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ORIGINAL RESEARCH PAPER

Ethylamine as new derivatization reagent differentiating reducing from non-reducing saccharides

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Abstract
Typical derivatization reagents for saccharides in high-performance thin-layer chromatography, like 2-naphthol sulfuric acid, aniline diphenylamine orthophosphoric acid, or p-aminobenzoic acid, generally detect both reducing and non-reducing saccharides. A new reagent was found with ethylamine, specifically reacting with reducing saccharides on normal-phase silica gel plates, resulting in strongly fluorescent zones after heating the plate at 150 °C for 15 min. In contrast, non-reducing saccharides generally did not reveal fluorescent signals tested with 26 different saccharides. Optimal chromatographic separation was achieved with a mixture of 2-propyl acetate, methanol, and water with 1 mg/mL natural product reagent A when the plate was twofold developed. The high sensitivity of the ethylamine derivatization was shown with mean limits of detection and quantification of 10 and 30 ng per zone, respectively, calculated by different methods for selected mono- and disaccharides. The developed method has exemplarily been used for the digestion control of starch by α-amylase, the determination of lactose in lactose-free milk, and for the quantitative and qualitative study of honey. The analysis of honey gave an excellent example of the advantageous consecutive derivatization with ethylamine and aniline diphenylamine orthophosphoric acid reagent as reagent sequence to detect the coelution of reducing and non-reducing saccharides.

Keywords High-performance thin-layer chromatography · HPTLC · Saccharide · Sugar · Derivatization · Ethylamine

1 Introduction

Saccharides typically lack suitable chromophores or fluorophores, making selective and specific chromatographic analysis by high-performance liquid column chromatography (HPLC) impossible, except for electrochemical [1] or mass-selective [2] detection. In contrast, analysis by high-performance thin-layer chromatography (HPTLC) has the great advantage of highly selective derivatizations of saccharides, as has been thoroughly and very recently reviewed [3]. Therefore, only a few aspects should be mentioned. The aniline diphenylamine orthophosphoric acid reagent was widely used, providing different colors supporting the identification of saccharides in addition to their relative migration distances [4–8]. Additionally, aniline diphenylamine orthophosphoric acid reagent resulted in a sucralose-specific fluorescent signal, while other saccharides did not [5]. The colorful differentiation of saccharides is not observed with the 2-naphthol sulfuric acid reagent, generally resulting in brownish zones, but this reagent is well equivalent in terms of sensitivity [5, 9, 10]. p-Aminobenzoic acid, resulting in strongly fluorescent zones of saccharides, clearly outperforms the former reagents in sensitivity [11, 12]. Identical reagent-free fluorescent signals were obtained by heating the chromatogram developed on a silica gel amino plate [5, 13, 14].

All reagents used so far have in common that they detect both reducing and non-reducing saccharides equally. In 1904, *Wöhlk* reported a color reaction of lactose and maltose, which gave a specific deep red color when heated in the presence of aqueous ammonia, while other reducing saccharides gave only faint yellow solutions [15]. However, non-reducing saccharides like sucrose and raffinose did not result in any color. Forty years later, *Fearon* replaced ammonia with methylamine or ethylamine and observed comparable color reactions [16]. These old color reactions encouraged us to study the suitability of simple amines for the specific detection of saccharides on normal-phase silica gel plates.

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2 Experimental

2.1 Chemicals and materials

L-Arabinose (Ara), D-cellobiose (Cel), L-fucose (Fuc), D-fructose (Fru), D-galactose (Gal), *N*-acetyl-D-galactosamine (GalNAc), D-glucose (Glc), *N*-acetyl-D-glucosamine (GlcNAc), D-mannose (Man), D-melibiose monohydrate (Mel), D-raffinose pentahydrate (Raf), L-rhamnose (Rha), D-ribose (Rib), L-sorbose (Sor), D-sucrose (Suc), and D-trehalose (Tre) (all saccharides ≥ 98%), acetonitrile (HPLC grade), 2-propyl acetate (> 99%), methanol (HPLC grade), natural product reagent A (≥ 98%), sodium dihydrogenphosphate monohydrate (> 98%), and sodium chloride (> 99.5%) were delivered by Carl Roth (Karlsruhe, Germany). D-galacturonic acid (GalU), D-glucosamine hydrochloride (GlcN), sodium D-glucose-6-phosphate (GlcP), D-lactose monohydrate (Lac), D-melezitose monohydrate (Mlz), D-sorbitol (Sol), stachyose monohydrate (Sta), sucralose (Sul) (all saccharides ≥ 98%), ethylamine (aqueous solution 70%), and α-amylase from human saliva (Type IX-A, lyophilized powder, 1,000–3,000 units/mg protein) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Purified water was prepared by a Destamat Bi 18E (Heraeus, Hanau, Germany). HPTLC plates silica gel 60 were from Merck (Darmstadt, Germany). Wheat starch, wheat flour, spring honey, summer honey, blossom honey, and the lactose-free milk samples were purchased from a local supermarket.

