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ORIGINAL RESEARCH PAPER

Reagent sequence for planar chromatographic analysis of eight sweeteners in food products approved in the European Union

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Abstract

Sweeteners, which are regulated as food additives in the European Union, are used as tabletop sweeteners or added to foods for sweetening with the aim of reducing the calorie content. For their simple analysis, a quantitative high-performance thin-layer chromatography multi-imaging (HPTLC−UV/Vis/FLD) method was developed, which used a reagent sequence to detect eight important sweeteners in eight different food products. The samples were dissolved or diluted and separated on HPTLC plates silica gel 60 F_{254} with a mixture of ethyl acetate, methanol, and acetic acid 5:1:1, *V*/*V*. Due to the different structures and detectabilities of the sweeteners, different post-chromatographic derivatization reagents were compared for multi-detection of the sweeteners on the same plate. First, the UV absorbance was detected, and then the derivatization reagent sequence was performed with the primuline reagent, then ninhydrin glacial acetic acid reagent, and finally 2-naphthol sulfuric acid reagent. It was important to arrange and use the reagents according to their increasing acidity. Zones of interest can be confirmed by mass spectrometry. Compared to the status quo analysis of sweeteners, the whole method is simple, robust, and rapid considering the minimalist sample preparation and reagent sequence applied on the same plate. In addition, the influence of food sample matrix on the results is easily understood due to the image-giving nature and multi-detection.

Keywords Planar chromatography · HPTLC–UV/Vis/FLD · Derivatization reagent sequence · All-on-one plate

1 Introduction

European countries are steadily improving their food quality systems. Food additives are of particular interest as they are regulated and approved by the European Commission, Parliament, and Council in the European Union [1]. Most of these have specified a maximum allowable concentration or usable dose depending on the food type. Among these, sweeteners are used as tabletop sweeteners or added to foods (instead of saccharides) with the aim of reducing the calorie content. Due to the diversity of their structures, a variety of powerful analytical methods have been reported for the analysis of approved sweeteners. The current method of choice for the analysis of sweeteners in foods is high-performance liquid chromatography combined with electrospray ionization mass spectrometry (HPLC–ESI-MS) or even tandem mass spectrometry (HPLC–ESI–MS/MS) [2]. For example, HPLC−ESI-MS was used for analysis of nine sweeteners with quantification limits of down to 1 mg/kg [3] or 0.1 mg/kg [4]. In combination with more selective tandem mass spectrometer (HPLC − MS/MS), cyclamate [5] or further nine sweeteners [6] were detected even down to 0.1 µg/L, which is more than needed. Using the evaporative light scattering detector (HPLC–ELSD), detection limits were below 15 mg/kg [7], and for diode array detection (HPLC–DAD), these ranged down to 0.2 mg/L [8]. Also peroxide oxidation and derivatization with trinitrobenzene-sulfonic acid was used for analysis of cyclamate by HPLC–UV down to 1 − 20 mg/kg [9]. HPLC ion chromatography with electric conductivity detector was even lower in detectabilities [10].

Although there are various sensitive analytical methods, HPTLC could be used especially in combination with a reagent sequence to detect the structurally different

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sweeteners for simple and cost-effective screening. In particular, sample preparation could be performed minimalistically even for matrix-rich food samples due to the matrix robustness of HPTLC [11]. Optional quantification and identification by MS can be performed, only if required. Considering these aspects, it was investigated whether HPTLC could be advantageous for food quality control.

