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A THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF D-MANNOSE, D-GLUCOSE AND D-GALACTOSE IN AQUEOUS SOLUTION

BARRY B. PRUDEN and GILLES PINEAULT

Department of Chemical Engineering, University of Ottawa, 770 King Edward Avenue, Ottawa K1N 6N5, Ontario (Canada)

and

HASSAN LOUTFI

Irving Pulp and Paper, P.O. Box 300, Station B, Saint John, N.B. (Canada)

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SUMMARY

A method for the determination of D-mannose, D-glucose and D-galactose is described for commercially available glass thin-layer plates. The silica gel GF layer on the plates was activated by a combination of heating and treatment with sodium bisulphite and a citrate–phosphate buffer. Plates were developed twice with an acetone–*n*-butanol–water (53:40:7) mixture, with heat treatment between developments. Spots were colored by treatment with ceric sulphate and subsequent charring, and quantitative analysis was made with a densitometer. The time required for analysis of one plate (5 samples) was 3 h, and standard errors of estimate were 2.8%, 3.6% and 5.6% for the three sugars, respectively, for 10 replicates at levels of up to 20.3 g/l mannose, 8.0 g/l glucose and 4.5 g/l galactose.

INTRODUCTION

The polysaccharide portion of Waste Sulphite Liquor (WSL) from the pulp and paper industry contains D-mannose, D-glucose and D-galactose as the main hexose components[1] which are fermentable to alcohol and yeast[2]. A fast, sensitive and specific method of analysis is necessary if the concentration of all components is to be followed during the course of reaction. For this reason the thin-layer chromatographic (TLC) method described in this work was developed. It has been used in an engineering study to analyze these three hexoses in over 25 fermentation runs[3] in which an average of 10 samples was taken for each run and analyzed in duplicate or triplicate.

Many TLC methods for the separation of sugars have appeared since the publication of Stahl and Kaltenbach[4] in 1961. Recent publications[5–8] give qualitative and quantitative methods for carbohydrates, and books[9–14] and reviews[15,16] also give TLC methods for carbohydrates.

The TLC method described here is a new analytical method for the quantitative

determination of D-mannose (mannose), D-glucose (glucose) and D-galactose (galactose) in water solution. It is an improvement on existing methods using a commercially available pre-coated glass plate. The improvement involves activating the silica gel GF245 layer by drying, spraying with 0.1 *M* sodium bisulphite, drying, spraying with citrate–phosphate buffer (pH 4), and drying before use. Another modification was the use of a second development, after drying under a stream of air, to ensure the best separation. The developing solution suggested by other authors[17], acetone–*n*-butanol–water (5:4:1), was modified to the ratio 53:40:7. Finally, a syringe was used with a commercial spotter to obtain controlled diameter and density spots.

Scanning of the developed and charred chromatograms was done horizontally using a densitometer.

EXPERIMENTAL

Apparatus

The densitometer was a Kontes Glass Company Chromaflex K-49500 with a photographic filter (Photar, ND. 6 series 4) on the light source. The unit was operated in the filtered, visible-light, reflectance mode at a scanning speed of 10 cm/min and an attenuation of 200. It was connected to a Fisher Recordal recorder with a 10 mV span and chart speed of 5 in./min.

A Kontes K-416330 Chromaflex spotter with an air-flow regulator was used, together with a Hamilton 10-μl syringe (No. 701). Glass plates pre-coated with 250 μm of silica gel GF, size 20 × 20 cm, supplied by Fisher Scientific (Pittsburgh, Pa., U.S.A.) (Cat. No. 6-601A), and two Gelman (Ann Arbor, Mich., U.S.A.) developing chromatography chambers (Model 51325-1), supplied by Fisher were used. The conditioning and buffering chemicals were sprayed with a Spratool Aerosol (No. 15-233). The ceric sulphate powder was supplied by BDH (Toronto, Canada). The phosphate–citrate buffer was from Fisher Scientific (Cat. No. SO-P-82).

