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COOLING PLATE FOR CELLULOSE THIN-LAYER ELECTROPHORESIS AND ITS APPLICATION TO AMINO ACID ANALYSIS

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SUMMARY

A stainless-steel water-cooled plate has been designed for the electrophoresis of small and intermediate sized molecules on plastic based cellulose thin layers. The upper cooling surface of the plate was formed by milling out five shallow grooves aligned to the direction of electrophoresis. Insulation of the plate was achieved by dipping it in a suspension of electrostatically charged plastic particles. Electrophoresis was carried out by soaking five thin-layer strips in the electrolyte (4%, v/v, formic acid), and laying them with the layer face down along the cooling surface so that the sample spot was sighted over the centre of a groove. Electrophoretic separation then took place along the uncooled zone covering the groove. Adjustment of the electrolyte concentration and the voltage made it possible under these conditions to separate 23 amino acids when developed by chromatography in the second dimension using 15 × 18 cm layers.

INTRODUCTION

Methods using electrochromatography for the separation of charged molecules of small and intermediate size on thin layers have been available for 20 years[1–4]. Although many of these procedures have been subsequently superseded by developments in high-performance liquid chromatography (HPLC), the thin-layer procedures still have an important role to play as an adjunct to HPLC, in studies involving rapid screening of large numbers of samples, and in the analysis of radioisotope tracer distribution in metabolites. One of the reasons why the application of electrophoresis to thin layers has not been used more frequently may be attributed to the fact that equipment specifically designed for the purpose is not generally available. This paper describes an attempt to rectify this deficiency by the development of a simple cooling system which fulfils the requirements of jointly controlling the temperature of the layers, and limiting the diffusion of the solutes. The plate is designed to take five 15 × 4 cm layers or two 15 × 18 cm layers at one time. The device has been applied mainly to the analysis of amino acids. This design is not suitable for application to gel electrophoresis.

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MATERIALS AND METHODS

Materials

Ninhydrin and the amino acids were obtained from Calbiochem (Australia). Tween 20 and the plastic-based cellulose thin-layers (20 × 20 cm) were obtained from Sigma (St. Louis, MO, U.S.A.). Whatman chromatography paper was obtained from W. & R. Balston, U.K. The cooling plate was insulated by Atractaseal (East Brisbane, Australia).

Preparation of the thin layers

The cellulose layers were placed one-by-one in a Pyrex dish and washed successively in 2 *M* acetic acid, and three times each in ethanol and water. The layers were allowed to dry at room temperature. Immediately after drying they were cut into 15 × 4 cm strips and wrapped in thin plastic for storage in the deep freeze. A store of layers ready for immediate use could thus be built-up.

Protein precipitation and determination

All samples containing protein were deproteinised. 12 ml of concentrated acetic acid was diluted to about 50 ml and 5 ml of Tween 20 was added. This solution was then titrated to pH 5.3 with concentrated ammonia. The solution was made-up to 100 ml to give a final approximate concentration of 2 *M* ammonium acetate.

A volume of 0.5 ml (in the case of a dried tissue sample or an acetone powder) or an equal volume (in the case of a physiological fluid) of the above reagent was added to the test sample and the mixture was heated in a boiling water bath for 3 min to bring about protein coagulation. On cooling the solution, two volumes of chloroform were added, and the mixture was emulsified on a vortex mixer to precipitate the remaining protein. The tubes were allowed to stand for 10 min and mixed a second time. The two phases were then separated by centrifugation for 5 min at 3000 rpm. The aqueous phase was removed with a Pasteur pipette, and retained for analysis.

The protein precipitate was digested in 0.1 *M* sodium hydroxide in sealed tubes overnight, and determined by the method of Lowry *et al.*[5].

