Journal of Chromatography, 271 (1983) 127–192
Chromatographic Reviews
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHREV. 167

APPLICATIONS OF TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

MONA ZAKARIA, MARIE-FRANCE GONNORD and GEORGES GUIOCHON*
Laboratoire de Chimie Analytique Physique, Ecole Polytechnique, Route de Saclay, 91128, Palaiseau Cedex (France)

(First received January 5th, 1983; revised manuscript received January 25th, 1983)

CONTENTS

1. Introduction . . . 128
2. Stationary phases and retention mechanisms . . . 129
   2.1. Inorganic adsorbents . . . 130
   2.2. Organic adsorbents . . . 131
      2.2.1. Polyamide . . . 131
      2.2.2. Cellulose . . . 132
   2.3. Reversed phases . . . 132
   2.4. Ion exchangers . . . 133
   2.5. Thin-layer gel chromatography phases . . . 133
   2.6. Stationary phases for two-dimensional thin-layer chromatography . . . 133
3. Detection . . . 134
4. Applications . . . 137
   4.1. Lipids . . . 137
      4.1.1. Fatty acids and their esters . . . 139
      4.1.2. Steroids . . . 139
      4.1.3. Phospholipids . . . 141
      4.1.4. Glycolipids . . . 144
   4.2. Pigments . . . 149
   4.3. Alkaloids . . . 153
   4.4. Proteins and their constituents . . . 160
      4.4.1. Amino acids . . . 160
      4.4.2. Dimethylaminonaphthalenesulphonyl (Dns) and dinitrophenyl (DNP) amino acids 163
      4.4.3. Peptides and oligopeptides . . . 165
      4.4.4. Proteins . . . 165
   4.5. Carbohydrates . . . 167
   4.6. Glycopeptides . . . 167
   4.7. Nucleic acids and their constituents . . . 169
      4.7.1. Bases, nucleosides and nucleotides . . . 169
      4.7.2. Oligonucleotides and nucleic acids . . . 172
   4.8. Environmental pollutants . . . 175
   4.9. Pesticides . . . 177
   4.10. Inorganic compounds . . . 180
   4.11. Miscellaneous . . . 181
5. Conclusion . . . 187
6. Summary . . . 188
References . . . 188

0378-4355/83/$19.80

1 INTRODUCTION

Bi-directional chromatography, which is a more specific term than two-dimensional chromatography when referring to flat paper or thin-layer beds[1], is an analytical technique that is becoming increasingly important in the separation of mixtures of varying complexity. This method consists in spotting a sample at the corner of a flat bed and allowing the migration of a solvent in one direction, followed by another migration at right-angles to the first.

When the same solvent is fed successively in the two directions of a uniform adsorbent layer, all chromatographed spots align on a diagonal. The sole advantage of the method is then a very slight increase in resolution corresponding to an increase, by a factor of $\sqrt{2}$, in the distance of migration of the spot The realization of more efficient systems, by means of which a larger number of spots can be resolved[2], implies the use of the entire chromatographic area available and requires the selection of appropriate different mobile phases for each direction.

Similar efficiencies are attained when the same mobile phase is fed successively, at an orthogonal angle, to a chromatographic plate that has been coated with two adsorbents. Different retention mechanisms dictate the behaviour of the solutes chromatographed for instance, on a reversed-phase layer in one direction and a normal-phase layer in the other. The solute R_F values are then different in each direction and the spots are dispersed on the chromatographic plate.

The tremendous separation potential afforded by this technique is apparent from the flexibility available in combining various stationary and mobile phases for use in either direction.

In 1944, Consden, Gordon and Martin[3] first reported a bi-dimensional separation of amino acids on paper. The eluents used were collidine–water in one direction and phenol–water in the second The chromatography took 5 days and resulted in the resolution of 15 of the 22 amino acids in the sample mixture. This technique was subsequently described by Overell[4], Cheftel *et al* [5] and Nordmann *et al.*[6,7], who successfully achieved the separation of various organic acids.

The importance of this method increased in the early 1950s as bi-directional chromatography became instrumental in the elucidation of several biochemical pathways[8]. Calvin[9,10], for instance, used two-dimensional paper chromatography in combination with autoradiography to assay algal extracts kept in air containing $^{14}CO_2$. After the identification of the various sample components, a kinetic study of ^{14}C incorporation helped to suggest a scheme for carbon reduction during photosynthesis. The same technique enabled Beloff-Chain *et al.*[11,12] to differentiate between the conversion of glucose into oligosaccharides, polysaccharides and lactate in muscles and that into amino acids and lactate in the brain. Chain *et al.*[13,14] also studied the effect of insulin on glucose metabolism in various types of animal tissue such as the diaphragm, liver, brown adipose tissue, brain and the pituitary.

Although two-dimensional thin-layer chromatography (2-D TLC) has been used for almost 40 years (Fig. 1), there has been no review of its various applications prior to this paper Several workers have reported past and present developments of classical TLC and its future trends[15–20]. The performance and spot capacity of two-dimensional TLC have been described elsewhere[21], and also recent developments in TLC instrumentation[22]

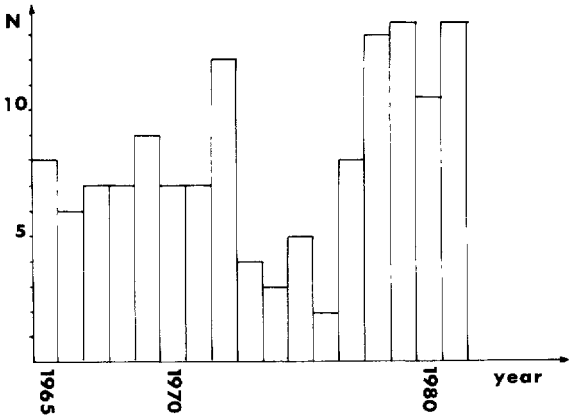


Fig 1 Histogram showing the number of original two-dimensional TLC publications in the last 18 years

This paper briefly reviews the different types of stationary phases available for chromatography, and the effective mechanisms of solute retention on each phase, prior to discussing the various applications of the 2-D technique. A short discussion on the methods available for spot detection is also included.

We have tried to cover the literature as thoroughly as possible. Authors who have not been cited are kindly asked not to take offence but rather to inform us of their work. More importantly, this review aims to demonstrate the efficiency of the method in resolving compounds of widely different nature, polarity or class, and the importance of the judicious selection of chromatographic conditions such that different mechanisms may govern solute retention in each direction

2 STATIONARY PHASES AND RETENTION MECHANISMS

Various powders are commercially available that may be slurried alone or admixed with agents for specific applications, and spread on glass, aluminium foil or plastic supports. Pre-coated TLC plates are also available without or with a binder for increased adhesion and mechanical stability of the layer, or an indicator for solute detection

The thickness of the layer is often 0.1 0 3 mm for analytical applications and 0.5 2.0 mm for preparative work. The particle size varies, a small diameter being less critical than a narrow size distribution in achieving high efficiencies and good resolution[2]· the narrow size range, 5 μm "HPTLC" packings have disadvantages such as a slow flow-rate of the mobile phase, a long development time and spot diffusion, whereas packings with the same size distribution and a larger diameter (10–20 μm) afford faster and still adequate separations[2 23], as is obvious from results obtained with good but conventional TLC plates made with particles *ca.* 11 μm in diameter[24].

The type of stationary phase needed to resolve a given mixture depends, to a large extent, on the properties of the sample components. In general, stationary phases may be inorganic sorbents (silica, alumina, magnesia), organic sorbents (cellulose, polyamide), ion exchangers (ionic moieties substituted on to an organic adsorbent) and reversed phases (non-polar, organic groups chemically bonded often to an in-

organic silica backbone). These thin layers may be impregnated with a specific reagent to enhance or create a particular type of solute–stationary phase interaction, or coated side-by-side on a support, a practice often used in bi-directional TLC.

2.1. Inorganic adsorbents

Inorganic oxides such as silica, alumina and magnesia are polar adsorbents which, according to their degree of activation, may be used to separate polar or moderately polar compounds. Procedures for adsorbent activation by heat or deactivation by addition of a moderator such as water have been standardized to ensure reproducibility of separations.

Heat activation rids TLC plates of physically adsorbed water, thus freeing the adsorptive surface for interactions with various solutes. Adsorptive sites in silica and magnesia are essentially hydroxyl groups. Thus, heating these two adsorbents to temperatures above 200°C for silica and 100°C for magnesia, a process whereby Si–OH and Mg–OH groups are converted into Si–O–Si and Mg–O–Mg, respectively, results in the loss of chromatographic activity[25]. In alumina, active adsorptive sites are thought to be aluminium atoms, strained Al–O bonds, basic oxide ions and cationic centres. The loss of most of the surface hydroxyl groups early in the heating process does not reduce the activity of alumina, which actually increases with increasing temperature up to about 800°C and is lost only at 1000°C[25].

In chromatographic systems that involve weak or moderately strong solvents, adsorption is described primarily by the following relationship[25]:

$$\log K = \log V_a + \alpha (S^0 - A_s \varepsilon^0) \quad (1)$$

where K, the distribution coefficient, is the ratio of the solute concentration in the adsorbed state to that in the mobile phase, V_a is the volume of a monolayer of solvent adsorbed on the surface, α, the activity of the adsorbent, is proportional to its average surface energy and related to its water content, S^0 is the adsorption energy of the solute in the reference solvent and on an adsorbent of standard activity ($\alpha = 1$), A_s is the molecular area required for the adsorption of a solute molecule on the adsorbent surface and ε^0 is the solvent strength parameter[25].

Hence, the extent of retention by adsorption on a given phase increases primarily with the activity, average surface energy and surface area of the adsorbent. In addition, retention increases with S^0, the adsorption energy of the solute molecule, which is approximately equal to the sum of the individual adsorption energies contributed by each constituent group. Certain functional groups interact more strongly with polar adsorbents than others. The retention, for the corresponding solutes, increases in the order saturated hydrocarbons < aromatic hydrocarbons ≈ halogen derivatives < ethers < esters ≈ aldehydes ≈ ketones < amines ≈ alcohols < carboxylic acids.

Stronger solvents (larger ε^0) decrease the solute retention. This effect is more important for larger solute molecules (large A_s, eqn. 1). The polarity of the mobile phase needed to achieve adequate R_F values is determined by that of the solutes studied. Polar compounds often require the presence of water in the mobile phase to ensure the *in situ* deactivation of the plate and prevent the occurrence of tailing or

irreversible chemisorption caused by strong adsorbent–solute interactions. In addition, desorption is ensured by the favoured interactions of the solute with the polar eluent. However, when the amount of water in the eluent exceeds 16%, solute partition is effective to a certain extent[26].

Other factors that affect adsorptivity are the acidic or basic functionalities of the adsorbent, as well as steric factors. Each adsorbent is characterized by a definite geometrical arrangement of adsorptive sites, favouring interaction with certain types of solutes. For instance, alumina is better suited than silica for the separation of aromatic hydrocarbons with different functional groups or steric arrangement. Moreover, although the adsorptive properties of alumina are similar to those of magnesia, the latter adsorbent has a much higher affinity for carbon–carbon double bonds and is especially suitable for the separation of planar aromatic hydrocarbons.

In general, solute interactions with a given adsorbent may involve London dispersion forces, orientation or induction dipole forces, hydrogen bonding and weak covalent bonding (acid–base interactions and complex formation). The extent to which any of these occurs depends on the particular chromatographic system used. Adsorbents may often be impregnated with specific reagents that may complex reversibly with the solute or covalently bond to it Impregnation with boric acid, for instance, is used for the separation of vicinal dihydroxy isomers which are retained by forming cyclic boric acid derivatives. Argentation chromatography or impregnation with silver nitrate allows the separation of unsaturated compounds owing to rapid and reversible complexation of their π double bond electrons with the impregnated silver ions. Similarly, the separation of nitrogenous bases on plates impregnated with Zn, Cd or Mg involves the occurrence of selective interactions between the metal ion used and the free nitrogen electrons[19]

2.2. Organic sorbents

In addition to the aforementioned inorganic sorbents, various organic macromolecular stationary phases are also commercially available. Of these, polyamide and cellulose are the most commonly used, and are particularly suitable for the separation of hydrophilic, strongly polar substances.

