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## NEW SPREADER FOR THE PREPARATION OF GRADIENT LAYERS IN THIN-LAYER CHROMATOGRAPHY

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### SUMMARY

The preparation of gradient layers with reproducible separating properties suffers from various difficulties, so the Desaga gradient spreader, which has been known since 1964, has been redesigned. The new spreader allows the suspension to be passed directly through a special dividing wall into the cylindrical trough where mixing takes place. The layers thus prepared are highly sensitive and yield reproducible results. If chromatography is carried out transverse to the gradient (T-gradient), sensitive and substance-specific curves are obtained; chromatography parallel to the gradient improves separations to give selective focusing.

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### INTRODUCTION

The advantages of gradient techniques in liquid chromatography have been known for many years. One can distinguish between two possible procedures: one method is the use of a gradient in the mobile phase, as proposed in 1949 by Tiselius<sup>1</sup>, and known as gradient elution. Optimal results are achieved in the separation of mixtures with a wide range of polarities and of substances that have non-linear adsorption isotherms. In thin-layer chromatography (TLC), gradient elution was used nearly 20 years ago, e.g., by Wieland and Determann<sup>2</sup> and Rybicka<sup>3</sup>.

In this paper, we are interested in the second possibility, that is, of preparing a gradient in the stationary phase. In 1963, Berger *et al.*<sup>4</sup> prepared layers from strips of different adsorbents, thus obtaining a stepwise gradient technique with a discontinuous gradient. Nowadays, a simplified form of this technique is used in preparing prefabricated plates with a concentration zone by applying at the starting region a strip of an inactive sorbent and next to it the actual separating layer. The advantages of such layers are described in several manufacturers' publications<sup>5-7</sup>.

The preparation of a continuous gradient in the layer was made possible with the gradient spreader described by Stahl in 1964 (ref. 8). The gradient layers thus obtained yield three different gradients within the length of the run, with correspondingly differing properties (Fig. 1). One of the possibilities is development transverse to the gradient (T-gradient technique) (Fig. 1A). The substance is applied in a band and

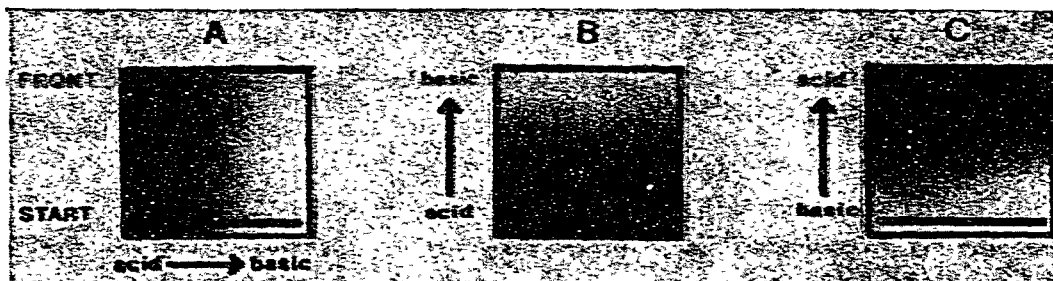


Fig. 1. The three different possibilities for the use of a gradient layer. The direction of the gradient is shown by the shading: grey = acidic, white = basic.

substance-specific curves are obtained. It is also possible to chromatograph in two directions parallel to the gradient, as carried out hitherto in column chromatography (Fig. 1B and C). A single solvent and a single layer thus bring about separations of substances with widely differing polarities if chromatography of a mixture is carried out parallel to the gradient. This technique is very suitable for screening tests. Chromatography parallel to the gradient is also theoretically superior to gradient elution. As Snyder and Saunders<sup>9</sup> demonstrated in 1969, the effective number of separation steps is much higher for the gradient in the stationary phase than in gradient elution. The gradient layer thus not only has methodological advantages, but gives better separations on account of the greater number of separation steps that can be expected, given simple and, above all, reproducible preparation of the gradient layers. Intangible factors and a lack of skill in the preparation of pH-gradient layers in particular have often resulted in non-uniform layers and unsatisfactory gradients. Leakages in the trough, which could not be controlled on account of the arrangement of the slide and diagonal separating wall, are a particular cause of these problems, which have been overcome with the development of the new spreader described here.