Application of the samples to the layers

Samples were applied to a point which was 1.5 cm from each side of a selected corner of the layer with an Absoluter Micro-pipettor (Tri-Continental Scientific, U.S.A.) of 2–10-μl capacity. A volume of 2 μl of 2,4-dinitrophenyl (DNP)-lysine (1 m*M*) was first applied as a marker, and was followed by the test samples. All the applications were dried by blowing air at room temperature. Heating of the layers before electrophoresis was strictly avoided. The short side nearest the application point now becomes the anode end of the layer, and the long side nearest the application point becomes the bottom side of the layer.

On completion of the sample applications, all the spots were concentrated by dipping the top side of the layer in 0.01 *M* hydrochloric acid, allowing the fluid front to cross the DNP-lysine ring, and then drying the layer. A second concentrating run was usually required to move the DNP-lysine into a sharp bar about 8 mm from the bottom side of the layer.

Electrophoresis

The design of the apparatus. The design of the apparatus and the arrangements for electrophoresis are shown in Fig. 1.

The stainless-steel cooling plate (235 × 160 × 3 mm) was prepared by milling-out five grooves along the upper surface (140 × 7 × 1.5 mm, approximately) at 37 mm apart and parallel to the short sides of the plate (Fig. 1b and c). Shallow steps (235 × 15 × 0.05 mm, approximately) were then milled-out along the long sides of the plate. The remaining raised surfaces of the plate were ground flat with emery paper, and all corners were smoothed and rounded-off by filing so as to ensure good attachment of the insulating material.

A copper box section (200 × 130 × 11 mm) was then prepared with inlet and outlet pipes silver soldered into one of the short sides. This assembly was then soft soldered onto the under-side of the stainless-steel plate, thereby forming the water circulation compartment for the cooling plate. Cooling water was supplied from a cold tap with the flow-rate set fairly high.

The completed cooling plate was then electrically insulated by dipping it into a fluid containing a suspended electrostatically charged plastic powder. This process provided a hard thin layer of insulating plastic over the entire plate.

A waterproof marker pencil was then used to draw black lines (3 cm) in the middle of each groove at the anode end of the plate.

Preparation of the electrophoresis vessel. The wicks and connecting strips (Fig. 1) were washed three times in the electrolyte (4%, v/v, formic acid), in which they were allowed to soak until required. A volume of 100 ml of the electrolyte was added to each of the electrode compartments. The electrode partitions and the paper connecting strips were then positioned in the electrophoresis vessel. The connecting strips were blotted-off just prior to the addition of the thin layers.

Application of the electrolyte to the thin layers. The anode end of each thin layer was dipped into the electrolyte, and the fluid front was allowed to move as far as the middle of the DNP-lysine spot. The layer was then removed and excess fluid was blotted-off the dipped edge. The opposite end of the layer was then placed in the electrolyte, and the layer fully immersed until the fluid level was about 1 cm from the spot. The two electrolyte fronts were allowed to meet, and the layer was held in this position for about 30 s so as to ensure ample irrigation of the layer. On removal of the layer from the electrolyte, it was given a good shake to remove most of the excess fluid by knocking the plastic backing against the edge of the bench with the cathode end held down. Excess fluid on the cathode end was then blotted-off with tissue and the layers were placed on the cooling plate with the cellulose layer face down, and the DNP-lysine spots centred over the black lines in the grooves. The ends of the layers were pressed firmly against the connecting strips so as to establish good fluid contact with the connecting strips, and soak-off any remaining excess electrolyte on the layers. When all the layers were in position on the cooling plate, the wicks were then squeezed to remove excess fluid and placed in the electrode compartment. The layers were then covered with a piece of clear plastic, followed by a glass plate (225 × 160 × 2 mm) which was placed so that it rested on top of the two wicks. The plate was pressed firmly down so as to ensure a good fluid junction between the layers and the wicks. The apparatus was then closed, and the current turned on. It was usual to run the electrophoresis at 200 V for the first 7 min, and