2.2 Standard solutions

Saccharide stock solutions were individually prepared in water (10 mg/mL, stored at 4 °C for maximal one week) and freshly diluted with water to 1 mg/mL to obtain individual working standard solutions. Standard mixes for quantifications (0.1 mg/mL) were prepared by pipetting 100 µL of the respective working standard solutions, filled up to 1000 µL with water.

2.3 Sample preparation

For the digestion of starches, 100 mg of soluble starch, wheat starch, and wheat flour were weighed into 25-mL falcon tubes and suspended in 10 mL of buffer (20 mM sodium dihydrogen phosphate with 6.7 mM sodium chloride, pH 6.7). The falcon tubes were placed in a boiling water bath for 15 min to inactivate enzymes. A total of 1 mL of each freshly vortexed suspension was pipetted into a 2-mL Eppendorf tube, followed by the addition of 1 U α-amylase; except for native samples (0 min). Incubation was carried out at ambient temperature for 1, 3, 5, 7, and 9 min using a Vortex Genie 2 with multi-tube holder (Scientific Industries, Bohemia, NY, USA) at speed 10. After incubation, α-amylase was deactivated in a boiling water bath for 15 min. The cooled samples were made up to a total volume of 2 mL with water, vortexed, and centrifuged at 17,000*g* (Heraeus Pico 17, Thermo Fisher Scientific, Schwerte, Germany).

Milk samples (100 µL) were pipetted into a 10-mL volumetric flask and filled up with methanol. After vortexing, the flask was allowed to stand for 10 min to settle down the proteins, whereafter the supernatant was filtrated through a 0.45-µm membrane filter (cellulose acetate) into an HPTLC vial.

Honey samples (1 g) were weighed into 25-mL falcon tubes, followed by the addition of 10 mL of water. After 15 min on a vortex with multi-tube holder at speed 10, the sample solutions were filtrated through a 0.45-µm membrane filter (cellulose acetate) into a 2-mL Eppendorf tube. For the qualitative and quantitative analyses, the honey solutions were diluted 1:25 and 1:250, respectively, with water in an HPTLC vial.

2.4 High-performance thin-layer chromatography

HPTLC instrumentation (CAMAG, Muttenz, Switzerland) consisted of Automatic TLC Sampler (ATS 4), Automatic development chamber (ADC 2), Derivatizer, TLC Visualizer 2, TLC Scanner 4, and Plate Heater III. The instruments were controlled by visionCATS software version 3.2 SP2. HPTLC silica gel 60 plates were prewashed with methanol and dried for 10 min in an oven at 110 °C. Samples and standard solutions were applied as 6-mm bands with a track distance of 7 mm, distance from the lower edge 8 mm, and left edge minimal distance of 12 mm with the following settings: water as sample solvent type, standard (qualitative analyses) or quantification (quantitative analyses) as filling/rinsing quality, and 25 µL syringe volume. During the method development, standard solutions (1 mg/mL) were applied at 0.2 µL. For quantifications, standard solutions (0.1 mg/µL) were applied at 0.2, 0.5, 0.8, 1.4, and 2.0 µL. Starch samples were applied at 6 µL, milk samples at 1.0, 3.0, and 5.0 µL, and honey samples at 0.5 µL and 1.5 µL (1:25 and 1:250 dilutions).

After plate preconditioning for 20 min in a 20 cm × 10 cm twin-trough chamber (using filter paper and 10 mL mobile phase in the opposite trough), the plate was developed with 10 mL 2-propyl acetate–methanol–water (7:3.5:1.5, *V/V*) containing 1 mg/mL natural product reagent A up to 60 mm, taking 12 min, and thereafter dried inside a fume hood for 15 min. A second development was performed analogously. Alternatively, development was performed in the ADC 2 with chamber saturation (using filter paper) for 5 min and plate preconditioning for 15 min with 20 mL and 10 mL mobile phase, respectively, followed by plate drying for

10 min. Relative humidity of the surrounding air during experiments was < 50%.