2.2.1. Polyamide

With aqueous or polar eluents, this sorbent behaves as a reversed phase, whereupon partition is thought to be involved, at least to some extent, in solute retention. With non-polar developers, polyamide is a normal phase on to which chromatographed substances may adsorb. This adsorption may entail the function of hydrogen bonds between proton-donating groups such as hydroxyl, amino, carboxylic or phosphoric in the solute molecule and carbonyl oxygens in the sorbent. Additional interactions may occur between electrophilic nitro compounds, quinones or aldehydes and amino groups in the polyamide phase. Compounds with delocalized π electron systems are also found to exhibit strong affinity for the sorbent. Clearly, chromatography on polyamide is a process of simultaneous e^- donor–e^- acceptor interactions, the extent of which depends on the chemical nature of the solute, the composition of the desorbing eluent and the presence of free –NH– and $>C=O$ groups on the adsorbent surface[26].

2.2.2. Cellulose

In paper chromatography, one often witnesses rapid spreading of substances along cellulose fibres. The powdered sorbent used in TLC, however, is characterized by very short fibres which afford sharper spots and a better resolution. TLC cellulose exists in two forms: native fibrous (fibre length 2–25 μm, average degree of polymerization 400–500) and microcrystalline (particle size 20–40 μm, average degree of polymerization 40–200). In cellulose, most glucose units constitute crystalline regions, joined together as fibrils These are bound into fibres by amorphous β-glucoside linkages. Whereas the crystalline surface is impermeable to most solvents, the amorphous regions hold imbibed water Both structural types are responsible for solute retention, the former involving adsorption and the latter partition

As partition involves the distribution of a solute between two immiscible liquids, it may be surprising at first to evoke this mechanism when solvents miscible with water, if not water itself, are used as developers. However, the water present in the amorphous regions has different properties to bulk water: it is linked by hydrogen bonds to the cellulose, forming a "liquid gel" with high apparent density and low velocity of diffusion.

The partition coefficient, a measure of solute retention, is determined by the extent of solute incorporation in the gel and interaction, by means of hydrogen bonds, with the "liquid gel" water. Molecular size and shape and the position and nature of hydrophilic groups in the chromatographed substance are important in this process[26].

Partition is not, however, solely responsible for chromatographic behaviour, as the migration sequence of several solutes differs from that based on partition coefficients alone. Adsorption and, to some extent, ion exchange are thought to participate in the chromatographic process

2.3. Reversed phases

TLC layers for reversed-phase applications were first obtained by impregnating polar plates with organic solvents such as paraffin[27]. Normal phases were later chemically modified, *e.g.*, by the *in situ* acetylation of cellulose or silanization of silica plates. The resulting phases could withstand development in a wide variety of solvents, and ensured reproducibility of separations[28–30] The number of commercially available reversed-phase plates increased subsequent to the more recent development of non-polar powders, in which different aliphatic chains are chemically bonded to a silica backbone Halpaap *et al.*[31] tested such phases with respect to their chemical and physico-chemical characteristics, wettability and chromatographic retention data

The mechanisms of solute retention on these various packings are diverse. partition is responsible for chromatography on paraffin-coated silica layers; with acetylated cellulose, although adsorption cannot be excluded, solutes are thought to partition, when aqueous eluents are used, between the mobile phase and the water-impregnated cellulose fibres; adsorption due mainly to solvophobic forces is responsible for solute retention on silanized or alkyl-chain bonded silica phases However, when water is used in large amounts, especially with small-chain reversed phases, mixed-mode mechanisms are believed to occur.

2.4 *Ion exchangers*

Polystyrene sulphonic (Dowex 50) or amine (Dowex 1) resins have long been used in TLC work. However, polyacrylamide gels or carbohydrate (cellulose and dextran) polymers, whether chemically modified or impregnated with liquid ion exchangers, are more suitable for the separation of bulky solutes, such as proteins and nucleic acids. Compared with resin exchangers, these matrices have larger pores which allow a greater capacity and a better transfer of macromolecules. In addition, the greater spacing between the active sites helps to achieve selective desorption under milder conditions than with resin phases.

The moderately strong polyethyleneimine (PEI) and weak diethylaminoethyl (DEAE) cellulose basic anion exchangers are commonly used in TLC applications, as well as the strong sulphoethyl (SE) and weak carboxymethyl (CM) Sephadex (polydextran) acidic cation exchangers. Inorganic cation exchangers such as zirconium(IV) phosphate and ammonium molybdate, are also commercially available.

The affinity of a compound for an ion exchanger is known to be proportional to its charge. Besides the electrostatic forces which contribute mainly to chromatographic retention in ion exchange, additional interactions are often operative as in the physical adsorption of hydrophobic solute moieties on the skeleton of the exchanger. With weakly acidic and weakly basic phases, the pH of the eluent may affect selectivity as the exchanging groups ion pair with H^+ or OH^- ions in the solution[32].

2.5. *Thin-layer gel chromatography phases*

Dextran gels such as Sephadex G-10, admixed with silica gel or cellulose, have been used as TLC layers for the separation of purine and pyrimidine nucleotides and bases, for instance. While the character of the separations remains that of Sephadex, the layer has mechanical properties similar to those of silica gel or cellulose. Polyacrylamide gels (Biogel P) have also been used in TLC[33].

2.6. *Stationary phases for two-dimensional thin-layer chromatography*

Different sorbents may be slurried together and spread homogeneously on a plate for two-dimensional TLC purposes. The composition of the eluents is chosen such that, in each direction, a different sorbent interacts primarily with the solutes. Sorbents have also been coated side-by-side, forming coupled, two-phase plates; chromatography is then performed on a different phase in each direction

The development of a plate in a given solvent is well known to affect the solute R_F values obtained from a subsequent development in another direction (Fig. 2) In addition, if the second solvent is very different from the first, its ascent in the orthogonal direction may be irregular, leading to distorted spots. In order to correct for those problems, Randerath and Randerath[35], after developing a PEI-cellulose plate in a buffer solution, washed away the residual salts by immersing the plate in methanol prior to a second elution. Bond[36] chose to transfer the spots obtained from the first development of a cellulose sheet on to another plate by clamping both thin layers face to face and developing with the second solvent, in a direction perpendicular to the first elution. The resulting chromatogram showed no interferences In addition, the solute R_F values were unaffected by the previous run.

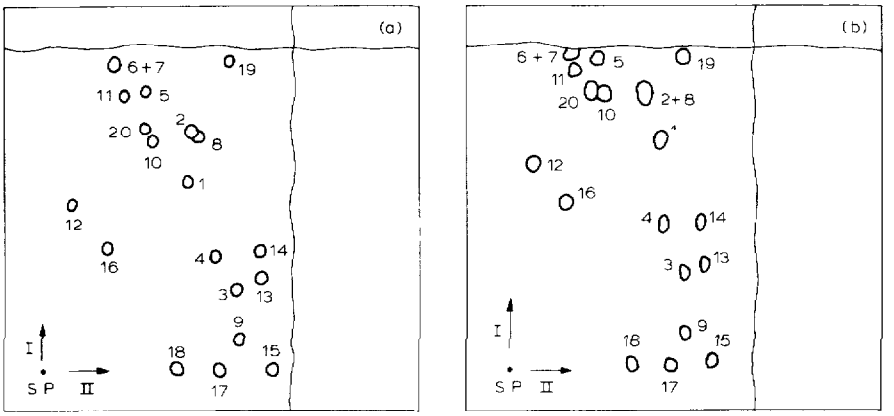


Fig 2 (a) Theoretical and (b) experimental two-dimensional chromatograms of dinitrophenyl (DNP) amino acids on RP-18 plates Eluents in the first direction, hexane ethyl acetate acetic acid (80:18 2), in the second direction, 1 *M* ammonia + 3% potassium chloride in 60% methanol S P = starting point 1 = DNP-Gly, 2 = DNP-Ala; 3 = DNP-Ser, 4 = DNP-Thr; 5 = DNP-Val, 6 = DNP-Leu, 7 = DNP-Ile, 8 = DNP-Pro, 9 = DNP-Met-O_2, 10 = DNP-Trp, 11 = DNP-Phe, 12 = Di-DNP-Tyr, 13 = DNP-Asp, 14 = DNP-Glu, 15 = DNP-$CySO_3Na$, 16 = Di-DNP-Lys, 17 = α-N-DNP-Arg, 18 = Di-DNP-His, 19 = DNP-OH, 20 = DNP-NH_2 Reproduced from ref 34 with permission

3 DETECTION

The localization, characterization and identification of spots are essential aspects of TLC. The techniques used in two-dimensional TLC are identical with those used in one-dimensional TLC, except that the whole surface of the plate has to be scanned, which makes the problems much more difficult. Detection may therefore require a very long time and very sophisticated instrumentation, contrasting with the great simplicity of the separation method. Techniques for recovering or displaying the two-dimensional separation have benefitted from recent improvements in image processing made possible through the availability of powerful, yet cheap, computers.

In two-dimensional chromatography the exact position of the spot must be determined for qualitative analysis and the whole spot profile, in both directions, must be integrated for quantitative analysis. The problem is therefore much more difficult than in conventional TLC, where the centre of the spot is on the vertical of the sample spot and the solute concentration profile in the direction perpendicular to that of the development is devoid of interest and can be integrated at once, as is done with the use of a narrow slit perpendicular to the scanning direction in photodensitometers for TLC Such devices cannot be used in two-dimensional TLC; the spatial resolution of the scanner, whether an optical beam, an electronic beam or any other system, must be small compared with the spot size, about 10–20 times smaller in dimension than the spot base width. Typically, this means that it should not exceed 0.1–0.5 mm in diameter, depending on the TLC conditions If ω is the width of the sensor beam and L the development length, a number L/ω of parallel scans must be carried out. This number varies from about 100 to around 2000 depending on the conditions, but in any case is large. Convenient software must be available to align these scans, detect the spots and segment and integrate them.

In the following we review the various detection methods used in conventional TLC and discuss how they can be applied to the special problem of two-dimensional TLC.

A laborious detection technique consists in scraping off the parts of the adsorbent layer containing each spot of interest and extraction from the adsorbent or *in situ* elution of the spot. Although some commercial equipment is now available (for example, Eluchrom Automatic Elution System from Camag), which considerably reduces the amount of solvent necessary for recovering the sample, this technique is inconvenient, time consuming and not quantitative. A final measurement step must also be added, such as a spectrophotometric determination. To apply this method the spot must first be located precisely.

One-dimensional TLC has sometimes been coupled with gas detection devices: detection of compounds eluted on a Chromarod can be performed either with a flame-ionization detector[37] or by direct insertion into a thermoionic[38] or mass spectrometric[39] detector. However, these techniques are not suitable for two-dimensional TLC because of their lack of spatial resolution and/or the considerable amount of time necessary for the scans

Spots may be revealed after performing adequate chemical tests. Reagents for these tests are often sprayed on the layer The non-uniformity of the spray may drastically affect the detection of the linearity of response Therefore, whenever possible spots are rendered visible by exposure to gaseous reagents, which act more uniformly.

Coloured or fluorescent spots can be easily detected visually under white or UV light. If the compound itself is not visible or fluorescent, detection can be performed by applying gaseous, colorimetric complexing agents or even radioactive markers, or by using the quenching effect of the compound on the fluorescence of an additive in the chromatographic bed. The compound then appears as a black spot on a brilliant background. This method permits the accurate determination of the position of the spot, but not of its size or concentration. The latter are determined by the use of one of the photometric methods of TLC classified as densitometric and fluorimetric techniques.

