MOLECULAR SIZE DISTRIBUTIONS OF LIGNOSULPHONATES BY THIN LAYER CHROMATOGRAPHY

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The work described in this paper arose from a need to find a rapid method of determining the molecular size distribution of lignosulphonates. These polydisperse macromolecules are compact, internally cross-linked, gel particles with molecular diameters in the approximate range 10–100 Å. Several familiar techniques for measuring molecular weight distributions and of general application to polymers, have been described and reviewed previously[1]. None of them was entirely satisfactory for our purposes because they were too slow or good technical performance was allied to very high capital cost of equipment.

It is the intention of this paper to demonstrate how application of the technique of thin layer chromatography (TLC) allied to gel permeation chromatography (GPC) led to rapid quantitative determinations of the molecular size distribution of lignosulphonates. It will be shown that the method can measure 20 or 30 samples each day; only small quantities (of the order of 100 μg) of polymer are required, results are reproducible to within a few percent and the method measures the size of polymer molecules rather than their molecular weights. Finally the cost of the apparatus is not unreasonably high–about £1500, of which about £1100 is general purpose equipment.

Determinations of molecular weight distributions of polymers are commonly based upon fractionation procedures. These various procedures have been reviewed several times[1]. They are tedious because separate fractions must be isolated and characterised.

A fairly recent but rapidly developing method, called gel permeation chromatography (GPC) (often referred to as gel filtration), is characterised by the capacity of certain gel-like substances to obstruct the passage of large molecules in solution, through them. A review of the technique with special reference to cross-linked dextrans[2] was published by PORATH. It is a process which fractionates molecules according to their sizes[3–5]. The measurements described in this present paper are based on a GPC process and result in a molecular size distribution. Separate characterisations of fractions are not required; only a calibration procedure.

FLODIN was early in the field of GPC using hydrophilic gels such as cross-linked dextrans of which "Sephadex" is the commonly available example[6]. He applied the technique to such substances as the globular proteins. MOORE worked with lightly cross-linked polystyrenes as the gel substances[7] and developed his method with solutions of hydrophobic polymers such as polystyrenes. Since that time GPC by column chromatography at least, has expanded greatly so that a variety of suitable

gel substrates are available for studying polymer solutions that are both hydrophilic and hydrophobic. Quantitative, if empirical, ralationships between molecular weight or molecular size, and rates of movement of the molecular species down the column of gel have been evolved by several authors. ANDREWS, in particular[4], obtained roughly linear relationships between the logarithm of molecular weight and elution volume of mixtures of proteins separated on columns of Sephadex. He extended these data to separations carried out on thin layers of Sephadex. A few months later MORRIS[8] published data on the separation of proteins by a similar technique.

It was not, therefore, a big step to the idea of measuring the molecular weight distribution of lignosulphonates by the same kind of thin layer chromatographic method. However, ANDREWS and MORRIS only required to identify the position to which a substance had migrated on a plate. They were not concerned with measuring the amount of substance at a given locus on the plate.

By contrast in order to determine the molecular weight distribution of polymer, it is necessary to measure the distribution of polymer concentration with distance, along the plate. In order to obtain useful quantitative data it was required to obtain a very uniform layer of gel and to devise a method of accurately measuring concentrations of polymer solutions at selected points on a TLC plate.

The second of these objectives was achieved by spectrophotometric means. The results quoted in this paper are for lignosulphonates which have a characteristic absorption maximum in the ultraviolet at 282 mμ, and this wavelength was used for measurement. Also there was required precise location of the sample on the TLC plate. Finally, it was essential that the sample was eluted along the plate on an accurately predictable locus, preferably parallel to the edge of the plate. Optically uniform layers of gels varying by no more than about 0.005 absorbance units, were achieved by careful use of modified conventional techniques.

It should be remarked in conclusion to this introduction that the technique described below suffers from one recognised disadvantage: it requires a rather high degree of manipulative skill, which is not, in our judgement, easily acquired by untrained personnel.

EXPERIMENTAL

Lignosulphonates

Thin layer chromatography was carried out on solutions of sulphite lye "C.A. Liquor" supplied by Wargons A.B., Vargön, Sweden. The fractions needed for calibration purposes were obtained from the same material.

