THIN-FILM CHROMATOGRAPHY IN THE STUDY OF CARBOHYDRATES*

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Thin-film silicic acid chromatography[1] has been developed by STAHL and coworkers[2-5] into an important analytical technique of great adaptability[6-8] which has been modified for micropreparative use[9] and quantitative analysis[9,10].

The method has found application in the study of a broad range of compounds including terpenes[1,2,11], organic peroxides[3], protein hydrolyzates[12,13], pyrethrins[14], indole and its derivatives[15], alkaloids[5,16], lipids[17-22], steroids[23,24], 2,4-dinitrophenylhydrazones[25], and in inorganic analysis[7]. Apart from its application in the separation of mixtures of a few simple sugars[26], digitalis and podophyllum glycosides[27], mono-O-methyl, mono- and di-O-isopropylidene[28], O-acyl[29-31], and 2,4-dinitrophenylhydrazone[32] derivatives, the technique has had only limited usage in the carbohydrate field. This paper reports the extension of thin-layer silicic acid chromatography to the qualitative investigation of a variety of partially or completely derivatized, low molecular weight carbohydrates and to the quantitative determination of the composition of a mixture of methylated sugars.

Three solvent systems have been employed. Benzene–ethanol–water–ammonium hydroxide (200:47:15:1)[33] (solvent A) is preferred for the separation of mixtures of O-methyl sugars and their glycosides. 1-Butanol–acetic acid–ethyl ether–water (9:6:3:1) (solvent B) is employed for the separation of carbohydrates with low degrees of substitution, whereas 1-butanol–acetic acid–water (2:1:1)[34] (solvent C) gives satisfactory resolution of sugar acids or acid derivatives.

In general, the R_F values of fully derivatized compounds are high (see Tables IV, V and VI), a decrease in R_F being observed as the number of free hydroxyl groups in the molecule is increased. Thus, the R_F of tetra-O-methyl-D-glucopyranose is 0.38 in solvent A, those of the tri-O-methyl-D-glucoses are of the order of 0.15 whereas the di-O-methyl-D-glucoses exhibit R_F values of approximately 0.05 (see Table III).

The separation of similarly derivatized diastereoisomers is slight. For example, in solvent A, the R_F of methyl tetra-O-acetyl-β-D-glucopyranoside is 0.76 whereas the R_F of methyl tetra-O-acetyl-β-D-mannopyranoside is 0.79 (see Table II). An exception to this generalization is the excellent separation of D-glucosaccharo-1,4-lactone from D-glucosaccharo-3,6-lactone in solvent C, the R_F of the former being 0.43 whereas the R_F of the latter is 0.85 (see Table VII).

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The pentoses and their derivatives have higher R_F values than the hexose analogues. However, this relationship is reversed in the case of the respective glyconic acids. Accordingly, L-arabinose and D-glucose have R_F values of 0.57 and 0.49 respectively in solvent B (see Table I), whereas the R_F value of L-arabonic acid is 0.35 and that of D-gluconic acid is 0.40 in solvent C (see Table VII).

The development of the chromatoplates requires 0.5 to 3.5 hours depending upon the relative volatility of the solvent and the room temperature. Solvent A affords the most rapid and solvent C the slowest development.

It is observed that the migration of a particular compound is decreased by repeated use of the solvent. For this reason it is recommended that fresh solvent be employed each time a chromatoplate is developed.

The compounds are detected by spraying the developed plates with concentrated sulfuric acid or alkaline potassium permanganate[35,36] and heating until suitable color formation occurs.

Sulfuric acid is effective in detecting all carbohydrate compounds. Sugar alcohols, glyconic acids, inositol and their derivatives require stronger heating than reducing sugars and their derivatives, a property useful in differentiating compounds having similar R_F values. Glycerol and glyceryl hexosides give transient pink spots which change to brown on standing at room temperature. Pentoses yield spots with a purple cast whereas sulfur-containing derivatives give yellow-green spots. Hexoses, hexose-containing oligosaccharides and their derivatives give yellow-brown to black spots.

