Journal of Chromatography, 193 (1980) 333–337

CHROM. 12,664

Note

Fluorodensitometric microdetermination of reducing sugars on thin-layer chromatograms

JUNJI IWAKAWA, HIROSHI KOBATAKE, IZUMI SUZUKI and HIDEO KUSHIDA
Kyoto General Medico-Chemical Laboratory, Misasagi Bessho-cho 95, Yamashina-ku, Kyoto (Japan)
(Received January 3rd, 1980)

Thin-layer chromatography (TLC) and gas–liquid chromatography (GLC) have found extensive application in the micro-analysis of sugars. GLC analysis, however, requires troublesome pretreatment including chemical reduction of reducing sugars to volatile alditol acetate derivatives or transformation into trimethylsilyl derivatives which generally produce at least two isomers, α- and β-anomers, complicating the quantitative evaluation of sugars.

On the other hand, TLC gives simplicity of operation and good separation of minute amounts of sugars. Thus, development of better micro-determination can be expected by combination of TLC with densitometry using highly sensitive equipment.

Fluorogenic reagents are known to provide high sensitivity and often a more simple treatment procedure, as compared with chromogenic reagents. As fluorogenic reagents for detection of carbohydrates on thin-layer plates, the following have been used: fluoroglycine[1], *m*-phenylenediamine[2], 2-thiobarbituric acid[3], aniline–H_3PO_4[4], aniline citrate[5], chromotropic acid–H_2SO_4[6], Dns hydrazine[7], *o*-phenylphenol[8], and ethylenediamine sulfate[9].

Ethylenediamine sulfate reagent (EDS reagent) was employed by Honda *et al.*[9] for the detection of sugars separated on paper chromatograms and was superior to other reagents in sensitivity and simplicity of handling.

We have found that this reagent is also excellent when applied in TLC, and that its combination with densitometry gives a new method of detecting and determining reducing sugars in minute amounts with high sensitivity.

EXPERIMENTAL

Materials

Chromatoplates with a 0.25 mm layer of silica gel G (type 60; Merck, Darmstadt, G.F.R.) were used for chromatography. The plates were activated at 110° for 30 min prior to use.

All chemicals and most of sugars used as samples were purchased from several commercial sources. 2-O-Methylarabinose and 6-deoxyglucose (quinovose) were prepared in our laboratory[10,11].

Chromatography

Sample solutions dissolved in redistilled water were applied to the starting points, 2 cm away from one edge of the plate. The plate was developed to a distance of 17 cm from the origin at room temperature with a solvent system, ethyl acetate–pyridine–water (8:2:1), in a chromatographic chamber. After drying, the plate was subjected to repeated development for an improved separation of the sugars.

Detection of spots

Visualization of sugar spots with EDS reagent was carried out according to the procedure of Honda *et al.*[9] in paper chromatography. After the multiple development process (two developments), the plate was thoroughly dried and sprayed uniformly with 10% aqueous EDS solution and heated at 120–130° for 10 min in an oven. The sugars appeared as fluorescent violet spots on a dark blue background, under UV irradiation at *ca.* 365 nm with a Manasulu lamp (Tokyo, Japan).

Densitometry

Quantitative determination of the fluorescence intensity of sugar spots was carried out directly on the thin-layer plates with a Shimadzu dual-wavelength TLC scanner CS-900 (Shimadsu Seisakusho, Kyoto, Japan). The fluorescence intensity on the chromatogram was measured at the excitation wavelength of 365 nm and the emission wavelength of 450 nm. Integrated values of the fluorescence intensity were derived by use of a Shimadzu Chart Recorder U-225 MCS.

RESULTS AND DISCUSSION

Fig. 1 shows thin-layer chromatograms of some pentoses, hexoses, methylated derivatives and maltose applied in 0.05, 0.1, 0.2, 0.5, and 1.0 μg amounts. In all cases examined, as little as 0.05 μg can be detected with the naked eye. Monomethyl sugars, which have recently been found to occur in organisms[12,13], were also tested and were detectable at nearly the same level as the free sugars; phosphorylated sugars, such as glucose-1-phosphate, ribose-5-phosphate, glucose-6-phosphate, mannose-6-phosphate and galactose-6-phosphate, were also detected at similar sensitivity.