2 Experimental

2.1 Chemicals and materials

Sucralose (Suc, E 955, > 99%) was provided by Innosweet, Braunschweig, Germany. Neohesperidin dihydrochalcone (Neo, E 959, ≥ 85%) was delivered from SAFC Supply Solution, Jerusalem, Israel. Saccharine sodium (Sac, E 954, > 98%) was obtained from Roth, Karlsruhe, Germany. Aspartame (Asp, E 951, p. a.) was provided by Barentz, Oberhausen, Germany. Acesulfame potassium (Ace, E 950, > 99%) and aspartame-acesulfame salt (E 962, 2:1 mixture) were provided by Fluka, Buchs, Switzerland. Sodium cyclamate (Cyc, E 952, > 99%) and HPTLC plates silica gel 60 F_{254} (20 cm × 10 cm) were supplied by Merck, Darmstadt, Germany. The steviol glycosides (E 960) rebaudioside A (Reb, 97.3%) and stevioside (Ste, 97.7%) were obtained from Phytolab, Vestenbergsgreuth, Germany. Organic solvents, sulfuric acid, glacial acetic acid, ninhydrin, and 2-naphthol, all analytical grade, were obtained from Sigma-Aldrich Fluka, Darmstadt, Germany. Primuline (p. a.) was purchased from Waldeck, Münster, Germany. Bidistilled water was prepared with Destamat Bi 18E, Heraeus, Hanau, Germany. Food samples were bought on the local market (Table 1).

2.2 Standard solution mixture

Individual standard solutions of the eight sweeteners E 950, E 951, E 952, E 954, E 955, E 959, E 960, and E 962 were prepared by dissolving 10 mg in 10 mL methanol each (1 µg/µL). A methanolic standard solution mixture was prepared that contained Ace 600, Suc 100, Sac 100, Asp 50, Ste/Reb 30, Neo 75, and Cyc 800 ng/µL.

2.3 Sample preparation

The solid samples (ID 1, 2, 7 and 8; Table 1) were crushed, and 1 g each (5 g for ID 8) were dissolved with 0.5 mL hot water in a 5-mL volumetric flask and filled up with methanol to the mark. ID 1 was further diluted with methanol 1:1. For the semi-solid yoghurt sample (ID 4), 20 g was dissolved in 10 mL methanol. Liquid samples (IDs 5 and 3) were directly used, whereby ID 6 was diluted 1:2 with methanol. If needed, centrifugation may be used at 3000 × *g* for 5 min.

2.4 Chromatographic method

HPTLC instrumentation (CAMAG, Muttenz Switzerland) was operated and data were processed with winCATS software, version 1.4.5.2027. The solutions were sprayed as 8-mm band (Automatic TLC Sampler ATS4) allowing 18 tracks to be applied on HPTLC plates silica gel 60 F_{254} (20 cm × 10 cm) with a 10-mm distance between bands, 14-mm distance from the left side and 8-mm distance from lower edge. For calibration, 1 – 20 µL of the standard solution mixture was applied on the plate (30 ng – 16 µg per

Table 1 Overview on food samples investigated, their labeling, and contents found

ID	Food product	Manufacturer	Sweetener labeled	Found (mg/100 g)
1	Lemon balm candy	Ricola, Laufen, Switzerland	Acesulfame-K Aspartame	3 7
2	Sage candy	Dallmann, Wiesbaden, Germany	Aspartame	10
3	Sugar free energy drink	Red Bull, Fuschl am See, Austria	Acesulfame-K Aspartame	114 5
4	Strawberry/red currant yoghurt, 0.1% fat	Bauer, Wasserburg, Germany	Sucralose	6
5	Coca Cola zero drink	The Coca Cola Company, Coca Cola Erfrischungsgetränke, Berlin, Germany	Acesulfame-K Aspartame Na-cyclamate	18 7 [a]
6	Energy shot energy drink	Red Bull, Fuschl am See, Austria	Sucralose	15
7	Wick blue icebergs, extra strong, chewing gum	Procter & Gamble, Schwalbach, Germany	Acesulfame-K Aspartame	87 149
8	Em-eukal lemon candy	Dr. C. Soldan, Nürnberg, Germany	Sucralose	0.2