Derivatization was carried out with 1 mL ethylamine solution (15% in water, Derivatizer, yellow nozzle, level 2), followed by plate heating at 150 °C for 15 min. Chromatograms were captured under 366 nm followed by fluorescence measurements (366 nm > 400 nm, measurement slit 5 mm × 0.2 mm). An optional second derivatization was performed with 2 mL aniline diphenylamine orthophosphoric acid reagent (yellow nozzle, level 5), followed by plate heating at 110 °C for 10 min. Chromatograms were documented at white light illumination (transmittance mode).

2.5 Validation

Limit of detection (LOD) and limit of quantification (LOQ) were determined for glucose-6-phosphate, maltose, lactose, fructose, and rhamnose. The standard solutions (0.1 mg/mL) were applied with 0.2, 0.5, 0.8, 1.4, and 2.0 µL (20–200 ng), followed by development, derivatization, and fluorescence measurement. The calculations followed the Deutsches Institut für Normung (DIN) method [17], the International Council for Harmonisation (ICH) guidelines [18], and the United States Pharmacopeia (USP) procedure [19] (available in visionCATS).

3 Results

3.1 Selectivity of ethylamine for reducing saccharides and reagent sequence

Both ethylamine and methylamine are available as aqueous solutions and can be used for derivatization by simple dilution. However, the vapor pressure of ethylamine (114 kPa at 20 °C) is three times lower than the vapor pressure of methylamine (300 kPa at 20 °C), which is why ethylamine was selected for the present study. The first experiments quickly showed that reducing mono- and disaccharides on the silica gel plate formed a dye, which was absent with the non-reducing sucrose (Fig. 1a). However, lactose and maltose did not give the described red color [16]. Instead, all reducing saccharides showed a non-specific ochre color, and this was only detectable when relatively high amounts (> 500 ng) were applied. This initially disappointing result turned positive when the plate was viewed under 366 nm illumination. Except for sucrose, the exemplarily applied saccharides revealed strongly fluorescent zones (Fig. 1b) comparable with the p-aminobenzoic acid reagent. The non-reducing sucrose could be detected after consecutive derivatization with the aniline diphenylamine orthophosphoric acid reagent reagent (Fig. 1c). For these promising saccharide-specific

