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CONTINUOUS RECORDING OF DENSITY VARIATIONS IN FLOWING LIQUIDS

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SUMMARY

An apparatus for continuous recording of density variations in streaming liquids is described. The apparatus is based on Archimedes' principle and adapted for flowing liquids. The liquid is pumped through a glass measuring cell, into which a glass plumb is suspended from the arm of a sensitive automatic electrobalance. A recorder connected to the balance provides a continuous registration of the buoyancy of the plumb. Substances can easily be detected with this apparatus in quantities corresponding to changes in the specific gravity of 10^{-5} g/ml in the chosen eluting liquid. The apparatus has been used as a detector in gel chromatography.

INTRODUCTION

In the course of exploratory investigations of chromatography on Sephadex LH-20 in organic solvents we encountered the need for a general method of continuous analysis of the effluent solution: a method that would be applicable to any solvent and sample substance. It occurred to us that a method based on Archimedes' law should have the desired general applicability. Therefore, we decided to construct a sensitive flow densitometer for the continuous recording of density variations in the effluent from chromatographic columns. The design, performance, and some practical applications of such an instrument are described below.

EXPERIMENTAL

Principle

The column effluent is guided through a measuring cell containing a plumb that is suspended from the beam of a sensitive microbalance by means of a very thin wire. A recorder connected to the balance provides continuous registration of the *effective* weight of the plumb, and thus indicates changes in the specific gravity of the liquid flowing through the cell.

Although the principle is easily stated, it is obvious that such an arrangement will respond readily to forces other than simple buoyant uplift, and therefore cannot be expected to provide a smooth record of the very small density variations involved

in chromatographic work unless mechanical disturbances of the plumb and its suspension can be almost completely prevented. Variations in the rate of flow through the measuring cell or in the pattern of flow within it cannot be tolerated. The establishment of temperature gradients that would initiate convective flow must thus be avoided, and the apparatus must be completely isolated from mechanical vibrations.

Apparatus

Fig. 1 is a schematic drawing of the experimental arrangement used for the application of the above principle to the analysis of effluents from Sephadex columns. The eluent is stored in an insulated container equipped with a thermometer that is calibrated to 0.02°. A capillary tube inserted through the stopper serves as a vent for the intake of air as liquid is drawn from the reservoir. The eluent is pumped from the reservoir into the bottom of the column via a well-insulated Teflon capillary tube (I.D., 0.8 mm). Since even minute fluctuations in the rate of liquid input are transmitted through the entire system and disturb the balance of the plumb in the measuring cell, a very steady input rate is an essential requirement for a smooth baseline. If a pump is used its output must be almost perfectly flat. The Perpex peristaltic pump (LKB 10200 Perpex pump, LKB-Produkter AB, Stockholm, Sweden) has given satisfactory results at the sensitivity levels required for the studies described herein. The silicone tubing used in the pump is resistant to most organic solvents commonly used for chromatography.

The chromatographic column used (4.5 × 51 cm) was similar in design to that described by PORATH AND BENNICH[1], except that the chromatographic tube was made of glass and the end pieces and porous membranes were of Teflon.