The alkaline permanganate spray readily detects all compounds with free hydroxyl groups or compounds which are derivatized with alkali-labile groups. The spray gives bright yellow spots on a purple background which fade to white spots on a brown background on standing at room temperature. This spray is preferred for the detection of polyols and inositol.

With partially derivatized carbohydrates ammoniacal silver nitrate[37], *p*-anisidine–trichloroacetic acid[38] or aniline hydrogen oxalate[39] may be used. The sensitivity of the ammoniacal silver nitrate is lower on silicic acid than on chromatography paper. Acylated derivatives are readily detected (*cf.* ref. 29) as the ferric hydroxamates[40].

A mixture of 3-O-methyl-D-glucopyranose, 2,3,6-tri-O-methyl-D-glucopyranose and 2,3,4,6-tetra-O-methyl-D-glucose can be resolved using solvent A, and quantitatively eluted[10] and estimated using the phenol–sulfuric acid colorimetric determination[41]. This procedure permits a rapid determination of the molar ratios of the mono-, di-, tri- and tetra-O-methyl components in the hydrolysate of a methylated polysaccharide.

Thin-layer silicic acid chromatography affords a simple, rapid and sensitive method for the qualitative and quantitative study of low molecular weight carbohydrates. It is of particular value for carbohydrates which are not readily detected by conventional chromatography. By this means reactions in carbohydrate chemistry involving the preparation or transformation of derivatives can be followed with speed and facility, and, as a result of the sensitivity of this technique, very low concentrations of side products or impurities can be detected.

EXPERIMENTAL

(*a*) *Solvents*

The following solvents were used for the separation of partially or completely derivatized carbohydrates:

Solvent A[33]. Benzene–ethanol–water–ammonium hydroxide (sp. gr. 0.8), 200:47:15:1 parts by volume. The upper phase of this two phase system is used. It has been observed that the substitution of glacial acetic acid for the ammonium hydroxide gives slightly sharper bands or spots.

Solvent B. 1-Butanol–acetic acid–ethyl ether–water, 9:6:3:1 parts by volume (single phase system).

Solvent C[34]. 1-Butanol–acetic acid–water, 2:1:1 parts by volume (single phase system).

(*b*) *Spray reagents*

Spray 1: Concentrated sulfuric acid. Spray 2[35,36]:0.5 % potassium permanganate in *N* NaOH.

(*c*) *Preparation of plates*

A slurry of Silica Gel G (Merck and Co.) (30 g) in water (66.5 ml) is applied to 5 × 20 cm or 20 × 20 cm smooth glass plates using the apparatus of STAHL[2,4].

The plates are dried overnight at 135°. The serrated plates, after drying, are scraped with a straight-edge to remove adsorbent from the ridge peaks.

(*d*) *Chromatographic procedure*

The compounds are dissolved in suitable solvents and applied to the chromatoplate with glass capillaries[3]. The plates are developed in pre-equilibrated battery jars or wide-mouth bottles by the ascending technique.

(*e*) *Detection of carbohydrate compounds*

The developed plates are dried in the air and sprayed with either concentrated sulfuric acid or a solution of 0.5 % potassium permanganate in *N* sodium hydroxide. The plates are then heated in a suitable oven at about 100°. With the alkaline permanganate spray heating for 0.5 to 2 min is required whereas if the plates are preheated before the spraying, the color develops almost instantaneously. With the sulfuric acid spray a heating time of 5 to 10 min is usually sufficient. Whereas reducing sugars and their derivatives can be detected at about 100°, sugar alcohols, glyconic acids, inositol and their derivatives require heating to approximately 150°.

(*f*) *The separation of partially or completely derivatized sugars, polyols, glyconic and glycuronic acids and inositol*

The results of this study are summarized in Tables I to VII.

(*g*) *The quantitative determination of a mixture of 3-O-methyl-D-glucopyranose, 2,3,6-tri-O-methyl-D-glucopyranose and 2,3,4,6-tetra-O-methyl-D-glucose*

3-O-Methyl-D-glucopyranose (0.00404 g, 2.0 μmole), 2,3,6-tri-O-methyl-D-glucopyranose (0.00135 g, 0.61 μmole) and 2,3,4,6-tetra-O-methyl-D-glucose (0.00061 g, 0.25 μmole) are dissolved in methanol (approx. 0.2 ml).

