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QUANTITATIVE ESTIMATION OF SUGARS IN BLOOD AND URINE BY PAPER CHROMATOGRAPHY USING DIRECT DENSITOMETRY

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SUMMARY

A system of quantitative paper chromatography is described in which compounds are directly scanned on the paper after a locating colour reaction. Sources of error arising at different stages are analysed and controlled by modifications in technique. Reproducibility is improved by using paper with uniform solvent flow characteristics, and the colour reaction controlled by choice of optimal reagent concentration, a modified dipping technique and rotation of the chromatogram during heating in the hot-air oven. After paper clarification by oiling, spots are scanned transversely, across the development axis, by double reflectance densitometry using a Joyce-Loebl Chromoscan, and calculations are based on comparison of peak areas. Most of the principles involved are generally applicable to paper chromatography. Sugar estimations in plasma and urine have a coefficient of variation between $\pm$ 2.0 and 3.5% when applications around 20 μg are used without replication. Sugar recoveries from urine are between 99 and 108% provided the desalting resin is correctly prepared; but from plasma and haemolysed whole blood recovery is higher due to a factor arising at the stage of deproteinization. The method has been used to estimate stachyose, raffinose, melibiose, lactose, lactulose, palatinose, sucrose, galactose, glucose, fructose, mannose, fucose, xylose, xylulose and 3-O-methyl glucose, either singly or in various combinations, in concentrations down to 1 mg/100 ml. The advantages of the method, in relation to other available techniques, are discussed.

INTRODUCTION

Semi-quantitative estimations by direct visual comparison of test and standard spots on the same paper chromatogram is simple and has been widely used[1–9]. Quantitative estimations can be made of sugars eluted after separation by paper chromatography; unfortunately additional labour and recovery problems are involved. Direct spot measurements have also been suggested for quantitation of chromatograms (*e.g.* ref. 10), but precision and accuracy have often been considered inadequate[11]. Nevertheless, the advantages of this approach are distinct, and the present study comprises a systematic evaluation of possible sources of error in the estimation of sugars by direct densitometry of paper chromatograms, and control

of important factors by simple modifications in technique. The method was developed to study absorption and renal excretion of a wide range of sugars in relation to the measurement of intestinal disaccharidase activity, hexose transport and oligosaccharide permeability in human subjects with gastrointestinal disease.

EXPERIMENTAL

Apparatus and materials

Desalting resin. Zerolit DM-F, Permutit (formerly Biodeminrolit) and glacial acetic acid (for resin preparation) were supplied by B.D.H. Chemicals Ltd., Poole, Dorset.

Deproteinization reagent. Sulphosalicylic acid was supplied by B.D.H. Chemicals Ltd.

Solvents. The following solvents were used: pyridine, methanol, ethyl acetate, acetone, and butan-1-ol; all reagent grade from B.D.H. Chemicals Ltd.

Locating reagents. The reagents used were: *o*-dianisidine (Hopkin & Williams Ltd., Chadwell Heath, Essex); and 4-aminobenzoic acid, orthophosphoric acid, and citric acid, from B.D.H. Chemicals Ltd.

Oiling reagent. Liquid paraffin, B.P., and light petroleum, b.p. 60–80° (B.D.H. Chemicals Ltd.) were used as oiling reagents.

Sugars. Stachyose, isomaltose, palatinose, and 3-O-methyl glucose were obtained from Koch-Light Laboratories, Colnbrook, Essex; lactose and sucrose from Hopkin & Williams Ltd., Chadwell Heath, Essex; D-glucose, D-galactose, D-fructose, D-arabinose, D-xylose, D-mannose, D-sorbose, L-rhamnose, L-fucose, and maltose from B.D.H. Chemicals Ltd.; D-melibiose from Sigma Chemical Company, St. Louis, Mo. Lactulose was a gift from Philips-Duphar, Weesp, The Netherlands.

Preservative. Preservative used was thiomersal (merthiolate) from B.D.H. Chemicals Ltd.

Chromatography paper. Whatman No. 3 chromatography, Whatman 3MM (uncoated), and Whatman 3MM (polythene backed) were supplied by H. Reeve Angel & Co., Ltd., London E.C.4.

Pipettes. "Drummond" microcaps, in 1-, 2-, 5-, 10-, and 20-μl sizes, were supplied by Shandon Scientific Co., Ltd., London N.W.10; 0.1-cm^3 ex. goldline glass pipette by E-MIL. H. J. Elliot, Ltd., Pontypridd, Glamorgan.

Chromatography tanks. 300, and 500 Panglas "Chromatanks" were supplied by Shandon Scientific Co., Ltd.

Industrial blower. B.S. 170/1939 from H. J. Latham, Ltd., Shoeburyness, Essex.

Hot-air oven and rotary motor. Chromatographic drying oven (Baird & Tatlock, Ltd., Chadwell Heath, Essex) and rotary electric motor, 10 r.p.m. (Crouzet, Thanet House, Brentford, Middlesex) were used.

Scanning densitometer. Chromoscan recording and integrating densitometer (1966 Model) was obtained from Joyce-Loebl & Co., Ltd., Team Valley, Gateshead, Durham.

Storage and preparation of samples and standards

Standard sugar solutions. A mixed standard solution of 100 mg/100 ml with respect to lactose, lactulose, sucrose, galactose, glucose, fructose and xylose in water,

with merthiolate (0.01% w/v) as preservative, has been used for 3 years. Aliquots were deep-frozen in glass polythene-capped bottles and the standard solution in routine use, which was kept at 4°, was replaced every few months.

Sugar survival during storage. Dilutions of 500 mg/100 ml stock aqueous monosaccharide and disaccharide solutions were made in distilled water, and urine, to produce 20 mg/100 ml final concentrations with respect to each sugar. Aliquots were stored in the dark, in 4-ml glass vials with polythene closures, (a) deep-frozen, (b) at 4° and (c) at room temperature. Survival was assessed by quantitative chromatography. When 0.01 g/100 ml merthiolate had been added, survivals of raffinose, lactose, lactulose, sucrose, galactose, fructose, xylose and 3-O-methyl glucose were above 88% after 18 months storage, at room temperature, with no appreciable losses from the frozen samples. Later studies[12] indicate that sugar survival in stored plasma is equally good when deep-frozen, but irregular losses occur at room temperature and 4° due to bacterial growth not prevented by 0.01% merthiolate.

Preparation of urine and plasma for chromatography. Desalting of urine and plasma was found to be necessary when applications above 40 µl were required. While the presence of much salt caused spot trailing, smaller amounts still produced some spot elongation capable of reducing the transverse-scan peak area measurement used for quantitation (see Fig. 10). Complete deproteinization of test solutions was also important, because even small concentrations of protein produce a sticky pellicle at the origin which makes large applications difficult, and causes spot trailing and distortion.

Desalting urines. For this purpose the mixed cation/anion-exchange resin Zerolit DM-F (formerly "Biodeminrolit") was used in the H^+acetate$^-$ state, employing a shaking rather than a column technique[13].