Gels

"Sephadex" Superfine, Grades G-25, G-50, G-75 and G-100 supplied by Pharmacia A.B. of Uppsala, Sweden, were used throughout the work. The G-25, 75 and 100 gels were sedimented at low concentrations in order to remove the relatively large and small particles. This procedure gave more uniform thin layers. It was found that several Sephadex samples contained substantial quantities of particles outside the nominal size range. Gels were swollen in the prescribed manner[6] with buffer solution (1 l, pH 6.80) containing disodium hydrogen phosphate (23.9 g), potassium dihydrogen phosphate (9.1 g) and sodium chloride (5.85 g). Gels of a thin consistency were found

to be satisfactory and this was judged by rotating them in bottles. The manner in which the gel film spread on the surface of the bottle was found a useful guide. No scientific standard for judging suitable gel consistency was found.

Spreading of gels on TLC plates

Gels were spread on polished silica plates (20 cm long, 5 cm wide and 0.2 cm thick), supplied by Thermal Syndicate Ltd. They were cleaned successively with chromic acid, water and acetone, and finally dried in an oven at 80° for 5–10 min. The thin layers were formed with an automatic plate leveller and spreader supplied by Q.V.F. Ltd. It was found necessary, in order to obtain uniform layers of gel, to separate individual plates (which had been racked horizontally in a series), from each other by means of double sided adhesive tape. The tape was fixed to the edge of each plate and the adjacent plate moved up to the free surface of the tape. The tape did not protrude above the top surface of the plates. In addition the ends of each plate were covered with a water resistant adhesive tape 0.64 cm wide and 0.016 cm thick. The Q.V.F. spreading box was then replaced by a similar "home-made" device formed in aluminium but with no relief to the plane bottom surface.

TLC plate elution chamber

The chamber is illustrated in Figs. 1 and 2. It was machined from a single block of PVC and covered with a lid made from plate glass. The lid was drilled with a

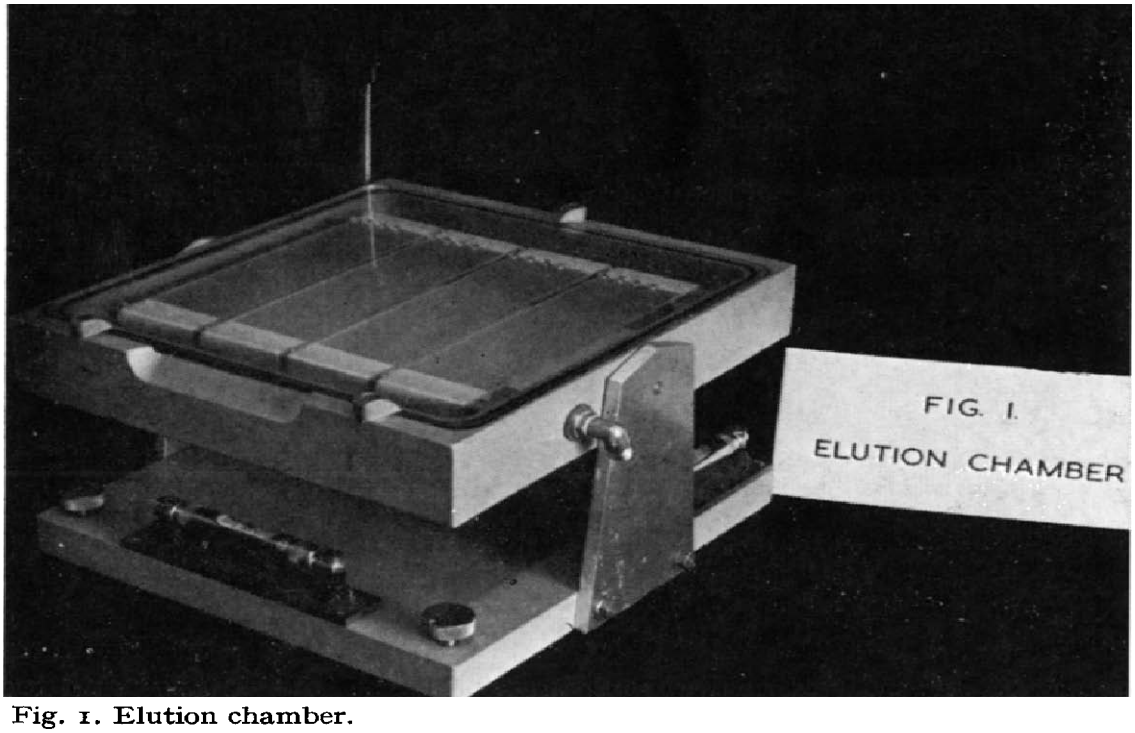

Fig. 1. Elution chamber.

series of accurately positioned 3/16 in. diameter holes as indicated in the figure and sealed to the chamber with a rubber 'O' ring. The whole assembly was pivoted on a stand which could be set up horizontally by means of two spirit levels at 90° to each other.