A portion of this solution is applied to a 20 × 20 cm glass plate coated[24] with Silica Gel G, using a capillary tube. The sample is applied at 1 mm intervals along a straight line 16 cm long equidistant from each side and 3.2 cm from the bottom of the plate. The plate is developed in solvent A by the ascending technique.

TABLE I

R_F VALUES FOR FREE SUGARS, POLYOLS AND *myo*-INOSITOL IN SOLVENT B

Compound	R_F	*Compound*	R_F
D-Ribose	0.59	Melibiose	0.15
L-Arabinose	0.57	Lactose	0.09
D-Xylose	0.57	Raffinose	0.13
D-Glucose	0.49	Glycerol	0.58
D-Mannose	0.55	Erythritol	0.52
D-Fructose	0.51	D-Mannitol	0.38
Maltose	0.29	D-Glucitol	0.39
Cellobiose	0.32	Galactitol	0.36
Isomaltose	0.16	Maltitol	0.10
Laminaribiose	0.26	*myo*-Inositol	0.27
Sucrose	0.25		

TABLE II

R_F VALUES FOR GLYCOSIDES AND DERIVATIZED GLYCOSIDES OTHER THAN O-METHYL ETHERS

Compound	R_F *in solvent*	
	A	*B*
Methyl β-L-arabinopyranoside	0.03	0.48
Methyl α-D-glucopyranoside	0.02	0.48
Methyl β-D-glucopyranoside	0.02	0.52
Methyl α-D-mannopyranoside	0.02	0.51
n-Amyl β-D-glucopyranoside	0.04	0.69
Phenyl β-D-glucopyranoside	0.03	0.69
Methyl β-maltoside	0.01	0.31
Methyl β-lactoside	0.00	0.17
Methyl α-isomaltoside	0.00	0.19
Methyl 3,6-anhydro-α-D-glucopyranoside	0.03	0.59
Methyl 6-O-benzyl-α-D-galactopyranoside	0.08	0.69
Methyl tetra-O-acetyl-β-D-glucopyranoside	0.76	0.79
Ethyl tetra-O-acetyl-β-D-glucopyranoside	0.78	0.80
n-Propyl tetra-O-acetyl-β-D-glucopyranoside	0.83	0.83
Phenyl tetra-O-acetyl-β-D-glucopyranoside	0.82	0.81
Methyl tetra-O-acetyl-β-D-mannopyranoside	0.79	0.81
Methyl hepta-O-acetyl-β-maltoside	0.74	0.74
Methyl 4,6-O-benzylidene-α-D-glucoside	0.21	0.81
Methyl 4,6-O-benzylidene-β-D-glucoside	0.21	0.79
Methyl 2,3-anhydro-4,6-O-benzylidene-α-D-alloside	0.83	0.76
Methyl 2,3-di-O-tosyl-4,6-O-benzylidene-α-D-glucoside	0.90	0.91

TABLE III

R_F VALUES OF O-METHYL ETHERS OF SUGARS AND POLYOLS IN SOLVENT A

Compound	R_F
2,3-Di-O-methyl-D-xylopyranose	0.15
2,3,4-Tri-O-methyl-D-xylopyranose	0.28
2,4-Di-O-methyl-D-glucopyranose	0.05
2,6-Di-O-methyl-D-glucopyranose	0.05
2,3,6-Tri-O-methyl-D-glucopyranose	0.18
2,4,6-Tri-O-methyl-D-glucopyranose	0.13
2,3,4,6-Tetra-O-methyl-D-glucopyranose	0.38
2,4,6-Tri-O-methyl-D-galactopyranose	0.17
2,4-Di-O-methyl-L-fucopyranose	0.11
1,2,5,6-Tetra-O-methyl-3,4-O-isopropylidene-D-mannitol	0.60