Analysis

Quantitative TLC analysis was performed on 20 × 20 cm glass plates pre-coated with silica gel GF245 (layer thickness 250 μm). The plates were activated and buffered before use. The plate was held for 1 h in the oven at 110°, and then sprayed with 0.1 *M* sodium bisulphite solution and dried at 110° for 10 min. The plate was then sprayed with the citrate–phosphate buffer (pH 4) and kept in the oven at 110° for 30 min. It was transferred to a desiccator until required.

The plate was lined up and a 5-μl sample was spotted. Four samples were placed on each plate together with a standard sample (spotted in the middle). To get the desired spot of controlled diameter and density, a sample was taken with the syringe which was then inserted in the glass tube of the Chromaflex spotter. This supported the syringe and directed the needle to the surface of the plate through the guide holes of the manifold. The manifold had 6 spotting positions, and each position consisted of a guide hole for the needle and 4 holes spaced evenly around 360° for directing the inert gas (nitrogen) uniformly around the needle and on to the surface of the thin-layer plate. The velocity of the gas flow through the manifold (0.5 l/min) regulated the size of the origin spot. The distance from the manifold to the plate surface (6 mm) was regulated by slide-guide and lock nuts.

With the plate in position and constant gas flow, the syringe was introduced in its guide so that the needle just touched the plate surface; the syringe plunger was then pushed down slowly at a constant speed, and the solution was spotted onto the thin-layer plate in a uniform manner.

The plate was developed at 25° with acetone–*n*-butanol–water mixture (53:40:7) in a saturated Gelman chromatographic chamber. The distance run was 15 cm and the elution time 55 min. After drying under a stream of air, a second development was made with the same conditions to ensure best separation.

The plates were dried in a stream of air, and sprayed with an acid solution of 1 g ceric sulphate dissolved in 100 ml of 10% sulphuric acid solution in water. It was important to use a uniform spray for best results. The plate was then heated at 110° for 15 min to char the sugars.

Quantitative measurements were made with the Kontes densitometer. Measurements were made by scanning the plate horizontally across columns: *i.e.*, all mannose spots were scanned on one pass, all glucose spots were scanned on the next, etc. The scanning was made twice and the average peak height was compared to known sugar concentrations to give calibration curves. Scanning could also be performed vertically, *i.e.*, the mannose, then the glucose and then the galactose spot could be scanned in order, to produce another set of calibration curves. However, vertical scanning had the disadvantage that the peaks became a multiple peak instead of three separate peaks and there was loss of accuracy.

RESULTS AND DISCUSSION

A schematic diagram of a chromatographic plate is given in Fig. 1, which shows the arrangement of the spots and the computation of R_F. Typical R_F values were 0.59, 0.50 and 0.41 for mannose, glucose, and galactose, respectively. The spots typically were 0.8–1.1 cm in length (parallel to flow), which required separations of over 1 cm, or R_F values which differed by more than 0.07 for the 13.6-cm solvent front. The spots widths were typically 0.7–1.0 cm, and five samples, or five spots, were run on each plate. Within the limits of overlap and edge effects, up to seven samples could have been run per plate; in this work, the number of samples on each 20 × 20 cm plate was limited by the spotter design.

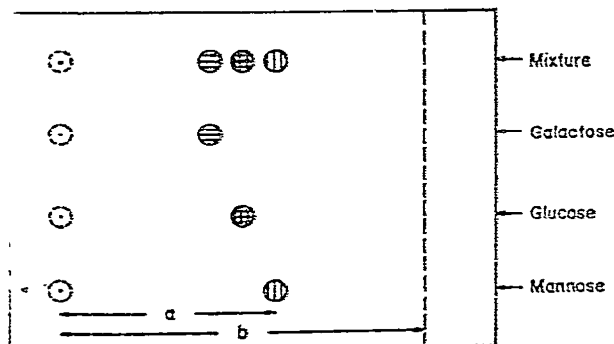


Fig. 1. Schematic diagram of chromatographic plate. R_F = a/b.