(a) Preparation of resin. Resin (500 g) is treated with acetic acid (3 l of 10% v/v) followed by washing with distilled water (4 l), preferably on a large column. The hygroscopic resin is then dried on an open tray at room temperature to minimise water dilution effects. A molecular sieve factor is also present, thus over-dehydration of resin can produce sugar recoveries above 100% with a differential related to molecular size (see Table I). The degree of dehydration can be measured by observing the percentage expansion of a column of resin in a test-tube on addition of distilled water (referred to as "percentage water gain"). "Water gains" of between 20 and 35% give sugar recoveries near to 100%, but the performance of each batch of resin should be checked.

TABLE I

EFFECT OF TREATMENT WITH RESIN ON CONCENTRATION OF SUGAR SOLUTIONS

Sugar concentrations in a solution treated with Zerolit DM-F are altered by a combination of water dilution and molecular sieve factors, which have opposing effects. Progressive physical disruption of resin caused by drying alters the molecular sieve effect, thus the differential related to molecular weight disappears as dehydration increases.
The concentration of the sugars was 100 mg/100 ml. Zerolit DM-F was added to 70% of fluid level.

Hydration of resin		*Recovery (%)*			
w/w% water	*% "water gain"*	*Stachyose*	*Raffinose*	*Lactulose*	*Galactose*
42	15	104	96	93	89
35	35	116	109	110	105
29	50	115	113	112	110
0	150	112	113	113	112

(b) Treatment of urine with resin. To approximately 3 ml of urine in a stoppered tube, prepared resin is added until it occupies about 70% of the combined resin plus urine level (salt estimations indicate that more than a 50% level is required for complete desalting). After 3 min shaking the tube is centrifuged, and the clear supernatant is ready for application. Average recoveries from urine and pure aqueous solution treated with adequate amounts of prepared Zerolit DM-F were 103% (range 99–108%) and 102% (range 99–107%), respectively for eight different sugars.

Deproteinization of whole blood and plasma. Plasma or haemolysed whole blood (1 ml) is mixed with sulphosalicylic acid (1 ml of a 5% w/v aqueous solution), shaken, and stood for 10 min before centrifugation. The supernatant is then desalted, as described for urine, to remove salt and remaining sulphosalicylic acid prior to application.

It was noted that sulphosalicylic acid below a concentration of 2.5% w/v failed to deproteinize plasma or haemolysed whole blood completely when used in a 1:1 volume ratio, and above 10% resulted in persistence of acid after desalting with resulting recovery problems. Acetone (100%), ethanol (100%), and picric acid (saturated aqueous) were found to produce incomplete deproteinization of plasma when used in 1:1 volume ratio. Average sugar recoveries from plasma and haemolysed whole blood were 105.5% (range 102–108%) and 114.2% (range 106–123%), respectively when sulphosalicylic acid was used for deproteinization. Sugars, which are excluded from the space occupied by precipitated protein, become more concentrated in the protein-free supernatant than if the whole space was available. Calculated recoveries therefore tend to be greater than 100%, especially in haemolysed whole blood which has a higher protein content than plasma. Sugar recoveries from plasma treated with ethanol and acetone were lower (averages 93.5 and 89.3%, respectively) caused by spot distortion due to incomplete protein removal, and picric acid gave the least satisfactory results.

Application of samples and arrangement of the chromatogram

Arrangement of the chromatogram. Each chromatogram, dimension 29 × 51 cm, is cut from a standard 46 × 56 cm sheet of Whatman No. 3 chromatography paper, and marked out as shown in Fig. 1. Four test and two standard applications

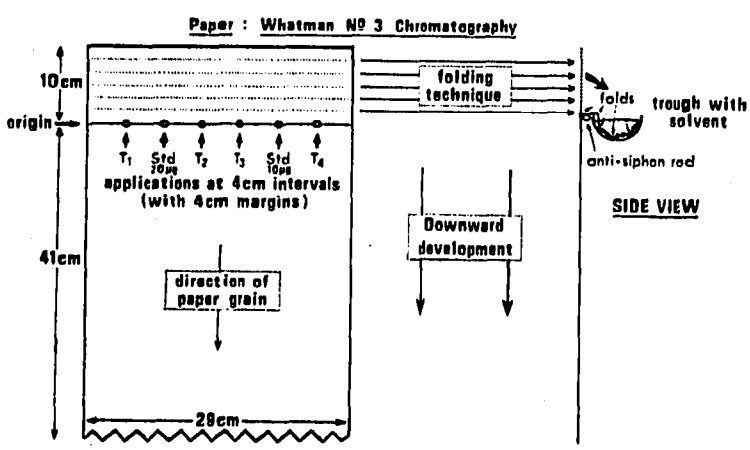

Fig. 1. Paper layout and arrangement for descending chromatography.

are made on each sheet to enable unidimensional development parallel to the machine direction. Full width sheets (46 cm wide) are awkward to dip using the draining-rod technique, but sheets cut to a width of 38 cm are convenient if a large tank (such as Shandon 500 Panglas Chromatank) is available, and allow seven test plus two standard applications.

Pipetting technique. Application volumes above 40 μl are delivered between marks from 0.1 ml graduated glass pipettes. Drummond glass microcapillaries are more suitable for volumes between 1 and 40 μl. A polythene tube with mouthpiece, attached to the Drummond capillary mount as a sucker, provides simple control and enables high precision to be rapidly acquired by the inexperienced. Coefficients of variation for 10-μl applications, made by five different workers, were $\pm$ 0.4, 0.43, 0.47, 0.57 and 1.2% (based on weights of forty consecutive deliveries).

Application volume and quantity of sugar. Solutions are applied in 20-μl aliquots, with consecutive drying for multiples, amounts less than 20 μl being "made up to volume" by superimposing distilled water before drying. Standards containing 10 and 20 μg of each sugar (*i.e.* 10 and 20 μl of the 100 mg/100 ml mixed standard solution) are applied at positions 2 and 5 on the origin of each chromatogram. These quantities lie within the more linear portion of the standard curve relating peak area to sugar quantity shown in Fig. 2, but amounts greater than this show a variable and progressive departure from linearity. Test solution application volumes are chosen to produce spots of similar intensity to the 20-μg standard, thus, for anticipated concentrations of 10, 100, and 1000 mg/100 ml appropriate volumes would be 200, 20, and 2 μl, respectively. The 10-μg standard should be used when test spots are much below 20-μg intensity to avoid overestimation caused by deviation of the standard curve from linearity.

Hot-air drying during application. Hot-air drying is convenient to speed the application of large volumes. Pure sugars (stachyose, raffinose, lactose, lactulose, sucrose, galactose, glucose, fructose and xylose) applied to the chromatogram were shown to survive quantitatively when heated for 5 min with an industrial blower

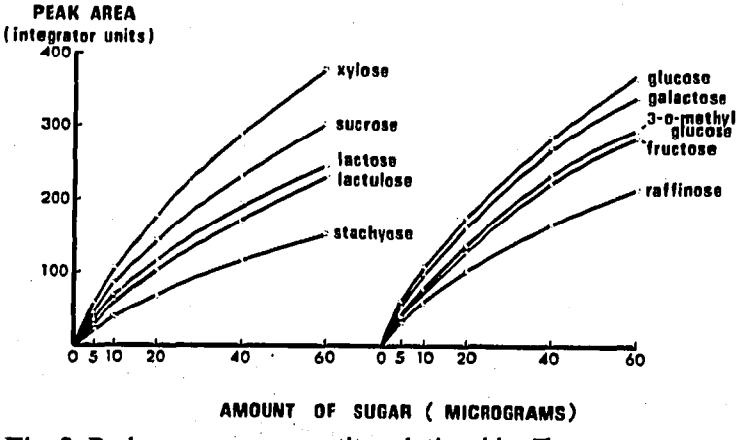

Fig. 2. Peak area–sugar quantity relationship. Transverse-scan peak areas (by reflectance densitometry) of sugar spots developed on Whatman No. 3 chromatography paper, located with 4-aminobenzoic acid reagent and clarified by oiling, are plotted against sugar quantity.