Densitometry can be carried out in the transmission or the reflection mode. It measures the intensity of the light transmitted through the TLC plate or reflected by it. Quantitation is based on the decrease in light transmitted or reflected by the spot in comparison with the neighbouring background[40]. It requires background suppression and assumes a relationship between the signal and the concentration of a compound. This relationship is linear only at very low concentrations[41]. For higher concentrations it is not given by the conventional Beer's law but by the Kubelka–Munk equation[40], because of diffusion of light by the particles in the layer. The response factor of a compound depends on its coefficients of light adsorption and scattering[40]. Obviously the densitometric measurements must be made using a monochromatic incident beam, and the wavelength must be optimized depending on the compounds of interest. Transmission densitometry has been shown theoretically[42] to be more sensitive than reflectometry, although layer uniformity is more critical in the former method. Both non-uniformity of the layer and poor light intensity affect the signal-to-noise ratio. However, such problems may be overcome to some extent with a double-beam instrument. Reflectometry is more widely used because of the higher

optical density of most compounds, especially in the UV range. Frei[40] published an extensive study on reflectance spectroscopy. Using double-beam instruments, one can also compensate for the background effects due to the layer.

In fluorimetry, the incident radiation excites the various compounds, and those which emit secondary radiation are detected This emitted light is characterized by a longer wavelength than the incident radiation. The non-spotted, background or base regions of the layer do not fluoresce. The excitation wavelength can be adjusted to optimize the sensitivity of the measurement for a given compound. The most commonly used wavelengths are 254 and 360 nm.

Fluorescence measurements can also be carried out in the so-called quenching mode A phosphor additive is mixed to the sorbent slurry before coating the plate When the developed plate is irradiated with UV light the base regions of the plate emit a uniform visible light, while the sample compounds appear as dark spots. This method is more sensitive when the light is transmitted from the back of the plate

Many commercially available instruments are single-wavelength devices in which a monochromatic beam is used to scan the plate surface. They can be applied to all modes of densitometry or fluorimetry, with the use of suitable mirrors and filters. In the fluorescence mode, better instruments permit the adjustment of both the excitation and emission wavelengths In these instruments the scanning optical beam is supposed to illuminate on the plate a rectangle with a very large aspect ratio, the long dimension (*ca* 1 cm) being perpendicular to the scanning direction. This yields a signal proportional to the light intensity perceived, *i.e.*, integrated over the whole slit length If the relationship between light intensity and spot concentration is non-linear, this results in quantitative errors when the concentration is not constant along the slit. On the other hand, this permits integration of the fluctuations due to layer heterogeneity over a larger area and decreasing the noise. Hence, the signal should be integrated over the slit to linearize the characteristic response of a single slit in terms of concentration[43]. Knapstein *et al* [44] obtained a linear calibration graph for steroids detected on silica gel in the quenching mode

With dual-wavelength densitometers, fluctuation of the light source is balanced and a flying spot can replace a fixed slit aperture, which leads to greater accuracy in quantitation. The complexity of dual-wavelength densitometers requires computer-assisted processing of the signal[45] Speed, like resolution, is expensive and rapid evaluation of a one-dimensional thin-layer chromatogram requires the scanner to be connected either to a recorder or to a video screen. Speed can also be achieved at the expense of a lower sensitivity if the signal noise depends on the integration time.

In 1976, Devenyi and co-workers[46,47] introduced video techniques in the densitometric evaluation of thin-layer chromatograms The whole chromatogram is irradiated by a suitable light source of relatively homogeneous intensity and an image is taken by a Vidicon camera This is extremely fast and also reasonably accurate and sensitive, and the rather sophisticated processing such as background subtraction or spot integration is easy to carry out in an interactive mode

Generally, the HPTLC scanners available for photodensitometric measurements cannot be adapted easily to two-dimensional TLC. The speed of detection is an extremely critical factor as all of the plate surface has to be scanned. Scanning a conventional TLC plate takes a few minutes, but scanning a two-dimensional plate would take from several hours to one day. No software is available to align all these scans and process them to obtain quantitative information.

Two-dimensional detection devices have resulted mainly from two-dimensional gel electrophoretic analyses[48,49] and from the TLC of radioactive compounds[8]. In both instances an autoradiograph is obtained and then scanned with a very narrow visible light beam (spatial resolution around 0.1 mm or below). Complete data acquisition and processing systems have been developed by several groups[48–50]. These systems offer possibilities of data acquisition, filtering, background subtraction, calibration and data reduction, and provide for spot detection, segmentation, integration, pairing and data file handling on thousands of spots.

Video-densitometric techniques have been applied to two-dimensional TLC. A two-dimensional matrix of pixels has sometimes been used[47,51] together with a Vidicon camera to obtain both normal two-dimensional and spectral images. Both fluorescence and absorption measurements can be carried out with this technique The sensitivity of this type of detector is in the nanomole range Although its main drawbacks are cost and complexity, it is still more attractive to the analyst than the classical spectrodensitometer, the use of which is tedious and time consuming and necessitates image reconstitution after hundreds of one-dimensional scans of a two-dimensional plate.

Recently, Guiochon and co-workers[52,53] conceived the use of a detection device on-line with a two-dimensional liquid chromatographic system. Detection is now performed in the elution mode, in the second direction, with a photodiode array Reticon camera used as a series of individual UV cells parallel to the exit edge of the plate. In this system, the solvent is pumped through the chromatographic bed under pressure and no longer moves under the influence of capillary forces. At the exit, the solvent is forced through a slot which constitutes the detector cell.

A monochromatic light passes through this slot and is focused on the diode array of the Reticon camera A time-dependent signal similar to the line signal of an autoradiography scan is recorded and processed through a data acquisition system, then displayed on a colour TV screen. HPLC elution conditions may thus be reproduced and quantitation is no longer affected by plate characteristics and chromatographic bed diffusivity as in regular TLC

4 APPLICATIONS

The separations that have been accomplished using two-dimensional TLC are classified in this section under lipids, pigments, alkaloids, proteins and their constituents, carbohydrates, glycopeptides, nucleic acids and their constituents, environmental pollutants, pesticides and miscellaneous We have tried to emphasize the peculiarities of various assays by analysing the chromatographic behaviour of solutes. The retention and chromatography of compounds may involve adsorption, partition, ion exchange or restricted diffusion (gel permeation) Although one particular chromatographic mechanism may be emphasized for the retention of solutes under given conditions, other concurrent processes should not be forgotten.

4.1. Lipids

"Lipids" is a term used to qualify various classes of compounds that are characterized in general by solubility in organic solvents and insolubility in water. In this

TABLE 1

SEPARATION OF FATTY ACIDS AND THEIR ESTERS

N is the number of spots resolved in the best published chromatogram Compounds· name of classes, groups or individual compounds studied Stationary phase. composition of the thin layer used as the chromatographic bed Also includes dimensions and activation conditions when available When two different phases are used, 1 refers to the phase used for the first development, usually a narrow strip along the edge of the plate, and 2 to the second stationary phase SSP sample spot position; distance from the edges of the plate Solvent 1 composition of the solvent used for the first development Sometimes several successive developments are carried out using different solvents Their compositions are given in the order a, b, c, etc. *t/L.* time (min) or length (cm) of the first (with solvent 1) and second (with solvent 2) developments Drying conditions under which the plate is dried after the first development is finished Solvent 2 same as solvent 1, but for the second development. Ref. also includes figure numbers, if one is reproduced here

N	*Compounds*	*Stationary phase*	*SSP (cm)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref*
	Alkenyl acyl-, alkyl acyl- + diacyl glyceryl acetates	Si gel G, 110°C, 1 h		Skelly F -diethyl ether (88·12)	14 cm	N_2 dry	Toluene		54
11	Fatty acids, prostaglandins, thromboxanes	Si gel, 20 × 20 cm		Diethyl ether–CH_3OH acetic acid (90 1·2)			Chloroform–methanol–acetic acid–water (90 8 1 0.8)		55
13	Fatty acids esters	Si gel D.B , Camag, 20 × 20 cm, 0 25 mm, wash, 139-142°C, 30 min	2 5	Light petroleum diethyl ether–acetic acid (75 25 2 5)	70–80 min, 16 cm	15 min, room temp	Diethyl ether light petroleum–acetic acid (70·30 1)	65–75 min, 15 cm	56
8	Ginsenosides	Silanized Si gel 60 F254, 20 × 20 cm, 0 25 mm	2 0	(a) Chloroform–methanol–ethyl acetate–butanol–water (4 4 8 1 2) (b) Chloroform butanol methanol–water (4 8 3 4)	10 cm	air dry, 10 min	(c) Chloroform methanol water (13 7·2)	10 cm	57
13	Mercury(II) adducts of unsat esters (mono-, di-, tri-, tetra-, penta-, hexa-enes)	(1) Si gel G-Kieselguhr (3·7), 20 × 20 cm (2) Impregnate with 10% paraffin in light petroleum		On (1) isobutanol–formic acid–water (8 8 5)	Top	1 h air, 10 min H_2S chamber	On (2)· formic acid–acetonitrile–acetone (8 8 5)		58
16	Trialkylglyceryl esters, dialkoxyglycerides, alkoxyglycerides + *n*-glycerides	(1) Si gel G, 4 × 20 cm, 0 25 mm (2) $AgNO_3$-impregnated Si gel G, 16 × 20 cm, 0 25 mm. 110–120°C, 1 2 h	2 0	On (1) petroleum hydrocarbon–diethyl ether (90 10) (need 50% relative humidity)			On (2) petroleum hydrocarbon–diethyl ether (90 10) (2 ×) (need 50% rel humidity)		59 (Fig. 3)
4	Positional isomers of hydrostearic acid	Si gel G, 0 25 mm		Benzene–acetone (20 1)			Benzene–acetone (20·1)		60

section, four main groups of substances are considered: fatty acids and their esters, steroids, phospholipids and glycolipids. The different two-directional assays are briefly discussed in the text and detailed chromatographic conditions are given in Tables 1–5.

4.1 1. Fatty acids and their esters (Table 1)

Separations of various glyceryl acetates[54] and of some fatty acids from prostaglandins and thromboxanes[55] have been achieved on silica plates owing to the different selectivity of the solvent system in each direction. Fatty acid esters have also been resolved on silica[56] with a more polar eluent in the second than in the first direction.

A special category of compounds, the ginsenosides (triterpenoid saponins of the dammarane type), have been assayed on silanized silica gel using a less polar mobile phase in the second than in the first direction[57].

El-Zeany and Ahmed[58] devised an original method to resolve fatty acid esters. They first separated the mercury(II) adducts of these unsaturated esters on a 3.7 silica gel–Kieselguhr adsorbent with isobutanol–formic acid–water, based on the different degree of solute unsaturation. Then, the plate was positioned in a hydrogen sulphide chamber; mercury(II) sulphide was deposited as black spots and the esters were liberated. Chromatography in the second direction was accomplished after impregnating the plate with paraffin. The esters partitioned between that phase and the formic acid–acetonitrile–acetone eluent. Their mobility was a function of chain length Whereas is this method mercury(II) adducts of solutes were chosen to achieve separations based on different degrees of unsaturation, more commonly unsaturated compounds are resolved according to their ability to form coordination complexes with ions (such as silver) which impregnate the stationary phase Schmid *et al* [59], for instance, after separating glycerol esters into four classes according to basic structure, type and number of functional groups on plain silica gel, differentiated each class according to the number and configuration of the double bonds in the constituent compounds, on silica gel impregnated with silver nitrate (Fig 3).

