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High-performance thin-layer chromatography analysis of steviol glycosides in *Stevia* formulations and sugar-free food products, and benchmarking with (ultra) high-performance liquid chromatography

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ABSTRACT

A high-performance TLC (HPTLC) method was newly developed and validated for analysis of 7 steviol glycosides in 6 different types of food and *Stevia* formulations. After a minimized one-step sample preparation, 21 samples were developed in parallel, allowing an effective food screening. Depending on the sample application volume, the method was suited to analyze food sample concentrations in the mg/kg range. LOQs of stevioside in natural yoghurt matrix spiked at 0.02, 0.13 and 0.2% were determined by the calibration curve method to be 12 ng/band (peak height). ANOVA was successfully passed to prove data homogeneity in the working range (30–600 ng/band). The accuracy (recovery tolerance limit, 92–120%), repeatability (3.1–5.4%) and intermediate precision (4.0–8.4%) were determined for stevioside in milk-based matrix including sample preparation and recovery rates at 3 different concentration levels. For the first time, the recording of HPTLC–ESI-MS spectra via the TLC-MS Interface was demonstrated for rebaudioside A. HPTLC contents for rebaudioside A were compared with results of two (U)HPLC methods. The running costs and analysis time of the three different methods were discussed in detail with regard to screening of food products.

1. Introduction

The sweetest plant in the *Stevia* genus of about 240 species [1] is *Stevia rebaudiana.* Its sweetness and appropriate taste balance depends on the ratio of the single diterpene glycosides, *i.e.* steviol glycosides, predominantly present in the leaves. The glycosilation of the diterpene skeleton at both, C13 and C19 (Table 1), is essential for the intensity of its sweetness of up to 450 fold if compared to sucrose [2]. About 20 different steviol glycosides were discovered in Stevia leaves [2–5] and in new cultivars even up to 30 steviol glycosides were reported [6]. According to our opinion it is not proven whether all 30 steviol glycosides found so far are natively present in the leaves or artifacts formed during the extensive extraction and isolation of steviol glycosides from the leaves. However, stevioside (mostly *ca.* 6–10%) and rebaudioside A (mostly 2–4%) were the two major steviol glycosides found in dried leaves, and thus, most frequently found in *Stevia* formulations.

Since December 2011, steviol glycosides (E 960) have been permitted for use as food additive and a sweetener in the EU. For steviol glycosides, a daily intake of 4 mg/kg body weight, expressed as steviol equivalents, was defined as acceptable [7]. Its use in various food categories is regulated, *e.g.*, in flavored fermented milk products, ice cream, chocolate products, fine bakery wares, marmalades, fruit nectars, flavored drinks, decorations, table top sweeteners, food supplements and dietary foods for special medical purposes and weight control. The allowed maximal additions specified (expressed as steviol equivalents) range from 20 mg/kg in processed nuts or potato-, cereal-, flour- or starch-based snacks to 3.3 g/kg in chewing gums. Tabletop sweeteners can even contain up to 12 g/kg steviol glycosides [7]. Studies focused on sample preparation for major steviol glycosides and their stability [3,4,8–10]. However, degradation of steviol glycosides was evident for an acidic milieu at elevated temperatures over a prolonged time [8]. Having in mind all the self-made cooking recipes found in the internet, there is especially need for profound stability studies in acidic

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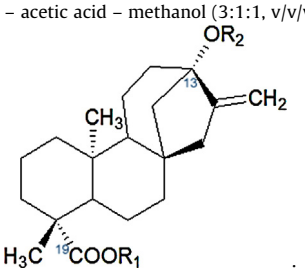
Table 1
Structure of the diterpene skeleton as well as seven steviol glycosides with different glycosilation at C13 and C19 and respective hR_F values obtained with ethyl acetate – acetic acid – methanol (3:1:1, v/v/v) as mobile phase on HPTLC silica gel plates.



Steviol glycoside	R_1	R_2	hR_F
Dulcoside A	β-Glc	β-Glc-α-Rha (2 → 1)	58
Rebaudioside A	β-Glc	β-Glc-β-Glc (2 → 1) I β-Glc (3 → 1)	25
Rebaudioside B	H	β-Glc-β-Glc (2 → 1) I β-Glc (3 → 1)	54
Rebaudioside C	β-Glc	β-Glc-α-Rha (2 → 1) I β-Glc (3 → 1)	34
Rebaudioside D	β-Glc-β-Glc (2 → 1)	β-Glc-β-Glc (2 → 1) I β-Glc (3 → 1)	11
Steviolbioside	H	β-Glc-β-Glc (2 → 1)	77
Stevioside	β-Glc	β-Glc-β-Glc (2 → 1)	36

or fermented, heat-treated food. The strict, regulated use through EU regulations [7] is in contrast to an unrestrained private use, which is questionable with regard to the formation of steviol and its pharmacologically active derivatives.