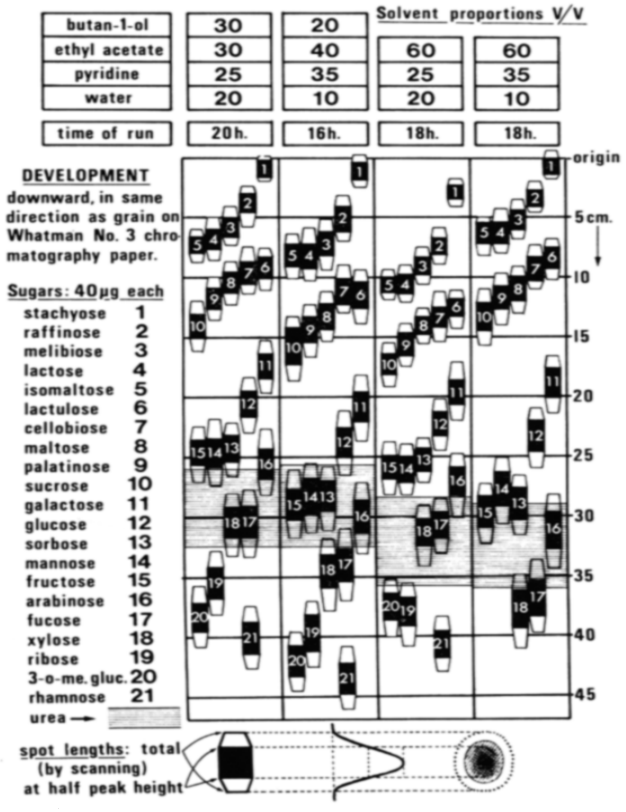

Fig. 3. Sugar separations produced by versions of the ethyl acetate–pyridine–water solvent system with or without butan-1-ol.

(maximum paper temperature 105°). However, in the presence of sulphosalicylic acid, which may persist after deproteinization when desalting is incomplete, heating caused considerable degradation of sugar and also release of pentoses, glucose and various oligosaccharides from the paper.

Development of the chromatogram

Solvent system. This can be adjusted to suit individual requirements. In the case of sugars, development of paper chromatograms with an ethyl acetate–pyridine–water (EA–P–W) solvent mixture produces a generally better separation than many other systems[14,15]. Addition of butan-1-ol to this system was found to assist the removal of pyridine prior to location, and, though the speed of development was reduced, a better final sugar resolution was obtained. Details of sugar separation related to different versions of the butan-1-ol–EA–P–W system are given in Fig. 3. For reliable quantitations using the transverse scanning technique (see Fig. 10) the central 1 cm of a sugar spot should not be overlapped by part of another sugar spot or compound capable of modifying the sugar locating reaction. Solvent proportions and development time are chosen according to practical requirements, but certain sugar combinations cannot be sufficiently separated to allow estimation in the same

specimen unless a specific locating reagent is available. Urea, which interferes with several sugar locating reactions and is not completely removed from urine by the desalting technique described, can be made to superimpose on either fructose or xylose areas by using butan-1-ol–EA–P–W in 20:40:35:10 or 30:30:25:20 proportions, respectively (see Fig. 3).

Paper grain and texture. These are important because they influence the shape, frequency of distortion, and transverse alignment of spots. The regularity of solvent flow was frequently found to vary between different batches of the same grade of paper, and to depend on the direction of development in relation to the paper grain (= machine direction). Choice of paper with optimal flow characteristics is aided by observing the way in which pigment lines, rules onto the paper with a fibre pen (a black Platignum Penline fiber pen was used), descend during chromatographic development. Close similarity exists between spot alignment and the incidence of distortion, and the appearance of the pigment lines, as shown in Fig. 4.

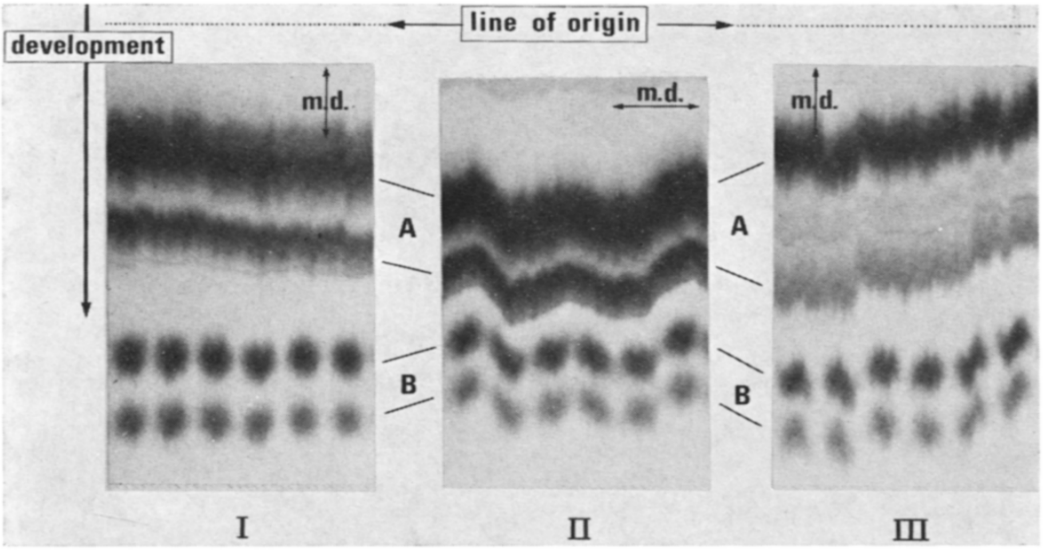

Fig. 4. Solvent flow characteristics of paper demonstrated by penline technique. Transverse alignment and incidence of spot distortion (B) are closely related to the appearance of pigment lines (A) ruled onto the chromatogram origin and developed similarly. Examples I and II, demonstrating good and poor flow patterns, were run parallel to, and at 90° to the machine direction (m.d.), respectively, on Whatman No. 3 chromatography paper. Example III is from a batch of Whatman 3MM with very poor flow characteristics.