then at 500 V for the remaining 23 min. With all five of the smaller strips in place on the cooling plate, 500 V gave rise to a constant current of 25 mA. The purpose of an initial run at 200 V is to stabilise the system before applying the full voltage. Occasionally, depending on the source of the sample under test, the DNP-lysine might be seen to form two spots. This characteristic does not normally affect the other amino acids, and is not necessarily an indication of trouble in the electrophoresis. For the final separation of the amino acids, they were subjected to chromatography in the other dimension. This was effected by 2 developments using 42 ml of butanol–0.4% (v/v) pyridine–acetic acid (22:10:10, v/v/v). Between the two runs 0.3 g ninhydrin was dissolved in an equal volume of the developing solvent and added to the chamber immediately prior to the second run. On completion of chromatography, the layers were exposed to cold air in the fume hood for 1 h by which time the ninhydrin colour was well developed. The strips were then placed in a drawer and allowed to complete

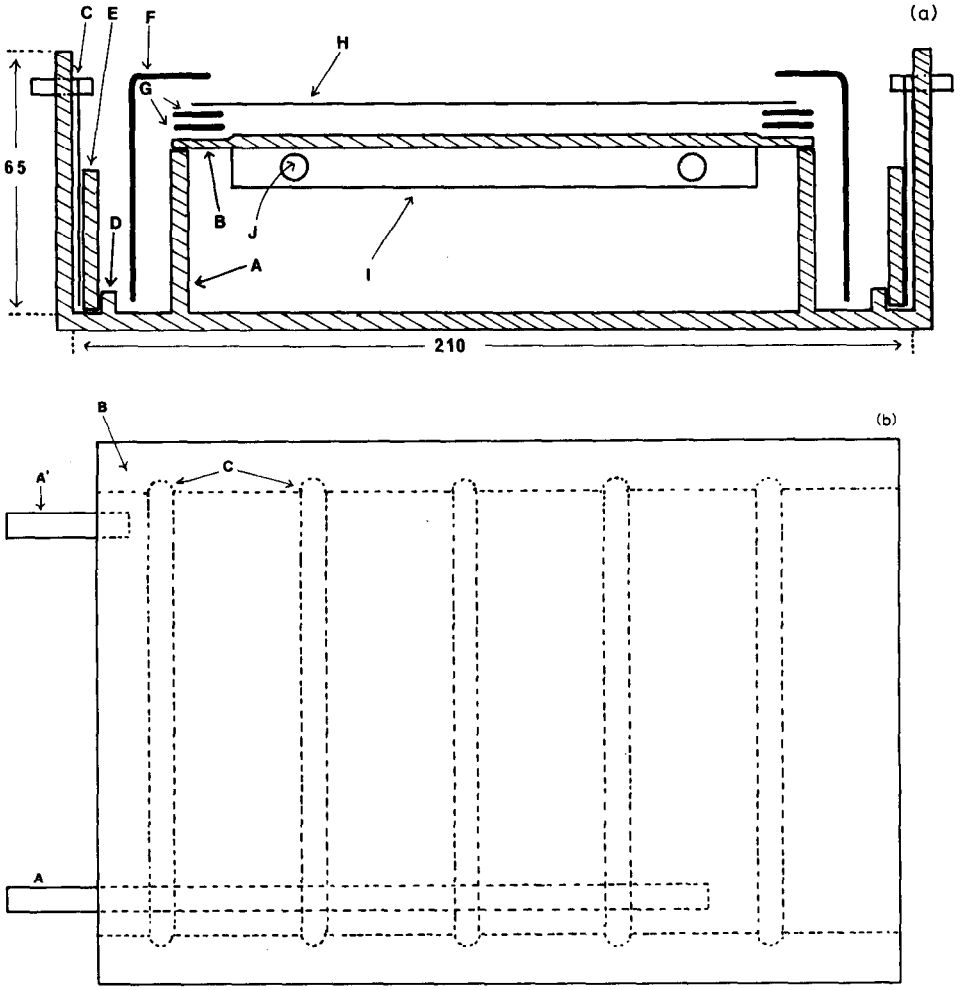

Fig. 1.