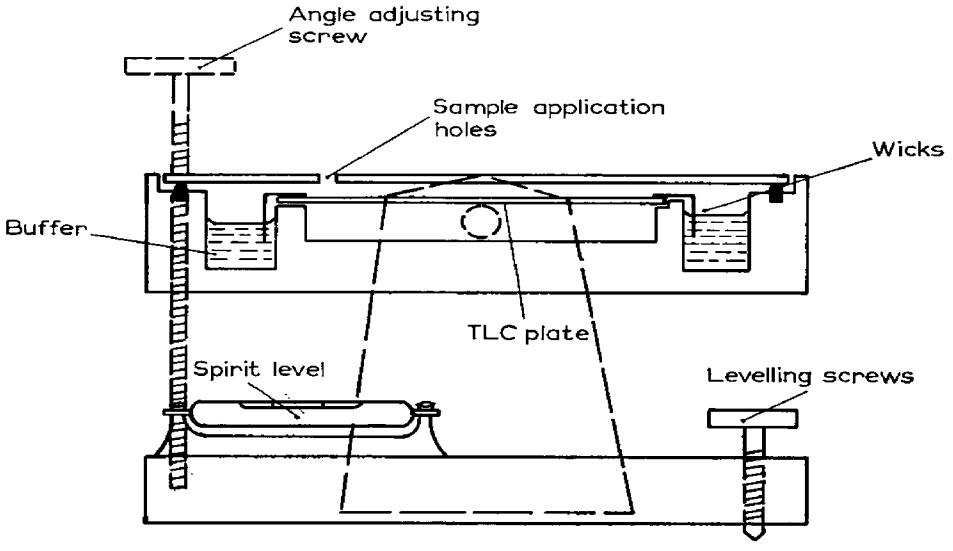

Fig. 2. Elution chamber, side view.

The rate of solvent travel along the plates (~ 1.0 cm/h) was controlled by tilting the chamber through a fixed angle. This was achieved by a screw shown in the figures. The levels of buffer solution in both reservoirs were equalised before each run by means of a brass bar fitted with pointed level screws.

Application of sample solutions to TLC plates

In order to obtain uniform results it was necessary before applying samples to the plates, to allow buffer solution to flow over them in the elution chamber for at least 24 h. The chamber was then adjusted to an horizontal position. A drop of sample solution (3–9 %, w/v of lignosulphonate; 1 μl) was formed on the plastic coated tip of a 10 μl Hamilton syringe. The syringe was fitted with a perspex spigot, Fig. 3.

The syringe was then inserted into one of the holes drilled in the glass lid of the elution chamber and the drop of sample solution was removed by the horizontal gel surface. Having applied the sample solution, the plates were adjusted to the appropriate angle, *e.g.* 2°–3° for G-50 plates.

Apparatus for scanning TLC plates

A line diagram illustrating the apparatus is given in Fig. 4, and Fig. 5 illustrates an ancillary calibration circuit.

The apparatus, which was situated in a room kept at 20 $\pm$ 1/2°, consists of a spectrophotometer (Unicam SP. 500) to which was rigidly attached a light tight housing containing a front surfaced aluminised mirror at 45° to the vertical and above it a biconvex silica lens of focal length 6 cm. The light beam was focussed on to the horizontally held plate. The plate holder shown in Fig. 6 also carried two cells of known absorbance for calibration purposes.

Light transmitted through the plate was received on a Hilger Gilford photocell. The signal from the photocell, which was roughly linear in absorbance, was then "linearised" by means of the Hilger-Gilford absorbance converter and the final output read from a recorder (Smith's Servoscribe Type R.E. 511). Associated with the linearising device was a further circuit which assisted in calibrating the recorder, Fig. 5.