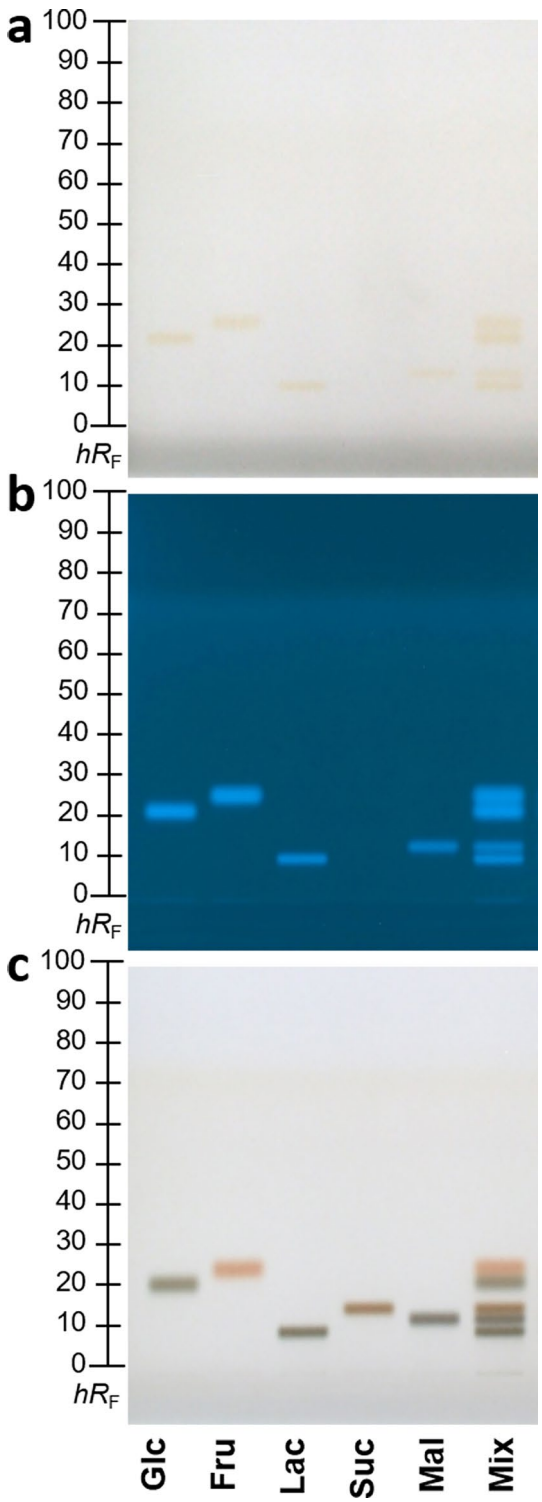

Fig. 1 HPTLC–Vis/FLD chromatograms of glucose (Glc), fructose (Fru), lactose (Lac), sucrose (Suc), maltose (Mal), and a mixture of them (each 500 ng/band), on HPTLC silica gel 60 plates developed once with 2-propyl acetate–methanol–water (7:3.5:1.5, *V/V*) with 1 mg/mL natural product reagent A. Plate images were captured after derivatization with ethylamine under white light (**a**) and 366 nm illumination (**b**) as well as after consecutive derivatization with aniline diphenylamine orthophosphoric acid reagent under white light illumination (**c**)

derivatizations, the optimal conditions for the detection and separation of saccharides were evaluated.

3.2 Screening of 26 reducing and non-reducing saccharides

The optimal ethylamine concentration (15%), reagent volume (1 mL, applied via the Derivatizer), and heating temperature (150 °C) were determined (data not shown). Volumes > 1.5 mL of the aqueous reagent resulted in zone broadening due to a too-wet layer. The new derivatization reagent was validated with 26 reducing and non-reducing saccharides, including acids and amines. As a mobile phase, acetonitrile–water (4:1, *V/V*) [20] containing 1 mg/mL natural product reagent A [21–23], improving the separation, especially of fructose and glucose, was first tested. This planar hydrophilic interaction liquid chromatography has a short development time and results in the separation of the most saccharides tested (Fig. 2a) compared with the widely used mixtures of n-butanol–2-propanol–acetic acid. Again, all reducing saccharides showed a nice fluorescence, while the non-reducing saccharides, like stachyose, melezitose, trehalose, sucralose, and sucrose, did not. The exemplarily applied sugar alcohol sorbitol was also not detectable. But with increasing hR_F, the zones became broader, and galacturonic acid migrated into the front with this solvent system. Therefore, mobile phase mixtures of 2-propyl acetate, methanol, and water [7] were studied in different ratios, also allowing rapid development. The optimum was found for a mixture of 7:3.5:1.5 (*V/V*) when the natural product reagent A was added at 1 mg/mL, and the plate was developed two-fold (Fig. 2b). By the second development with the same mobile phase, the zones became sharper, galacturonic acid now showed an hR_F of 45, glucose and fructose were still separated, and N-acetylglucosamine and N-acetylgalactosamine more clearly provided double zones, most likely diastereomers.

Generally, the separation of maltose and cellobiose could not be achieved, and the separation of both the pentoses and hexoses could be more satisfactory. But regarding the strong blue fluorescence, these monosaccharides seem overloaded at 200 ng per zone and lower amounts applied would provide better selectivity. However, separating such a number of saccharides is generally challenging. Appropriate adjustment of the mobile phase can be made for samples where specific separations are essential.

3.3 Limits of detection and quantification

The LOD and LOQ were exemplarily determined for glucose-6-phosphate, maltose, glucose, lactose, fructose, and rhamnose to cover the whole hR_F range using derivatization via the ethylamine reagent. Depending on the calculation method, LODs ranged 6–10 ng per zone and LOQs ranged 14–30 ng per zone (Table 1), which is quite comparable with the literature [5, 11, 12].

