Thin-layer chromatography on buffered silica gel in the analysis of sugars in acid hydrolysates

Isolation and identification of the sugar component of nucleosides, nucleotides and nucleic acids in microgram quantities are usually carried out by chromatography of their acid hydrolysates prepared by heating with *N* sulfuric acid at 100° for 1 h[1]. According to our experience, most chromatographic procedures on paper[2–4] and thin layers[5,6] do not give satisfactory results unless applied to previously neutralized and desalted samples. This preliminary treatment is not easily applicable to the micro scale since it always brings about a certain loss of material.

A thin-layer method has recently been suggested[7] which allows good resolution of sugars even in the presence of considerable amounts of salts; this system is however more suitable to hexose than to pentose separation.

WALDI[8] separates neutral sugar samples on kieselguhr layers impregnated with phosphate buffer, pH 5. The conditions described by this author are not suitable to the analysis of the sugars tested by us in the presence of acid. Ribose, lyxose and xylose do not separate and tailing is observed with rhamnose and deoxyribose.

In the present study conditions have been defined for the chromatography of sugars in acid samples, with special emphasis upon the separation of pentoses. The method is applicable to acid hydrolysates of nucleosides, nucleotides and nucleic acids and can be extended to the analysis of hydrolysates of natural heterosides and oligosaccharides.

The application of various solvent systems and color reagents is evaluated and discussed.

Experimental

Samples. The following sugars were tested: D-ribose, D-lyxose, D-xylose, D-arabinose, 2-deoxy-D-ribose* and D-glucose. The sugar solutions were made by dissolving 10–20 mg of sugar in 10 ml of *N* sulfuric acid; a mixture of the sugars tested was prepared in the same way.

A ribonucleic acid hydrolysate was obtained by heating 50 mg of ribonucleic acid (sodium salt, from yeast, B.D.H.) with 10 ml of *N* sulfuric acid, in a sealed vial, at 100° for 1 h.

Preparation of the plates. 20 × 20 cm plates were used; a slurry obtained by mixing 25 g of Kieselgel G (E. Merck, A.G., Darmstadt, Germany) with 50 ml of 1/15 *M* phosphate buffer, pH 8[7], was applied to the plates at a thickness of 0.25 mm using an automatic coating device. The plates were allowed to dry at room temperature overnight and stored in a desiccator over anhydrous calcium chloride.

Chromatographic procedure. Aliquots (1–2 μl), containing 1–4 μg of sugars and their mixtures in *N* sulfuric acid, was spotted on the plates at 1.5 cm intervals with a microsyringe. The spots were dried at room temperature. The origin was 2.5 cm above the bottom edge of the plate.

Plates were developed by ascending technique at 20° until the solvent front had reached 1 cm from the upper edge of the plate, then air dried in a horizontal position.

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* 2-Deoxyribose was examined in the presence of acid, as was done for ribose and other pentoses, although it is altered under the conditions of acid hydrolysis.

In some cases a multiple development technique was used in order to sharpen separations.

Several solvent systems have been examined, most of which were unsuitable for a separation under the conditions described above; among those which gave the best results the following can be listed:

I. Methyl ethyl ketone–acetic acid–water saturated with boric acid (9:1:1, v/v)[4],
II. Phenol–water (75:25, w/v)[9],
III. Methyl ethyl ketone–formic acid (99%)–water saturated with boric acid (9:0.5:1.5, v/v),
IV. *n*-Butanol–acetone–water (4:5:1, v/v)[7,10].

Detection of the spots. Various spray reagents were used to detect the sugar spots:
A = *p*-Anisidine hydrogen phthalate[6],
B = Diphenylamine–aniline phosphate[11],
C = Indole–sulfuric acid[12],
D = 3-Methylindole–sulfuric acid[12],
E = Ammonium molybdate, reagent B of EL KHADEM AND GIRGIS[13].

Results and discussion

Silica gel impregnated with phosphate buffer was the preferred layer; other types of layers and other buffers gave unsatisfactory results.

Solvent system I* on the above mentioned layer permits the separation of all the sugars tested even in the presence of acid. Spots are well defined, with little or no tailing; multiple development technique with the same solvent system can be applied to improve separation[14] without noticeable diffusion of spots. Best results are obtained by a threefold development (Fig. 1).

With solvent system II very compact and nearly circular spots are formed; R_F values for lyxose, xylose and arabinose are very close together and a complete separation is seldom achieved even with multiple developments. This solvent system is useful for the separation of ribose from arabinose and glucose in acid samples.

Good resolution is also obtained with solvent system III, a modification of solvent I; xylose and lyxose, the latter with a slightly higher R_F value, separate incompletely.

In solvent system IV xylose, lyxose and ribose run in a single spot; this solvent is however interesting since it allows the separation of ribose from rhamnose** which run together in solvents I and II. Spots are round and with little tailing. Since R_F values are high, the multiple development method is not advantageous[14].

Among the detection reagents tested, the best results are given by reagents A and B. An almost colorless background and brighter colors result when plates are heated, immediately after spraying, at 120° for 10–20 min. Observation of fluorescence under long-wave U.V. light (350 mμ) greatly increases the sensitivity. Reagents C and D give a colored background which interferes with the localization of the spots, while reagent E, under the conditions used, shows a low sensitivity.