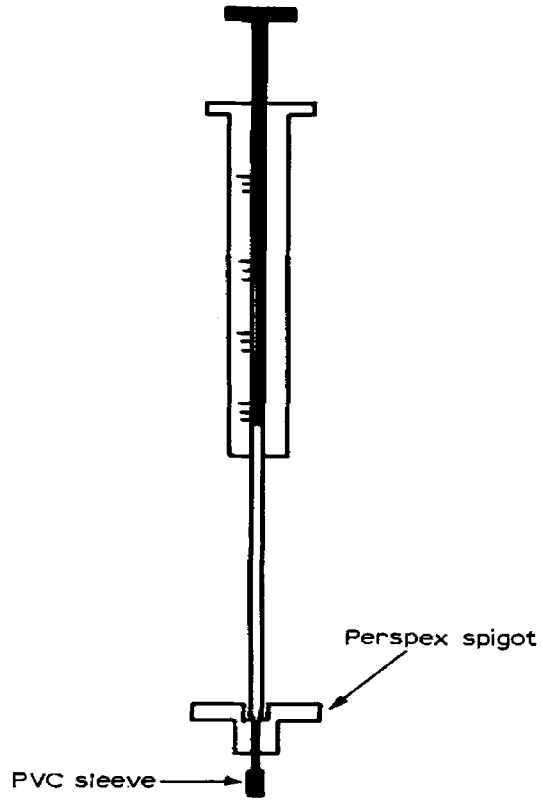

Fig. 3. Hamilton syringe, modification.

The TLC plate was well located kinematically, in a horizontal rack totally enclosed in a light and vapour tight chamber which also contained buffer solution. The plate was moved across the light beam by means of a motorized rack and pinion fitted with a clutch. Four tracks could each be selected on each plate by means of a selector rod.

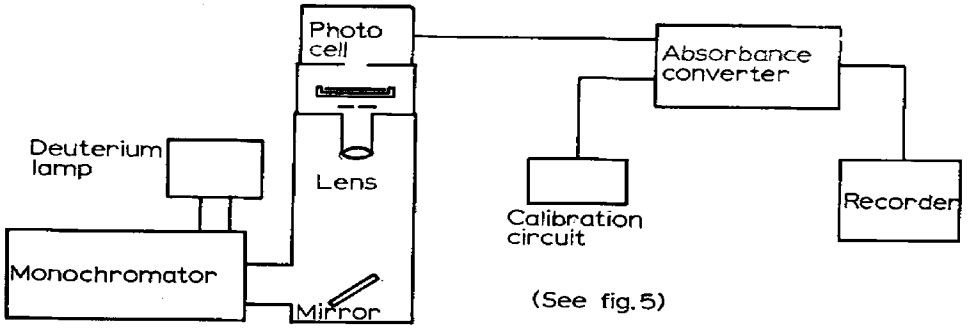

Fig. 4. Scheme for scanning thin layer chromatographic plates.

Procedure for scanning plates

A plate was taken from the enclosed elution chamber and rapidly transferred (about 10 sec) to the scanning apparatus. Even during this short period some water evaporates from the gel layer, with consequent increase in optical absorbance. The plate was therefore allowed to re-equilibrate in the scanning apparatus and equilibrium