After removal of solvent by evaporation at room temperature the center of the plate is covered with a band of polyethylene film 12 cm wide situated 4 cm from each side of the plate and covering its entire length, so as to leave a 2-cm strip, containing a portion of the sugar mixture, on each side of the protective covering. The exposed areas of the plate are sprayed with sulfuric acid and the plate heated for 5 min at 100° to develop color. The bands are located and marked. The silicic acid of the appropriate areas containing each component is then sucked off the plate through a drawn out tube into distilled water (3 ml) in a test tube by means of a water aspirator. The glass tubing and rubber connections used to conduct the silicic acid into the water are rinsed with distilled water (1 ml) and the two solutions combined. The silicic acid is separated by centrifugation and aliquots (1 ml) of the clear, colorless supernatant are used in the determination of carbohydrate by the phenol–sulfuric acid method[41]. The absorbancies of triplicate samples are determined at 485 mμ in a Coleman Jr. spectrophotometer[41]. The average absorbance is used to determine the weight of carbohydrate present by reference to standard curves previously constructed for the purpose.

A blank experiment revealed the presence on the developed chromatoplate of two readily visible, narrow yellow bands having R_F values of 1.00 and 0.78. The colored

TABLE IV

R_F VALUES OF O-METHYL ETHERS OF GLYCOSIDES

Compound	R_F *in solvent*	
	A	*B*
Methyl 4-O-methyl-α-D-glucopyranoside	0.05	0.57
Methyl 6-O-methyl-α-D-galactopyranoside	0.06	0.45
Methyl 2,3-di-O-methyl-α-D-glucopyranoside	0.13	0.64
Methyl 2,6-di-O-methyl-α-D-glucopyranoside	0.15	0.62
Methyl 4,6-di-O-methyl-α-D-glucopyranoside	0.23	0.64
Methyl 3,4-di-O-methyl-α-D-mannopyranoside	0.21	0.57
Methyl tetra-O-methyl-α-D-glucopyranoside	0.52	0.75
Methyl tetra-O-methyl-α-D-mannopyranoside	0.54	0.69
Methyl hepta-O-methyl-β-maltoside	0.76	0.65

TABLE V

R_F VALUES OF ESTERIFIED SUGARS, POLYOLS AND *myo*-INOSITOL

Compound	R_F in solvent	
	A	B
Tetra-O-acetyl-D-xylopyranose	0.84	0.84
Penta-O-acetyl-α-D-glucopyranose	0.82	0.79
Penta-O-acetyl-β-D-glucopyranose	0.81	0.79
Penta-O-acetyl-β-D-galactopyranose	0.81	0.77
Hexa-O-acetyl-D-mannitol	0.83	0.73
Hexa-O-acetyl-*myo*-inositol	0.79	0.82
3,4,5,6-Tetra-O-acetyl-D-glucosazone	0.74	0.88
Tri-O-acetyl-D-glucal	0.83	0.79
Hexa-O-acetylxylobiose	0.72	0.84
Octa-O-acetyl-α-maltose	0.79	0.85
Octa-O-acetyl-β-cellobiose	0.64	0.84
Octa-O-acetyl-β-laminaribiose	0.62	0.81
Octa-O-acetyl-sucrose	0.63	0.77
Octa-O-propionyl-α-maltose	0.92	1.00
6-O-Tosyl-tetra-O-acetyl-β-D-glucopyranose	0.84	0.87
6-Deoxy-6-iodo-tetra-O-acetyl-β-D-glucopyranose	0.88	0.87
Tetra-O-benzoyl-D-fructofuranose	0.87	0.87