4 1.2. Steroids (Table 2)

This class of lipids, of which cholesterol is a common representative, includes physiologically important compounds such as the bile acids, the androgens and the oestrogens, and essential metabolic regulators, such as the adrenocortical hormones. Thus, the separation of these substances and their quantitation in biological samples are essential, especially for diagnostic purposes

Using silica, Bouillé *et al.*[61] first eluted the impurities associated with cortisone in plasma and adrenal extracts Chromatography in the orthogonal direction was achieved with a more polar mobile phase containing water, which resulted in the migration of corticosterone from its original position on the plate. Also using a silica phase, Bicknell and Gower[62] succeeded is resolving a mixture of polar and non-polar compounds. Two successive developments in the first direction with benzene–diethyl ether (9:1) permitted the separation of the non-polar C_{19} 16-unsaturated steroids 5α-androst-16-en-3β-ol, 5β-androst-16-en-3α-ol, 5α-androst-16-en-3α-ol, 1,3,5-[10],16-oestratetraen-3-ol and 5α-androst-16-en-3-one. The use of a more polar eluent, benzene–methanol (9·1), for development in the second direction permitted

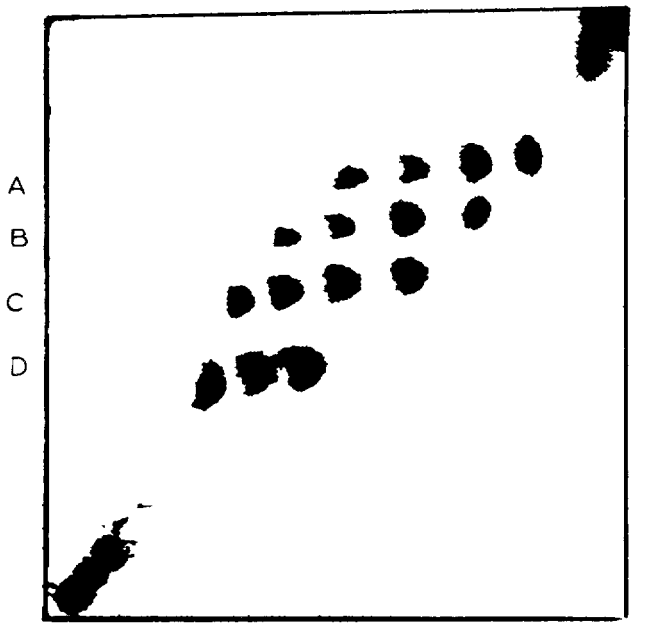


Fig 3 Two-dimensional chromatogram of a model mixture containing the following compounds: (A) trialkyl glyceryl ethers trioctadecyloxypropane, 1,2-dioctadecyloxy-3-octadecenyloxypropane, 1-octadecyloxy-2,3-dioctadecenyloxypropane, trioctadecenyloxypropane, (B) dialkoxyglycerides, 2,3-dioctadecyloxy-1-O-octadecanoylpropanol, 2,3-dioctadecyloxy-1-O-octadecenoylpropanol, 2,3-dioctadecenyloxy-1-O-octadecanoylpropanol, (C) alkoxydiglycerides, 3-octadecyloxy-1,2-di-O-octadecanoylpropanediol, 3-octadecenyloxy-1,2-di-O-octadecanoylpropanediol, 3-octadecyloxy-1,2-di-O-octadecenoylpropanediol, 3-octadecenyloxy-1,2-di-O-octadecenoylpropanediol, (D) triglycerides tri-O-octadecanoylpropanetriol(tristearin), 1,2-di-O-octadecanoyl-3-O-octadecenoylpropanetriol (oledodistearin), 1-O-octadecanoyl-2,3-di-O-octadecenoylpropanetriol (stearodiolein), tri-O-octadecenoylpropanetriol (triolein) Solvents first direction, petroleum hydrocarbon diethyl ether (90 10), developed once on plain silica gel G strip, second direction, petroleum hydrocarbon diethyl ether (90·10) developed twice on silver nitrate-impregnated silica gel G Indicator charring after spraying with chromic sulphuric acid solution Reproduced from ref 59 with permission

the separation of the more polar Δ^4-3-oxosteroids testosterone and progesterone and of the Δ^5-3β-hydroxysteroids pregnenolone and 5,16-androstadien-3β-ol.

Taylor[63] separated thirteen polar Δ^4-3-oxosteroids and oestrogens on silica with chloroform methanol–water (94·6:0.5) in the first direction, followed by cyclohexane–ethyl acetate (50.50) in the second direction (Fig. 4). Cavina *et al.*[64] achieved similar resolutions of oestrogens and progestagens on silica by combining appropriate different developing systems. Clearly, the polarity of the mobile phase, its constituents and the degree of deactivation of the stationary phase achieved by use of water in the eluent are all factors that affect such assays.

Issaq[65] resolved two isomeric pairs, namely 7α- from 7β-hydroxycholesterol and 5β,6β- from 5α,6β-epoxycholesterol on a special plate with two different sorbents coated side-by-side Chromatography in the first direction was accomplished on silica gel with diethyl ether as the eluent. Orthogonal elution was then achieved for a few centimetres on silica and, for the remaining part, on silanized silica, with a non-polar heptane–HMDS (98.2) as the mobile phase

Similarly, Levitt and Touchstone[66] separated six bile acids on a two-phase plate where a 3 × 20 cm reversed-phase strip had been coated at the side of a 17 × 20 cm silica layer. Reversed-phase chromatography was carried out on the thin strip with an acid–organic eluent mixture and resulted in the separation of the bile acids into three classes: cholic, deoxycholic and chenodeoxycholic. Orthogonal development with the mixed aqueous (acetic acid–water) and organic (chloroform–methanol) solvent on the silica phase further separated the taurine from the glycine conjugates in all three classes of compounds. It is important to remember that these bile acids have functionalities that interact strongly with silica unless the adsorbent is modified through bonding with alkyl chains (reversed-phase strip) or deactivated by the presence, in the mobile phase, of components such as acetic acid or water, which compete strongly with the solute for the available adsorptive sites.

Vidrine and Nicholas[67] resolved two different lipid classes, *viz.*, steroids and unsaturated aliphatic hydrocarbons (terpenoids), on a mixed thin-layer plate coated with a slurry of silica, silanized silica and calcium sulphate. The eluent was relatively non-polar in the first direction, and retention could be attributed to adsorption In the second direction, however, a more polar eluent was used, containing 25% of water. Under these conditions, a partition–adsorption mechanism is probably in effect.

Finally, Curtius and Müller[68] achieved an original, truly two-dimensional GC TLC separation of a hormone mixture. The first dimension involved a separation on a GC column with a split effluent, 10% directed to a flame-ionization detector and 90% spotted on a TLC silica plate which moved linearly with time. The development of this plate with chloroform–ethanol (90:10) represented the second dimension and improved the resolution obtained with GC alone (Fig. 5) Clearly, the second separation is not especially helpful with the twelve components shown here, which are sufficiently well resolved by the GC step for quantitative analysis. It is, however, of greater use with more complex mixtures that cannot be resolved by GC but are resolved by the two-dimensional separation scheme.

4.1.3. Phospholipids (Tables 3 and 4)

This lipid class includes the phosphoglycerides and sphingolipids, all important membrane constituents. Chromatography of these compounds is often accomplished on silica gel, in its pure form or impregnated with either boric acid, ammonium sulphate, magnesium acetate, magnesium silicate or magnesium hydroxycarbonate.

In general, separations of phosphoglycerides (Table 3) require development of the plate with a water–organic eluent containing acetic acid in one direction and a base such as ammonia solution in the other [69–73]. Thus, the mobility of the molecules in each direction is affected by the differing degree of ionization, *i.e.*, the extent of amine protonation and phosphate and/or carboxyl dissociation, as well as by the competition of the acid or base in the mobile phase for adsorptive sites on the silica plate. Moreover, Jain and Subrahmanyam[74] have reported that appropriate amounts of ammonium sulphate in the stationary phase and acetic acid in the eluent are important for obtaining spots with no tailing and good resolution.

It is interesting that, on a phase containing boric acid[75], phosphatidylinositol, which has vicinal *cis*-hydroxyl groups in its sugar moiety, is retained the longest (Fig 6). This may exemplify the selective affinity of boric acid for *cis*-diol compounds.

TABLE 2

SEPARATION OF STEROIDS

Column headings as in Table 1.

N	*Compounds*	*Stationary phase*	*SSP (cm)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref.*
	Corticosterone purification	Kieselgel GF 254 (Si), 20 × 20 cm, 0.4 mm	2.0	(a) *n*-Hexane–ethyl acetate (4:1) (b) Ethyl acetate cyclohexane toluene (10:10:1)	20–30 min, 10 cm 30 min 10 cm	room temp.	Ethyl acetate–chloroform–water (90:10:1)	30 min, 10 cm	61
17	Δ^4-3-Oxosteroids, Δ^5-3β-hydroxysteroids, C_{19}-16-unsat. steroids	Kieselgel G (Merck), 20 × 20 cm, 0.5 mm	2.0	Benzene diethyl ether (9:1) (2 ×)	16.8 cm		Benzene methanol (9:1)	16.8 cm	62
13	Δ^4-3-Oxosteroids, oestrogens	Kieselgel GF 254, 20 × 20 cm, 0.5 mm		Chloroform methanol water (94:6:0.5)	14.4 cm from spot		Cyclohexane ethyl acetate (50:50) (2 ×)	18 cm	63 (Fig. 4)
6 or 8	Estrogens, progestagens	Si gel 60 F_{254}, 20 × 20 cm, 0.25 mm	2.0	Toluene–95% ethanol (90:10)	18 cm	Air dry, 20 min, dark	Butyl acetate–light petroleum acetic acid (70:30:1)	~18 cm	64

4	7α-Hydroxycholesterol, 7β-hydroxycholesterol, 5β,6β-epoxycholesterol, 5α,6α-epoxycholesterol	(1) 20 × 20 cm Si gel (2) 10 cm dipped in HMDS, 100°C, 2 h	(1) Diethyl ether	13 cm	Dry	(2) Heptane HMDS (48.2)	~17 cm	65
6	Bile acids	2-D plate, 20 × 20 cm, CS Multi K (Whatman) (1) 3 cm KC 18 strip (2) remainder, Si gel	(1) Ethanol 0.01 *M* KH_2PO_4 (pH 2.5) (1:1)		Dry	(2) Chloroform–methanol acetic acid water (75:25:5:5)		66
8	Cholesterol, lanosterol, dihydrolanosterol, oxidosqualene, geraniol, *trans,trans*-farnesol, *cis,trans*-farnesol	Si gel G silanized Si gel H $CaSo_4$ $\frac{1}{2}$ H_2O (50:43:7), 20 × 20 cm	Hexane–ethyl acetate (90:10)	45 min, 14 cm		*p*-Dioxane–water (75:25)	2 h	67
12	Male and female hormones	(1) GC column impregnated with 3% XE-60 (2) Kieselgel G	(1) N_2, 50 ml/min, 210 → 265°C		Spray plate with HCl, split trimethylsilyl-esters	(2) Dichloromethane–acetone (70:30) or chloroform–ethanol (90:10)		68 (Fig. 5)