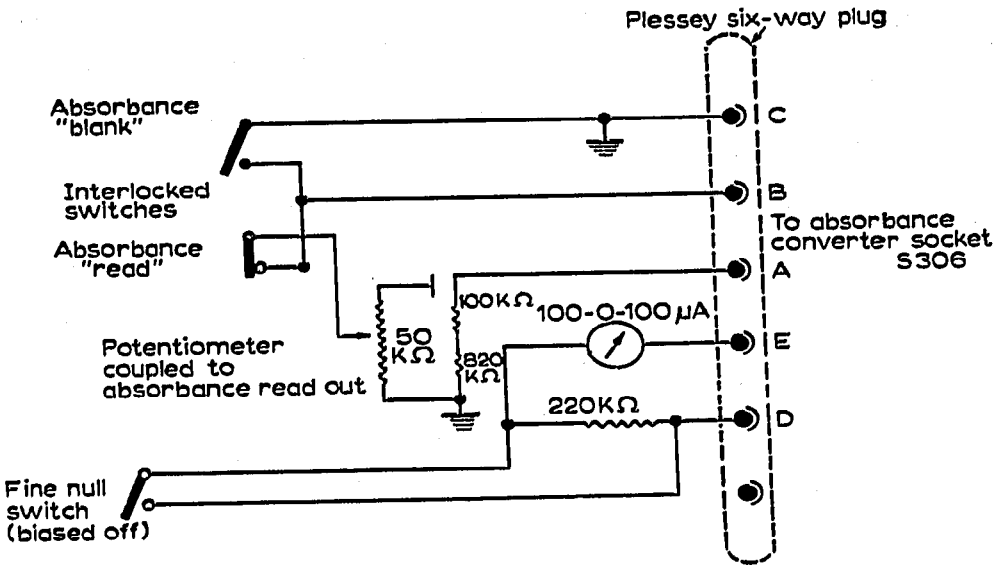

Fig. 5. Calibration circuit.

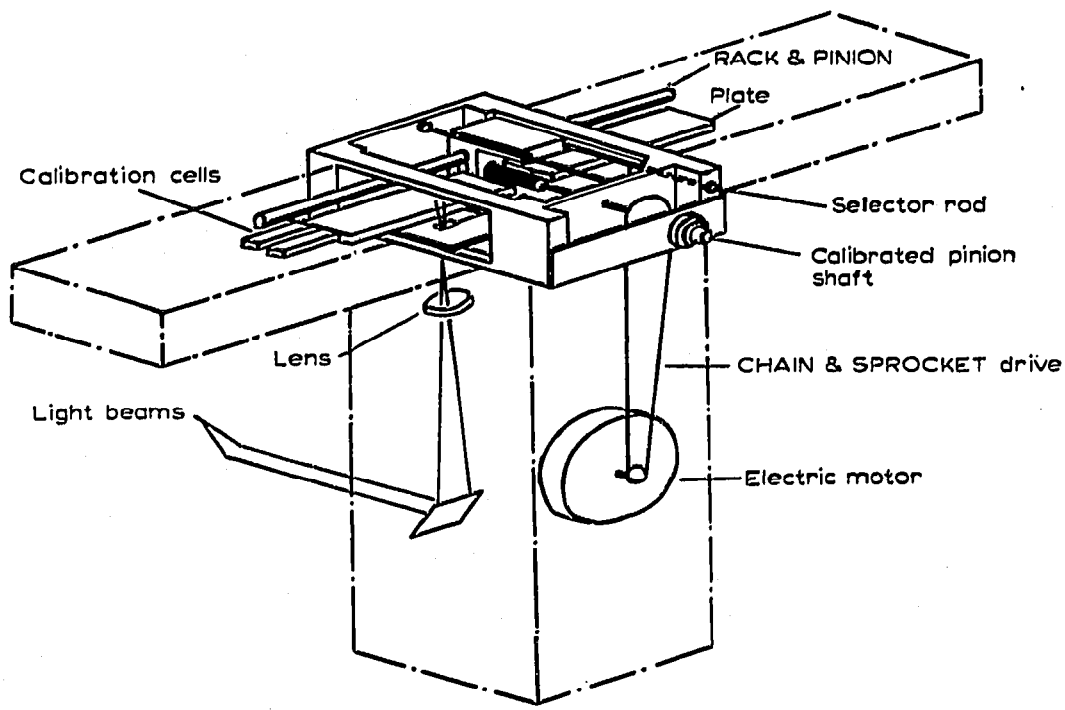

Fig. 6. Thin layer chromatographic plate holder.

was indicated by a constant reading from the recorder. This procedure usually took about 10 min. The apparatus was then calibrated by means of the two cells containing standard solutions, held in the same rack as the TLC plate. Finally, the absorbance of the four sample tracks on the plate was measured.

RESULTS

Fig. 7 shows a typical chromatogram together with the absorbance of the same track before a sample was applied to the plate.

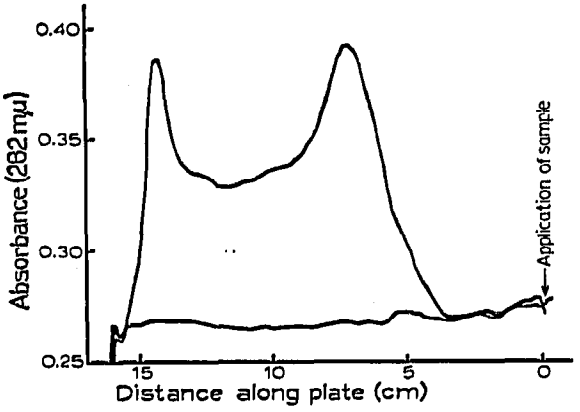

Fig. 7. Thin layer chromatography of lignosulphonate on G-50 Sephadex.