[a]Not quantified

band, depending on the sweetener). For quantification, sample application volumes (as in Fig. 3) were slightly adjusted to be between 2 and 20 µL/band, *e.g.,* 10/2 µL for ID 1, 5/2 µL for ID 3, 3 µL for ID 4, 20/5 µL for ID 5, and 20 µL for ID 8. Development was performed in the twin trough chamber or Automated Developing Chamber 2 at a relative humidity of the air of about 30% with a mixture of ethyl acetate, methanol, and acetic acid 5:1:1, *V/V*, up to 65 mm. The plate was dried after application (1 min) and development (3 min). Plate images were recorded (DigiStore 2 Documentation System) at UV 254 nm, FLD 366 nm and white light illumination (Vis). Densitometry (TLC Scanner 3) was performed via absorbance measurement at selected wavelengths (Table 2), which were determined by recording UV spectra between 200 and 400 nm. Quantification was performed by peak height or area. For post-chromatographic derivatization, the plate was three times immersed (TLC Immersion Device III, vertical speed 4 cm/s and immersion time 1 s) according to the following reagent sequence, followed each by plate heating (TLC Plate Heater III) and image documentation or densitometry, if needed.

1. Primuline reagent (100 mg primuline dissolved in 20 mL water and 80 mL acetone), followed by solvent evaporation and detection at 366 nm.
2. Ninhydrin reagent (0.3 g ninhydrin dissolved in 95 mL isopropyl alcohol and 5 mL glacial acetic acid), followed by heating at 120 °C for 5 min and detection at Vis.
3. 2-Naphthol sulfuric acid reagent (1 g 2-naphthol dissolved in 90 mL ethanol and 6 mL 50% sulfuric acid added dropwise), followed by heating at 120 °C for 5 min and detection at Vis.

The reagents stored in the refrigerator were stable for several months. For optional recording of full-scan HPTLC–ESI-MS spectra (*m/z* 100–1000; MSD, Agilent, Waldbronn, Germany), respective zones were directly eluted (TLC–MS Interface) into the ESI spray chamber using methanol at a flow rate of 0.2 mL/min (HP 1100 pump, Agilent). The capillary voltage for positive and negative ionization was set to be + 4 kV and − 4 kV, respectively. The nebulizer gas pressure was 20 psig, and drying gas temperature and flow rate were 300 °C and 10 L/min, respectively.

3 Results and discussion

3.1 Chromatographic method

Individual standard solutions of eight EU-regulated sweetener, which were available in the laboratory, were applied (2 µL/band) on the HPTLC plates silica gel 60 F_{254} and different mobile phases were tested using the primuline reagent for detection. The mobile phase development and separation was best with the mixture of ethyl acetate, methanol, and acetic acid 5:1:1, *V/V*, up to 65 mm. In parallel, 18 analyses took about 30 min. The separation worked fine for relative humidities of the air up to about 30% and was negatively influenced by high relative humidities. The 14% acetic acid content was crucial for separating the acid-sensitive sweeteners (Sac, Ace and Cyc) to obtain a good peak shape and selectivity. With acetic acid in the mobile phase, these three sweeteners migrated comparatively faster than the other sweeteners. Hence, the accurate acetic acid portion in the mobile phase and the humidity of the air were identified as very important factors of influence, which must be ensured for a reproducible separation.

Next, various post-chromatographic derivatization reagents were tested for selective detection of the sweeteners (Fig. 1). Selective derivatization can substantially increase the separation efficiency by seeing only the analyte(s) and related compounds in the food matrix (Table 1), rather than everything. Performing selective derivatizations in sequence on the same plate is an even stronger feature of planar chromatography. A derivatization reagent sequence may take longer, but only this broadens the range of possible sweeteners that can be detected with less expensive UV/Vis/FLD detectors, instead of the commonly used MS/MS detector. It was important to arrange and use the reagents according