TABLE VI

R_F VALUES OF ACETAL AND MERCAPTAL DERIVATIVES OF SUGARS AND POLYOLS

Compound	R_F in solvent	
	A	B
1,2-O-Isopropylidene-D-glucose	0.10	0.79
1,2,5,6-Di-O-isopropylidene-D-glucose	0.49	0.90
1,2,3,4-Di-O-isopropylidene-D-galactose	0.47	0.84
6-O-Tosyl-1,2,3,4-di-O-isopropylidene-D-galactose	0.90	0.89
6-Deoxy-6-iodo-1,2,3,4-di-O-isopropylidene-D-galactose	0.93	0.89
3,4-O-Isopropylidene-D-mannitol	0.05	0.68
1,2,3,4,5,6-Tri-O-isopropylidene-D-mannitol	0.92	0.88
4,6-O-Ethylidene-D-glucitol	0.02	0.61
1,2-O-Isopropylidene-3,5-O-benzylidene-6-O-acetyl-D-glucofuranose	0.83	0.87
D-Galactose diethyl mercaptal	0.05	0.66
L-Arabinose diethyl mercaptal	0.12	0.77
L-Fucose diethyl mercaptal	0.18	0.76
L-Rhamnose diethyl mercaptal	0.21	0.78

TABLE VII

R_F VALUES OF SUGAR ACIDS AND THEIR DERIVATIVES

Compound	R_F *in solvent C*	*Compound*	R_F *in solvent C*
D-Ribonic acid	0.38	Calcium D-galactonate	0.38
D-Ribono-γ-lactone	0.61	D-Galactono-γ-lactone	0.61
L-Arabonic acid	0.35	D-Galactono-δ-lactone	0.47
Calcium L-arabonate	0.33	D-Galacturonic acid	0.32
L-Arabono-γ-lactone	0.64	D-Mannuronic acid	0.36
L-Arabono-δ-lactone	0.58	D-Mannurono-γ-lactone	0.53
L-Arabonic phenylhydrazide	0.62	L-Rhamnono-γ-lactone	0.64
D-Gluconic acid	0.40	D-Gulonic acid	0.31
D-Glucono-γ-lactone	0.68	D-Gulono-γ-lactone	0.53
D-Gluconic phenylhydrazide	0.60	D-Gulono-δ-lactone	0.43
D-Glucurono-γ-lactone	0.58	α-D-Glucoheptonic-γ-lactone	0.43
D-Glucosaccharo-1,4-lactone	0.43	Ascorbic acid	0.57
D-Glucosaccharo-3,6-lactone	0.85	Isoascorbic (D-araboascorbic) acid	0.57
D-Galactonic acid	0.38		

compounds in these bands are water-soluble and the aqueous solutions when treated with the phenol–sulfuric acid reagent show some absorbance at 485 mμ. Hence, this colored material would therefore interfere with the colorimetric determination of carbohydrate compounds with the same R_F values. The other areas of the chromatoplate contain no water-soluble materials which interfere with the phenol–sulfuric acid assay and no blank is required for the determination of carbohydrate compounds located outside of the two areas of the colored bands referred to above.

The results of two separate determinations are as follows:

Experiment 1. Found: 3-O-Methyl-D-glucopyranose, 111.2 γ (0.575 μmole); 2,3,6-tri-O-methyl-D-glucopyranose, 43.2 γ (0.194 μmole); 2,3,4,6-tetra-O-methyl-D-glucose, 19.2 γ (0.0815 μmole). This corresponds to a molar ratio of 7.0:2.4:1.0.

Experiment 2. Found: 3-O-Methyl-D-glucopyranose, 176.0 γ (0.909 μmole); 2,3,6-tri-O-methyl-D-glucopyranose, 67.6 γ (0.304 μmole); 2,3,4,6-tetra-O-methyl-D-glucose, 30.0 γ (0.127 μmole). This corresponds to a molar ratio of 7.1:2.4:1.0. The molar ratio of the three sugars applied was 8.0:2.4:1.0.

Recovery of carbohydrates from silica gel is quantitative.

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SUMMARY

Thin-film silicic acid chromatography has been demonstrated to be a useful technique for the separation of a wide variety of partially and completely derivatized carbohydrates. The procedure can be applied to the quantitative separation and estimation of mixtures of O-methyl sugars.