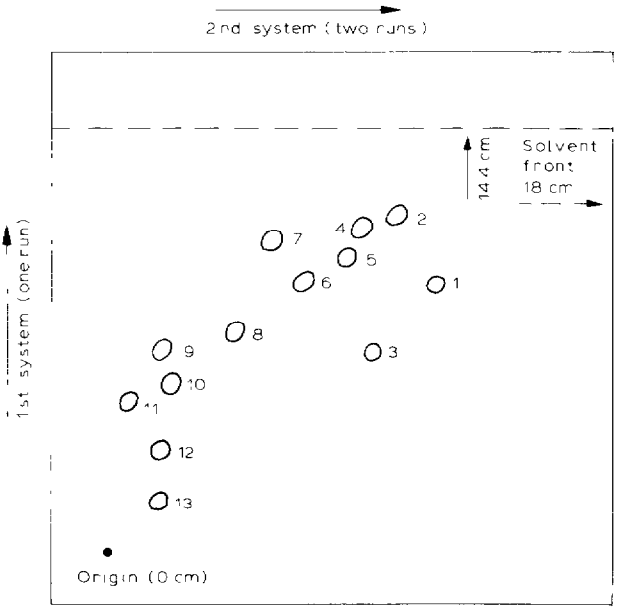


Fig 4 Two-dimensional thin-layer chromatogram of steroids on silica gel Solvent systems (1) chloroform–methanol–water (94 6 0 5), (2) cyclohexane–ethyl acetate (50.50) 1 = Oestrone [3-hydroxy-1,3,5(10)-estradien-17-one], 2 = progesterone(4-pregnene-3,20-dione), 3 = oestradiol [1,3,5(10)-estratriene-3,17β-diol], 4 = androstenedione (4-androstene-3,17-dione), 5 = 17α-hydroxyprogesterone (17α-hydroxy-4-pregnene-3,20-dione), 6 = testosterone (17β-hydroxy-4-androsten-3-one), 7 = deoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione), 8 = 11-deoxycortisol (17α,21-dihydroxy-4-pregnene-3,20-dione), 9 = corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione), 10 = cortisone (17α,21-dihydroxy-4-pregnene-3,11,20-trione), 11 = aldosterone (18,11-hemiacetal of 11β-21-dihydroxy-3,20-dioxo-4-pregnen-18-al), 12 = cortisol (11β,17α,21-trihydroxy-4-pregnene-3,20-dione); 13 = oestriol [1,3,5(10)-estratriene-3,16α,17β-triol] Reproduced from ref 63 with permission

Pollet *et al.*[77] achieved the separation of different lipid classes, namely cholesterol, cerebrosides, sulphatides, phospholipids and gangliosides, on plain silica gel by repetitive elution with chloroform–methanol solutions of varying polarity in orthogonal directions (Table 4). Radwan[78] and Ikawa and Goto[79] resolved mixtures of fatty acids, steroids, phospholipids and other compounds on silica with an organic -acidic phase in one direction and an organic–basic phase in the other. Hubmann[80] was able to separate these various substances using different eluents for successive developments in each direction, owing to the different selectivity of each organic modifier with respect to the various solutes (Table 4).

4 1.4. Glycolipids (Table 5)

Glycolipids, such as the cerebrosides and the more complex gangliosides, are sugar-containing membrane lipids The separation of such compounds was performed on silica gel by Ohashi[81], who used chloroform–methanol–aqueous ammonia to develop the plate in the first direction and *n*-propanol–aqueous ammonia in the second. Ledeen *et al* [82], and subsequently Hunter *et al* [83], in assaying for brain gangliosides, modified these conditions to a certain extent. ammonia was included in one dimension only, while salt was added to either or both solvents.

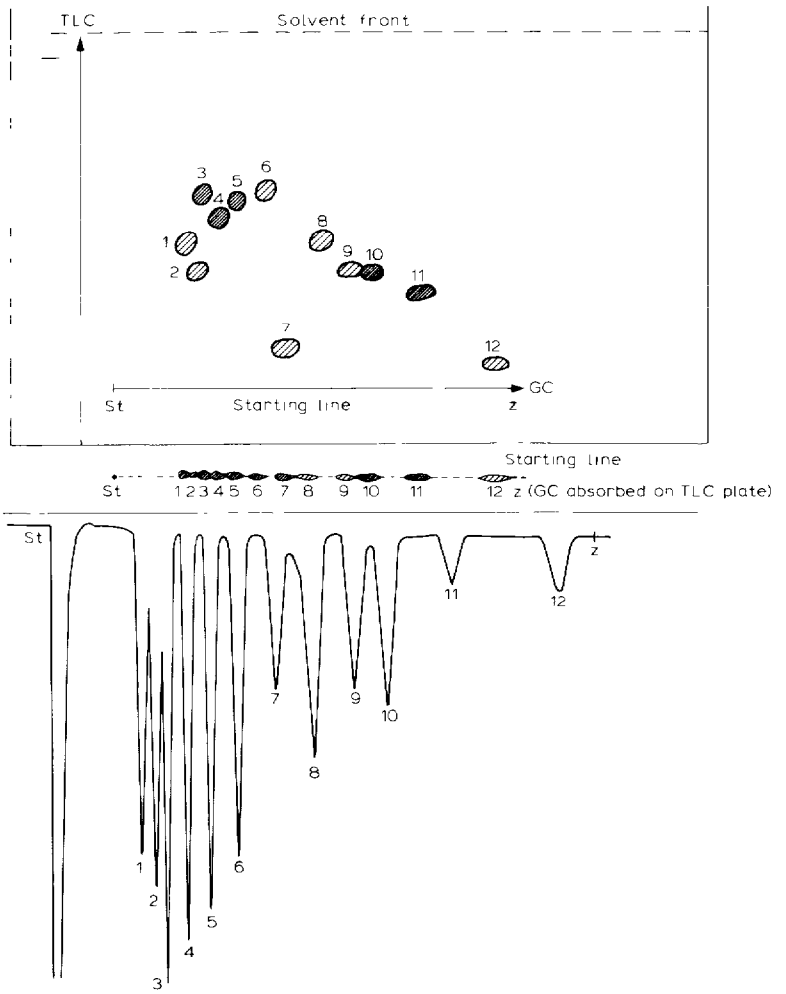


Fig 5 Two-dimensional chromatography of twelve steroids (trimethylsilyl derivatives) using GC and TLC in combination The lower part of the figure represents the gas chromatogram and the upper part illustrates the TLC separation after plate elution It may be noticed that solutes 1, 2 and 3 are completely resolved after the TLC step. 1 = Allopregnandiol, 2 = pregrandiol, 3 = androsterone, 4 = etiocholanolone, 5 = dehydroepiandrosterone, 6 = pregnanolone, 7 = pregrantriol, 8 = 11-ketoandrosterone; 9 = 11-ketoetiocholanolone, 10 = 11-hydroxyandrosterone, 11 = 11-hydroxyetiocholanolone, 12 = pregnantriolone St = starting point Reproduced from ref 68 with permission

Cardenolides, which are glycosides with a steroid moiety, constitute a different category of sugar-containing lipids. Thirty-two cardenolides were separated by Clarke and Cobbs[84] on silica with a different solvent system for each direction. A correlation between the chromatographic mobility of the compounds and their chemical structure was observed The number and position of the hydroxyl substituents played an important role in solute retention (Fig 7).

TABLE 3

SEPARATION OF PHOSPHOLIPIDS

Column headings as in Table 1.

N	*Compounds*	*Stationary phase*	*SSP (cm)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref.*
6	Phosphatidyl (P) glycerol (G), pserine (PS), P-ethanolamine (PE), pinositol (PI), leathin (L), sphingomyelin (S)	Si gel H with 5% ammonium sulphate, 20 × 20 cm		Chloroform–methanol–water–gl. acetic acid (65:25:4:8)	10 cm	70°C, 5 min	THF–methylol–methanol–2 *M* ammonia (40:28.5:7.8:4.2)	10 cm	69
6	PG, PE, PI, PS, L, S (in amniotic fluid)	Si gel H with 50 g/l ammonium sulphate, 20 × 20 cm, 110°C, 30 min		Chlofororm–methanol–water–acetic acid, 17.4 *M* (162.5:65.0:10.0:0.5)	10 cm	70°C, 5 min	THF–methylol–methanol–2 *M* ammonia (120:80:35:10)	10 cm	70
13	PE, PC, PS, PI, L, S, and others, cerebrosides and sulphatides (in brain and mitochondrial inner membrane)	Si gel H (20 g) with magnesium acetate (1.5 g), 0.25 mm, N_2, 50% humidity, 30 min		Chloroform–methanol–28% aq. ammonia (65:25:5)		Dry, 10 min, N_2	Chloroform–acetone–methanol–acetic acid–water (3:4:1:1:0.5)		71

10	PI, PS, PE, PG, etc. (in erythrocytes)	Si gel with 1% alkaline magnesium silicate or 2% magnesium hydroxycarbonate		Chloroform–methanol 7 *M* ammonia (90:54:11)		Dry, 15 min; if rel. hum. >65%, dry 1 h *in vacuo*	Chloroform–methanol–acetic acid–water (90:40:12:1)		72
11	PI, PS, PG, etc. (in yeast)	Si gel H, 20 × 20 cm, 0.25 mm		Chloroform–methanol–acetic acid–water (52:20:7:3)	60–90 min	Air dry, 20 min, wash with diethyl ether, air dry, 20 min	Chloroform–methanol–40% aq. methylamine–water (13:7:1:1)		73
9	PG + derivatives, cardiolipin	Si gel G (25 g) with 0.4 *M* boric acid (50 ml), 20 × 20 cm, 0.25 mm, 120°C, 1 h		Chloroform–methanol–H_2O conc. NH_3 (70:30:3:2)		Dry, 30 min, N_2	Chloroform–methanol–water (63:35:5)		75 (Fig. 6)
	L, S from PI, PS, PE, etc.	Si gel-impregnated glass-fibre sheets (ITLC type SG), 100°C, 30 min	2	Dichloromethane–ethanol–water (100:25:3)	6 cm, 3 min	Air dry, 5 min	Chloroform–methanol–ammonia (170:20:3)	4 cm, 2 min	76

TABLE 4

SEPARATION OF PHOSPHOLIPIDS AND OTHER LIPID CLASSES

Column headings as in Table I

N	*Compound*	*Stationary phase*	*SSP (cm)*	*Solvent 1**	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref*
11	Phospholipids, cerebrosides, sulphatide (plasma, liver, brain)	Si gel impregnated with ammonium sulphate, 20 × 20 cm, 0.5 mm, 110°C, 1 h		Chloroform methanol acetone–water (60 25 8 4)	Top	50°C, 5 min	Chloroform methanol acetic acid–water (50 35 8 1)	Top	74
11	Cholesterol, cerebrosides, gangliosides, phospholipids (cellular membranes)	Si gel HPTLC 60 F 254 (Merck), 10 × 10 cm	2	(a) Chloroform methanol–water (70 30 4)	Top	Bench dryer, few min	(b) Chloroform methanol (1·4)	2/3 of the plate	77
							(c) Chloroform methanol (2 1)	Up to the top	
				(d) Chloroform methanol (2 1)		Bench dryer, few min			
17	Triglycerides, fatty acids, steroids, phospholipids (plant leaves)	Si gel H, 20 × 20 cm, 0.3 mm		Chloroform methanol 7 *N* ammonia (65 30 4)			Chloroform methanol–acetic acid water (85 10 10·2)		78
17	Triglycerides, fatty acids, steroids, phospholipids (plant leaves)	Si gel H impregnated with 2% aq ammonium sulphate, 20 × 20 cm, 0.3 mm, 110°C, 1 h		Acetone benzene water (91 30 8)			Chloroform methanol acetic acid water (85·10 10 2)		78
15	Bile acids and salts, fatty acids, sterols, phospholipids (gall bladder)	Kieselgel H (type 60), 20 × 20 cm, 0.25 mm, 120°C, 1 h	1.5	Chloroform–ethanol 28% aq ammonia (25 35 1)	15 cm	Air stream, 30 min	Chloroform ethanol ethyl acetate–acetic acid water (8.6 5·4·1)		79
11	Fatty acids, triglycerides, steroids, phospholipids (serum)	Si gel, 4.5 × 2.6 cm		(a) Chloroform–methanol water (65.25·4)	3 cm		(c) *n*-Hexane–diethyl ether gl acetic acid (85 20·2) (for lower right-hand side of plate)	2.6 cm	80
				(b) *n*-Hexane diethyl ether gl acetic acid (85 20 2)	4.5 cm		(d) Butanol gl acetic acid–water (60 20 20)	2.6 cm	

* (a), (b), (c), (d) indicate the sequence of developments.