Drying and storage of chromatograms prior to location. Pyridine is the only component of the solvent system found to inhibit colour production during sugar location, *o*-dianisidine phosphate and especially 4-aminobenzoic acid reactions being affected. Prolonged drying, even in the hot-air oven, fails to remove pyridine completely from the paper, even though the odour disappears. Drying after treatment with an alcohol such as butan-1-ol displaces pyridine from the paper. When the developing solvent contains butanol, a single further butanol dip is sufficient to

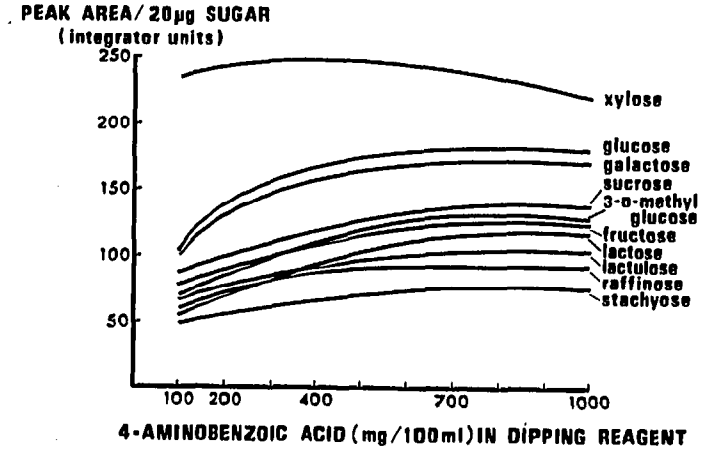

Fig. 5. Colour–concentration curves for 4-aminobenzoic acid location of sugars. Except for xylose, most sugars require a reagent concentration above 500 mg/100 ml to produce maximum colour, 700 mg/100 ml 4-aminobenzoic acid in methanol being chosen for routine purposes.

complete pyridine removal; otherwise it is necessary to repeat the butanol treatment three times.

Chromatograms kept in a dark drawer inside polythene bags showed no evidence of sugar loss, even after prolonged storage.

Location of sugars on the chromatogram

Locating reagents employed. *o*-Dianisidine phosphate and 4-aminobenzoic acid were studied. Both are sensitive and produce stable colours for most sugars, however, *o*-dianisidine phosphate has the disadvantage of being unstable in solution, requiring fresh preparation before use, and is considered potentially carcinogenic. Reagent

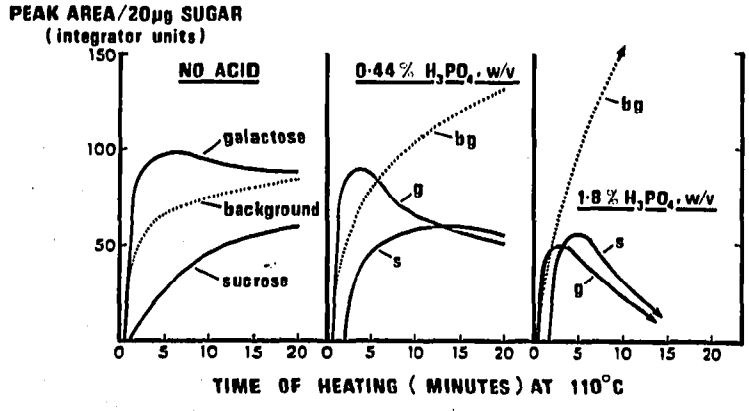

Fig. 6. Colour reaction related to acid concentration. The colour–time curves plotted relate peak areas for 20 µg of galactose and sucrose (aldose and ketose reactions, respectively) to concentration of free H_3PO_4 in *o*-dianisidine reagent (600 mg/100 ml in methanol). Acid concentration must be carefully standardised to obtain reproducible results, the level chosen giving a convenient speed of reaction without rapidly rising background colour and loss of sensitivity.

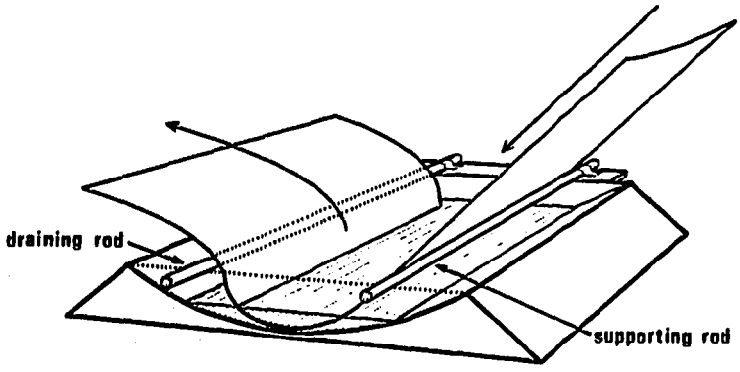

Fig. 7. Dipping technique with draining rods. The trailing (R-hand) end of the paper should be held with an upward convexity, as illustrated, and gently thrust downwards over the supporting-rod into the tray. The chromatogram, manipulated to perform a tight U-bend between the rods, is passed slowly and evenly through the dipping reagent. On leaving the tray, the undersurface of the paper is thus firmly thrust against the draining rod, most of the free surface-fluid being removed.

concentrations related to the plateau-portion of the colour–concentration curves, shown in Fig. 5, have been chosen to minimise the effect of non-uniform reagent distribution on spot reproducibility and also give maximum colour production. Acid concentration must be carefully controlled since it influences the speed and sensitivity of the colour reaction in the hot-air oven, and the amount of background colour produced, as shown in Fig. 6. Volatile acids (*e.g.* acetic acid) are best avoided.

Preparation of o-dianisidine phosphate reagent. Free *o*-dianisidine, 600 mg, dissolved in about 50 ml of acetone, is converted to the phosphate derivative by adding 0.6 ml of orthophosphoric acid (88% w/v) in a stoppered cylinder. The resulting sticky precipitate is washed in the same cylinder with further acetone, and dissolved without delay in 90 ml of methanol. Citric–phosphoric acid solution is added (10 ml of 2.2% H_3PO_4 plus 20% w/v citric acid in acetone), and the reagent is ready for use. Citric acid temporarily stabilises the reagent.

Preparation of 4-aminobenzoic acid reagent. Concentrated stock solution containing 2 g/100 ml of 4-aminobenzoic acid in methanol is stable at room temperature. 35 ml of stock solution plus 0.4 ml of 88% H_3PO_4 are diluted to 100 ml with methanol for use, giving a final reagent concentration of 700 mg/100 ml.

Application of locating reagent to the chromatogram. A dipping rather than a spraying technique was chosen because uniformity of reagent distribution and concentration can be better controlled, and danger from toxic constituents is less. The dipping technique, illustrated in Fig. 7, employs a draining rod to remove free surface-fluid which otherwise runs down the paper producing non-uniform reagent distribution.

Redistribution of locating reagent during drying. Movement of solute towards the paper surface, which accompanies solvent evaporation during the drying stages of chromatography, may produce an irregular distribution of locating reagent, especially if drying occurs in an air current[16]. Chromatograms are therefore better dried in still air after the reagent dip.