Densitometric recordings of 0.5 μg of each sugar on the chromatogram are shown in Fig. 2. The fluorescence intensities of methyl derivatives and rhamnose were slightly weaker than that of the others. Typical calibration curves obtained with hexoses shown in Fig. 2 gave a linear relationship between the peak and the sugar amount in the range 0.04–1.0 μg.

The average R_g (g = glucose) values calculated from a number of chromatograms are summarized in Table I.

In order to explore precision of this method, nine mixtures made from 0.5 μg each of glucose, quinovose, fucose, rhamnose, arabinose, and 3-O-methylglucose were applied and the fluorescence intensities were measured as described. The results (Table II) indicate that the method allows sugars to be easily measured with good reproducibility.

The value of the standard deviation, in general, appears to become larger as the R_g value increases[14]. It should be kept in mind that fucose tends to give a spread spot in the developing process in TLC, resulting in a larger standard deviation, al-

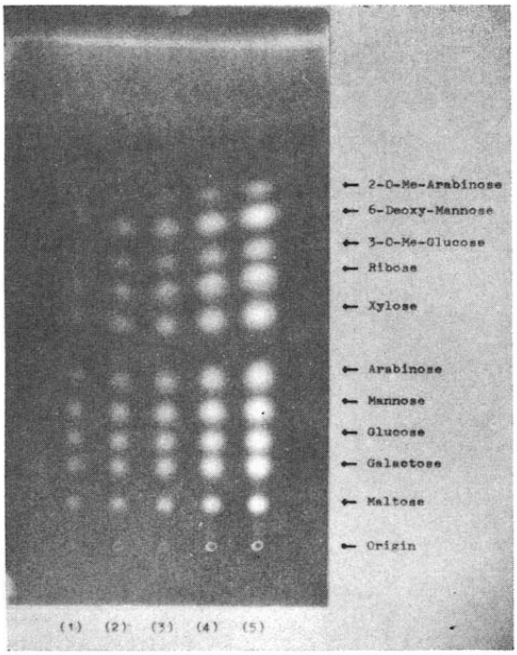

Fig. 1. Thin-layer chromatogram on silica gel of sugars visualized with ethylenediamine sulfate reagent. A double development technique was used. The solvent system was ethyl acetate–pyridine–water (8:2:1). 1 = 0.05 μg; 2 = 0.1 μg; 3 = 0.2 μg; 4 = 0.5 μg; 5 = 1.0 μg.

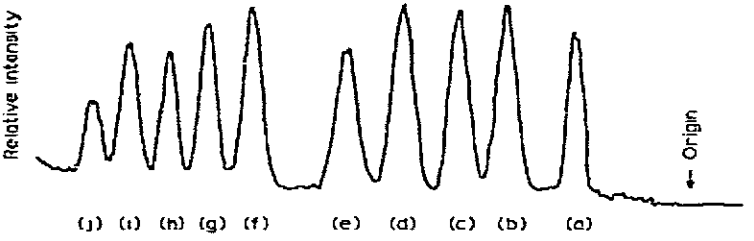

Fig. 2. Densitometric recording of the thin-layer plate. From right to left the peaks represent maltose (a), galactose (b), glucose (c), mannose (d), arabinose (e), xylose (f), ribose (g), 3-O-methylglucose (h), rhamnose (i), 2-O-methylarabinose (j). 0.5 μg of sugar was deposited. See Fig. 1 for the solvent and detection.

though it gives a relatively small R_g value. Other 6-deoxyhexoses (rhamnose and quinovose) did not show such a property.

The variation with time of fluorescence intensity of spots of glucose and rhamnose (0.5 μg each) is shown in Fig. 3. The fluorescence intensity reached a maximum 0.5–1 h after the plate was heated, and then increased slightly, indicating that it is reasonably stable in the dark. From these results, it is recommended that the fluorescence intensity of sugar spots is determined 1 h or more after the completion of plate heating.

TABLE I

AVERAGE R_g VALUES OF SUGARS

See Fig. 1 for the solvent system.

Type of sugar	R_g
2-O-Methylarabinose	3.22
Rhamnose	2.90
Quinovose	2.76
3-O-Methylglucose	2.63
Ribose	2.42
Fucose	2.15
Xylose	2.10
Arabinose	1.57
Mannose	1.29
Glucose	1.00
Galactose	0.75
Maltose	0.37
Glucose-6-phosphate	0.03

* g = Glucose.