Wing and BeMiller[6] and Anderson and Stoddart[8] reported R_F values for the analysis of mannose, glucose and galactose in mixtures of other sugars. These are given in Table I, and are for various irrigation solvent systems and thin-layer materials and methods. Most of the combinations give good separation of two of the three sugars, so that we could have selected two sets of conditions and run two thin-layer analyses for each sample. However, because of the large number of samples, we attempted to find a method which would give good separation of the three compounds on one plate. Mannose differs from glucose in that it has an axial 2-OH group instead of an equatorial 2-OH group in its pyranose chair formation, and galactose has an axial 4-OH group compared to an equatorial 4-OH group for glucose. Superficial analysis of structure might suggest that the compounds would elute in the order galactose–glucose–mannose, or mannose–glucose–galactose, since the first and last compounds are the least alike, and all have identical molecular weights. For both of Anderson and Stoddart's[8] analyses, the order of elution was mannose–glucose–galactose (highest to lowest R_F value). This order was found in nine out of fourteen of the analysis methods of Wing and BeMiller[6], and in the other five analysis methods, where the order was either glucose–mannose–galactose or mannose–galactose–glucose, the R_F values were too close for quantitative analysis of the three together. This information helped in our search for a good quantitative method, in that if the order of elution for a particular solvent or plate preparation was not mannose–glucose–galactose we did not attempt further improvements.

Early experiments showed that the commercial plates would have to be treated to make them more selective. after trying many solvent systems in various proportions (Table II) with silica gel G, silica gel GF, and cellulose MN, and with these layers with various buffer solutions[11] such as 0.02 M sodium acetate, 0.02 and 0.1 M boric

TABLE I

LITERATURE R_F VALUES FOR MANNOSE, GLUCOSE AND GALACTOSE

Thin-layer material	*R_F values*			*Reference*
	Mannose	*Glucose*	*Galactose*	
Polycarbonate	0.31	0.18	0.13	8
Silica gel	0.43	0.41	0.32	8
Silica gel	0.41	0.28	0.32	6
Silica gel	0.60	0.61	0.53	6
Silica gel	0.57	0.47	0.47	6
Silica gel	0.53	0.48	0.39	6
Cellulose	0.23	0.19	0.17	6
Silica gel	0.44	0.48	0.40	6
Silica gel	0.19	0.13	0.12	6
Silica gel	0.29	0.21	0.17	6
Silica gel	0.60	0.46	0.40	6
Silica gel	0.26	0.20	0.18	6
Silica gel	0.45	0.41	0.41	6
Silica gel	0.23	0.29	0.17	6
Silica gel	0.52	0.54	0.40	6
Cellulose	0.30	0.25	0.21	6
Silica gel	0.46	0.41	0.36	17

TABLE II

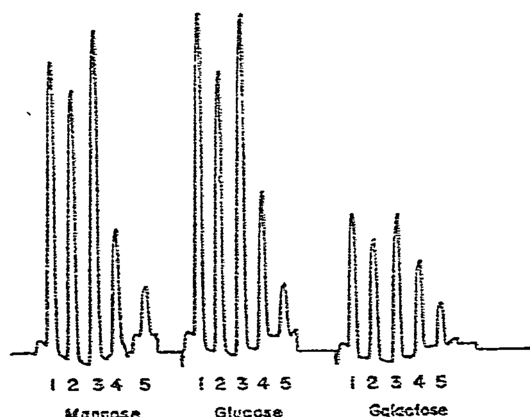
SOLVENTS TRIED IN TLC OF A SOLUTION OF MANNOSE, GLUCOSE AND GALACTOSE

Mixture	*Composition (v/v)*	*Reference*
Ethyl acetate–65% isopropanol	65:35	11
Methanol–chloroform–acetone–conc. ammonium hydroxide	42:16.5:25:16.5	11
1-Butanol–acetic acid–ether–water	9:6:3:1	6
Ethyl acetate–acetic acid–methanol–water	12:3:3:2	6
1-Propanol–water	17:3	6
Acetone–methanol	9:4	6
Ethyl acetate–methanol–water	37:40:23	7
Ethyl acetate–pyridine–water	40:20:40	11
	100:35:25	7
Chloroform–acetone–95% ethanol	38:38:24	11
Chloroform–methanol	90:10	6
Chloroform–96% ethanol	5:4	7
n-Butanol–acetone–water	4:5:1	8
n-Butanol–acetic acid–water	4:1:4	8