Control of heating in the hot-air oven. The colour reaction of *o*-dianisidine phosphate or 4-aminobenzoic acid with sugars in the presence of acid requires heating

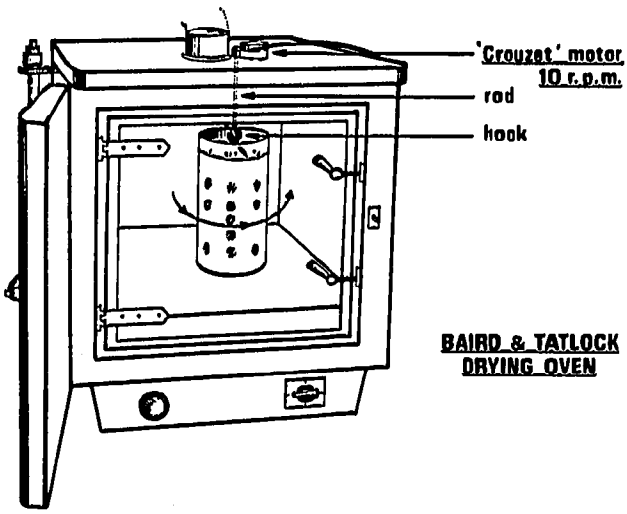

Fig. 8. Rotating the chromatogram in the hot-air oven. After dipping in locating reagent and drying, the chromatogram is clipped to form a cylinder (lateral margins stapled to the sides of a 2.5-cm wide paper strip) which can then be rotated in the hot-air oven as illustrated. This ensures uniform heating of each sugar series (standards plus tests) during the period of the colour reaction.

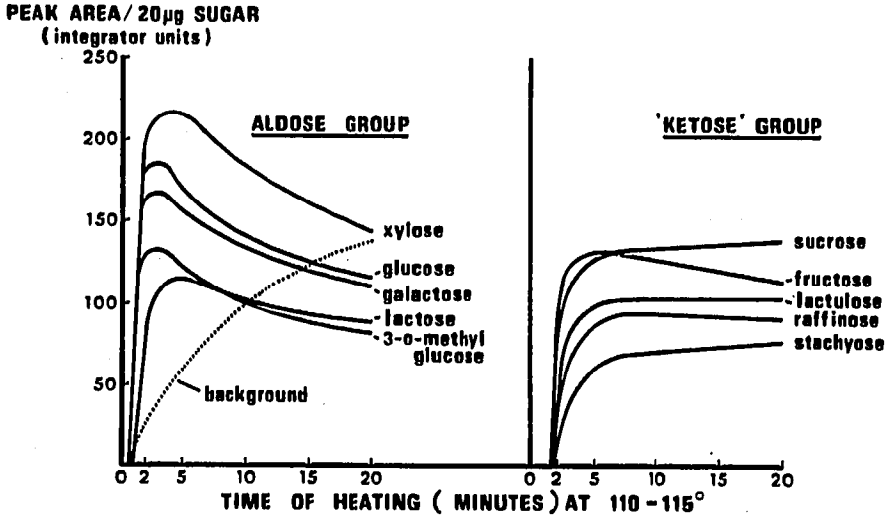

Fig. 9. Colour–time curves for 4-aminobenzoic acid sugar locating reaction. After dipping in colour reagent (700 mg of 4-aminobenzoic acid + 0.4 ml of 88% H_3PO_4 w/v in 100 ml of methanol) replicate strips were cut from the chromatogram and rotated on a cylindrical frame in the hot-air oven at 110 to 115°. Single strips were removed at 5-min intervals, and later oiled and scanned to measure the rate of the colour reaction for different types of sugar. Background intensity was measured as peak area, 1.5-cm strips being scanned against non-located paper. Sugar measurements have been corrected for background colour.

of the chromatogram above 100°. Control of both temperature and duration of heating in the hot-air oven are necessary for a reproducible colour reaction. Irregular heat radiation from oven walls and temperature layering of hot air, however, make uniform heating difficult. This may be overcome by rotating the chromatogram, stapled in the form of a cylinder inside the oven, by means of an electric motor, as shown in Fig. 8. Though upper and lower ends of the cylinder are not necessarily at the same temperature, spots of the same sugar type (*i.e.* the standard and test spots to be compared) are exposed to similar heating conditions when rotated with an equatorial disposition.

Time and temperature of heating. Aldose sugars (lactose, galactose, glucose, xylose, 3-O-methyl glucose, etc.) react quickly to produce maximum colour within 3 min at 110 to 115°, but "ketose" sugars (stachyose, raffinose, lactulose, sucrose, fructose, etc.) require 5 to 10 min at this temperature to reach the same stage using the locating reagent recipes already given (see Fig. 9 for colour–time curves with 4-aminobenzoic acid). Heating for 5 to 10 min at 110 to 115° is suitable when both sugar groups are to be measured on the same chromatogram; at this time aldose colours, though past their peak value, are only slowly decreasing, "ketose" colours are maximal, and the background colour is increasing less rapidly. Near maximum sensitivity for both sugar groups is thus obtained, and the sloping portions of the colour–time curves unfavourable to spot and background reproducibility avoided.

Modified oiling technique. After location the chromatograms are dipped in a mixture of liquid paraffin and light petroleum, b.p. 60–80°, (55:45, v/v) and allowed to dry in the fume cupboard. The use of diluted liquid paraffin facilitates dipping, and prevents excessive uptake of oil and air trapping by the paper, which is ready for scanning after 15 min drying. The increased paper translucency due to oiling was found to improve sensitivity by about 100%, measured as spot peak areas for several sugars by reflectance scanning, and to improve spot reproducibility by eliminating the effect of irregular disposition of colour between the paper surfaces.

Stability of the chromatogram after location. After location a slow increase in background colour occurs at room temperature which is hastened by exposure. The pink pentose–4-aminobenzoic acid colour soon changes to brown, but in other respects sugar colours with this reagent and *o*-dianisidine phosphate change very slowly at room temperature when chromatograms are stored in a dark drawer inside individual polythene bags. Storage in the deep-freeze gives best colour stability but is only necessary when scanning is much delayed. Background changes tend to be more rapid at paper margins; for this reason it is unwise to cut out spots until the time of scanning.

Measurement of chromatographic spots

Scanning technique. Several different types of direct spot measurement show a quantitative relationship to the amount of compound present[19]. Of these, peak areas obtained by scanning transversely across the spot at 90° to the direction of development (see Fig. 10) have been employed for reasons to be discussed. Measurements were made by reflectance densitometry using a Chromoscan double-beam recording and integrating densitometer (Joyce-Loebl, 1966), which traces the absorption profile of the spot, and integrates the peak area in arbitrary units. Both the pen deflection and integrator counting rates of this instrument are proportional to optical density.