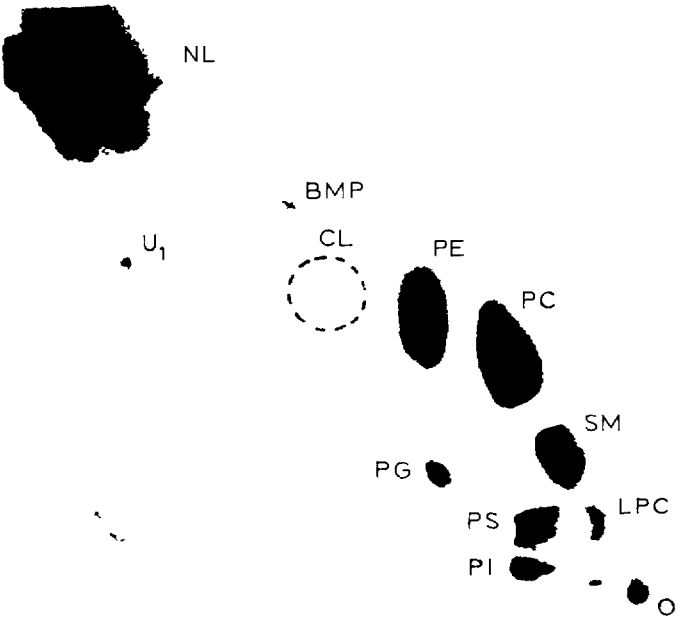


Fig 6 Two-dimensional thin-layer chromatogram of rat lung liquids on silica gel impregnated with boric acid Abbreviations NL = neutral lipids; UI, unidentified, BMP, bis(monoacylglyceryl)phosphate, CL = cardiolipin, PE = phosphatidylethanolamine; PC = phosphatidylcholine, PG = phosphatidylglycerol, SM = sphingomyelin, PS = phosphatidylserine, PI = phosphatidylinositol, LPC = lysophosphatidylcholine; O = origin The solvent for development in the vertical direction was chloroform methanol water concentrated ammonia (70 30 3 2) and in the horizontal direction chloroform–methanol water (65 35 5) Reproduced from ref 75 with permission.

4.2. Pigments

This general group contains the various carotenes, xanthophylls, porphyrins and anthocyanidins, *i.e.*, all natural pigments, and the synthetic tannins. Detailed conditions for their two-dimensional separation are reported in Table 6.

Xanthophylls were chromatographed by Knowles and Livingston[86] on a 1:4 magnesium oxide Celite strip in the first direction and a 1.6 silica gel G–calcium hydroxide layer in the second Adsorbability increased with the number of hydroxyl groups on the cyclic moieties of the solutes, the presence of epoxide functionalities especially at the 5,8-position and the length of the conjugated double bond system (Fig. 8). Other separations of carotenoids have been achieved by Tirimanna[87] and Sherma and Zweig[88] The hydrocarbonaceous carotenes exhibited the largest R_F values on silica Owing to its greater number of conjugated double bonds, zeaxanthin was more retained than lutein[87]

Porphyrin methyl esters were resolved by Elder[89] on a silica gel plate. A quaternary solvent system was used in the first direction. Retention was increased by carboxylic acid substituents at positions 2 and 4 of the porphyrin ring, as witnessed by the smaller R_F for coporphyrin in comparison with mesoporphyrin. Additional ethanoic acid substituents at positions 1, 3 and 5, as in uroporphyrin, further enhanced the adsorption interactions of the solute with the stationary phase. Of all the

TABLE 5

SEPARATION OF GLYCOLIPIDS

Column headings as in Table 1.

N	*Compounds*	*Stationary phase*	*SSP (cm)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref.*
	Gangliosides (rabbit brain)	Si gel 60, 20 × 20 cm, 0.25 mm		Chloroform methanol 0.2% aq. $CaCl_2$ (50:40:10) or Chloroform methanol–0.25% aq. KCl (50:40:10)	4 h		Chloroform methanol 25 *M* aq. ammonia (50:40:10) or Chloroform–methanol–0.25% KCl in aq. ammonia (50:40:10)	5 h	82
11	Gangliosides (tissues)	Si gel (Merck), 10 × 10 cm		Chloroform methanol–28% aq. ammonia–water (2 ×)		Over P_2O_5, 2 h, *in vacuo*	*n*-Propanol–28% aq. ammonia–water (75:5:25) or chloroform–methanol–water (60:40:9)		81
32	Cardenolides (leaf)	Kieselgel 60 DC Fertigplatten, 20 × 20 cm, 0.25 mm	2	Ethyl acetate dichloromethane methanol–water (120:72:7:4)	2 h	Air	Dichloromethane methanol (9:1)	2 h	84 (Fig. 7)
	Steriosidc (leaf diterpene glycoside) purification	Kieselgel G type 60 impregnated with boric acid, 20 × 20 cm, 0.5 mm		*n*-Butanol ethyl acetate isopropanol–water (35:100:60:30)			Ethyl acetate acetic acid–water (8:4:1)		85

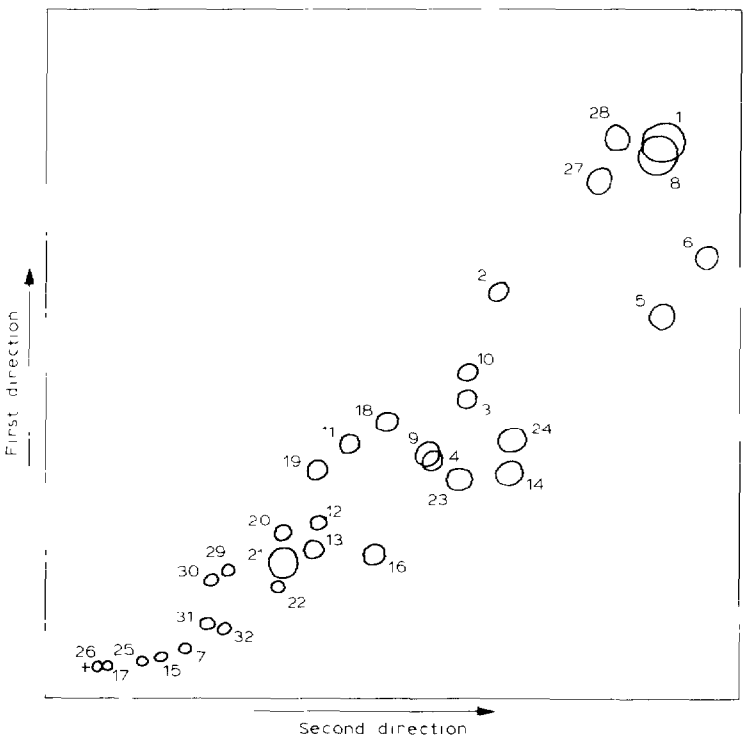


Fig 7 Chromatogram of a mixture of cardesolide standards on silica gel 1 = Digitoxigenin, 2 = digitoxigenin monodigitoxoside, 3 = digitoxigenin bisdigitoxoside, 4 = digitoxin, 5 = α-acetyldigitoxin, 6 = β-acetyldigitoxin, 7 = lanotoside A 8 = gitaloxigenin, 9 = gitaloxin, 10 = gitoxigenin; 11 = gitoxigenin monodigitoxoside, 12 = gitoxigenin bisdigitoxoside, 13 = gitoxin, 14 = β-acetylgitoxin, 15 = lanatoside B 16 = strospeside, 17 = digitalinum verum, 18 = digoxigenin, 19 = digoxigenin monodigitoxoside, 20 = digoxigenin bisdigitoxoside, 21 = digoxin, 22 = digoxoside, 23 = α-acetyldigoxin; 24 = β-acetyldigoxin, 25 = lanatoside C, 26 = deacetyl lanatoside C, 27 = α-anhydrodigoxigenin, 28 = β-anhydrodigoxigenin, 29 = diginatigenin, 30 = diginatigenin monodigitoxoside, 31 = diginatigenin bisdigitoxoside, 32 = diginatin Solvent in the first direction, ethyl acetate dichloromethane methanol water (120 72 7 4), and in the second direction dichloromethane methanol (9 1) Reproduced from ref 84 with permission

compounds studied, haematoporphyrin was the only porphyrin with two hydroxyl groups and it was retained the most on the silica plate (Fig 9)

Saitoh *et al* [90] achieved the separation of various metal tetraphenylporphyrin chelates on a reversed-phase plate. The first development was carried out with acetone propylene carbonate (20·80) and the second with acetone alone Retention may be related to the ratio of the central metal electronegativity to its ionic radius, $En./_{r_i}$[91] Larger $En./_{r_i}$ values correspond to more delocalized chelate–metal bonds and smaller R_f values on the reversed-phase plate. In addition, chromatographic behaviour is affected by the configuration of each complex, whether planar, pyramidal or distorted octahedral, and its stability in each of the eluents used

Anthocyanidins were resolved on cellulose by Mullick[92] A methanol acid-water solvent mixture did not differentiate between the methoxy and hydroxy group substituents on the anthocyanidin aromatic ring. A formic acid–hydrochloric acid or

TABLE 6

SEPARATION OF PIGMENTS

Column headings as in Table 1

N	*Compounds*	*Stationary phase*	*SSP (cm)*	*Solvent 1*	*t/L*	*Dryuing*	*Solvent 2*	*t/L*	*Ref*
14	Xanthophylls	(1) MgO–Celite (1 4) stripe (2) Si gel G $Ca(OH)_2$ (1 6) layer	2 5 cm from end of MgO–Celite stripe	On (1) hexane acetone (7·3) + ethoxyquin	40 min	Dry dark, 1–2 min, N_2	On (2) 2% butanol in benzene + ethoxyquin	1 h 20 min	86 (Fig 8)
17	Carotenes, xanthophylls (seeds)	Si gel G, 0 35 mm, 100°C, 30 min		Chloroform–ethyl acetate (40 10)			Diethyl ether		87
7	Carotene, xanthophylls, chlorophyll	Si gel sheets, Eastman K 301 R, 15 × 15 cm		Benzene–acetone (3 1)	Top		Isooctane–acetone–diethyl ether (3 1 1)	Top	88
7	Carotene, xanthophylls, chlorophyll	Cellulose sheets		1% *n*-propanol in light petroleum			Light petroleum benzene–chloroform–acetone–isopropanol (50 35 10 5 0 17)		88
11	Porphyrin methyl esters	Si gel, Camag D 5, 20 × 20 cm, 0 3 mm, dry at 110°C, 18 h exposure to air	3	Carbon tetrachloride dichloromethane–methyl acetate methyl propionate (2·2·1·1)	Top		(a) Benzene butanone (40·3) (b) Chloroform kerosene ethanol (200.100.17)	Top Top	89 (Fig 9)
7	Metal tetraphenylporphyrin chelates	RP-18 F 254s (Merck), 10 × 10 cm, 0 2 mm, 1 h at 110°C, cool in desiccator	1 5	Acetone–propylene carbonate (20 80) (2 ×)	7 5 cm		Acetone	7 5 cm	90
6	Anthocyanidins	Microcrystalline cellulose, Avicel SF, 20 × 20 cm, 0 25 mm		Formic acid–4 *N* HCl (2.1)	4 5 h		Methanol–conc HCl–water (190 1 10)	2 h	92
6	Anthocyanidins	Microcrystalline cellulose, Avicel SF, 20 × 20 cm, 0 25 mm		Methanol conc HCl–water (190·1 10)	2 h		*n*-Amyl alcohol acetic acid–water (2 1 1)	8 h	92
29	Tannin A or B constituents	Silica (Merck) GF 254, 110°C, 30 min		*n*-Butanol–acetic acid–water (4 1 5)	2 h, 15 cm		*n*-Butanol–ethanol–ammonia water (75 10·15·10)	1 5 h, 15 cm	93