## EXPERIMENTAL

### *Apparatus*

The former gradient spreader (Fig. 2) consisted of two main parts: the basic device with mixing shaft, and the divider trough, containing the diagonal separating

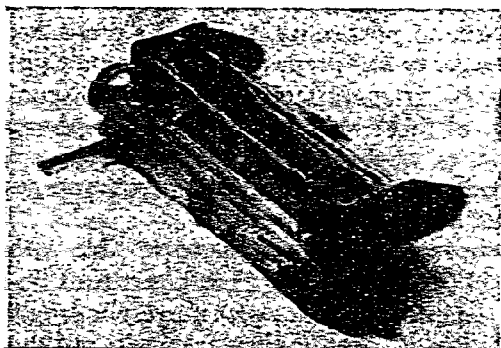


Fig. 2. Former gradient spreader with built-in mixing shaft and mounted divider trough.

wall which was positioned on the cylindrical trough. To prevent failures with this device, we tried to develop a new spreader without a separate trough in order to eliminate leakages, and some problems had to be solved first.

In the new spreader (Model 85; Desaga, Heidelberg, G.F.R.) (Fig. 3), the cylindrical trough also serves as a divider trough. A specially formed separating wall fitted to the rounded bottom of the cylindrical base is placed diagonally in the trough to separate the suspensions. Consequently, the "split" spreader can be filled directly with both suspensions, and the separating wall pulled up and replaced with a special mixing shaft, which is inserted by means of a plastic holder. A magnetic coupling at one end of the shaft connects it firmly with the separate electric motor drive. Normally, 15 sec at 470 rpm is a sufficient mixing time. Each revolution is accompanied by a small lifting motion of 2 mm. After mixing, the cylindrical trough and the shaft within it are switched to the emptying position to allow the suspension to flow through the slit of the spreader. The suspension normally covers five  $20 \times 20$  cm plates on the levelling table.

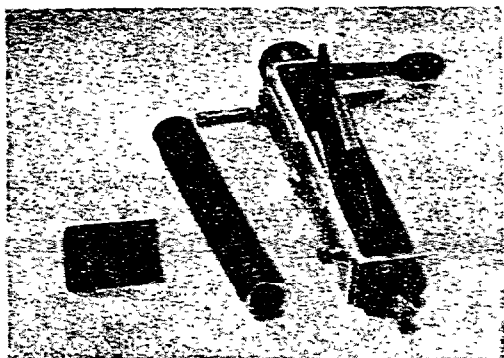


Fig. 3. New Desaga Model 85 gradient spreader with diagonal separating wall. Centre, the mixing shaft which is placed in the cylindrical trough after the diagonal separating wall has been removed; left, the special holder.

#### *Preparation of pH-gradient layers*

A 23-g amount of silica gel GF<sub>254</sub>, Type 60 (Merck, Darmstadt, G.F.R.) is weighed into each of two 100-ml erlenmeyer flasks. To one is added 50 ml of 0.5 *N* sodium hydroxide solution and to the other 50 ml of 0.5 *N* sulphuric acid from burettes. The flasks are stoppered and shaken vigorously for 10 sec. Each suspension is then placed in the corresponding chamber of the dividing trough and the surfaces are carefully levelled with a spatula. The separating wall is then pulled up and the mixing shaft placed in the cylindrical trough with the help of the plastic holder. With the motor drive mixing is carried out for 15 sec in one direction only. The lever of the spreader is then turned 90° upwards and the suspension is spread uniformly on to the five plates. The layers are left on the levelled table until the watery shine has disappeared; they are then dried for 15 min in a current of warm air and heated for 30 min at 110°.

*Test solutions*

A 0.1% solution of dimethyl yellow, methyl red and phenol red in methanol and a 0.1% solution of methyl red in methanol are used.

*Application of the test solution*

A 0.04-ml volume of test solution is applied as a 17-cm narrow band transverse to the gradient with a Desaga Autoliner 75 and then developed.