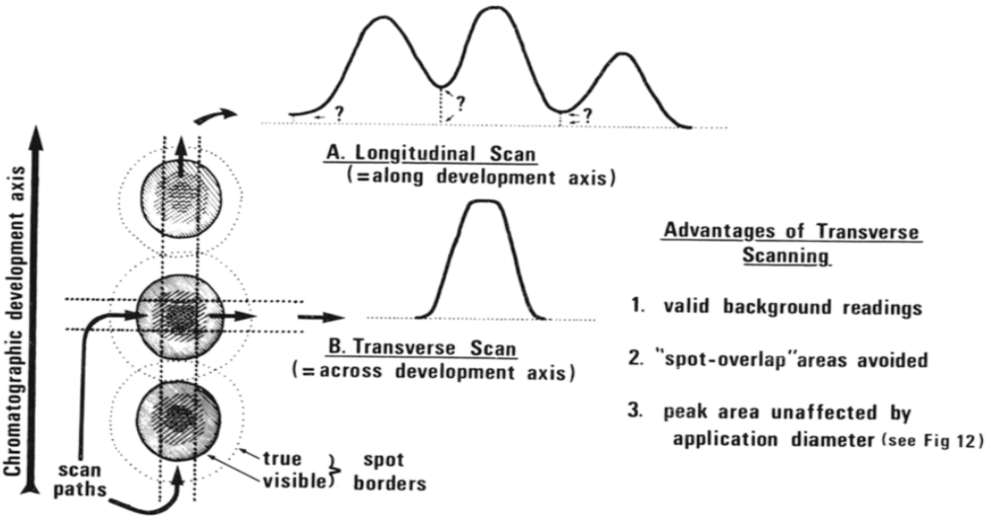

Fig. 10. Longitudinal *versus* transverse scanning.

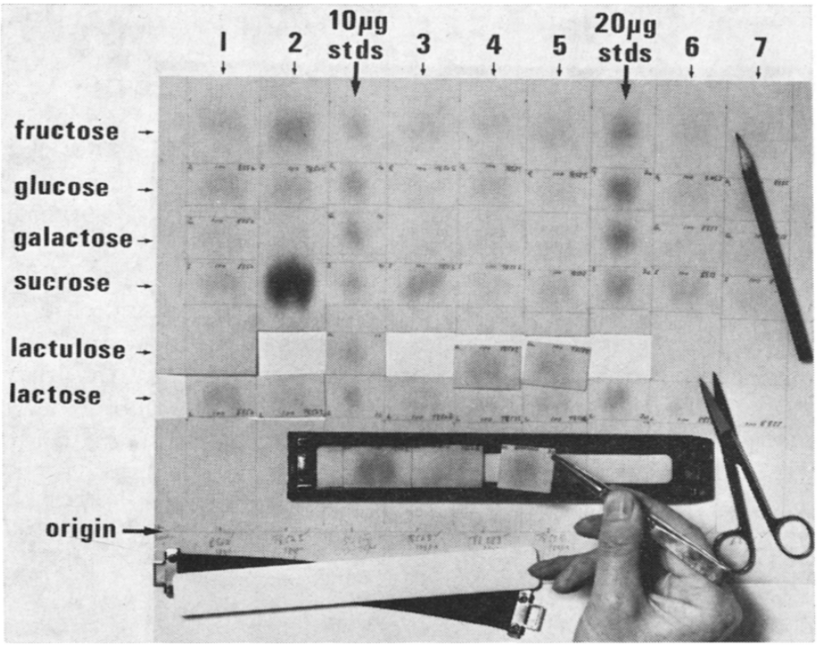

Fig. 11. Cutting out and mounting spots on the Chromoscan sample-holder. Test and standard spots have been marked out in pencil using the glass sample-holder slide, so that they can be correctly centered for transverse scanning (at 90° to the development axis).

The principles are similar to those of conventional absorptiometry, *e.g.* selection of complimentary colour for illumination, but the response relationship follows Kubelka's rather than Beer's Law, and approximates to the latter only in the lower range of colour intensity[10].

Alignment of spots on the chromatogram was seldom good enough to allow a spot series to be scanned unless they were first cut out and individually centered on the Chromoscan sample-holder, as shown in Fig. 11. The yellow-brown or red-brown spots, of average diameter 30 mm, were scanned against an opaque matt-white formica backing, using quartz-iodine light source, blue filter, and 10 × 1 mm light slit, with the sample-holder drive in 1:1 gear ratio so that peak and spot dimensions were the same. Peak areas were recorded using the digital integrator, and background corrections, measured by running the recording pen along the base of each peak, deducted. Corresponding test and standard spots were always scanned together.

Calculation of sugar concentration. In the system used, provided test spots were near to the intensity of either 10- or 20-μg standard spots, peak areas could be assumed to be proportional to the amount of sugar present (see Fig. 2), and the following formula applied:

$$\text{Sugar concentration in test solution (mg/100 ml)} = \frac{\text{Test P.A.}}{\text{Std. P.A.}} \times \frac{\text{Std. A.V.}}{\text{Test A.V.}} \times \text{Sugar concentration in standard solution (mg/100 ml)}$$

where P.A. = peak area (integrator units)
A.V. = application volume.

Performance of the scanning instrument. When peak areas, as distinct from peak heights are to be measured using a time-based integrator, it is essential that spots mounted in the sample-holder should pass the incident light beam at uniform velocity otherwise false variations in peak width, and hence area, will occur. The particular instrument used had a coefficient of variation of $\pm$ 3% when the integrator was used to measure peak areas. Non-uniform movement of the sample-holder, arising from the elaborate gearing system was mainly responsible, and a laborious correction involving repeated scanning with rotation of spots through the available slide positions was found to reduce this error to below $\pm$ 1% (ref. 16). Estimations of spot reproducibility quoted have been corrected in this way, and therefore do not include the instrument bias.

Sensitivity. Adequate desalting and deproteinization allow at least 400 μl of urine or plasma to be applied without deterioration in sugar resolution. This enables concentrations of 1.0 mg/100 ml, or lower for some sugars, to be measured, though best precision would be obtained at 4 mg/100 ml or above. Reflectance densitometry applied to a translucent medium, such as oiled paper, scanned against a white backing, referred to as double reflectance scanning because the light beam traverses the specimen twice, doubles the response of the instrument.

Reproducibility. Spot reproducibility was calculated from the transverse-scan peak areas for 20-μg amounts of seven different sugars. For each sugar a series of twenty-four spots were scanned (four chromatograms with six applications each), and the percentage differences from the average peak area for the corresponding chromatogram used to calculate the coefficient of variation. Spot reproducibility was best using Whatman No. 3 chromatography paper with development parallel to the machine direction (paper grain), 4-aminobenzoic acid location with rotation in the hot-air oven, and paper clarification by oiling. Under these circumstances coefficients

of variation were ± 2.4, 2.5, 1.8, 2.3, 2.3, 1.4, and 1.9%, respectively, for lactose, lactulose, sucrose, galactose, glucose, fructose, and xylose, with an average of ±2.0%. In practice, because sugar quantitation involves comparison of test with standard spots, both of which are independently subject to variation, the final variance will be doubled giving an average coefficient of variation of ± 2.9% (range ± 2.0 to 3.5%) for repeated estimation, without replication, when applications contain around 20 μg of an individual sugar. Replication of test and standard should give the usual improvement in precision[17].