3.4 Applications

As proof of principle, the presented method has exemplarily been used for the digestion control of starch by

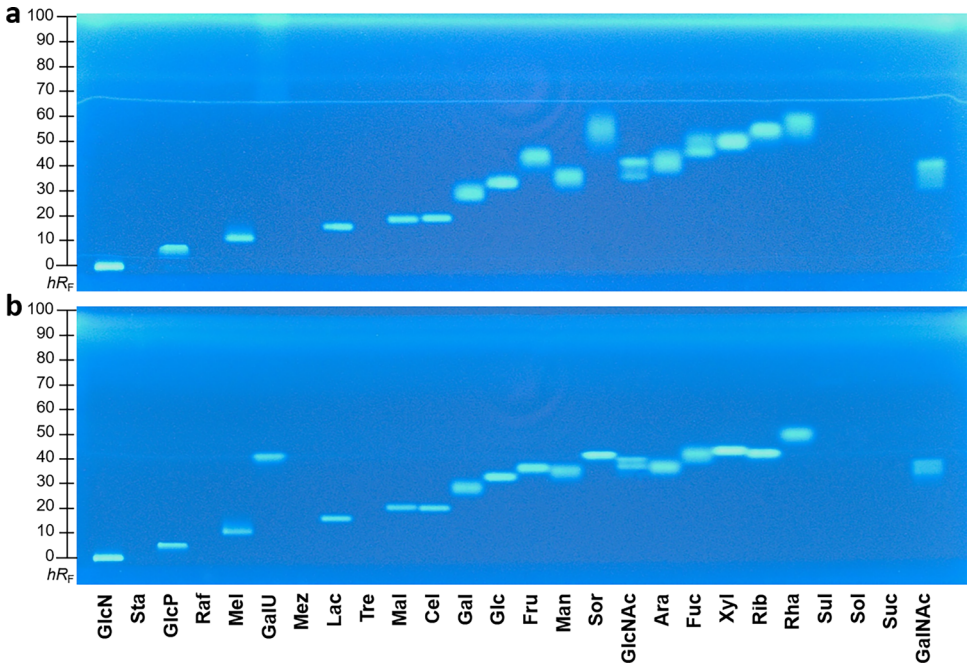

Fig. 2 HPTLC–FLD chromatograms of 26 saccharides (listed in section 2.1; 1 mg/mL, 0.2 µL applied) on HPTLC silica gel 60 plates developed with acetonitrile–water (4:1, *V/V*) containing 1 mg/mL natural product reagent A (**a**) and two-fold developed with 2-propyl acetate–methanol–water (7:3.5:1.5, *V/V*) with 1 mg/mL natural product reagent A (**b**) detected at 366 nm after derivatization with ethylamine

Table 1 Saccharides with limits of detection (LOD) and quantification (LOQ) calculated according to different guidelines [17–19]

Saccharide	LOD (ng per zone)			LOQ (ng per zone)		
	[17]	[18]	[19]	[17]	[18]	[19]
Glucose-6-P	9	22	6	34	66	13
Maltose	3	8	9	14	24	18
Lactose	4	10	14	17	31	29
Fructose	4	9	7	15	26	14
Rhamnose	11	28	8	43	84	18

α-amylase, the detection of saccharides in lactose-free milk, and in the quantitative and qualitative analysis of honey.

3.4.1 Saccharide analysis of digested starch

The time course of enzymatic starch degradation was simply studied in vials with different starch sources, soluble starch, wheat starch, and wheat flour. Alternatively to in-vial degradation, an all-in-one nanoGIT system can be used, recently reported for the simulated static oral and intestinal on-surface amylolysis of flours and soluble starch [24]. To check for the presence of starch degradation products initially present in the samples, native sample extracts without adding α-amylase were also applied. The influence of enzymes in wheat flour was excluded due to the sample preparation (heating step). Starch degraded mainly into maltose (hR_F 23) besides other oligosaccharides over time (Fig. 3). The most intense zones of maltose were found in the wheat flour, followed by the soluble starch and wheat starch. However, besides traces of glucose (hR_F 35), maltose was also detected in the blank wheat flour extracts, naturally present in the flour.

3.4.2 Saccharide analysis of lactose-free milk

The HPTLC analysis of lactose-free milk was already reported with different reagents for derivatization on silica gel plates, whereby the p-aminobenzoic acid was found to perform best [11]. Three commercial milk samples labeled lactose-free (< 0.1%) were analyzed with the present method for their lactose content. In all samples, lactose (hR_F 19) was visually almost slightly detectable at the highest application volume (5 µL), while galactose and glucose expectedly were strongly detected (Fig. 4). Thus, without densitometric measurement, the lactose concentration of the three milk samples was rated below the lowest lactose standard applied (20 ng per band), resulting in < 40 mg/100 mL lactose in milk, thus fulfilling the declarations.

