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THIN-LAYER ELECTROPHORETIC BEHAVIOUR OF OLIGO- AND MONOSACCHARIDES, URONIC ACIDS AND POLYHYDROXY COMPOUNDS OBTAINED AS BIOMASS DEGRADATION PRODUCTS

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SUMMARY

A recently developed thin-layer electrophoretic method has been applied to the determination of biomass degradation products comprising mono-, oligo- and polysaccharides and their derivatives as well as phenolic compounds. The separation was carried out using 0.3 *M* aqueous borate solution as the buffer and silanized silica gel as the support, the surface of the latter being covered with a thin film of octanol-1. For visualization of the carbohydrates, specific sulphuric acid-containing naphthoresorcinol and orcinol reagents were applied. The mobility of hydrolyzed products is determined by the number and position of their reactive sites and, to a less extent, by the size of their molecule. The good separations obtained confirm this method as an alternative to the commonly utilized chromatographic procedures.

INTRODUCTION

Zone electrophoresis is a method frequently applied in carbohydrate analysis[1–3]. Ionic compounds, such as uronic acids or sugar phosphates, migrate even at neutral pH, but electrically neutral substances (mono-, oligo- and polysaccharides) have to be converted into an ionic form. Borate ions have proved to be suitable for this purpose, as they react with vicinal hydroxyl groups forming negatively charged complexes[2–5]. Steric hindrance by large side-chains and interaction with other ligands may promote or block the formation of complexes[3]; therefore a wide variety of mobilities can be found. A special advantage of using borate complexes is their high mobility under the influence of an electric field, up to ten times higher than the mobilities of other charged carbohydrate systems[1].

The choice of the carrier materials is a central problem. Good separations of mono- and oligosaccharides were obtained by high-voltage electrophoresis on chro-

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matography paper[6]. For the analysis of polysaccharides, foils of cellulose acetate[7] and glass fibre paper[8] are suitable. The use of gel materials (acrylamide, agarose, starch), frequently utilized in protein and nucleic acid chemistry, is not possible here because of the molecular sieve effect and the polydispersity of the polysaccharides[9].

The following factors have additionally reduced the applicability of electrophoresis to this case: (a) the limited possibilities for visualization (for chromatography paper and cellulose acetate); (b) the adsorption and electroendoosmosis effects (for glass fibre paper)[8,10]. The first limitation is especially relevant to non-reducing oligo- and polysaccharides, as the specific reagents containing sulphuric or phosphoric acids cannot be used because of simultaneous side-reactions with the carrier materials, such as chromatography paper, cellulose acetate or gels. These reagents may be applied on glass fibre paper, but adsorption and endoosmosis effects cause distortion of zones and complex conditions for migration. When silanized glass fibre paper is used, these phenomena can be suppressed and good results are obtained for polysaccharides, but this carrier material is mechanically unstable and difficult to handle[8].

To overcome these disadvantages, silanized silica gel, which has been used in thin-layer chromatography (TLC)[11], can be used as a support. Its surface is covered with a thin film of an organic fluid not miscible with water[12]. With this system, stable thin layers are obtained, adsorption and endoosmosis effects are low and chemically aggressive reagents can be employed. The carbohydrate-containing zones are detected by using special phenol–sulphuric acid reagents. Sulphuric acid degrades carbohydrates to furfural derivatives, which form coloured compounds with phenols[13].

The present work describes the evaluation of this technique and its application to the special problem of analyzing degradation products obtained by hydrothermolysis of biomass. In this procedure, the biomass components are solubilized in pure water under pressure at temperatures of 170 to >300°C. Hemicellulose, cellulose and lignin can be dissolved step by step[14,15]. This treatment leads to aqueous solutions containing a complex mixture of poly-, oligo- and monosaccharides as well as other polyhydroxy compounds, furan derivatives and phenolcarboxylic acids. For the determination of these substances, high-performance liquid chromatography (HPLC)[16], ion-exchange and gas chromatography[17], TLC[18] and isotachophoresis (for phenolic components of the lignin)[19] have been employed. Electrophoresis could therefore be a suitable supplement to these commonly utilized methods.

MATERIALS AND METHODS

Instrumentation

In all experiments a Bio-Rad horizontal electrophoresis cell (for 15 cm × 10 cm plates) with a power supply and Desaga equipment for the manufacture of TLC plates (with variable adjustment for thickness of the layer) were used.