The $R_G \times 100$ values with solvent systems I, II and III are listed in Table I

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* This solvent system was introduced by REES AND REYNOLDS[4] for the paper chromatographic separation of glucose and sorbitol; it separates pentoses on paper only as desalted samples.
** Relative R_F value of ribose to rhamnose in this solvent system is 0.79.

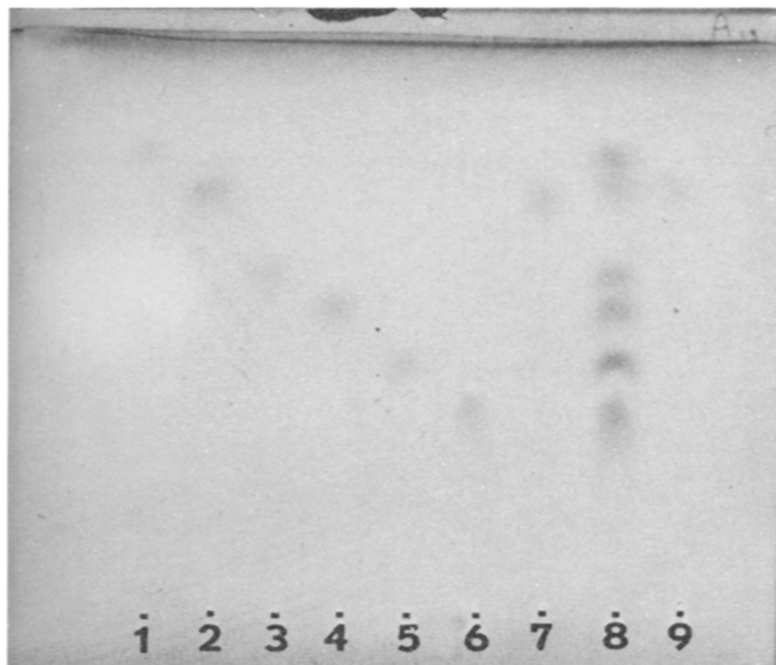

Fig. 1. Separation on a buffered silica gel layer with solvent system I, threefold elution; the spots were detected with reagent A. 1 = 2-Deoxy-D-ribose; 2 = D-ribose; 3 = D-lyxose; 4 = D-xylose; 5 = D-arabinose; 6 = D-glucose; 7 = RNA acid hydrolysate; 8 = mixture of 1, 2, 3, 4, 5, 6; 9 = D-ribose.

together with the colors developed by reagents A and B and with the time required for a single run.

It is worthy of note that in solvent systems I, II and IV deoxyribose is faster than ribose, but in solvent system III an inversion occurs; this solvent, as indicated

TABLE I

	$R_G \times 100$			*Colors*	
	Solvent I	*Solvent II*	*Solvent III*	*Reagent A*	*Reagent B*
2-Deoxy-D-ribose	241	266	326	brick red	bright purple
D-Ribose	221	180	361	various shades of red-brown	yellow-green
D-Lyxose	174	154	216		grey-green
D-Xylose	155	144	202		green
D-Arabinose	125	143	149		grey-green
D-Glucose	100	100	100	yellow-brown	grey-blue
Number of developments	3	2	2		
Time (h) for a 15-cm run at 20°	2–3	5–6	2–3		

above, differs from solvent I only in the acid component, *i.e.* formic acid instead of acetic acid. The sequence for the other sugars is constant in all solvent systems.

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Quantitative micro thin-layer chromatography of fatty materials

Quantitative separation by gas–liquid chromatography (GLC) has become an almost indispensable analytical tool in many fields including lipids. For certain fatty materials, such as those containing hydroxy or epoxy groups or conjugated systems, there is a risk of alteration of the structure at the high temperatures necessary for resolution by GLC. Thin-layer chromatography (TLC) is a mild, room-temperature technique with remarkable powers of resolution, and its quantitation could overcome some of these limitations of GLC. Macro-quantitation of TLC has been achieved by several workers (for a review, see MANGOLD[1]). In the present paper a procedure is reported for quantitation on microscope slides (microchromato plates) with several variations of the TLC technique, *viz.* direct, reversed-phase, boric acid-coated, silver nitrate-coated, etc., which are useful for specific separations. The technique matches GLC in speed, requiring only 3–5 min for the separation and about 30 min for quantitation thereafter.

Experimental

Materials. The materials used in this investigation were prepared and purified by conventional methods.

Method. A slurry of silica gel G (30 g) was prepared in a solution of chloroform–methanol (80:20, v/v)[2]. Two glass microchromatoplates (2.5 × 7.5 cm) held flat together were dipped in the slurry and withdrawn. After drying at room temperature, they were activated by heating at 110° for 30 min.

These plates were further treated for the special separations. Reversed-phase plates were obtained by impregnation by the ascending technique with a 5 % silicone oil–ether solution[3]. Spraying with a 6.25 % solution of silver nitrate yielded argentated plates. Boric acid plates were obtained by upward development in a clear saturated aqueous solution of boric acid. The plate was spotted with a chloroform solution of the mixture to be analysed *ca.* 1 cm from the bottom at the centre. Development was conducted in small covered glass jars or glass beakers for *ca.* 3 min.