acid, 0.02 *M* sodium borate, sodium phosphate (pH 5), and 0.1 *M* sodium bisulphite. During these trials, however, it became apparent that the best solvent system was a mixture of acetone, *n*-butanol and water, with which Prey *et al.*[17] found R_F values as in Table I, last line. Using this solvent system, the above buffers and a citrate–phosphate buffer (pH 4, commercially available) were tried in combinations. It was found that a marked improvement was made when the plate was first dried, then sprayed with 0.1 *M* sodium bisulphite, dried, and then sprayed with the citrate–phosphate buffer. From this point the proportions of acetone, *n*-butanol and water were optimized, and the method further improved by double resolution in the same direction.

Selection of the developing solution for the sugars was based on the nature of the thin layer and the binder. As an inorganic layer and binder were used here, a mixture of sulphuric acid and ceric sulphate in water was employed to char the organic sugar spots[18]. Good results were obtained with this method which produced dark spots on a white background.

A horizontal scan of a TLC plate is given in Fig. 2 for a fermentation run in which the three sugars were removed from solution with time. A standard containing 20.8 g/l mannose, 8.0 g/l glucose and 4.5 g/l galactose was spotted in position 3. The fermentation broth of the same composition was sampled after a few minutes (spot 1), and then at 1-h intervals (spots 2, 4 and 5). The peak height was measured from the mid-point of the base of the triangle formed from the peak and the shifting baseline. The peak heights were translated into concentrations using a calibration curve as in Fig. 3. This curve was obtained from TLC plates of sugar solutions of known concentrations. Fig. 3 was prepared for sugar concentrations up to 20.8 g/l mannose, 8.0 g/l glucose and 4.5 g/l galactose, and was derived from samples of pure sugars in distilled water and in the fermentation broth. Horizontal scans only were used in its preparation. Standard errors for the three sugars were respectively 2.8, 3.6 and 5.6%. Wing and BeMiller[7] gave standard errors of 3.1–17% for monosaccharides in corn



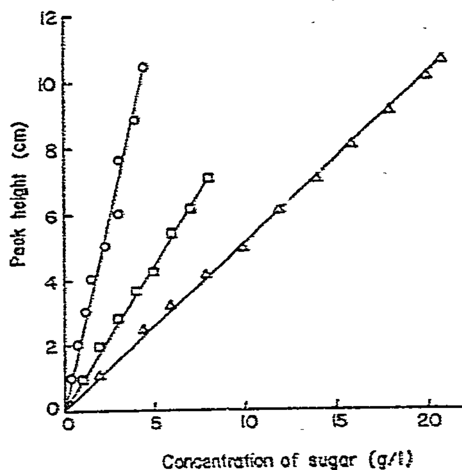


Fig. 2. Horizontal TLC scan. Position 3 has analysis 20.8 g/l mannose, 8.0 g/l glucose and 4.5 g/l galactose.

Fig. 3. Calibration curve for horizontal scanning of chromatogram. ○, Galactose; □, glucose; △, mannose.

syrups from 40.8% down to 7.3% by weight, the higher standard error being associated with the lowest percentage monosaccharide.

Vertical scanning has been discussed previously. A vertical scan for sugar solutions of known concentration is given in Fig. 4. Calibration curves prepared from this type of scan did not pass through zero and are not consistent. Accordingly, only horizontal scanning was used.