Chemicals

Special chemicals employed in the experimental procedures were silica gel 60 H, silanized (Merck Art. No. 7761), octanol-1 (p.a.), glass fibre paper (Whatman GF/C), 1,3-dihydroxynaphthalene (naphthoresorcinol), 3,5-dihydroxytoluene (orcinol), polyvinylpyrrolidone K 90 (molecular weight 340000; Fluka, Buchs, Switzer-

land) and xylan (mol.wt. about 25000; Serva Feinbiochemica, Heidelberg, F.R.G.).

The following chemicals were used as reference substances for the determination of the migration distances: DL-glyceraldehyde, methylglyoxal, 1,3-dihydroxyacetone and cellobiose (Fluka, Buchs, Switzerland); glycolaldehyde, furfural, 5-hydroxymethylfurfural, D-glucose, D-fructose, D-xylose (Merck, Darmstadt, F.R.G.); maltotriose (EGA-Chemie, Steinheim, F.R.G.); 1,6-anhydro-β-D-glucose (Sigma, St. Louis, MO, U.S.A.).

Buffer solution

Crystallized boric acid 18.54 g (0.3 mol) and Titriplex III (7.44 g, 0.02 mol) were dissolved in 200 ml of distilled water and 2 *M* sodium hydroxide solution was added until the pH was 10; then the solution was made up to 1000 ml with distilled water.

Thin-layer plates

A 15-g amount of silanized silica gel was suspended in 100 ml dichloromethane containing 4 g octanol-1. After stirring for 30 min the solvent was vacuum-evaporated. The remaining dry powder, which should be free from lumps, was mixed with 10 ml of a 2% solution of polyvinylpyrrolidone in the buffer and 35 ml pure buffer solution. Then the plates were coated to a thickness of 0.3 mm and placed in a desiccator. They were kept in an humid atmosphere (over the buffer solution) for 12 h.

Preparation of the solutions to be analyzed

Samples of wheat straw and poplar wood were chopped and ground, then degraded in the hydrothermolysis apparatus at 200 and 270°C (see refs. 14 and 15). The eluates were concentrated and dissolved in buffer solution (one to three times the quantity of the concentrate). Alternatively, standard solutions of pure substances and mixtures were prepared (0.5–3% solutions).

Electrophoretic procedure

The plates were taken from the desiccator and put immediately onto cooling block of the electrophoresis apparatus. The connection to the electrolyte vessels was made through strips of glass fibre paper, soaked with buffer. The plates were equilibrated for 30 min at the chosen voltage, then the samples (dissolved in buffer solution) were spread in small grooves (1 cm long) scratched into the layer with a needle. Electrophoretic conditions: 200–400 V for 60–120 min.

Preparation of visualization reagents

Naphthoresorcinol reagent (for carbohydrates). Immediately before use, 2.5 ml concentrated sulphuric acid were carefully added dropwise to 50 ml pure ethanol, then 1.5 ml octanol-1 were added. In this mixture, 0.4 g naphthoresorcinol were dissolved.

Orcinol reagent (for carbohydrates). Solution a (6% orcinol in pure ethanol containing 3–5% octanol-1) and solution b [1% iron(III) chloride in 10% sulphuric acid] were mixed in the ratio a:b = 1:10 before use.

Iron(III) chloride reagent (for phenolic compounds). 5% Iron(III) chloride solution in 0.5 *M* hydrochloric acid.

Visualization and preservation

After electrophoresis the plates were dried at 110°C for 15–30 min. The cool plates were sprayed with one of the reagents. In the case of carbohydrates, the plates were kept at room temperature (30 min) for prehydrolysis of the polysaccharides. They were then heated to 110°C until the coloured zones developed. The phenolic compounds appear immediately after spraying. The plates were then covered with a second glass plate of the same size and kept airtight by putting adhesive tape around them. They were stored in the dark (at 4°C); for documentation the electropherograms were photographed.