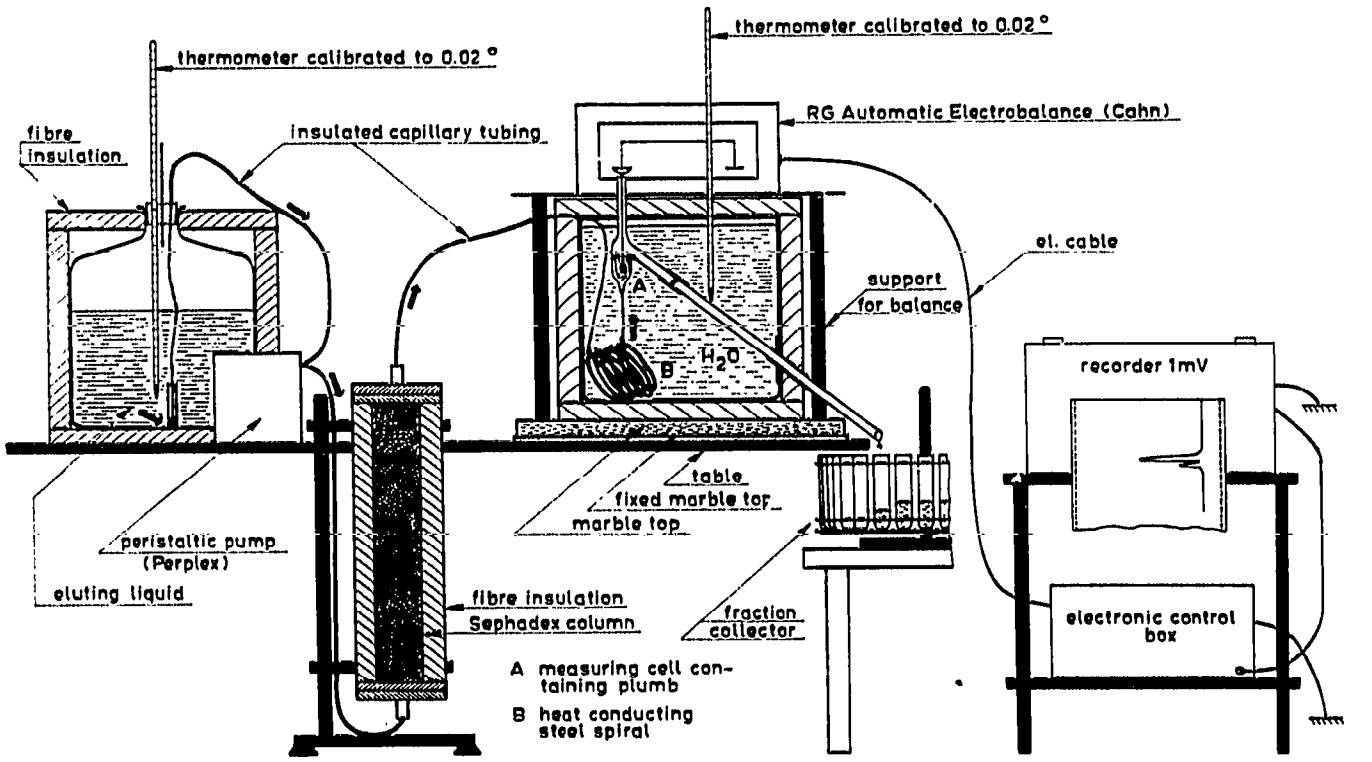

Fig. 1. Schematic drawing of the arrangement for the continuous recording of density alterations in the eluate from a chromatographic column.

The effluent from the insulated chromatographic column, packed with the appropriate adsorbant (*e.g.*, Sephadex LH-20, Pharmacia Fine Chemicals AB, Uppsala, Sweden), passes through a length of insulated Teflon capillary tubing into a spiral of steel capillary tubing (length, 2 m; I.D., 0.8 mm) that is immersed in a water bath. The Teflon capillary is butt-joined to the steel spiral by means of a short length of Viton tubing. The exit end of the steel spiral is connected to the glass capillary inlet of the measuring cell in the same fashion. This arrangement provides adequate temperature equilibration at moderate flow rates and a reasonably constant ambient temperature. It should be mentioned here that the water bath is not equipped with a temperature regulator, but merely serves as a ballast to level out ambient temperature variations.

Cells and plumbs of several different sizes and shapes have been tested. A vertical, open cell with a sidearm type overflow as shown in Fig. 2 has given satisfactory results with alcohol and water. The overflow sidearm is sufficiently wide (7.5 mm) to prevent siphoning and maintains the liquid surface at a constant level virtually independent of the surface tension. The measuring cell has a volume of 2.45 ml and is fixed to the wall of the water bath by means of the draining tube, the orifice of which is outside the water bath and is positioned above a fraction collector. The neck of the measuring cell passes through the lid of the water bath and is connected to the bottom plate of a Cahn Electrobalance (RG automatic electrobalance, Cahn Inst. Company, 15505 Minnesota Avenue, Paramount, Calif., U.S.A.).

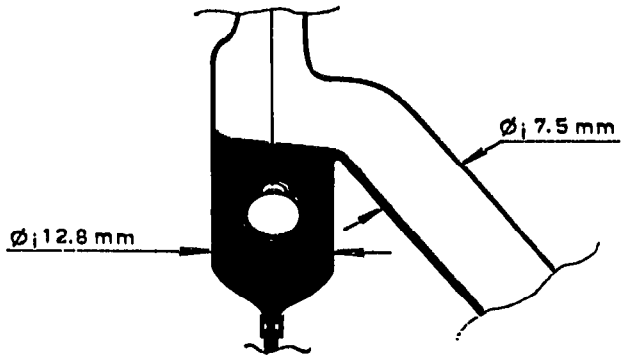

Fig. 2. The measuring cell with the suspended plumb and the draining tube.