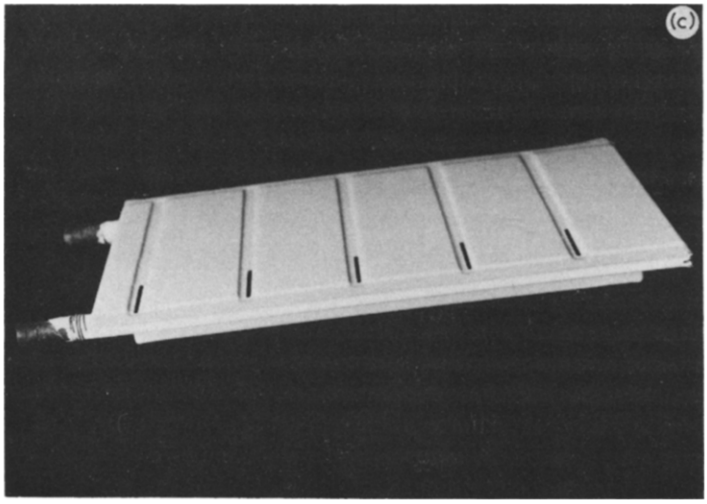

Fig. 1. The design of the electrophoresis vessel and its operation. (a) Cross-sectional side-view of the electrophoresis apparatus. The vessel was made of 4 mm perspex and has inner dimensions 245 × 210 × 65 mm. The electrode compartments at either end of the box are 245 × 25 mm, and the inner walls (A, 40 mm high) support either end of the cooling plate (B, stainless steel, 235 × 160 × 3 mm). The electrodes (C) are triangular pieces of stainless-steel sheet (140 mm at the bottom), which are attached by means of a centrally placed terminal to the top of the end-walls of the electrode compartments. Running along the bottom of each of the electrode compartments (7 mm from each end-wall) is a perspex rib (D, 5 mm high) which provides a retaining step for the electrode compartment partition (E), which consists of a perspex frame (243 × 35 mm) with an open mid-section, fitted into a piece of dialysis tubing (40 mm, flat width). The wicks (F) consist of a pair of 3MM Whatman strips (230 × 70 mm) inserted into dialysis tubing (75 mm flat width). Connection of the current from the wicks to the thin layers is effected at each end by a pair of 3MM Whatman strips (G, 230 × 15 mm). The thin layers (H, 15 × 4 mm) are placed face down on the cooling plate so that both ends overlap onto the connecting strips, and the wicks are then bent to overlap the thin layers. The outline of the copper box for the circulation of cooling water is shown (I), together with the water circulation ports (J). The apparatus is closed by a simple stepped lid. (b) The cooling plate viewed from above showing the inlet (A) and outlet (A′) ports, the slots for the linking paper strips (B), and the grooves (C). (c) Photograph of the cooling plate.

the colour development overnight. When large numbers of samples were being run it was usual not to add ninhydrin for the second chromatographic run, but to apply the ninhydrin on completion of chromatography by dipping the strips in a 0.3% (w/v) solution of ninhydrin in acetone containing 1% (v/v) pyridine. In this procedure the strips were usually placed directly into a drawer for overnight colour development.

The larger format of 15 × 18 cm was used only on those occasions when resolution of the two leucines was important.

The chromatograms can be preserved indefinitely by sealing in thin plastic film and placing in the deep freeze.

Quantitation of the amino acids. When this was required, the ninhydrin spots were removed from the chromatogram by rubbing off with a small steel spike centrally attached by means of a spring clip to the top of a test tube. With the thin layer held facing down the rubbings were quickly collected in the bottom of the test tube