Table 2 Calibration performance for the eight sweeteners

Sweetener	Calibration curve	Correlation coefficient	Absorbance/reagent, wavelength
Saccharin	$y = x^2 + 0.355\ x + 17.498$	0.9985	UV absorbance, 200 nm
Acesulfame-K	$y = x^2 + 3.526\ x + 1788.984$	0.9975	UV absorbance, 230 nm
Neohesperidin	$y = 0.001\ x^2 + 8.803\ x + 260.384$	0.9998	UV absorbance, 290 nm
Aspartame	$y = x^2 + 7.265\ x + 57.416$	0.9999	Ninhydrin, 500 nm
Stevioside	$y = x^2 + 0.260\ x + 23.625$	0.9971	2-Naphthol, 500 nm
Rebaudioside A	$y = x^2 + 0.249\ x + 24.924$	0.9991	
Sucralose	$y = x^2 + 10.707\ x + 1180.444$	0.9981	
Na-Cyclamate	$y = x^2 + 0.036 \times 365.534$	0.9766	2-Naphthol, 650 nm

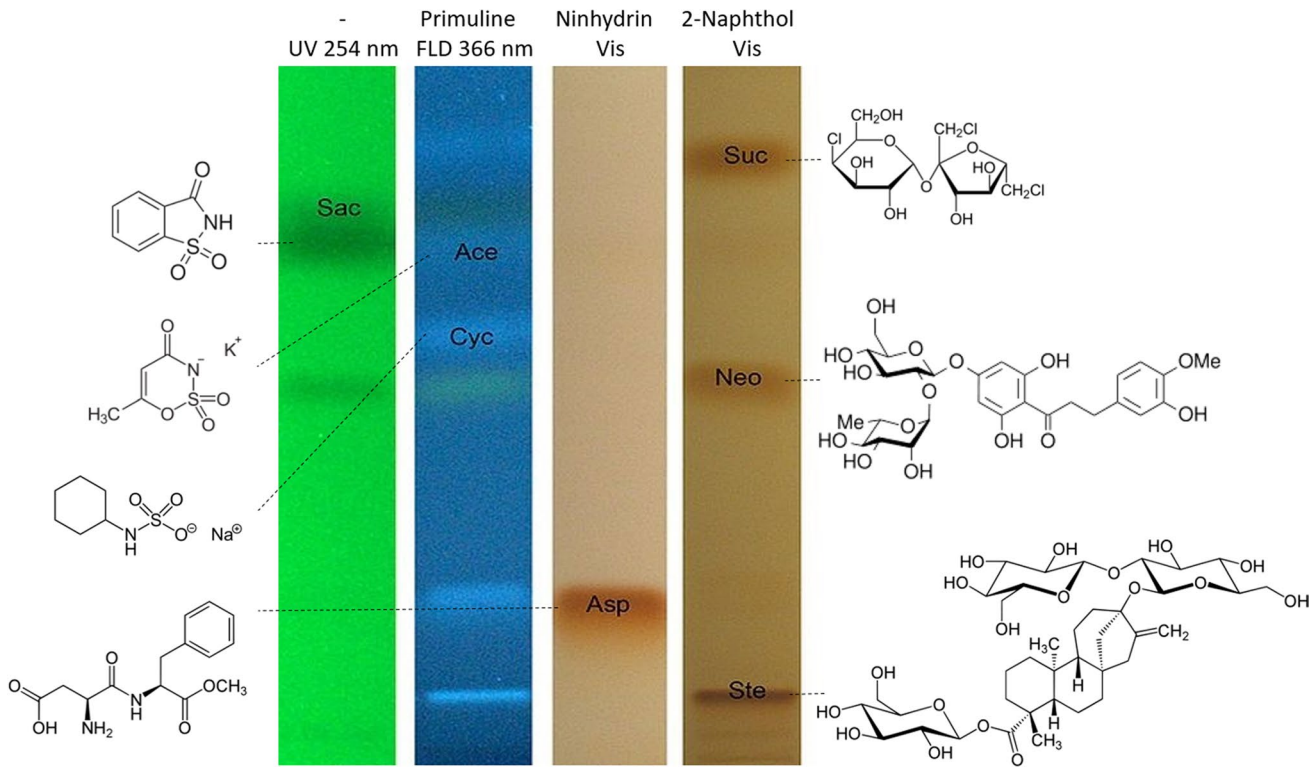


Fig. 1 Derivatization reagents among others studied to detect sweeteners of different structures separated on HPTLC plate silica gel 60 F_{254} with ethyl acetate, methanol, and acetic acid 5:1:1, *V/V*, and detected at UV/Vis/FLD (Reb was not applied)

