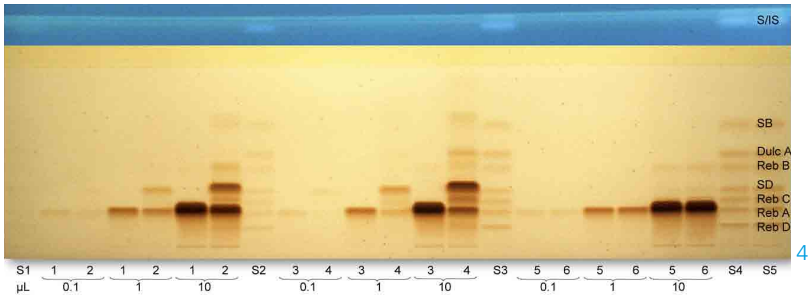
Chromatogram of Stevia rebaudiana leave extracts A and B (0.5, 1, 2 and 10 µL/band) besides standard solution (S1–S4; 33–198 ng/band; rubusoside: Rub) derivatized with the 2-naphthol reagent (white light illumination in reflectance mode); reprinted with permission from [1]

Although the use of steviol glycosides is strictly regulated for products, consumers often use E 960 in an unregulated fashion as evident on the internet. Isosteviol was described to be physiologically active [2] and may be formed in an acidic food matrix at elevated temperatures via acid hydrolysis of E 960. Thus secondly, the detection of the breakdown products steviol/isosteviol was integrated in this method. After two derivatizations from opposing sides, all steviol glycosides were detected between hR_F 11 and 56 and steviol/isosteviol at hR_F >95 as sum parameter.



3

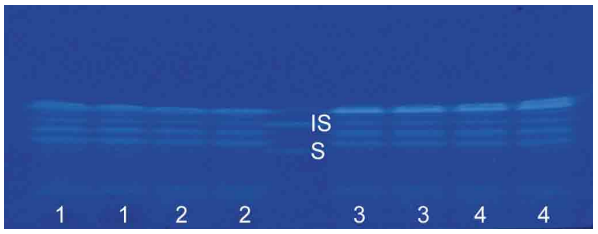
HPTLC-ESI-MS spectra for stevioside (SD, left) and rebausioside A (Reb A, right); reprinted with permission from [1]



4

Chromatogram of Stevia formulations 1-6 besides standard solution (S1–S5; 12–120 ng/band) after derivatization with 2-naphthol reagent (white light illumination in transmission mode) and primuline reagent for detection of steviol/isosteviol (S/IS, UV 366 nm); relevant parts of both images depicted together; reprinted with permission from [1]

As breakdown products are formed only under certain conditions with regard to matrix and food processing, the sum parameter should be a sufficient tool. However, for a baseline resolution of steviol and isosteviol, the following workflow is recommended: after chromatography and half-immersion (50 mm) into the 2-naphthol reagent (automated dipping/fixation of the whole plate was easier), the plate was cut at 52 mm. The upper non-derivatized plate part was further developed with *n*-hexane – acetic acid 19:1 up to 45 mm taking 10 min, followed by derivatization with the primuline reagent.



5

Baseline separation of steviol (S) and isosteviol (IS) of chocolates 1–4 after plate cut, chromatography (10 min) and derivatization with primuline reagent (UV 366 nm); reprinted with permission from [1]

To conclude, the performance data proved the quantitative HPTLC method to be highly suited for food control, along with its capacity for high sample throughput, robustness regarding varying matrices, cost-efficiency and fast analyses.

[1] J. P. Wald, G. Morlock, J. Chromatogr. A 1506 (2017) 109–119

[2] K. L. Wong *et al.* Planta Med. 70 (2004) 108–112

Thanks to PhytoLab, Vestenbergsgreuth, Germany, for standard compounds.

Further information is available from the authors on request.

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