The plumb is a solid glass sphere with a diameter of 5 mm and a weight of 0.3934 g and is provided with tiny handle. By means of a fine tungsten wire (0.02 mm diameter) the plumb is freely suspended from the beam of the microbalance. The wire is protected from draughts by the glass neck of the measuring cell. The electrobalance is held in position over the water bath by a rigid mount that rests on a vibration-free heavy marble base, as is shown in Fig. 1.

The electrobalance used is a null instrument. Changes in the force applied to the beam alter the amount of light striking a phototube. The photoelectric current is then amplified and is directed through a coil that is attached to the beam and which is surrounded by a magnetic field. The current flow gives rise to an electromagnetic restoring force. The correction is so swift that no movement of the beam is visually perceptible. The change in electromagnetic force is thus equal to the change in weight on the beam.

An earthed recorder (Hitachi, model OPD_{53}, Hitachi, Ltd., Tokyo, Japan) is connected to the electronic control unit of the balance (a 350 μF capacitor is connected across the recorder terminals to filter out rapid electrical noise). When the 1 mV channel is used, a full scale recorder deflection corresponds to a weight change of 0.2 mg. In all experiments described here we have used a paper speed of 2 cm/h.

The sensitivity of the balance and the recorder can be increased an additional 10-fold, but the noise increases in the same proportion unless temperature variations in the room are correspondingly lowered. No difficulty is encountered in *reducing* the sensitivity to accommodate larger changes in density or weight.

The elimination of disturbances

Owing to its nature and high sensitivity, the analytical arrangement described above is inherently very sensitive to mechanical and electrical noise. Mechanical vibrations are avoided by mounting the balance on a very heavy support that is fixed directly to the wall of the building and is not in contact with any other furniture or instrument, except, of course, the water bath that lies under the balance.

Electrical disturbances have been minimized by careful earthing and by connecting a condenser in parallel with the recorder input leads.

Temperature gradients within or around the apparatus can seriously disturb both the mechanical and electronic elements of the system and must be entirely avoided. For example, thermal convection currents within the measuring cell will directly affect the balance of the plumb. Therefore, the entire liquid system has been thoroughly insulated so that the maximum range of temperature variation is one degree above or below the prevailing ambient temperature. This approach of thermal insulation has been much more effective than attempts to *regulate* the temperature. If one attempts to use a thermoregulated water bath without a circulating device one will only create disturbing temperature gradients; while, on the other hand, any effective mechanical circulating device will inevitably produce disturbing vibrations. By the same token, any attempt to thermostat the entire room will also lead to temperature gradients or air currents.

Sensitivity

The densitometer described herein permits the detection of any solute peak in which the solute concentration at the peak maximum is sufficient to change the density of the eluting solvent by more than about 0.00001 g/ml. For example, the change in the concentration of H_2O required to produce this minimum usable density change when the solvent is 87.6 % ethanol is 0.0001 g/ml.

The use of the densitometer for the detection of solute zones in effluents from chromatographic columns

Before the start of an experiment the insulated water bath and the insulated eluent reservoir are filled with water and the chosen eluting solvent, respectively, both of which have been allowed to adjust to the ambient temperature. The peristaltic pump is then started. When the measuring cell has filled to the overflow level, the balance is turned on. After 1 h, the drift in the recorder reading is measured as described in the instruction manual for the Cahn electrobalance. The instrument should be re-calibrated whenever the eluting solvent is changed.

Before a sample can be applied, eluent must be pumped through the system until temperature equilibrium and a steady-state pressure are attained in the column, which can take as long as 6–10 h. The recorder chart drive is then started and the baseline is set at the desired level. The sample is then applied carefully so as to avoid introducing any gas bubbles into the column. A further sample can be applied as soon as all of the solute zones from the previous run have emerged from the column. The system can thus be kept in continuous operation for several weeks or months.