*Standardization of a definite activity*

After applying the solution, the layers are placed in a chamber with a relative humidity of 30% established by the use of a sulphuric acid solution. The transfer into the developing chamber is carried out quickly after 45 min to prevent "reacclimatization".

*Development*

Chloroform-methanol (85:15) is used as the solvent with chamber saturation and a 10-cm length of run. The dyes are identified from their natural colors; detection of the fluorescence chromatogram (Fig. 5) (not described here) is carried out under longwave UV light (365 nm) with a high-pressure lamp (Desaga HP-UVIS).

## RESULTS AND DISCUSSION

The quality of the gradient layers prepared with the former spreader was determined mainly by the leakproof nature of the divider trough and by the uniformity of descent of the suspension from the dividing trough into the actual mixing chamber.

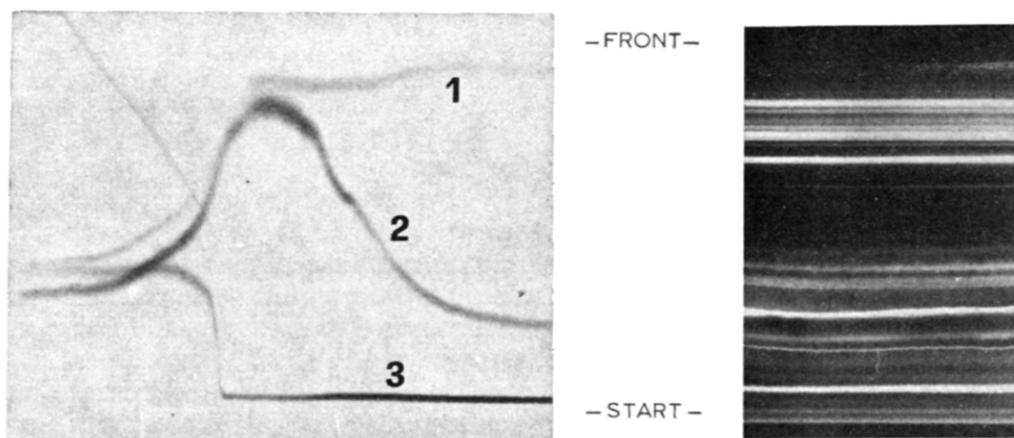


Fig. 4. A pH-gradient layer prepared with the new spreader. 1 = Dimethyl yellow; 2 = methyl red; 3 = phenol red. Characteristic curves for basic (1), amphoteric (2) and acidic (3) substances. Layer: acidic (left), basic (right).

Fig. 5. Chromatogram of fluorescent dyes with contaminants. Direction of the gradient: from acidic (below) to basic (above). More than 20 zones can be seen in a separation run of 18 cm. On a uniform neutral layer only 7 substances can be discerned.

Coherence of the material and adherence to the walls of the trough cause the suspension in the middle of the trough to pass faster into the mixing chamber. The suspension in the corners tends to be drawn towards the middle, which distorts the diagonal gradient, an effect which depends on the opening speed of the bottom of the dividing trough and on the viscosity of the suspension. One could obtain fairly good gradient layers with the former apparatus only when these parameters were constant. We overcame these difficulties by eliminating the separate dividing trough. This resulted in improved and, above all, reproducible gradient layers. We tested it with a mixture of pH indicator dyes, such as dimethyl yellow, methyl red and phenol red (Fig. 4).

The reproducibility of the gradient was determined by the position of the two inflections of the curve for methyl red. Five batches each of five  $20 \times 20$  cm plates were prepared and the same amounts of methyl red applied. Chromatography and evaluation took place under the same conditions. The former gradient spreader had a standard deviation of 2.5 cm, which was reduced to 0.5 cm with the new model.

We chromatographed fluorescent dyes in the gradient direction, going from acid to basic, and obtained very well formed zones, especially those of contaminants, in comparison with neutral layers. This can be considered as a focusing effect in the corresponding pH-gradient region (Fig. 5). A detailed report concerning this and other effects will be given in a subsequent paper.

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