RESULTS AND DISCUSSION

Separation of standard mixtures

Fig. 1a shows an electropherogram of standard substances: oligo- and monosaccharides, uronic acids, anhydro sugars and fragments of sugars such as polycarbonyl and polyhydroxy compounds. Their migration distances are spread over a wide range according to their borate complexes. The line electropherogram in Fig. 1b

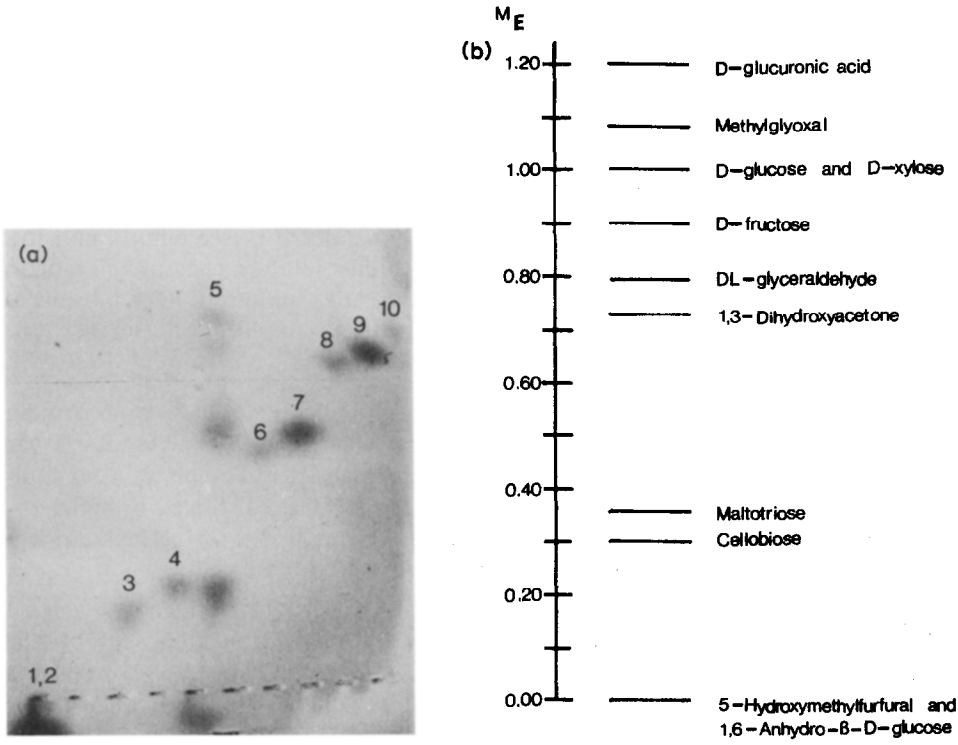


Fig. 1. (a) Electropherogram of standard substances occurring after hydrothermolytic degradation (350 V, 60 min; borate buffer pH 10). Detection with orcinol reagent. Substances: 1 = 5-hydroxymethylfurfural; 2 = 1,6-anhydro-β-D-glucose; 3 = cellobiose; 4 = maltotriose; 5 = mixture of all substances; 6 = 1,3-dihydroxyacetone; 7 = glyceraldehyde; 8 = glucose; 9 = methylglyoxal; 10 = glucuronic acid. (b) Line electropherogram showing the mobilities of the standard substances in M_E units (reference substance, D-glucose, M_E = 1.00). The lines mark the centres of the electrophoretic zones obtained.

TABLE I

RELATIVE MOBILITIES, M_E, OF BIOMASS DEGRADATION PRODUCTS

Reference substance: D-glucose, M_E = 1.00.

Zone	*Compound*	M_E	*Colour reactions*	
			With naphthoresorcinol	*With orcinol*
1	5-Hydroxymethylfurfural	0.00	Red*, blue-green	Yellow-dark brown
2	1,6-Anhydro-β-D-glucose	0.00	Blue	Dark brown
3	Cellobiose	0.30	Dark blue	Green-blue
4	Maltotriose**	0.35	Blue (weak)	Green-blue
5	1,3-Dihydroxyacetone	0.73	Blue-gray, brown	Yellow-light brown
6	DL-Glyceraldehyde	0.78	Red*, blue-gray	Dark brown
7	D-Fructose	0.90	Gray-blue	Reddish brown
8	D-Glucose	1.00	Blue	Green-blue
9	D-Xylose	1.00	Blue	Green blue
10	Methylglyoxal	1.08	Gray-green	Blue-violet
11	D-Glucuronic acid	1.20	Blue	Blue-green
	Furfural	–	Not detected	
	Glycolaldehyde	–	Not detected	

* Colour of zone before heating.