to their increasing acidity. The more acidic the reagent, the later applied in the sequence. As the reagent sequence with the highest number of detectable sweeteners and most confirmative information, the multi-detection of the sweeteners on the same plate was performed at their UV absorbance maxima (or image at UV 254 nm) as well as after derivatization with the primuline reagent, then ninhydrin glacial acetic acid reagent, and finally 2-naphthol sulfuric acid reagent. The chromatogram was subsequently immersed into three different reagents arranged according to increasing pH strength. The derivatization reagent sequence took about 20 min, which means about 1 min per analysis because all 18 tracks are derivatized simultaneously.

Absorbance spectra were recorded to find the optimal measurement wavelengths (Fig. 2). The 5-point calibration ranged 1:20 to cover a wide range of sample contents. It started approximately from the limit of quantification and was adjusted to the detectability of each sweetener using the most suited detection option. For example, it was from 30 – 600 ng/band for steviol glycosides (Ste/Reb) and ranged up to 0.8 – 1.6 µg/band for Cyc. The resulting calibration curves showed good performance characteristics (Table 2).

3.2 Sample analysis

Food samples (Table 1) were bought on the market and analyzed with the developed method. Only dissolution or dilution of each sample was needed. Application, chromatography, and detection via the reagent sequence took about 80 min, which means about 5 min per sample for 18 analyses in parallel. The high matrix load on the adsorbent is visible to the analyst and differs depending on the sample (Fig. 3). Thus, the influence of any sample matrix on the results is easily understood due to the image-giving nature and multi-detection. On the chromatogram after derivatization via the primuline reagent, all studied sweeteners were visible except for Asp (Fig. 3). Most were detected as bright blue zone in particular at higher amounts on the plate. Only Sac was darker blue than the bluish plate background. After derivatization of the same plate with the ninhydrin glacial acetic acid reagent, the Asp was selectively detected via its rusty color. Further, Ace was evident though weak in intensity. After final derivatization of the same plate with the 2-naphthol sulfuric acid reagent, almost all sweeteners were detected except for Sac and Asp. First via the applied