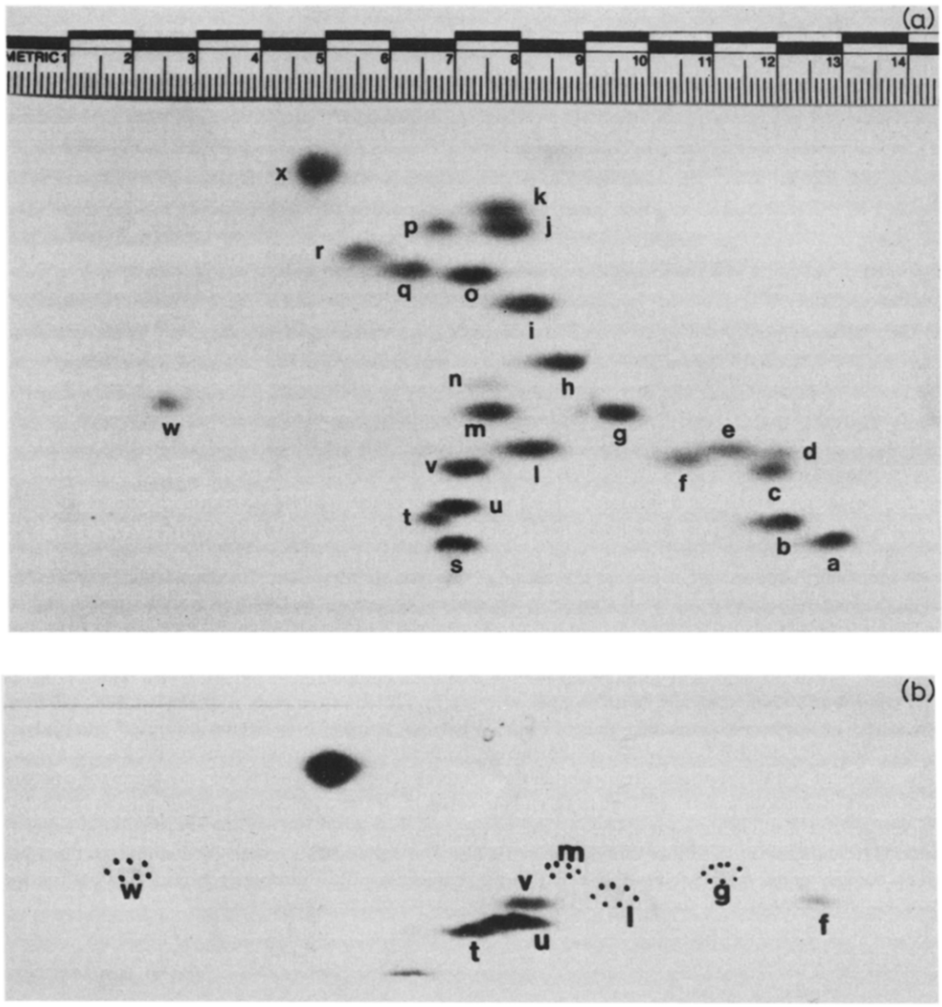

Fig. 2. Electrochromatograms of the amino acids. (a) 2 nmol of each of 23 amino acids were applied to a 15 × 18 cm layer. Letters in the figure refer to the following compounds: a = Lys; b = Arg; c = His; d = β-Ala; e = γ-Abu; f = Gly; g = Ala; h = α-Abu; i = Val; j = Ile; k = Leu; l = Ser; m = Thr; n = Pro; o = Met; p = Phe; q = Tyr; r = Try; s = Cys–Cys; t = Asp; u = Glu; v = Gln; w = Tau; x = DBP-Lys. (b) Separation of free amino acids present in an extract of HeLa cells. The HeLa cell acetone powder (*ca.* 1 mg protein) was extracted with 2 *M* ammonium acetate at pH 5.3, and 9 μl was applied to a 15 × 4 cm layer.

(5 s for each spot). The colour was taken-up in 1 ml butanol–ethanol–water (100:10:20, v/v/v) and measured by difference at 410 (peak) and 455 nm (isosbestic point) in a Cary 219 spectrophotometer. An internal standard consisting of 2 nmol norvaline or α-aminobutyric acid (α-abu) was included. The results were then expressed as norvaline or α-aminobutyric acid units, calculated as the ratio of the colour development of the amino acid to that of the internal standard. The range of measurement was 0.2–3 nmol.