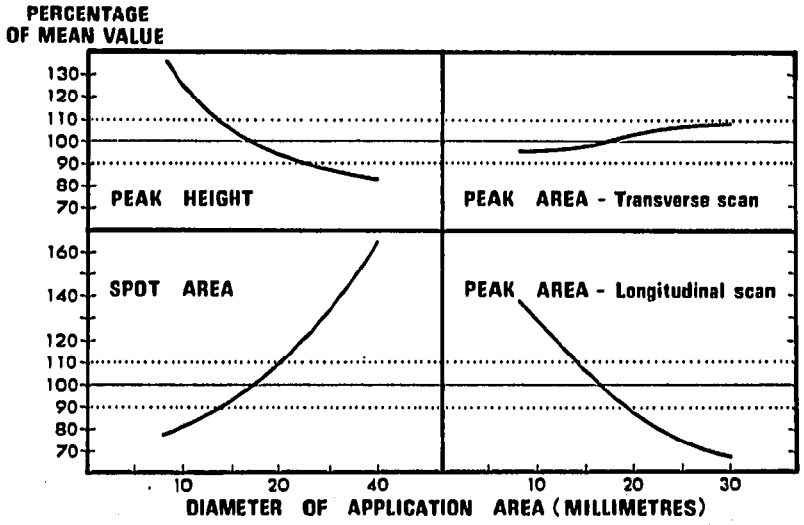

Fig. 12. Spot measurement related to application diameter. 20-μg sugar applications of 8, 12, 20, and 30 mm diameter produce spots of varying dimension (Fig. 13). Four different types of direct spot measurement advocated for quantitative estimation have been made, and the percentage variation is plotted in relation to application diameter. Variation in the size of the application area, which is difficult to prevent in practice, has least effect on the transverse-scan peak-area (Fig. 10).

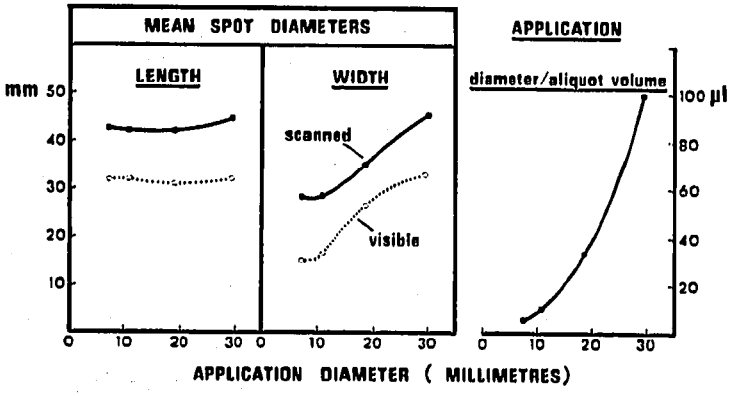

Fig. 13. Spot dimensions related to application diameter. Mean spot dimensions for duplicate 20-μg amounts of lactose, galactose and xylose have been plotted against application diameter, which was varied by using different application volumes.

Advantages of transverse-scan peak area. In practice, especially when widely different application volumes are necessary, it is difficult to control the size of the application area. It is therefore an advantage if the spot measurement chosen remains unaffected by variations in application diameter. The effect of variation in application diameter on spot area, peak height, and peak areas obtained by scanning across the spot both transversely and longitudinally (at 90° to, and parallel to the direction of chromatographic development, as shown in Fig. 10) are recorded in Fig. 12. Of these measurements, all of which have been suggested for direct quantitative analysis[19], only transverse-scan peak-area was not significantly affected by changes in dimension of the application area. This is because, as shown in Fig. 13, increase in application diameter produces an increase in width, but not length, of the final spot. In these circumstances, reduction in colour intensity and peak height due to increase in spot area, are accompanied by a compensatory increase in peak width only when the spots are scanned transversely.

Further advantages of transverse scanning (illustrated in Fig. 10) are (a), lack of interference with background readings caused by spot trailing or the presence of other compounds along the development axis, and (b), a transverse scan through the spot centre avoids areas of overlap which would make longitudinal scanning unreliable when spot separation is incomplete. For these reasons transverse-scan peak area is likely to be the most reliable measurement available.

Spot size and colour production. Relative colour production diminishes as compound concentration on the paper surface increases[18,19], and therefore tends to be better for larger spots than small intensely coloured ones. A more linear peak area–sugar quantity relationship exists when applications of larger diameter are made, as shown in Fig. 14. Relatively large applications (15-mm diameter = 20-μl aliquots) not only avoid the production of small, intense spots, but reduce the number of aliquots required for large application volumes without loss of resolution (since spot length is unaffected, see Fig. 13).

Spot distortion. (a) Oblique spots. This type of distortion, examples labelled "C" in Fig. 15, appears to be caused by irregular solvent flow during development. It interferes with quantitation by reducing the transverse-scan peak area, and is an

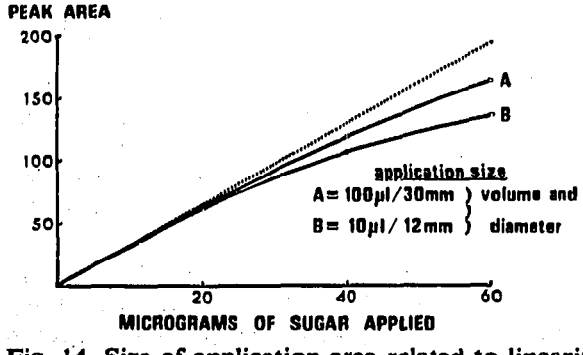

Fig. 14. Size of application area related to linearity of peak area–sugar quantity curve. Large, less intense spots resulting from applications of greater diameter demonstrate a more linear peak area–sugar quantity relationship (A) than the small, intense spots resulting from applications of smaller diameter (B).