The absorbance of the gel itself varied along the plate by about ± 0.005 absorbance units. However, this pattern of variation was accurately reproduced when the plate was repeatedly run through the scanning apparatus. Furthermore, the patterns of variation found for blank plates were reflected in the corresponding sample traces. Fig. 7 shows some indication of this. Consequently it became the practice to subtract the blank trace from the sample trace and to retain this graphically corrected data. The remaining data given below is expressed in this form and Fig. 8 shows "normalized" chromatograms of a single sample solution obtained from three separate tracks on three separate plates.

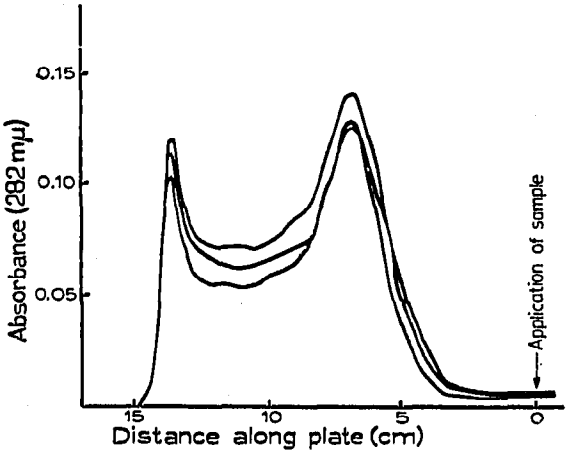

Fig. 8. Replicate chromatograms on three separate plates, using G-50 Sephadex.

The total areas under the curves vary substantially (± 10 % about the mean) due almost certainly to variation in the amount of sample applied to the plate. It was subsequently found that the 1 μl drops of sample were only precise to within ± 10 % (within 95 % confidence limits). But for any selected band width under any one of the separate curves, the proportional areas in fact vary by less than 5 %. Also the positions of the main peak to the right relative to the peak on the left, were consistent to one part in 100. Despite the precautions taken the distance of the extreme left-hand peak from the point of sample application on the right varied a little from plate to plate.

Therefore, the chromatograms in Fig. 8 were adjusted to the same elution length of 15 cm.

A similar practice is usually adopted in paper chromatography in order to normalise the variations of solvent migration on the paper. Following MORRIS's[8] example we took a further convention from paper chromatography in using R_F values which in this case were the ratios of distances travelled by polymeric species along the plate to the distance travelled by excluded (*i.e.* unfractionated) high molecular weight material. The distance travelled by this high molecular weight material is demonstrably equivalent to the distance which the buffer solution has travelled. This is because in GPC the largest molecules are, in effect, excluded by the gel and do not fractionate at all. It has been demonstrated by ANDERSON AND STODDART[9] that these R_F values are directly related to the usual GPC parameters.

It is a good general principle in chromatography to use samples which are small in quantity relative to the stationary phase; this is particularly true of hydrophobic GPC[10]. However, ANDREWS[4] found that concentration levels are less important with the hydrophilic Sephadex. He found no concentration effect for selected proteins in the range 0.01 to 0.5 %.

The TLC technique described here is limited to concentrations of lignosulphonate of 3 % or more because of the variation in absorbance (although small) of the thin gel layers themselves. Also, a further limitation comes from the diameter of the spot of sample applied to the plate, which is as much as 4 mm from 1 μl of solution. Despite this it was found that three solutions containing respectively 3, 6 and 9 % of lignosulphonate were eluted on one TLC plate with no substantial differences in the chromatograms when the areas under each were scaled in the ratios 1:2:3 (see Fig. 9).