RESULTS

Amino acids

Fig. 2a shows the resolution obtained for 23 amino acids, and Fig. 2b shows the separation of free amino acids present in an extract from HeLa cells. Fig. 3 shows the mean standard curve and standard deviation obtained for the ninhydrin colour reaction for eight of the amino acids on the one chromatogram. The standard colour values are expressed as α-aminobutyric acid units, and the standard deviations refer to the variance of the individual amino acids from the mean colour development for all. The mean coefficient of variation for all the amino acids tested at 2 nmol was 7%. Table I shows the comparison of results obtained for the concentrations of the major amino acids appearing in HeLa cells determined by the present procedure together with those obtained by two other groups of workers[6,7]. In other applications the system is being applied by other groups of investigators in this institution to the resolution of the amino acids and phosphorylated products resulting from photosynthesis in zooxanthellae[8], and the investigation of amino acid metabolism in Na^+-deficient plants[9].

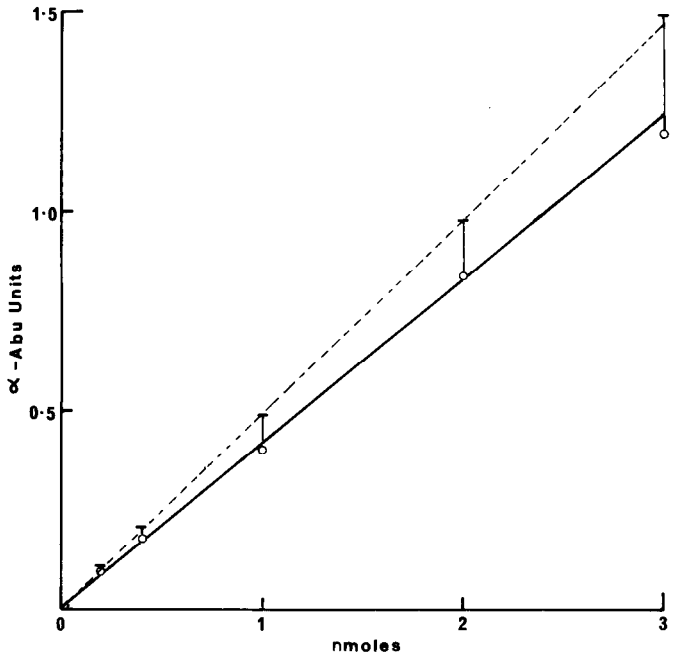

Fig. 3. Amino acid standard curve showing the development of ninhydrin colour. The figure shows the mean colour development and standard deviation for the following amino acids: Gly, Glu, His, Leu, Lys, Met, Phe, Tau. The colour development is expressed in α-Abu units, which was the internal standard in these experiments.

TABLE I

FREE AMINO ACIDS IN HeLa CELLS

	*Content ± S.D.** *(nmol/mg protein)*	*Concentration (mM)***	*Piez and Eagle*[6] *(mM)*	*Kabus and Koch*[7] *(mM)****
Asp	101 ± 12	15.0	1.3	16.0
Gln	80 ± 7	12.0	8.1	25.0
Glu	162 ± 12	24.0	10.8	24.0
Gly	37 ± 7	5.5	0.8	5.7
Tau	17 ± 2	2.5	14.3	na[§]
Others	10	–	–	–

* The monolayers on glass were exposed for 3 s to ice-cold isotonic sodium chloride and then fixed with acetone.

** The concentrations in cell water were estimated using the procedure of Piez and Eagle[6].

*** The concentrations in cell water were recalculated from the centrifugation into dibutylphthalate procedure of Kabus and Koch[7].

§ na = Not applicable.

DISCUSSION

Protein precipitation

The combination of heat coagulation and chloroform precipitation in the presence of a surface active agent was more efficient than any of the procedures using strong acids in that it completely eliminated trailing of the amino acids during electrophoresis, which was not the case when the proteins were precipitated by picric, phosphotungstic, trichloracetic or perchloric acids. This procedure has also the added advantage of avoiding the use of low pH values (of great importance in glutamine and asparagine determinations).