3.4.3 Saccharide analysis of honey

HPTLC methods for analyzing saccharides in honey or nectars were also already reported [4, 6]. With several different saccharides, honey is particularly well suited for demonstrating the selectivity of the ethylamine derivatization method. The main saccharides found in honey were glucose, fructose, melezitose, sucrose, and maltose [25]. Because only reducing saccharides can be detected with ethylamine, the two

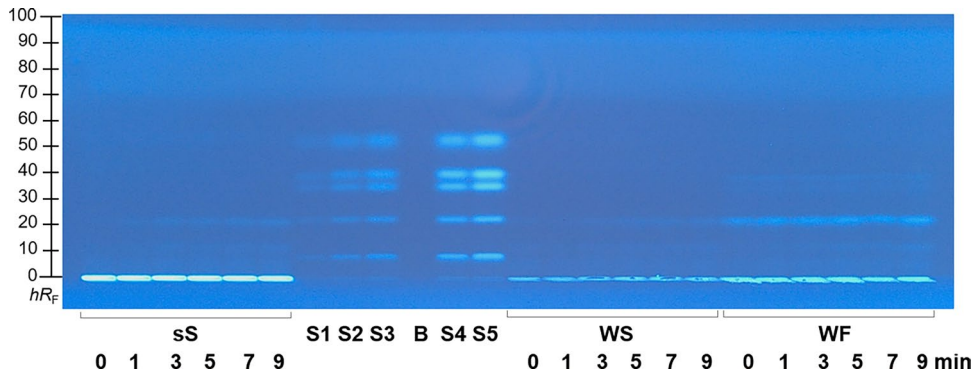

Fig. 3 HPTLC–FLD chromatograms of the saccharide analysis of digested starch samples, soluble starch (sS), wheat starch (WS), and wheat flour (WF) along with standard mixture (rhamnose, fructose, glucose, maltose, and glucose-6-phosphate with decreasing hR_F, each 0.1 mg/mL, applied at 0.2, 0.5, 0.8, 1.4, and 2.0 µL as S1–S5) and blank (B, buffer used for digestion). The plate was two-fold developed with 2-propyl acetate–methanol–water (7:3.5:1.5, *V*/*V*) containing 1 mg/mL natural product reagent A, and detected at 366 nm after derivatization with ethylamine

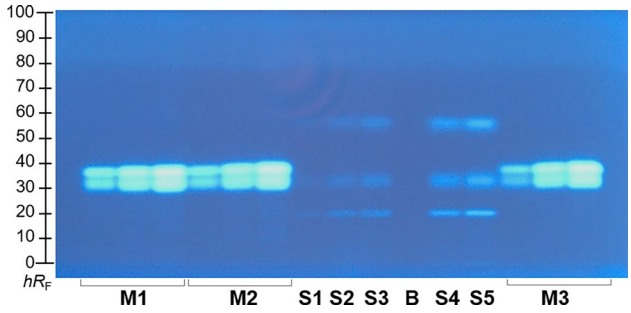

Fig. 4 HPTLC–FLD chromatograms of the saccharide analysis in lactose-free milk samples M1, M2, M3 (applied with 1, 3, and 5 µL) along with standard mixture (rhamnose, galactose, and lactose, decreasing hR_F, each 0.1 mg/mL, applied at 0.2, 0.5, 0.8, 1.4, and 2.0 µL) and blank (B, methanol used for milk dilution). The plate was two-fold developed with 2-propyl acetate–methanol–water (7:3.5:1.5, *V/V*) containing 1 mg/mL natural product reagent A, and detected at 366 nm after derivatization with ethylamine