** Reference substance, since cellotriose was not available, and maltose has a similar M_E to that of cellobiose.

enables a comparison of all the determined components; the corresponding values of the relative mobilities (reproducibility 5–7%) are given in Table I. In this table the colour reactions obtained by application of the naphthoresorcinol and orcinol reagents are also specified. Some compounds, *e.g.*, hydroxymethylfurfural and glyceraldehyde, can be identified even by their characteristic colour changes.

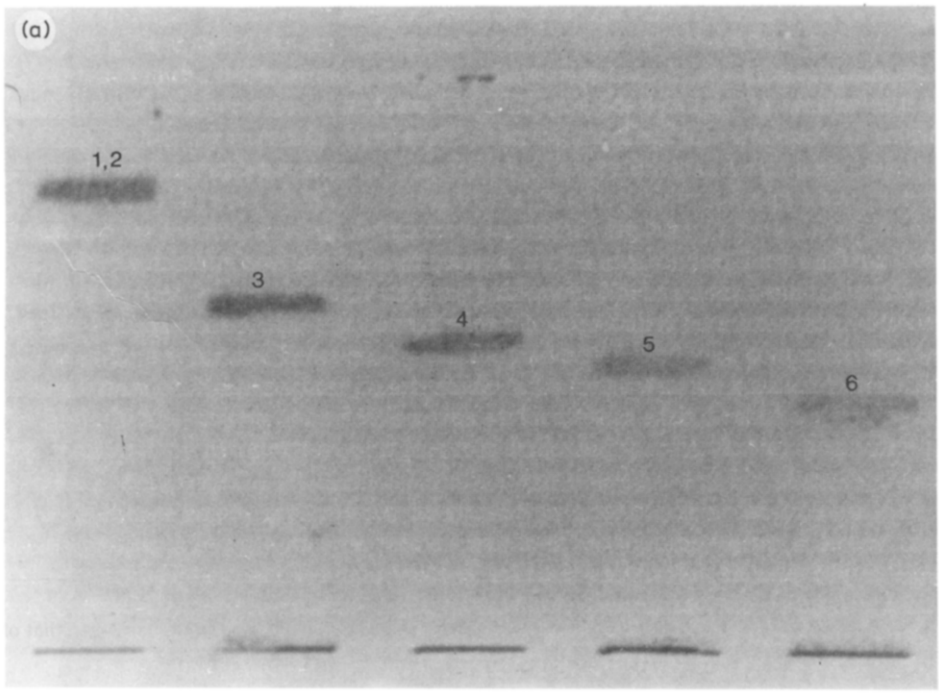
When lignocellulose-containing materials are degraded at temperatures higher

TABLE II

RELATIVE MOBILITIES, M_E, OF PHENOLS AND PHENOLCARBOXYLIC ACIDS

Reference substance: 3,4-dihydroxybenzoic acid, M_E = 1.00.

Compound	*Fluorescence*	*Colour of the iron complexes*	M_E
2,6-Dimethoxyphenol 1st fraction	–	Brown	0.0
2nd fraction	+	–	0.4
Sinapic acid 2st fraction	+	–	0.0
2nd fraction	+	Brown-gray	0.5
Ferulic acid 1st fraction	+	–	0.1
2nd fraction	+	Light brown	0.6
Acetovanillone	+	–	0.6
Homovanillic acid	–	Violet	0.6
4-Hydroxyphenylacetic acid	–	Reddish brown	0.7
4-Hydroxycinnamic acid	–	Orange	0.8
trans-3,4-Dihydroxycinnamic acid	+	Gray	1.0
3,4-Dihydroxybenzoic acid	–	Gray	1.0