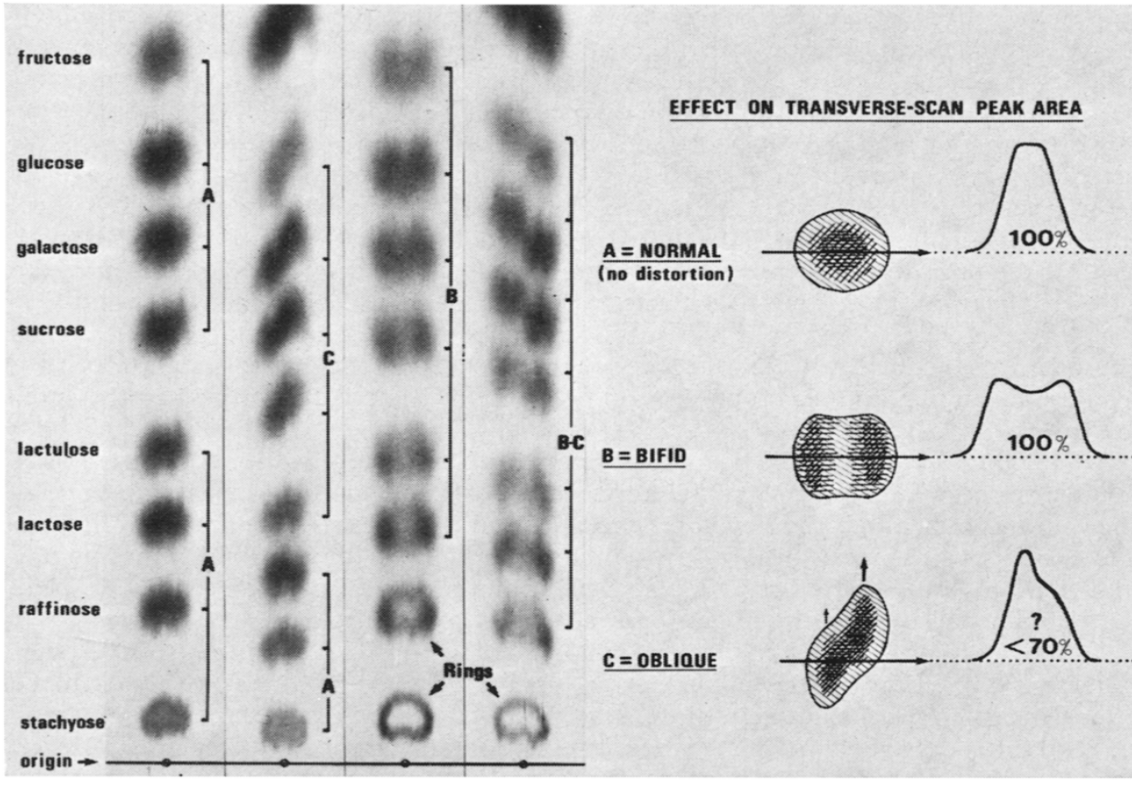

Fig. 15. Types of spot distortion. Oblique spot distortion, an important source of error, can be reduced by choosing paper with even solvent flow characteristics. Bifid distortions, the result of repeated applications to the same area, produce no quantitative error provided transverse-scan peak areas are used.

important cause of poor reproducibility. The incidence of this distortion can only be reduced by careful selection of paper with good solvent flow characteristics (see Fig. 4). Incidence of spot distortion often varies with the direction of development in relation to the machine direction of the paper (Fig. 4), for instance one batch of Whatman 3MM paper gave spot reproducibilities of $\pm$ 6.9 and 4.6, respectively for downgrain and acrossgrain developments.

(b) Bifid spots. This distortion, labelled "B" in Fig. 15, occurs when several aliquots have been superimposed during application, with consecutive drying, so that a ring-shaped zone of high sugar concentration is produced at the edge of the application area. Provided scanning is in the transverse direction there is no interference with quantitation, since both lateral components of the spot combine to give the same peak area as a normally shaped spot.

Alternative techniques for controlling spot reproducibility. Reproducibility was found to improve when the following techniques were applied to Whatman 3MM paper in place of clarification by oiling (values are quoted as average coefficients of variation for six different sugars).

(a) When each spot was scanned on both surfaces, as opposed to one surface, and the mean peak areas calculated, there was an improvement from $\pm$ 4.8 to 3.3%.

This method overcomes irregular distribution of colour in the paper by taking account of the intensity on either surface.

(b) The use of a polythene-backed version of Whatman 3MM paper improved reproducibility from ± 4.8 to 2.6%. The polythene layer prevents irregular movement of solute towards the paper surfaces during the drying stages of chromatography by confining solvent evaporation to one surface only.

Further improvement of spot reproducibility to ± 2.0%, when uncoated Whatman No. 3 paper was clarified by oiling, appears to be due to the following: first, the incidence of spot distortion was less for this type of paper than for most batches of Whatman 3MM; and second, paper clarification both increases sensitivity to reflectance scanning (double reflectance effect), and overcomes the problem of irregular colour distribution between the paper surfaces.

GENERAL DISCUSSION

Chromatographic procedures have earned an important place in the analysis of many groups of chemical compounds. There are few serious alternatives available for the quantitative estimation of mixtures of sugars, and several different chromatographic techniques are in use.

Gas–liquid and liquid–liquid procedures are available in which a detector system produces a continuous quantitative record of compounds leaving the column in the eluent stream[20–24]. Such automatic quantitation saves time and labour, but the flame ionisation and electron capture detectors used for gas–liquid methods lack the specificity possible with colour reactions on paper and thin-layer chromatograms. The inclusion of standards also presents a problem, and limitation to one analysis per column at a time is a disadvantage when many samples require evaluation, especially if the sample running time is long. The apparatus is also expensive and space consuming, and, in the case of liquid–liquid systems, non-versatile. Paper (PC) and thin-layer chromatography (TLC) overcome these difficulties, since several samples can be run in parallel with suitable standards on the same chromatogram, and the apparatus can be easily adapted for separation of many different groups of compounds. Constituents thus separated can either be eluted and then quantitated, or located and estimated *in situ* by direct densitometry. Several authors have favoured direct densitometry in preference to elution for reasons of convenience, labour and sensitivity[18,19,25–28], and the present study supports their claim that this method can in practice produce reliable quantitative results.

Reproducibility, important for any quantitative procedure, poses special problems in the case of direct densitometry of flat-bed chromatograms because of the large number of factors modifying spot shape, dimensions and colour intensity. Adequate control of these factors is essential, and has been approached in two different ways. Bush[18,27] and Boulton[28] have obtained adequate reproducibility by comprehensive control of conditions at all stages involved in PC of steroids and amino acids (respectively), resulting in a largely automated procedure. Others[19,29,30] have applied a more selective control of the factors considered relevant; this has been the approach used in the present study.

Pipetting errors, common to most chemical estimations, become particularly important when small volumes are involved. Fairbairn[29] stressed the imprecision of

manual techniques, and introduced an automated pipetting apparatus. However, the precision of the Drummond microcapillary technique, as used in the present study, compares well with that quoted for the automated pipette. The sample preparation technique described (desalting, and deproteinization when necessary) is simple and without the serious recovery problems that accompany preparation of sugar derivatives suitable for gas–liquid chromatography[21].

Reproducible colour reactions are achieved by attention to two main points: firstly, conditions are selected which minimise the effects of variation on colour production; and secondly, technical modifications are employed to increase the uniformity of these conditions over the surface of the chromatogram. Several technical refinements such as dipping with draining rods, rotation in the hot-air oven, paper clarification by oiling and the use of transverse-scan peak areas, as well as selection of paper with optimal solvent flow characteristics have already been discussed in the appropriate sections.

Many of the principles and factors discussed apply equally to chromatography on flexible thin layers, and these can be subjected to the same techniques (dipping, rotation and cutting out spots for alignment). Locations on rigid (glass-backed) thin layers are more difficult to control since they usually require spraying and cannot be heated with the same precision of temperature and time. Small intense TLC spots, though excellent for qualitative detection, are more difficult to center for scanning, and have limited linearity of quantity–colour relationship compared with the larger PC spots. The speed of TLC may not always be a clear advantage. Overnight development with less critical timing is often convenient and, in the case of sugars, which have relatively low R_F values, though rapid TLC methods are described[31], it is doubtful whether short one-dimensional upward development could produce such good resolutions as an overnight downward development with solvent over-run.

The fundamental aims have been to improve reproducibility and reduce labour, but there is considerable scope for further improvement. An alternative solvent system lacking pyridine but with equally good resolution to the butan-1-ol–EA–P–W system described would be more pleasant to use and avoid interference with the colour reaction. Reliable transverse spot alignment for scanning purposes, either by better control of chromatographic development, or automatic centering during densitometry, would allow the advantages of transverse-scan peak areas to be obtained without the inconvenience of manual spot alignment required at present. Lastly there is a requirement for chromatography paper with better solvent flow characteristics, and the need is still apparent for a simple, reliable reflectance scanning densitometer combining adequate precision, adaptable sample holder and both reasonable size and price, since available models tend to be inadequate and/or unnecessarily versatile, large and expensive.

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