REFERENCES

1 J. G. KIRCHNER, J. M. MILLER AND G. J. KELLER, *Anal. Chem.*, 23 (1951) 420.
2 E. STAHL, G. SCHRÖTER, G. KRAFT AND R. RENZ, *Pharmazie*, 11 (1956) 633.
3 E. STAHL, *Chemiker-Ztg.*, 82 (1958) 323.
4 E. STAHL, *Parfuem. Kosmetik*, 39 (1958) 564.
5 E. STAHL, *Arch. Pharm.*, 292 (1959) 411.
6 E. STAHL, *Z. Anal. Chem.*, 181 (1961) 303.
7 E. STAHL, *Angew. Chem.*, 73 (1961) 646.
8 E. G. WOLLISH, M. SCHMALL AND M. HAWRYLYSHYN, *Anal. Chem.*, 33 (1961) 1138.
9 F. J. RITTER AND G. M. MEYER, *Nature*, 193 (1962) 941.
10 H. K. MANGOLD AND R. KAMMERECK, *Chem. Ind. (London)*, (1961) 1032.
11 J. M. MILLER AND J. G. KIRCHNER, *Anal. Chem.*, 25 (1953) 1107.
12 E. MUTSCHLER AND H. ROCHELMYER, *Arch. Pharm.*, 292 (1959) 449.
13 E. NÜRNBERG, *Arch. Pharm.*, 292 (1959) 610.
14 E. STAHL, *Arch. Pharm.*, 293 (1960) 531.
15 E. STAHL AND H. KALDEWEY, *Z. Physiol. Chem.*, 323 (1961) 181.
16 K. H. MÜLLER AND H. HONERLAGER, *Arch. Pharm.*, 293 (1960) 646.
17 D. C. MALINO AND H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 37 (1960) 576.
18 H. JATZKEWITZ AND E. MEHL, *Z. Physiol. Chem.*, 320 (1960) 251.
19 J. J. WREN, *J. Chromatog.*, 4 (1960) 173.
20 J. J. WREN, *Chromatographic Reviews*, Vol. 3, Elsevier, Amsterdam, 1961, p. 111.
21 O. S. PRIVETT AND M. L. BLANK, *J. Lipid Res.*, 2 (1961) 37.
22 L. J. MORRIS, *Chem. Ind. (London)*, (1962) 1238.
23 H. WEICKER, *Klin. Wochschr.*, 37 (1959) 763.
24 M. BARBIER, H. JÄGER, H. TIBIAS AND E. WYAS, *Helv. Chim. Acta*, 42 (1959) 2440.
25 J. H. DHONT AND C. DE ROOEY, *Analyst*, 86 (1961) 74.
26 E. STAHL AND U. KALTENBACH, *J. Chromatog.*, 5 (1961) 351.
27 E. STAHL AND U. KALTENBACH *J. Chromatog.*, 5 (1961) 458.
28 V. PREY, H. BERBALK AND M. KAUSY, *Mikrochim. Acta*, (1962) 449.
29 M. E. TATE AND C. T. BISHOP, *Can. J. Chem.*, 40 (1962) 1043.
30 M. GEE, *J. Chromatog.*, 9 (1962) 278.
31 J. O. DEFERRARI, R. M. DE LEDERKREMER, B. MATSUHIRO AND J. F. SPROVIERO, *J. Chromatog.*, 9 (1962) 283.
32 E. F. L. J. ANET, *J. Chromatog.*, 9 (1962) 291.
33 G. A. ADAMS, *Can. J. Chem.*, 33 (1955) 56.
34 L. HOUGH, J. K. N. JONES AND W. H. WADMAN, *J. Chem. Soc.*, (1950) 1702.
35 E. PACSU, T. P. MORA AND P. W. KENT, *Science*, 110 (1949) 446.
36 D. R. BRIGGS, E. F. GARNER AND F. SMITH, *Nature*, 178 (1956) 154.
37 S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238.
38 D. R. BRIGGS, E. F. GARNER, R. MONTGOMERY AND F. SMITH, *Anal. Chem.*, 28 (1956) 1933.
39 S. M. PARTRIDGE, *Biochem. Soc. Symp. (Cambridge, Engl.)*, No. 3, (1948) 57.
40 M. ABDEL-AKHER AND F. SMITH, *J. Am. Chem. Soc.*, 73 (1951) 5859.
41 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.