Some illustrative experiments

A number of chromatographic experiments were performed on a Sephadex LH-20 column (4.5 × 50 cm) in 90 % ethanol. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals and the instructions given in their brochure were followed. The samples were dissolved in 10 ml of 90 % ethanol and the chromatograms were developed at a rate of 100 ml/h with the arrangements for measuring and recording shown in Fig. 1. The following samples were applied to the column: (1) a mixture of 50 mg phenylalanine methyl ester and 50 mg of *tert.*-butyloxy-carbonyl-valine (*t.*-Boc-valine); (2) 0.30 ml ethyl propionate; and (3) a mixture of 0.03 ml chloroform, 0.02 ml bromoform and 27 mg of iodoform. The chromatograms recorded are shown in Figs. 3–5.

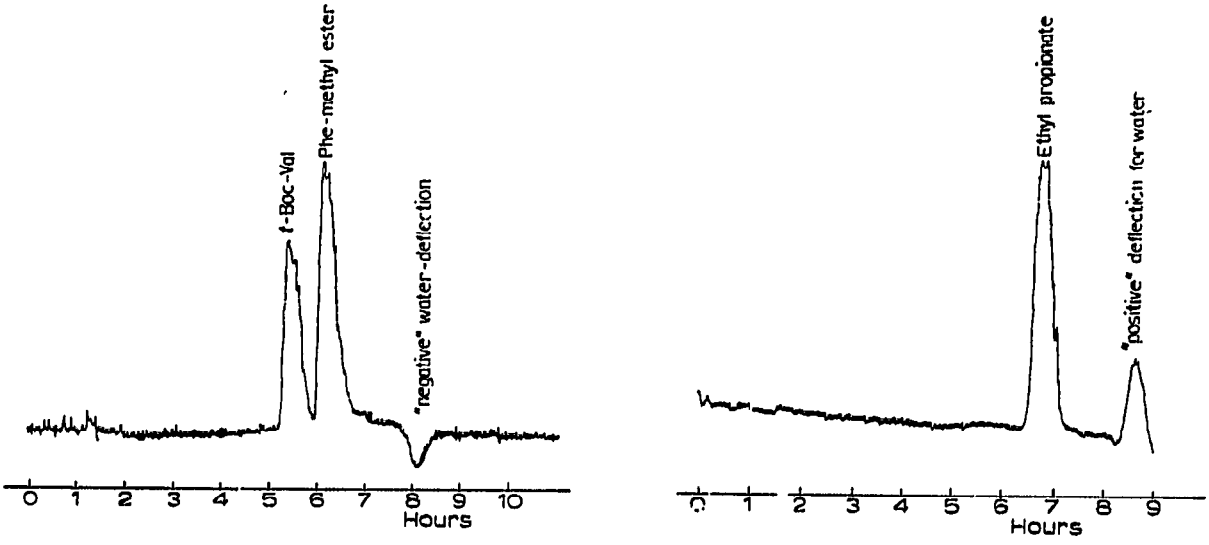

Fig. 3. Sephadex LH-20 chromatogram obtained for a mixture of *t.*-Boc-valine and phenylalanine methyl ester (50 mg of each). In this experiment, as in those referred to in Figs. 4–6, the elution was performed with 90% ethanol, at room temperature and the chart speed was 2 cm/h. For further details see the text. The separation was obtained by a combination of molecular sieving and adsorption effects. The "negative" water deflection is discussed in the text.

Fig. 4. Chromatogram of 0.15 ml of ethylpropionate on Sephadex LH-20 in 90% ethanol.

In order to test the effect of the amount of solute on the size and shape of the recorded curves some experiments were made with mixtures of *t.*-Boc-valine and phenylalanine in equal proportions by weight and in increasing quantities. In three experiments 25, 50 and 75 mg of each of the amino acid derivatives, respectively, were dissolved in 10 ml of 95 % ethanol. The chromatograms from the runs are shown