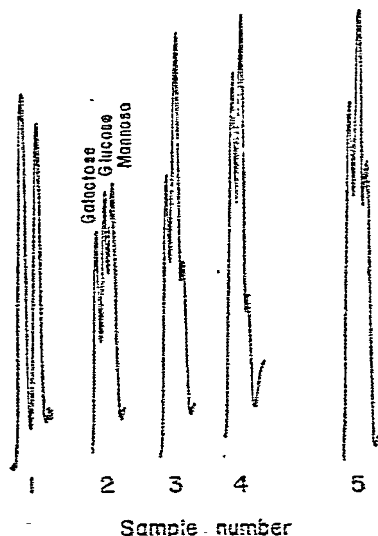


Fig. 4. Vertical scanning. On each scan the order is mannose–glucose–galactose from right to left Composition for the five scans in g/l in the order mannose–glucose–galactose was: (1) 21, 0, 3; (2 16, 2, 1.2; (3) 8, 8, 1.8; (4) 4, 9, 2.4; (5) 20.8, 10, 3.

A test for reproducibility, after the calibration curves had been established, gave recoveries of 100 ± 5% in 25 analyses with all three sugars present. The time required for this analysis was 3 h with plates prepared previously. Four samples plus a standard could be run, and two plates could be handled simultaneously. For a complete fermentation run, all samples could be analysed in one day.

CONCLUSIONS

A method has been developed for conditioning and developing a standard TLC plate so that mannose, glucose and galactose could be separated and quantitatively analysed. This method includes a double elution, with drying between elutions, and a system of buffering the commercially available silica gel thin layer.

A uniform and reproducible method of spotting was developed using a hypodermic syringe to measure the sample volume, and the Kontes Chromaflex spotter to ensure uniform application.

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REFERENCES

1 S. A. Rydholm, *Pulping Processes*, Interscience, New York, London, 1965, p. 823.
2 B. O. Russel and G. Isaiah, *Tappi*, 56, No. 9 (1973) 46.
3 M. H. Loutfi, *Biochemical Utilization of Waste Sulphite Liquor: Mixed Sugar Fermentation*, M.A.Sc. Thesis, University of Ottawa, Ottawa, 1975.
4 E. Stahl and U. Kaltenbach, *J. Chromatogr.*, 5 (1961) 351.
5 S. A. Hansen, *J. Chromatogr.*, 107 (1975) 224.
6 R. E. Wing and J. N. BeMiller, *Methods Carbohyd. Chem.*, 6 (1972) 42.
7 R. E. Wing and J. N. BeMiller, *Methods Carbohyd. Chem.*, 6 (1972) 54.
8 D. M. W. Anderson and J. F. Stoddart, *Carbohyd. Res.*, 1 (1966) 417.
9 J. M. Bobbitt, *Thin-Layer Chromatography*, Reinhold, New York, 1963.
10 K. Randerath, *Thin-Layer Chromatography*, Verlag Chemie, Weinheim/Bergstr., 1963 and 1966.
11 E. Stahl, *Thin-Layer Chromatography. A Laboratory Handbook*, Academic Press, New York, 1965.
12 E. Stahl, *Thin-Layer Chromatography*, Springer, Berlin, 1969.
13 J. G. Kirchner, *Thin-Layer Chromatography*, Wiley-Interscience, New York, 1967.
14 E. J. Shellard, *Quantitative Paper and TLC*, Academic Press, New York, 1968.
15 A. Niederwieser and G. Pataki, *Progress in Thin-Layer Chromatography and Related Methods*, Ann Arbor Sci. Publ., Ann Arbor, Mich., 1969.
16 R. M. Scott, *Thin-Layer Chromatography Abstracts*, Ann Arbor Sci. Publ., Ann Arbor, Mich., 1972.
17 V. Prey, H. Berbalk and M. Kausz, *Mikrochim. Acta*, (1961) 968.
18 K. Schreiber, O. Aurich and G. Osske, *J. Chromatogr.*, 12 (1963) 63.