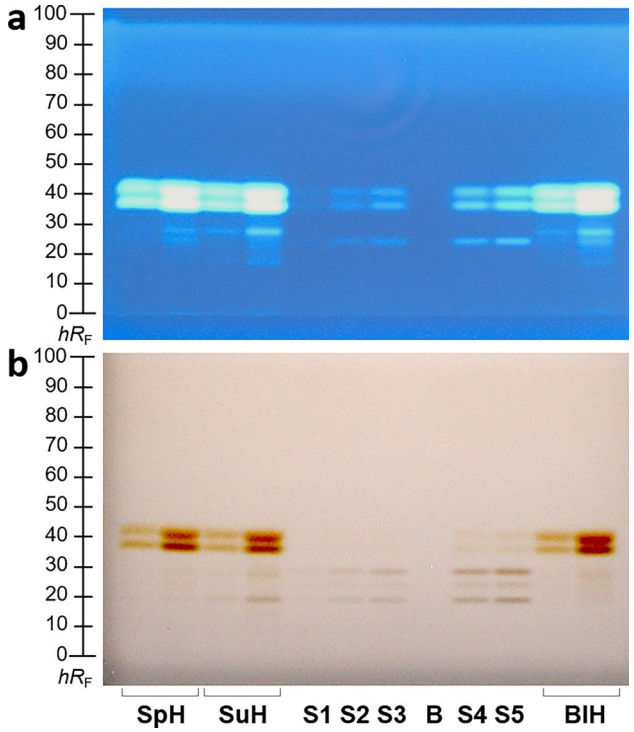

Fig. 5 Saccharide analysis of spring honey (SpH), summer honey (SuH), and blossom honey (BlH), diluted 1:250 and applied at 0.5 and 1.5 µL, along with standard mixture (fructose, glucose, sucrose, maltose, galactose, and melezitose, decreasing hR_F, each 0.1 mg/mL, applied at 0.2, 0.5, 0.8, 1.4, and 2.0 µL, S1–S5) and blank (B, water used for sample dilution). The plate was two-fold developed with 2-propyl acetate–methanol–water (7:3.5:1.5, *V/V*) containing 1 mg/mL natural product reagent A, and detected at 366 nm after derivatization with ethylamine (**a**) and under white light illumination after consecutive derivatization with aniline diphenylamine orthophosphoric acid reagent (**b**)

non-reducing saccharides (melezitose and sucrose) in the honey samples were detected first when the aniline diphenylamine orthophosphoric acid reagent was secondly applied as reagent sequence on the same plate.

Fructose (hR_F 39) and glucose (hR_F 35) were significantly present in all samples, and the tracks were overloaded with the aim also to record the saccharides that were only present in small amounts (Fig. 5a). Maltose (hR_F 22) was only detectable in the spring and blossom honey samples but not in summer honey. Furthermore, another fluorescent zone was visible in all honey samples directly above maltose. After derivatization with aniline diphenylamine orthophosphoric acid reagent, a relatively weak zone was detected in the honey samples at the same hR_F as sucrose in the standard mixture (Fig. 5b). However, the fluorescent zone in the honey samples (Fig. 5a, hR_F 28) could not be assigned to sucrose because the sucrose standard is not expected to show fluorescence when derivatized with ethylamine.

On derivatization with ethylamine, below maltose, both the summer honey and the blossom honey slightly revealed another zone of a saccharide (hR_F 18) not present in the standard mixture. At the same hR_F, the plate gave a signal for melezitose after derivatization with aniline diphenylamine orthophosphoric acid reagent. Since melezitose is also a non-reducing saccharide, it cannot be this saccharide. In both cases, the reducing turanose, formed by hydrolysis of melezitose, could be a candidate to be proven with an appropriate standard. Still, it could not be excluded that sucrose and melezitose were additionally present in these samples. Nevertheless, this coelution would not have been detected with aniline diphenylamine orthophosphoric acid reagent alone as a reagent for derivatization, giving a nice example of the advantageous consecutive derivatization as reagent sequence as presented. In sample dilutions of 1:250, glucose and fructose could easily be quantified in the studied honey samples (Table 2).

Table 2 Glucose and fructose contents of the studied honey samples

Saccharide	Content (%, *w/w*, $n = 1$)		
	Spring honey	Summer honey	Blossom honey
Glucose	29.0	32.5	32.6
Fructose	25.9	34.6	37.0

4 Conclusion

Ethylamine was shown not to produce colorful zones of saccharides separated on silica gel plates, but ochre color and in particular brightly blue fluorescent zones specific for reducing saccharides, allowing highly sensitive detection at 366 nm. The preparation of the ethylamine reagent was simple

and comparatively inexpensive and sustainable, since the concentrated aqueous solution only needed to be diluted with water. Exploiting a reagent sequence, non-reducing saccharides were additionally detectable after a consecutive derivatization with aniline diphenylamine orthophosphoric acid reagent on the same plate. The wide range of use of ethylamine for the specific detection of reducing saccharides was demonstrated via amylolysis of starches as well as analysis of lactose-free milk and honey. Advantageously, it also could be used to determine reducing saccharides without interference from a large excess of sucrose as a prominent saccharide in food.

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Declarations

Conflict of interest The authors, WS and GM are members of the Editorial Board of the journal. Therefore, the submission was handled by a different member of the editorial board, and they did not take part in the review process in any capacity.

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