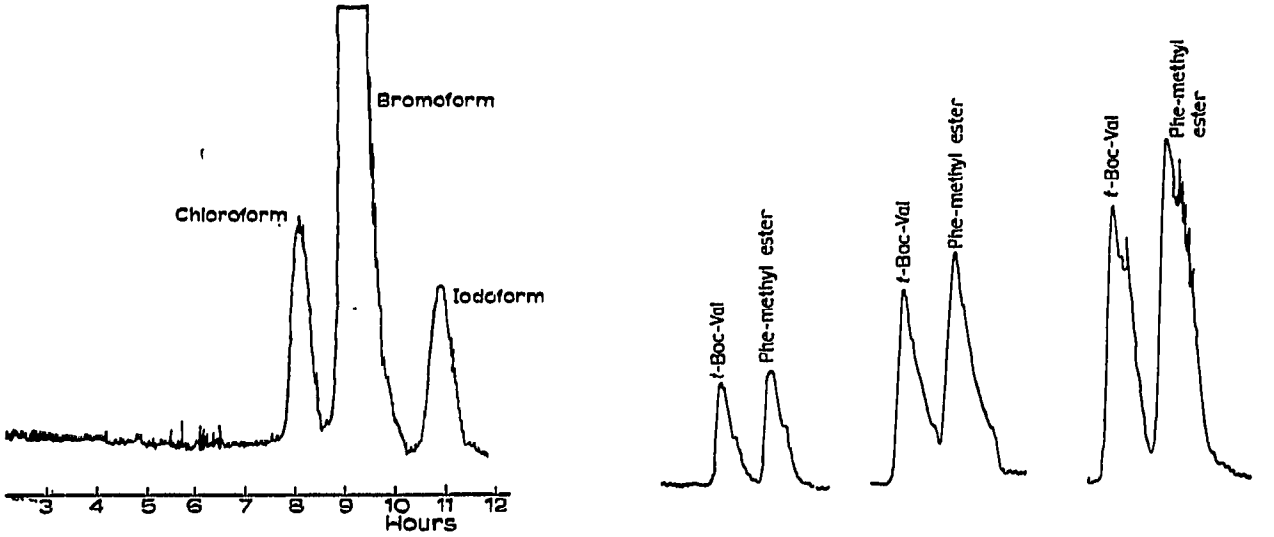

Fig. 5. Chromatogram of a mixture of chloroform, bromoform and iodoform obtained under the conditions described for Figs. 3 and 4.

Fig. 6. Three chromatographic experiments performed with increasing amounts of a mixture of *t.*-Boc-valine and the methyl ester of phenylalanine gave the patterns shown above. The amounts of each amino acid derivative were 25 mg (left), 50 mg (middle) and 75 mg (right).

in Fig. 6. The skew shape of the curves is presumably caused by a convective disturbance that occurs upon the passage of the rear part of the zone through the cell. This disturbance is usually not serious, as can be seen for the largest sample as well as from the curves in Figs. 3–5. The area under such curves can be used for quantitative evaluation of unknown samples provided calibration curves are available.

Pulses of samples made up from 87.6 % (w/w) ethanol to which had been added increasing quantities of water were introduced into the cell (column detached) and the deflections were measured.

In a series of such experiments 0.05, 0.10, 0.25 and 0.50 ml portions of water were added, each diluted to 100 ml with 87.6 % ethanol. The following reflections were recorded: 45, 75, 160 and 310 mm. The density of the injected samples was separately determined by a pyknometer. Since a deflection of 2.5 mm is well above the noise level, a density difference of 10^{-5} g/ml should be readily detectable.

DISCUSSION

Differential densitometry is more general in its application than any other non-destructive method which has been used for detection of solute zones in effluents from chromatographic columns. The method surpasses even differential refractometry in approaching universal applicability. Either of these methods can be especially valuable in situations where the light-absorbing properties of the solutes and solvent preclude the detection of solute zones by simple spectrophotometry.

The magnitude of the visible response (*i.e.*, the magnitude of the recorder pen deflection) of the densitometer unit to a given change in density is determined by such factors as the volume of the plumb, the relative densities of the plumb and the solvent medium, and the amount of electrical amplification applied. The sensitivity is thus

readily adjustable through several orders of magnitude. It is logical to define the *maximum usable sensitivity* in terms of the smallest density change which can be distinguished from baseline disturbances due to the various kinds of mechanical, electrical, and thermal noise to which the densitometer is very prone when operating at high sensitivity. We have succeeded in reducing the noise level to the extent that the *maximum usable sensitivity* of the instrument described herein is of the order of 0.00001 g/ml.