The layers

It is important to note that the layers prepared in Germany for Sigma were the only plastic-based thin layers which were found to be fully suitable for electrophoresis. Other layers, also from Germany, had a tendency to float off the plastic base when immersed in 2 *M* acetic acid. This problem can be solved by exposing the layers for 24 h to UV light after dipping in a 3% (v/v) solution of hydrogen peroxide in ethyl alcohol. This produced heavy oxidation in the layer, the products of which served to fix the layer to the plastic base. These layers were then thoroughly washed before use.

The apparatus

The design of the apparatus was dictated by three considerations.

(i) During electrophoresis on cellulose thin layers the separation of small and intermediate sized molecules suffers considerable loss of resolution if another surface (*e.g.* a cooling surface) besides that of the support surface is in contact with the layer. The reason for this is that the capacity of the layers to conduct high currents results in extensive inundation of the layers by the electrolyte, which in its turn gives rise to a second fluid phase (electrolyte only) forming between the cellulose matrix and the

other surface. Thus, solute molecules become distributed between the two phases, and smearing develops because those molecules in the electrolyte only phase migrate and diffuse much faster than the molecules in the cellulose matrix. In the procedure described above the solutes migrate through the cellulose matrix in the zone immediately above the groove along which excess electrolyte is continuously evaporated and condensed at the bottom of the groove. A second electrolyte phase therefore does not develop. Under these conditions smearing was eliminated, resulting in a considerable improvement in resolution compared to that obtained when the entire layer was exposed to a cooling surface.

(ii) Because the cellulose zones which lie over the grooves are not directly cooled, they run at a higher temperature than the surrounding areas of the layers. This gave rise to two effects: (1) the solutes separating along the uncooled zones migrated faster through the cellulose matrix; and (2) the evaporation of electrolyte from the uncooled zones and its condensation at the bottom of the grooves resulted in cooled electrolyte being drawn from the surrounding areas thereby squeezing the solutes in towards the middle of the uncooled zones. In this way lateral diffusion of the solute molecules was reversed to the extent that the longer the run, the greater was the degree of concentration of the solutes along the centre line of the uncooled zone. It was possible to obtain compact spots of uniform shape and size when equal amounts of the amino acids were applied to the chromatogram.

(iii) The final consideration in the development of the cooling plate was the search for a suitable coating which would insulate it electrically, whilst minimally affecting its capacity to transfer heat. The process described here made use of an electrostatic powder coating which provided theoretically an ideal solution to the problem. Two plates coated by this process have now been in operation in this laboratory for two years without any failure in the insulation. All the other plastic coatings tested failed because of the unavoidable presence of microscopic holes which provided charge conducting channels connecting with the metal surface. The continued passage of charge through these holes resulted in local heating which gradually melted away the plastic until the holes were large enough to fully short all charge to the cooling plate.

Quantitation of amino acids

The procedure described is useful in those circumstances where a suitably automated column procedure is not available, or when the procedure more easily resolves a particular mixture of amino acids. The advantage of the procedure over column procedures is that it will resolve most amino acid mixtures together with a large number of other metabolites which can be identified by their position if traced with a radioactive label. The use of dye elution as part of the procedure for the spectrophotometric determination of substances separated on chromatograms generally suffers from one major disadvantage which is that there is usually present large and variable amounts of impurities in the layers which absorb light across a wide band of wavelengths, and this is particularly true of cellulose. These impurities therefore contribute large and variable blanks to the determination depending on the position on the layer and the amount of layer eluted. This problem has been essentially overcome in the present procedure by the use of wavelength difference for the measurement of absorbance, with the chosen wavelengths being only 45 nm apart

and the 455 nm absorbance corresponding to the isosbestic point of the spectra for different concentrations of dye.

In conclusion the cooling plate has been designed specifically for cellulose thin layers, the matrix of which can support the rapid transfer of solute molecules, resulting in relatively high levels of current. For this reason the system is not suitable for polyacrylamide or agarose gels which tend to dry out along the uncooled zone[10]. The apparatus is reliable and flexible in operation and can routinely handle charges of up to 2000 V.

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