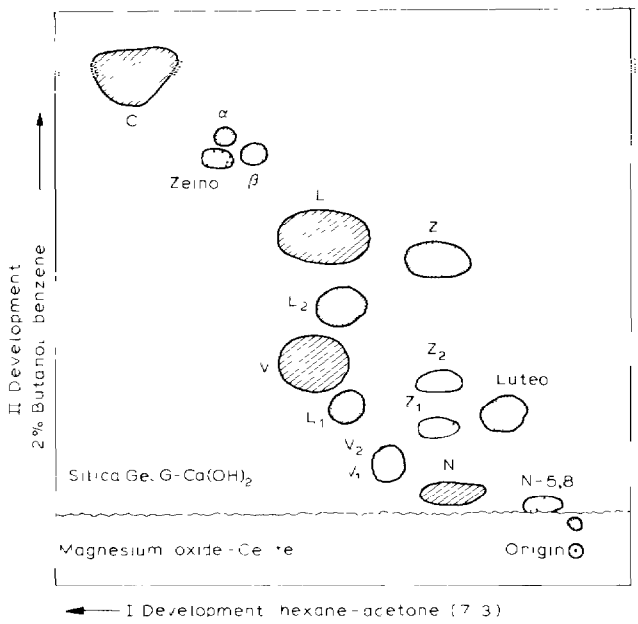


Fig 8 Two-dimensional, two-adsorbent TLC of a carotenoid mixture on a magnesium oxide Celite + silica gel G $Ca(OH)_2$ plate C = Carotenes, α = α-cryptoxanthin, β = β-cryptoxanthin, Zeino = zeinoxanthin, L = lutein, Z = zeaxanthin, L_1 and L_2 = lutein *cis*-isomers (corresponding to "neo-A" or "V" and "neo-B" or "U", respectively), V = violaxanthin, V_1 and V_2 = violaxanthin *cis*-isomers, Z_1 and Z_2 = zeaxanthin *cis*-isomers, Luteo = luteoxanthin, N = neoxanthin, N-5,8 = neoxanthin 5,8-epoxide The first elution is on the magnesium oxide Celite strip using hexane-acetone (7 3) and the second is on silica gel G-$Ca(OH)_2$ with 2% butanol in benzene Reproduced from ref 86 with permission

amyl alcohol–acetic acid water eluent used orthogonally did provide such a differentiation, and the retention increased with the number of hydroxyl substituents on the solute phenyl ring.

Finally, 29 intermediates in the synthesis of tannin A or B were separated on silica using an inorganic–acidic solvent mixture in one direction and an organic–basic eluent in the other. The migration sequence under alkaline conditions corresponded to different degrees of solute condensation or methylation whereas that under acidic conditions depended on the nature of the product[93]

4 3 Alkaloids

Heterogeneous nitrogenous substances produced by plants have been grouped under this heading. The two-dimensional chromatography of alkaloids pertaining to the cocaine, harmane, ergot and other families is considered here (Table 7)

Early attempts to separate opium[94], *Strychnos* and curare[95] alkaloids by two-directional paper chromatography were time consuming; development in one direction alone could require as long as 17 h

TLC enabled faster analyses to be achieved. Using a silica gel plate, Munier and Meunier[96] separated seven cocaine alkaloids Elution with a non-aqueous neutral mobile phase was followed by an orthogonal development in an acidic solvent

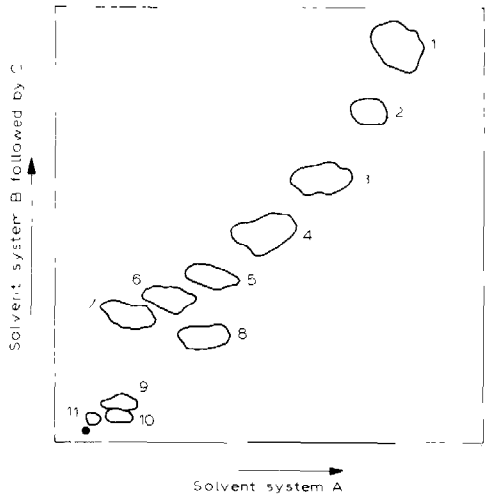
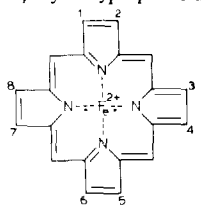


Fig 9 Separation of a mixture of porphyrin methyl esters on silica by two-dimensional TLC 1 = Mesoporphyrin IX, 2 = harderoporphyrin, 3 = coproporphyrin, 4,5,6 = penta-, hexa- and heptacarboxylic porphyrins, respectively, 7 = uroporphyrin, 8 = mono-β-hydroxypropionic monopropionic deuteroporphyrin IX, 9 = bis-β-hydroxypropionic deuteroporphyrin IX, 10 = haematoporphyrin IX, II = isohaematoporphyrin IX (all as their methyl esters) Basic structure



No	*Compound*	*Substituent in position*							
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	Mesoporphyrin	CH_3	C_2H_5	CH_3	C_2H_5	CH_3	$(CH_2)_2COOH$	$(CH_2)_2COOH$	CH_3
3	Coproporphyrin	CH_3	$(CH_2)_2COOH$	CH_3	$(CH_2)_2COOH$	CH_3	$(CH_2)_2COOH$	$(CH_2)_2COOH$	CH_3
7	*Uroporphyrin*	CH_2COOH	$(CH_2)_2COOH$	CH_2COOH	$(CH_2)_2COOH$	CH_2COOH	$(CH_2)_2COOH$	$(CH_2)_2COOH$	CH_3
10	Haematoporphyrin	CH_3	$CH(OH)CH_3$	CH_3	$CH(OH)CH_3$	CH_3	$(CH_2)_2COOH$	$(CH_2)_2COOH$	CH_3

Solvent system for the first direction (A) carbon tetrachloride–dichloromethane methyl acetate methyl propionate (2 2 1 1) Solvent systems for the second direction· (B) benzene–butanone (40 3) followed by (C) chloroform kerosene methanol (200 100 7) Reproduced from ref 89 with permission

TABLE 7

SEPARATION OF ALKALOIDS

Column headings as in Table 1

N	*Compounds*	*Stationary phase*	*SSP (cm)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref*
11	Major opium alkaloids	Acid-buffered Whatman paper No 1 immersed in 2% ammonium sulphate, 28 × 32 cm, 0 3 mm	2 5	Isobutanol glacial acetic acid (100·20) water to saturation	16-17 h		90 ml of diethyl ether saturated with aq. phase (water or 0 1 *M* acetic acid)	2 h	94
7	Cocaine alkaloids (similar polarity and structure)	Si gel 60 (Merck), 20 × 20 cm, 0 25 mm	2 5	Methanol–carbon tetrachloride (50 50)	155 min		Acetone–acetic acid–water (72.8·20)	180 min	96
5	Alkaloids of unequal polarity and structure, morphine, codeine, thebaine, etc	Si gel 60 (Merck), 20 × 20 cm, 0 25 mm	2 5	Ethyl acetate 2-nitropropane (50 50)	90 min		Methanol carbon tetrachloride (50 50)	138 min	96
19	Morphine, codeine and derivatives	Activated Si gel G, 20 × 20 cm, 0 25 mm	1 5	Methanol chloroform–ammonia (85 15 0 7)	15 cm	Air, 10 min	Hydrated diethyl ether acetone–diethylamine (85:8:7)	15 cm	97 (Fig 10)
13	Harmane alkaloids	Si gel 60 (Merck), 20 × 20 cm, 0 25 mm	2 5	Methyl ethyl ketone	98 min		Chloroform–methanol acetic acid (64 16 20)	185 min	96
15	Alkaloids from *Rauwolfia corynantha*, pseudocinchona	Si gel 60 (Merck), 20 × 20 cm, 0 25 mm		Diisopropyl ether methanol (85 15)	101 min	Dark, 30 min, 220°C	Acetone–acetic acid (90 10)	74 min	98
37	Ergot alkaloids	Si gel G, 20 × 20 cm, 0 25 mm, 110°C, 30 min	1 5	Methanol–chloroform (2 8)	15 cm	Air, 5 min	Diethylamine–chloroform (1 9)	15 cm	99
10	Ergot alkaloids	Si gel G in high-voltage electrophoresis apparatus, 20 × 20 cm, 0 25 mm	5 cm from anode, 4 cm from bottom edge	Acetic acid–pyridine buffer (pH 5 6), 1500 V, 45 mA	45 min	Fan dry, few minutes	Diethylamine chloroform (1 9)		99 (Fig. 11)

containing 20% of water. Retention involved adsorption under the former conditions and a mixed partition–adsorption mechanism under the latter. The mobility of each cocaine alkaloid was approximately inversely proportional to the number of functional groups that can be adsorbed simultaneously on the silica gel, and also to the strength of adsorption of each group Ecgonine, for instance, the only alkaloid in the mixture assayed to possess two strongly adsorptive groups (hydroxyl and carbonyl), on the same side of the six-membered ring, also exhibited the smallest R_F in both directions Pseudococaine (2α-carbomethoxy-3β-benzoxytropane), with its adsorptive functionalities in different planes although on the same side of the ring, was less retained than cocaine (2β-carbomethoxy-3β-benzoxytropane), where both substituents are co-planar in the β-position.

Munier and Meunier[96] also achieved the separation of morphine, codeine, thebaine, narcotine and papaverine, alkaloids of different polarity and structure, on a silica gel plate developed with a weakly polar solvent in one direction and a more polar one in the other Earlier work by Viala and Estadieu[97] involved the resolution of morphine, codeine and their derivatives by chromatography on activated silica. The first elution with methanol–chloroform–ammonia resulted in three spots, the most retained containing both morphine and codeine, the next corresponding to

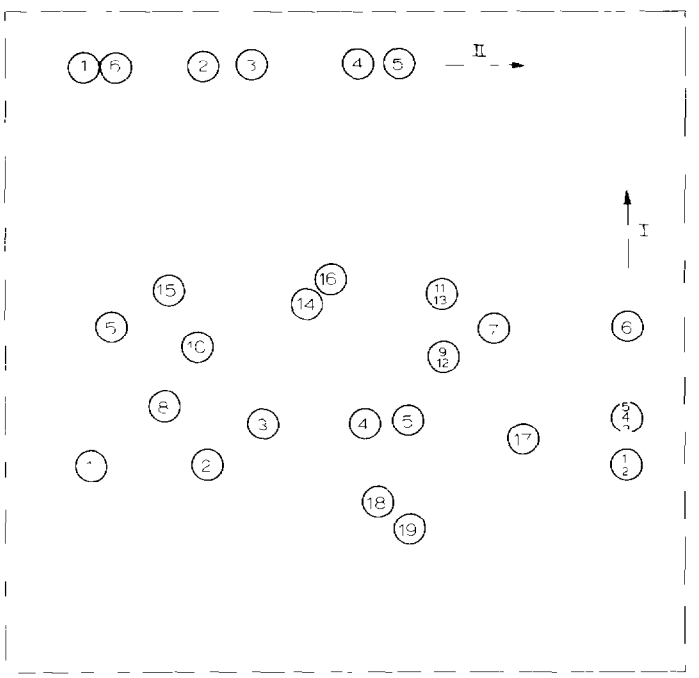


Fig 10 Two-dimensional separation of morphine, codeine and their derivatives on silica 1 = Morphine, 2 = codeine, 3 = O^6-monoacetylmorphine, 4 = diacetylmorphine, 5 = acetylcodeine, 6 = nalorphine, 7 = cocaine, 8 = quinine, 9 = nicotine, 10 = cotinine, 11 = methaqualone; 12 = diphenhydramine, 13 = mecloqualone, 14 = hydrochlorobenzethylamine, 15 = N-dimethyldiazepam, 16 = oxazepam, 17 = methadone, 18 = amphetamine, 19 = methylamphetamine Solvent in the first direction methanol chloroform ammonia (85 15·0 7), and in the second direction hydrated diethyl ether acetone diethylamine (85 8 7) Reproduced from ref 97 with permission

heroin and the least retained to O^6-monoacetylmorphine and acetylcodeine. Orthogonal development in a mixture of hydrated diethyl ether, acetone and diethylamine differentiated all solutes (Fig. 10).