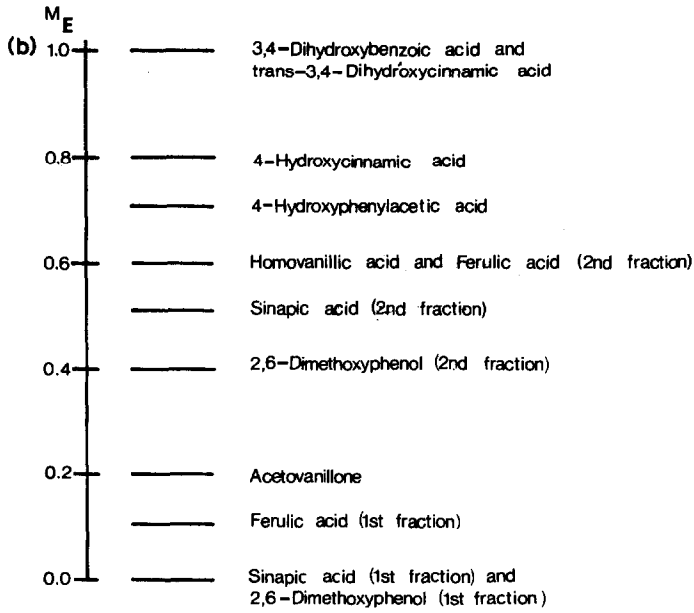


Fig. 2. (a) Electropherogram of phenolcarboxylic acids (200 V, 120 min; borate buffer pH 10). Detection with iron(III) chloride reagent. Substances: 1 = 3,4-dihydroxybenzoic acid; 2 = *trans*-3,4-dihydroxycinnamic acid; 3 = 4-hydroxycinnamic acid; 4 = 4-hydroxyphenylacetic acid; 5 = homovanillic acid; 6 = sinapic acid. (b) Line electropherogram showing the mobilities of phenols and phenolcarboxylic acids in M_E units (reference substance, 3,4-dihydroxybenzoic acid, M_E = 1.00). The lines mark the centres of the electrophoretic zones obtained.

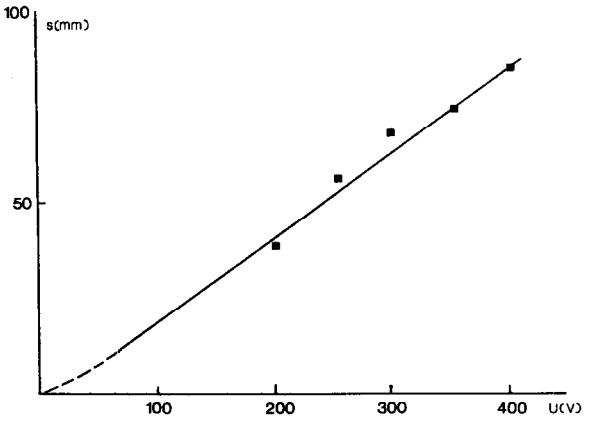


Fig. 3. Migration distance of D-glucose, *s*, *versus* the voltage between the electrodes, *U*, for constant electrophoresis time (60 min).

than 200°C; fragments of lignin will be dissolved, especially phenols and phenolcarboxylic acids. These compounds also form borate complexes, as shown in Fig. 2a. A wide variety of mobilities is found (see line electropherogram in Fig. 2b, Table II); some substances also form two complexes with different charges. Phenolic compounds are detected by application of the iron(III) chloride reagent and by their fluorescence in the UV range (254 nm).

It has been further shown that the basic relationship describing electrophoretic migration[20], namely the linear increase of the migration velocity with electric field strength (or voltage, when the distance between the electrodes is constant), is valid. Fig. 3 shows the mobility of D-glucose as a function of the voltage applied.

Separation of degraded biomass components

In Fig. 4 the electrophoretic behaviour of samples obtained by different decomposition procedures is shown. Fig. 4a shows hemicellulose from an alkaline degradation process, as compared to xylan (molecular weight *ca.* 25 000, Fig. 4b). Both components form zones with two maxima. This is very similar to the behaviour of a sample of wheat straw degraded by the hydrothermolysis process at 180–200°C, and indicates the heterogeneity of the polysaccharides (inhomogeneous fragments). In the electropherogram of a poplar wood sample (degraded at 270°C), products of lower molecular weight derived from the hemicellulose and cellulose parts of the biomass can be identified (Fig. 4c), such as hydroxymethylfurfural, oligosaccharides, glucose, xylose and fructose, and small amounts of undetermined byproducts (Table III).

Contrary to the chromatographic methods, the separation principle of this technique is based on the tendency of the compounds to form charged borate complexes, which depends on the positions of the hydroxyl groups. Their mobility is mainly determined by the relationship between the charge and the Stokes radius of the molecule, whereas their absolute size has no influence, *e.g.*, high-molecular-weight xylan has approximately the same mobility as cellobiose or maltotriose. Also the