TABLE II

REPRODUCIBILITY OF SUGAR DETERMINATION

Sugar	*Integrated value of fluorescence intensity in each area of sugar spot*									*Mean*	*±S.D. (%)*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*		
Glucose	36.8	37.0	36.2	37.4	36.7	35.4	34.6	35.5	36.3	36.2	0.89 (2.46)
Quinovose	36.9	37.7	35.1	38.2	36.4	33.4	33.1	34.3	35.9	35.7	1.82 (5.10)
Fucose	38.4	38.8	35.1	39.3	37.5	32.6	32.2	33.4	36.5	36.0	2.75 (7.64)
Rhamnose	24.5	25.7	26.2	25.3	24.3	23.2	24.1	22.6	23.5	24.4	1.19 (4.88)
Arabinose	30.9	32.2	32.7	31.4	30.8	29.6	30.6	29.3	30.5	30.9	1.10 (3.56)
3-O-Methylglucose	19.6	20.7	21.2	20.1	19.5	18.8	19.3	17.8	18.9	19.5	1.03 (5.28)

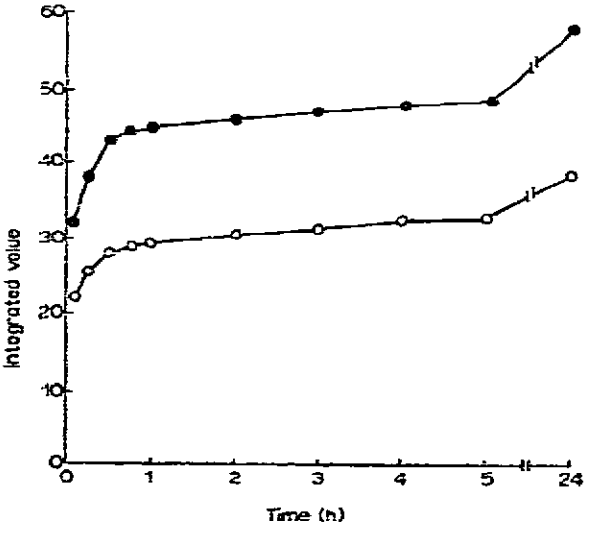

Fig. 3. Variation with time of fluorescence intensity of spots of glucose (●) and rhamnose (○) in the dark at room temperature.

These results show that the fluorodensitometric determination of sugars with EDS reagent on thin-layer plates is simple and accurate, and is especially useful when only a limited amount of sample is available.

ACKNOWLEDGEMENTS

The authors thank Dr. S. Kitaoka and Dr. Y. Nakano, Department of Agricultural Chemistry, Osaka-Prefectural University, Osaka, Japan, for their helpful advice.

REFERENCES

1 I. Smith, *Chromatographic Techniques*, Interscience, 1958, p. 168.
2 S. M. Partridge, *Nature (London)*, 158 (1946) 270.
3 E. Sawicki, C. R. Engel, W. C. Elbert and K. Gerlach, *Talanta*, 15 (1968) 803.
4 J. L. Garraway and S. E. Cook, *J. Chromatogr.*, 46 (1970) 134.
5 V. Vitek and K. Vitek, *J. Chromatogr.*, 143 (1977) 65.
6 T. Nakai, M. Koyama and H. Demura, *J. Chromatogr.*, 50 (1970) 338.
7 G. Avigad, *J. Chromatogr.*, 139 (1977) 343.
8 Y. Asabe, M. Suzuki and S. Takitani, *Anal. Biochem.*, 94 (1979) 1.
9 S. Honda, K. Kakimoto, K. Kakehi and K. Takiura, *Anal. Chim. Acta*, 64 (1973) 310.
10 G. J. Halliburton and R. J. Mcilroy, *J. Chem. Soc.*, (1949) 299.
11 O. Th. Schmidt, *Methods in Carbohydrate Chemistry*, Academic Press, New York, 1962, Vol. 1, p. 198.
12 H. Mayer and K. Framberg, *Eur. J. Biocgem.*, 44 (1974) 181.
13 R. L. Hall and E. J. Wood, *Biochem. J.*, 165 (1977) 173.
14 J. G. Kirchner, *J. Chromatogr.*, 82 (1973) 101.