Food industry develops food products in increasing numbers, sweetened with the regulated steviol glycosides or *Stevia* formulations. It is expected that the sorts of food products containing E 960 will rise in the EU in the next years. This upcoming multitude of food products will also impact food analysis. Robust high-throughput methods for different food matrices would be required, not only for food industry but also for food control and food safety. In most steviol glycoside studies, *Stevia* leaves were extracted with hot water followed by solid-phase extraction (SPE) [11–13], but also extraction with chloroform and methanol [14,15] or even supercritical fluid extraction were described [16,17]. In the food studies available, steviol glycosides were analyzed mainly in soy drink and dairy products, like milk, ice-cream and fermented milk drink. The fat from the food matrices was removed by centrifugation and the proteins were precipitated twice with acetonitrile. But also dry biscuits were extracted with ethanol, filtered, concentrated to dryness, taken up in 10% acetonitrile, centrifuged and filtered again. The supernatant was cleaned-up and concentrated on a RP18 SPE column and finally analyzed with HPLC. Food samples with a lower matrix load like jam were repeatedly extracted with water and centrifuged twice. [18] Carbonated beverages could directly be analyzed after filtration due to the low matrix load if compared to other sample matrices [19], however, it was found that the column lifetime was dramatically shortened and the appearance of the peaks and the baseline deteriorated [9]. Thus also for beverage samples, a RP18 SPE clean-up was preferred for routine hydrophilic interaction chromatography (HILIC)–HPLC analysis.

For steviol glycoside analysis in general, HPLC was applied using mostly NH_2 [11,14,15,20,21] and RP18 columns [12,13,22–25]. NH_2 columns have a high selectivity for all steviol glycosides, however, a poor reproducibility and longer equilibration times than the more robust reversed phase columns, which, on the other side, show a poor selectivity with regard to the separation of stevioside and rebaudioside A. This selectivity problem was overcome by gradient elution [22] or two columns in series [15,18,24] or even comprehensive HPLC × UHPLC [26] and HPLC × HPLC [27]. Recent papers report on the successful use of HILIC columns, which improved the resolution of stevioside and rebaudioside A at run times below 8 min [2,5,28]. But also hydrophilic packed columns working mainly according to size exclusion chromatography [29–31], silica gel [32] and carbohydrate columns [33] had been used in the early stages. Short run times of below 5 or 9 min were also shown using RP18-UHPLC–MS [34,35].

For complex food matrices, difficulties for the detection of the steviol glycosides at UV 200–210 nm [2,9,14,15,20,21,25] will be expected. When UV detection was performed, the shortest possible wavelength yielded the best sensitivity. UV 200 or even 190 nm was preferred, rather than 210 nm, however, these low UV wavelengths lack in selectivity for complex food sample matrices. An additional pre-derivatization with *p*-bromophenacyl bromide to enhance UV detection of steviolbioside and rebaudioside B in RP-HPLC was also reported [36]. Consequently, this general lack in selectivity and detectability led to the more selective and sensitive, but also more expensive use of mass spectrometry with all its different mass analyzers [2,5,9,28]. Using UHPLC–MS, a higher sensitivity in the negative mode was obtained by addition of dichloromethane as dopant to the mobile phase being a source of chlorine. For all the steviol glycosides, the chlorine adducts $[M+^{35}Cl]^-$ and $[M+^{37}Cl]^-$ were evident in the single ion reaction monitoring mode [34]. A qualitative LC-TOF method was also proposed to evaluate steviol glycosides [27].

Only few planar chromatographic methods were shown so far [32,37–39]. All were limited to the analysis of only few steviol glycosides and not applied to food analysis so far. The aim was to develop and validate a high-throughput HPTLC method with a minimized sample preparation and selective detection for the determination of major steviol glycosides in *Stevia* formulations and food products.

2. Materials and methods

2.1. Chemicals

Rebaudiosides A (97.3%), B (99.2%), D (93.7%) and stevioside (97.7%) were obtained from Phytolab, Vestenbergsgreuth, Germany. Rebaudoside C (99.0%), dulcoside A (99.6%) and steviolbioside (98.5%) were purchased from Chromadex, Irvine, CA, USA. Ethyl acetate, methanol, ethanol (all ≥99.5%) and D(−)-mannitol (≥99%) as well as HPTLC plates silica gel 60 and silica gel 60 F_{254} (20 × 10 cm, layer thickness *ca.* 200 μm) were delivered by Merck, Darmstadt, Germany. D(+)-Glucose (99%) and D(+)-galactose (>99%) were from vwr, Darmstadt, Germany. Isomalt (≥99.8%) was from BENEO-Palatinit, Mannheim, Germany. Acetic acid (purity 99–100%), sulfuric acid (50%), D(−)-sorbitol (>98%), xylitol (99%), D(−)-fructose (>99.5%) and D(+)-lactose-1-hydrate (≥99%) were obtained from Roth, Karlsruhe, Germany, and 2-naphthol from Fluka (Seelze, Germany). Distilled water was produced by Heraeus Destamat Bi 18 E (Thermo Fisher Scientific, Schwerte, Germany). *Stevia* formulations and food products (granulates, tablets, fluids/tinctures, yoghurts and teas) were bought at the local market, as well as refined sucrose (Südzucker, Mannheim, Germany). Sea buckthorn candies were produced from Cargill (Minneapolis, MN, USA) and obtained from Dr. Kienle, University of Hohenheim, Germany.