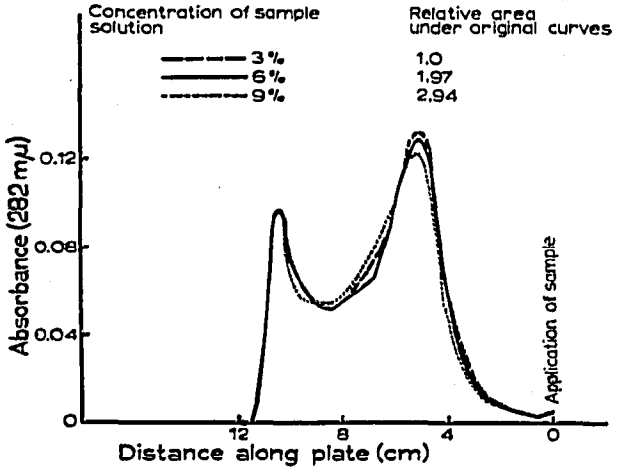

Fig. 9. Effect of concentrating, using G-50 Sephadex.

It is true that a different distribution may become apparent at concentrations lower than 3 % but these are beyond the capacity of the present method.

In order to obtain a full description of the molecular size distribution of a polydisperse polymer, it was a prerequisite that samples should be adequately fractionated. Lignosulphonates have molecular weights ranging from a few hundred to several million. Unfortunately, a single gel substance is not a satisfactory solution to this problem[6] and it was necessary to use four different Sephadex gels in order to obtain

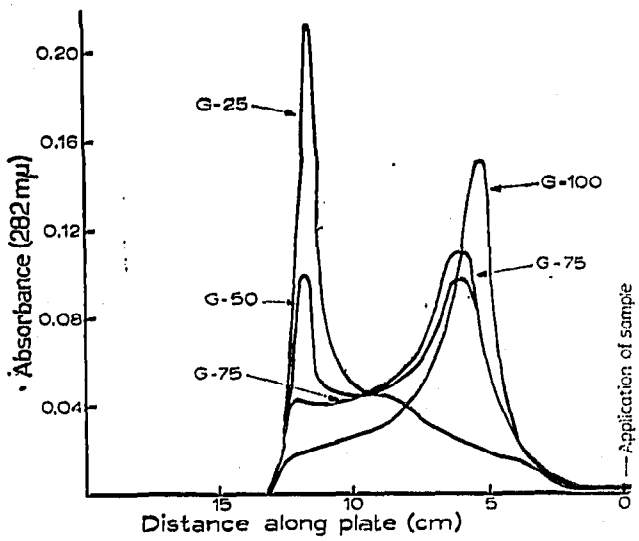

Fig. 10. Chromatograms on four Sephadex gels.

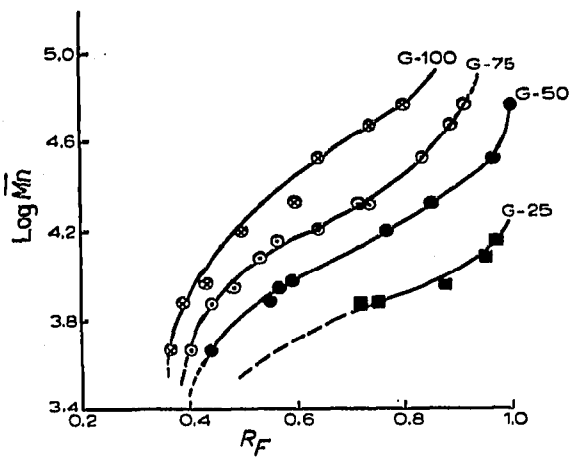

Fig. 11. Thin layer chromatographic calibration curves.

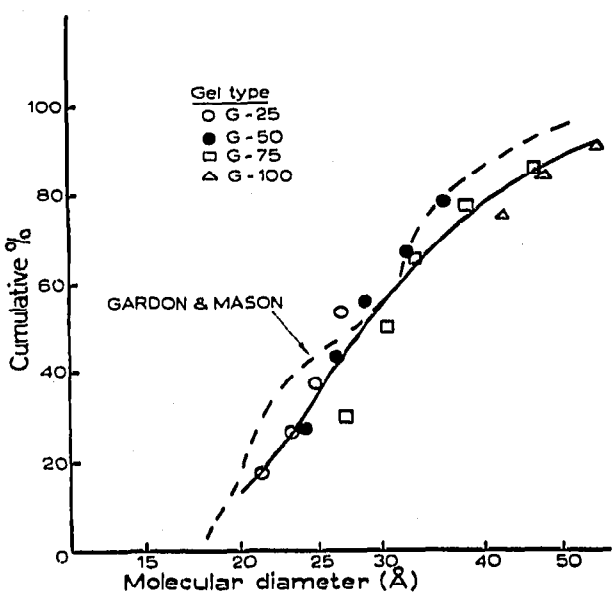

Fig. 12. Integral molecular size distribution of a lignosulphonate.

good coverage of a full range of molecular weights. Fig. 10 shows the results obtained from these gels.