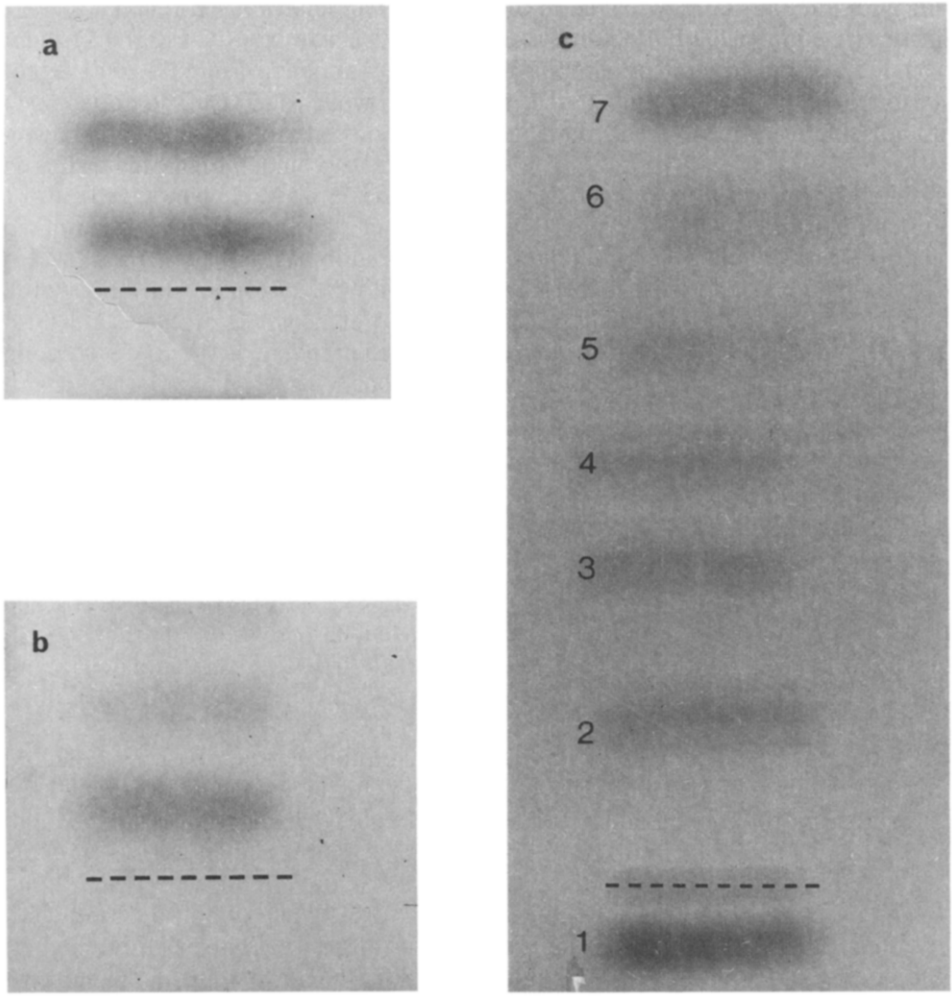


Fig. 4. Electropherograms of alkaline degraded hemicellulose (a) and xylan (b) (170 V, 120 min) and of poplar wood degraded hydrothermally at 270°C (c) (350 V, 60 min). Borate buffer pH 10; detection with naphthoresorcinol reagent.

TABLE III

RELATIVE MOBILITIES, M_E, OF THE ELECTROPHORETIC ZONES OF A POPLAR WOOD SAMPLE DEGRADED AT 270°C

Reference substance: D-glucose, M_E = 1.00.

Zone	M_E	*Corresponding compounds*
1	0.00	5-Hydroxymethylfurfural and 1,6-anhydro-β-D-glucose
2	0.2–0.35*	Cellobiose and oligosaccharides
3	0.50	Not identified
4	0.59	Not identified
5	0.71	1,3-Dihydroxyacetone
6	0.85	D-Fructose
7	1.00	D-Glucose and D-xylose

* Intensity maxima at 0.20 and 0.30.

steric positions of the hydroxyl groups are important, and compounds of comparable size having the same number of sterically suitable binding sites show similar mobilities, *e.g.*, glucose and xylose; glyceraldehyde and dihydroxyacetone.

After evaporation of the organic liquid covering the surface, the inactive support is suitable for the application of chemically aggressive detection reagents. This is a great advantage over previous supports. It enables visualization of the reducing as well as the non-reducing carbohydrates. Coverage of the surface of the support diminishes the adsorption effects and the electroendoosmosis[12].

A further advantage of this technique is the utilization of humid plates. This prevents concentration of the sample at the site of application and therefore possible chemical side-reactions.

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