The response of the densitometer to a given change in solute concentration is directly proportional to the density variation that occurs; and if we assume, for the moment, that the partial specific volume, $\bar{v}$, of the solute is independent of concentration at the relatively low levels of solute concentration ($< 1\%$) normally involved in chromatographic work, the following relation obtains:

$$\text{Densitometer response } \Delta D = k\Delta c\,(1 - \bar{v}\rho) \qquad (1)$$

where ΔD is the change in density, Δc is the change in solute concentration, $\bar{v}$ is the partial specific volume of the solute in the solvent (*i.e.*, the eluting medium) used, ρ is the density of the medium and k a proportionality constant. Increases in the concentration of solutes for which the product $\bar{v}\rho < 1$ or > 1 will increase or decrease, respectively, the density of the solution. According to the above relation, changes in the concentration of solutes for which $\bar{v}\rho = 1$ will have no effect on the density. The very broad applicability of the densitometer as a detector in chromatographic work is due to the fact that the latter situation is rarely encountered in practice and can, at any rate, be overcome by modifying the composition (*i.e.*, the density, ρ) of the medium. In principle, at least, one can attain a high sensitivity towards any particular solute simply by choosing a medium for which the value of the product $\bar{v}\rho$ is sufficiently greater than or less than unity. By the same token, one should be able to increase selectively the sensitivity toward certain constituents (*e.g.*, the lighter ones) of a complex sample, while at the same time decreasing the sensitivity toward other less interesting ones. For example, if one has performed an exploratory run in a medium where the interesting constitutents of the sample give only very tiny negative peaks while a few minor, but heavier salt contaminants give large positive peaks, one could expect to obtain a more representative pattern by using a medium of higher density. Unfortunately, the choice of solvent is often completely dictated by the requirements for the chromatographic separation, solute stability, ease of solute recovery, etc., so the degree to which the sensitivity can be manipulated in this fashion is rather limited in practice.

Since the selectivity cannot be controlled at will, the inherent ability of the densitometer to respond to almost any kind of variation in the composition of a column effluent is not an unqualified blessing in practical work, for only in exceptional cases is one really interested in observing all the zones of varying composition that might be generated in the course of a chromatography experiment. Even in outwardly simple runs with "known" reference substances one might often observe several unexpected positive or negative peaks the origin of which can be distressingly difficult to identify. Certain such "extra" peaks might be due to the presence of "trivial contaminants" in the sample. For example, a high sensitivity densitometer record obtained in a chromatographic analysis of a "pure" organic substance might show several peaks due to the presence of small amounts of inorganic salts, solvent used for

crystallization, moisture, etc., which one would not ordinarily regard as serious impurities. However, if the $\bar{v}\rho$ values for these various minor impurities are relatively far from unity, while $\bar{v}\rho$ for the main solute is very close to unity, the impurity peaks might be comparable in size to the main solute peak. Additional analyses of the isolated zones might then be required in order to identify those corresponding to the solute of interest. Peaks arising from "foreign" substances present initially in the sample material itself should not, of course, reappear upon rechromatography of material recovered from the main solute zone.

Unwanted "extra" peaks are especially difficult to avoid in chromatography experiments performed with an eluent containing more than one component. In such cases, one can obtain one or more positive or negative recorder peaks corresponding to zones that vary in composition only with respect to the relative proportions of the ingredients of the eluting medium itself. Such "solvent" peaks can arise either directly, as a result of an imperfect match between the sample medium and the eluting medium; or indirectly within the chromatographic column, as a consequence of the ability of one or more of the sample solutes to alter selectively the affinity of one or more of the ingredients of the eluting medium for the adsorbant.

The magnitudes of the deflections caused by the sample substances themselves are determined by the parameters given in equation (1). The larger the density difference the higher the sensitivity of the method. Obviously when complicated unknown solutions are to be measured, it is advisable to introduce into the system additional detectors. They should be placed prior to the densitometer.

In its present form the densitometer is only suitable for large columns such as those commonly used for preparative chromatography. For very large columns, however, the cell must be enlarged, or better still, a split flow arrangement could be introduced. For analytical purposes, with smaller columns and cells and with higher requirements of accuracy, a more elaborate temperature control system might be necessary to decrease further the temperature variations in the cell. For our present requirements, however, the simple apparatus described here has given an entirely satisfactory performance.

Finally, it should be pointed out that continuous densitometry should find applications outside the scope of chromatography, for example, the monitoring of distillation processes in industry.

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REFERENCE

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