Thin layers prepared from these various gels were calibrated by means of narrowly fractionated lignosulphonate samples. The calibration curves for these samples are shown in Fig. 11.

The narrowly fractionated materials were prepared by column chromatography of the polydisperse material on columns of Sephadex. They were shown to be narrow fractions by comparison of their weight and number average molecular weights. The ratio Mw/Mn was between 1.0 and 1.10. Hydrodynamic diameters of the lignosulphonates of narrow molecular weight distribution were calculated from their average molecular weights and partial specific volumes. A full description of the preparation and characterisation of these calibrants will be given elsewhere.

From the data given in the preceding figures it was possible to calculate a molecular size distribution of a total sample. Fig. 12 shows this. Certain corrections had to be made for the variation in the optical extinction co-efficients of the various lignosulphonate fractions: it was found that the co-efficients increased with decreasing molecular weight. These variations will also be dealt with elsewhere.

DISCUSSION

The data presented in this paper refer to lignosulphonates which are polydisperse polymers. It has been shown that a useful description of the molecular size distribution may be obtained rapidly by TLC using the gel permeation processes. The results obtained are similar to those published by GARDON AND MASON[11] who fractionated their samples either by precipitation methods or by dialysis. Their molecular weights were determined by a variety of classical procedures, such as osmometry, light scattering and ultra centrifugation. The agreement is satisfactory bearing in mind that samples used in this work were common to those described previously, only in the respect that they were obtained by sulphonation of spruce wood.

Having said this, it is worth remarking some detailed features of the distribution curve given in Fig. 11. There is an absence of data at the low and high molecular size extremities. This is because of difficulties which were encountered in preparing suitable calibrants from our chosen source of lignosulphonates.

It is also noticeable that the data diverges from the mean at the extremities of the fractionation ranges of the four gels. For example, the data obtained on G-25 layers diverges for molecules with diameters of greater than about 25 Å and the G-100 data diverges for molecules with diameters of less than about 35 Å. We have no satisfactory explanation of these divergencies but believe they are real. It could be, despite the evidence presented above on concentration effects, that concentration does play some part; one of which is most strongly marked at the extremities of a particular gel's fractionation range.

It would appear that the TLC method described above is not generally applicable to other polymer systems because it is limited by two factors: light absorbance of the gel substrate—even the cross-linked dextrans showed very little transmittance below 250 mμ and the hydrophobic gels such as cross-linked polystyrene absorb much more strongly in the ultra violet. Secondly, very few polymers have strong characteristic absorption bands in the visible or ultraviolet frequencies.

However, it is possible that quantitative staining or radio tracer techniques may be adapted to suit the method which has been described here.

ACKNOWLEDGEMENT

We should like to acknowledge the help given to us by Mr. H. GORDON of our Company, who manufactured several special pieces of the instrumentation.

SUMMARY

Thin layer chromatography has been previously applied to the separation and sizing of globular proteins. This paper describes an extension of the method to give rapid determinations of the molecular size distribution of lignosulphonates. These substances are water soluble polydisperse polymers which are well fractionated on columns of the hydrophilic gel substance "Sephadex".

Thin optically uniform layers of Sephadex were prepared on silica plates. Accurate location of samples on the plates yielded chromatograms which were measured spectrophotometrically by transmission at 282 mμ. The chromatograms were reproducible in all respects, to within ± 5 %. Although relatively high concentrations of polymer solutions were required for measurement (6–9 % of lignosulphonate) it is not thought that chromatograms were in error due to concentration effects.

The use of four different Sephadex gels: G-25, G-50, G-75 and G-100, together with a number of narrowly fractionated lignosulphonates of known molecular size, led to a complete molecular size distribution of the polydisperse samples.

Up to 32 chromatograms have been obtained in one day. The specialised equipment necessary to this method is not costly, but a good degree of manipulative skill is required.

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