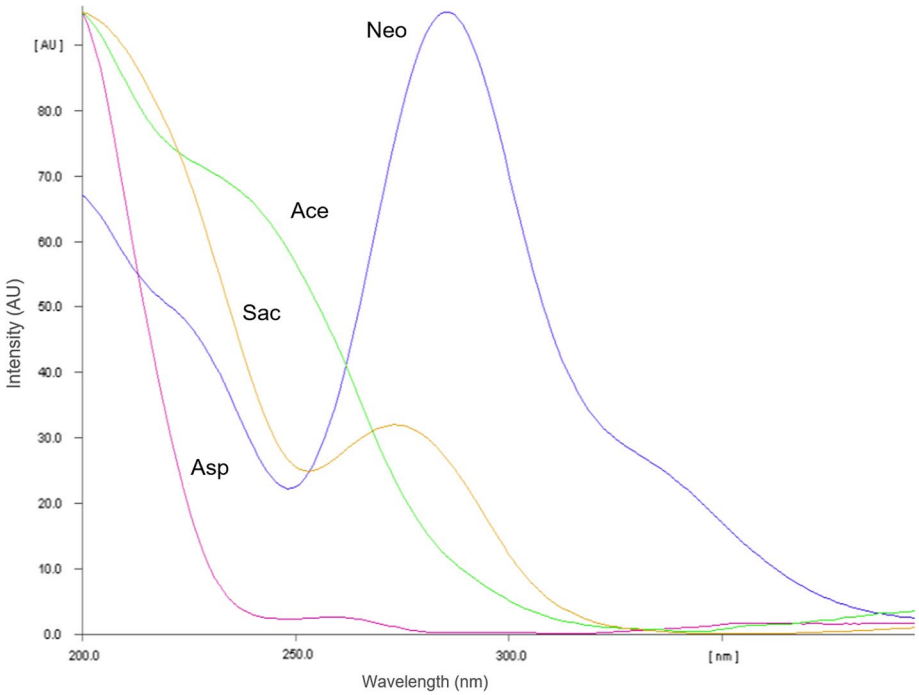


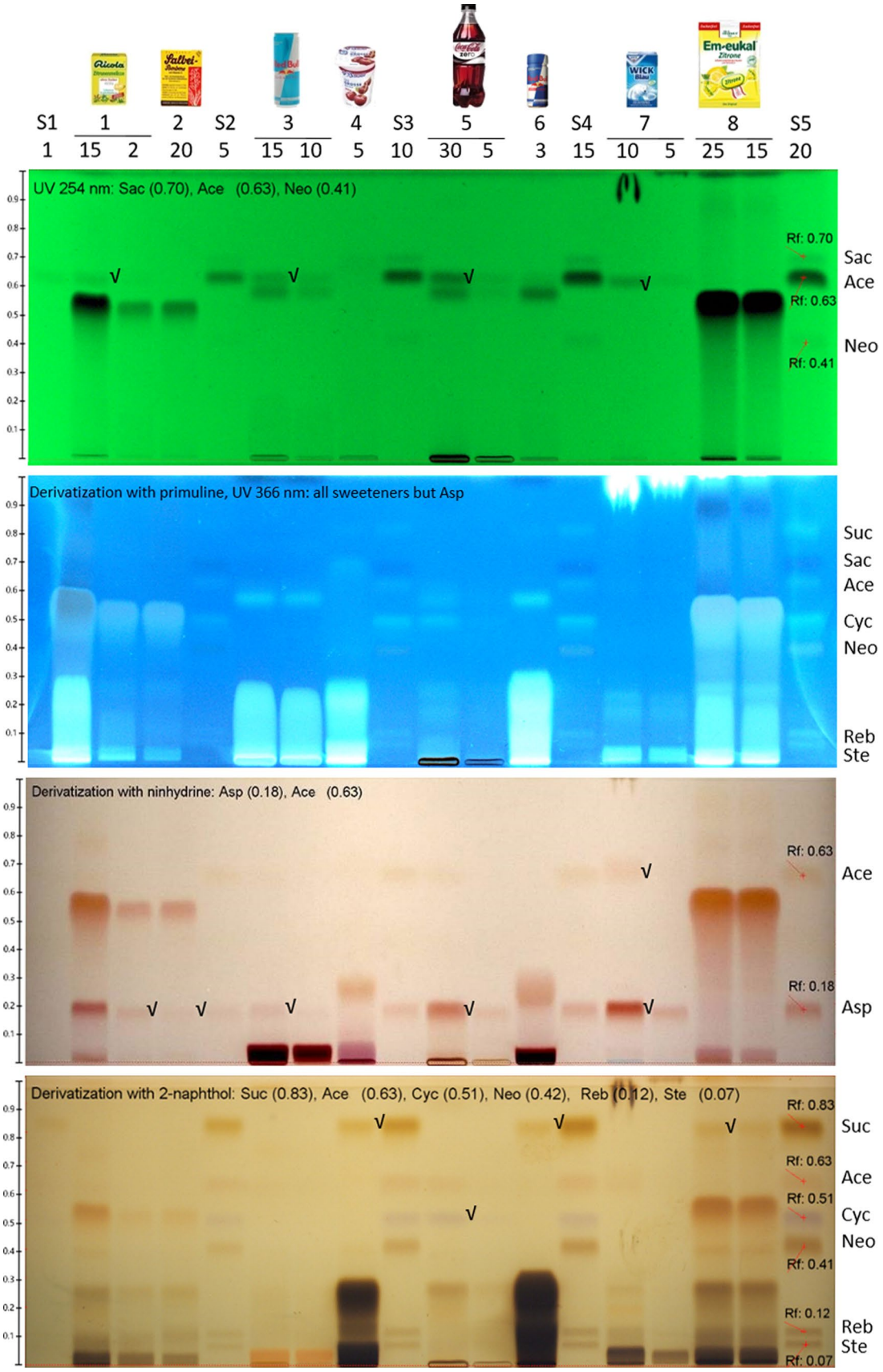
Fig. 2 Recorded UV spectra (200 – 400 nm) of selected sweeteners showing most absorbance maxima in the low UV range at or below 200 nm, making selective detection in the food matrix difficult

derivatization reagent sequence and through the influence of the different chemicals under plate heating, Cyc and Ace were observed as slightly blue and rusty zone, respectively, in the final chromatogram detected via the 2-naphthol sulfuric acid reagent.

The simplicity of the method was targeted, but conflicted with the fact that it still needs to be optimized for steviol glycoside analysis of some food product samples (Fig. 3, IDs 4, 6, and 8). A recently reported steviol glycoside separation [12] or an additional selective on-surface sample preparation step could be studied for such samples. The developed method for sweetener analysis has not been validated so far. However, its feasibility for quantification was studied. To better fit to the calibration curve, few sample volumes were slightly adjusted as mentioned. After densitometry, the obtained sample results were calculated to be between 0.2 and 149 mg/100 g (Table 2), depending on the sweetness of the sweetener and food product. These results in the per mil range down to the low mg/kg range were found to be conclusive considering the high sweetness and combined use of sweeteners in one food product. Optionally, zones of interest can be eluted for recording of mass spectra and confirmation by mass spectrometry.

4 Conclusion

Considering the minimalist sample preparation and multi-detecting reagent sequence applied on the same plate, the whole method is simple, robust, and rapid, if compared to the status quo analysis of sweeteners by HPLC–MS/MS. The method (application, chromatography, and reagent sequence detection) took about 5 min per sample, given for 18 samples analyzed in parallel. The operational costs were about 0.50 Euro per sample analysis. The influence of any sample matrix on the results is easily detected due to the image-giving nature and multi-detection. Still the method has to be optimized for steviol glycoside analysis of some sample matrices, and further sweeteners can be integrated into this method in future.


S1 1 15 2 20 S2 5 15 10 5 S3 10 30 5 3 S4 15 10 5 25 15 S5 20
UV 254 nm: Sac (0.70), Ace (0.63), Neo (0.41)
Rf: 0.70
Sac
Ace
Rf: 0.63
Neo
Rf: 0.41
Derivatization with primuline, UV 366 nm: all sweeteners but Asp
Suc
Sac
Ace
Cyc
Neo
Reb
Ste
Derivatization with ninhydrine: Asp (0.18), Ace (0.63)
Rf: 0.63
Ace
Rf: 0.18
Asp
Derivatization with 2-naphthol: Suc (0.83), Ace (0.63), Cyc (0.51), Neo (0.42), Reb (0.12), Ste (0.07)
Rf: 0.83
Suc
Rf: 0.63
Ace
Rf: 0.51
Cyc
Neo
Rf: 0.41
Rf: 0.12
Reb
Ste
Rf: 0.07

◂**Fig. 3** Developed derivatization reagent sequence for the analysis of eight sweeteners, shown for eight different food products: after absorbance detection at UV 254 nm, first the primuline reagent was used followed by FLD detection, then the ninhydrin glacial acetic acid reagent, and finally, the 2-naphthol sulfuric acid reagent, both chromatograms detected at Vis; separation on HPTLC plate silica gel 60 F_{254} with ethyl acetate, methanol, and acetic acid 5:1:1, *V*/*V*, detected at UV/Vis/FLD after each reagent for information on the food matrix and choice of the most selective detection mode

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Data availability Data are available on reasonable request from the authors.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Human participants and/or animals The research does not involve Human Participants and/or Animals.

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