Harmane alkaloids[96] and alkaloids from *Rauwolfia, Corynantha* and *Pseudocinchona*[98] were resolved on silica using a polar neutral mobile phase in the first direction and a more polar, acidic solvent mixture orthogonally. Other than the nature of the solvent itself, which selectively affects the retention order, the pH of the eluent, which determines the ionization state of the molecule as a function of its pK_a value(s), also influences the retention behaviour. Whereas the first development of the silica plate in a neutral solvent did not resolve harmane from norharmane[96], the subsequent development in the acidic eluent provided such a differentiation; nor-

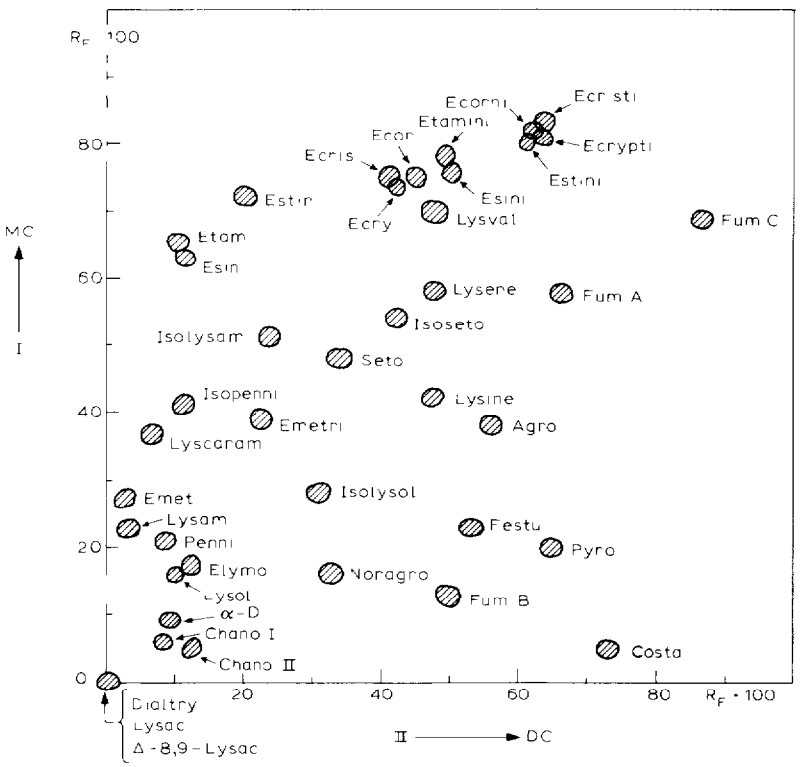


Fig 11 Two-dimensional chromatogram of ergot alkaloids separated on silica gel G Solvents: in direction I, MC = methanol chloroform (2 8), in direction II, DC = diethylamine chloroform (1 9) Agro = agroclavine, Chano I = Chanoclavine-(I); Chano II = chanoclavine-(II), Costa = costaclavine, α-Di = α-dihydrolysergol, Dialtry = DL-4-dimethylallyltryptophan, Elymo = elymoclavine, Ecor = Ergocornine, Econi = ergocorninine, Ecris = ergocristine, Ecristi = ergocristinine, Ecry = ergocrytpine, Ecrypti = ergocryptinine, Emet = ergometrine, Emetri = ergometrinine, Esin = ergosine, Esini = ergosinine, Estin = ergostine, Estini = ergostinine, Etam = ergotamine, Etamini = ergotaminine, Festu = festuclavine, Fum A = fumigaclavine A, Fum B = fumigaclavine B, Fum C = fumagaclavine C, Δ-8,9-Lysac = 6-methyl-$\Delta^{8,9}$-ergolene-8-carboxylic acid, Lysene = lysergene, Lysac = D-lysergic acid, Lysam = D-lysergic acid amide, Isolysam = D-isolysergic acid amide, Lyscaram = D-lysergic acid methylcarbinolamide, Lysine = Lysergine, Lysol = lysergol, Isolysol = isolysergol, Lysval = D-lysergyl-L-valine-methylester, Noragro = noragroclavine; Penni = penniclavine, Isopenni = isopenniclavine, Pyro = pyroclavine, Seto = setoclavine, Isoseto = isosetoclavine Reproduced from ref 99 with permission

TABLE 8

SEPARATION OF AMINO ACIDS

Column headings as in Table 1

N	*Compounds*	*Stationary phase*	*SSP (cm)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref*
13	Cystine, serine, glycine, threonine, alanine, lycosine, valine, phenylalanine, arginine, etc.	Filter-paper sheet, 18 × 22 in		Collidine	48 72 h		Water-sat phenol, in atmosphere containing NH_3		3,100
19	Amino acids	Zeokarb 225 (WR 1 5 2.0) equilibrated with 0 2 *M* (pH 3 1) buffer		0 2 *M* buffer (pH 3 1)	1 day		*m*-Cresol 0 3% ammonia, + over ammonia vapor		102 (Fig 12)
17	Amino acids	Whatman No 3 MM filter-paper, 7 × 6 in	1 5 cm from long paper edge, 4 5 cm from anode	Glacial acetic–formic acid buffer (pH 1 85), 20 kV, 100 mA	7 min	Dry, air, 40°C	2,4/2,5-Lutidine–ethanol water (11 4 5)	2 5 h	103
18	Amino acids	Whatman No 3 MM filter paper, 48 × 48 cm		Glacial acetic acid formic acid, 21 kV (pH 1 85)	70 min	Warm air	*n*-Butanol–acetic acid water (4·1·5)	16 h	104
20	Amino acids (protein hydrolysate)	Microcrystalline cellulose, Avicel, 20 × 20 cm, 0 5 mm, room temp , 24 h	1 5	1-Butanol acetic acid- water (3 1.1)	12 cm, 1 5 h	Air dry, room temp , 6 h	Phenol water (3.1)	12 cm, 2 h	105
12	Amino acids (protein hydrolysate)	Cellulose ECS 6064, 10 × 10 cm, 100°C, 30 min, cool, wash with solvent 1, dry, room temp.	1 5	Methanol-water–pyridine (4:1.2)	25–30 min	Air, ambient temp	Ethanol 1-butanol–water–propionic acid (5 5 2 5·1)	40 45 min	106

17	Amino acids (urine)	Cellulose + 2 cm ion exchange zone (desalting described)	On ion exchange zone	Pyridine–dioxane NH_3–H_2O (35 35 15.15) (2 ×)	90 min		*n*-Butanol acetone acetic acid water (35 35 10 20) (2 ×)	60 min	107
26	Polar + non-polar, natural + modified	Cellulose, short fibres, 20 × 20 cm, 0 25 cm		Propanol formic acid–water (75·5·20)	~20 cm		Methyl ethyl ketone 2-methylpyridine formic acid water (31.47 2 20)	16 cm	110 (Fig 13)
13	Bacterial cell-wall amino acids	Cellulose (Merck), 20 × 20 cm	1 5	Isopropanol acetic acid water (75 10 15)	3 h, 15.5 cm	Overnight, room temp , or rapid, 105°C	Methanol pyridine 10 *N* HCl water (64·8·2·14)	1.25 h, 15 5 cm	111
10	Amino acids	Si gel G (Merck), 20 × 20 cm, 0.35 mm		*n*-Propanol 34% ammonia (67 33)	18 cm	60°C, 30 min	0.2% ninhydrin in *n*-propanol–water glacial acetic acid (64 36 20)	18 cm	112
22	(Not all resolved) amino acids	Silufol (Kavalier), 15 × 15 cm	2	Ethyl acetate–isopropanol–water–ammonia (20 20 25 1 5)	13 cm	Air, 10 min, 110°C, 10 min, air, 10 min	Acetone water acetic acid–formic acid (50 15 12 3)	13 cm	113
36	Amino acids	Cellulose, 20 × 20 cm	2.5	*n*-Butanol acetone diethylamine water (10 10.2.5) (pH 12)	Top		Isopropanol–formic acid water (40 2 10) (pH 2 5)		115
13	Amino acids	Kieselgel G, 20 × 20 cm		2 *N* acetic acid 0 6 *N* formic acid buffer (1 1), pH 2, 460 V, 12.6 mA	1 h		*n*-Butanol glacial acetic acid–water (3.1.1)		114

harmane, with the larger pK_a value, exhibited a higher chromatographic mobility.

Finally, ergot alkaloids were separated on activated silica gel after elution with chloroform–methanol in one direction, followed by an organic solvent diethylamine in the orthogonal direction (Fig 11) The strong diethylamine base helped to desorb the alkaloids by competing for adsorption on to silanols[99]. Agurell[99] also resolved nine ergot alkaloids on silica by combining thin-layer electrophoresis in one direction with chromatography in the other.

4.4. Proteins and their constituents

The two-dimensional chromatographic assays of amino acids, peptides and proteins, important morphological and physiological cellular components, are discussed here. Conditions for their separation are given in Tables 8–10.

4.4.1. Amino acids (Table 8)

Early reports by Consden *et al.*[3] and Martin and Synge[100] described the bidirectional separation of several amino acids on paper The chromatographic behaviour of the solutes was found to be a function of their partition between the mobile phase and the water-saturated cellulose The analysis time was extremely long, one elution alone requiring 2–3 days. After the pioneering studies of Cohn on ion exchange[101], slightly faster separations of amino acids were achieved on sulphonated polystyrene resin-loaded paper, for instance[102]. In the assay reported by Knight[102], the first eluent was a 0.2 *M* buffer at a pH of 3.1 and cation exchange determined the elution order: compounds such as lysine, arginine, ornithine, histidine, tryptopan, cysteine, tyrosine and phenylalanine, which contain two amine groups, each proton-

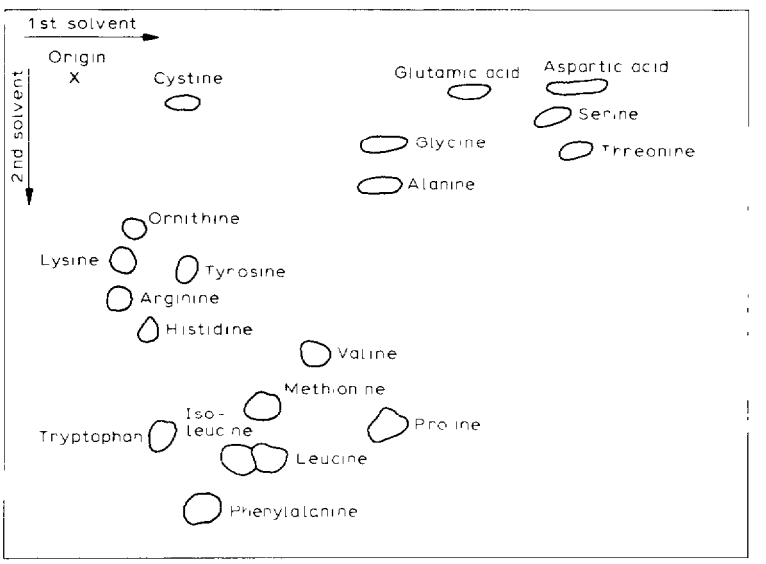


Fig 12 Two-dimensional separation of amino acids on cellulose Solvent used for the first development, 0 2 *M* buffer (pH 3 1), and for the second development, 0 3% ammonia in *m*-cresol Reproduced from ref 102 with permission

ated to a certain extent, were retained the most In the second direction, the cation-exchange phenomenon was suppressed by the eluent, which consisted of 0.3% ammonia in *m*-cresol. The amino acids were chromatographed in their anionic form Aspartic acid and glutamic acid, solutes with two negative charges, had the smallest R_F values (Fig 12). Nineteen proteinic amino acids were resolved.

Still faster amino acid separations on paper were achieved by electrophoresis followed by chromatography with a hydro-organic eluent orthogonally[103,104].

The development of short-fibre cellulose powder for TLC purposes facilitated the chromatography of amino acids and allowed rapid routine analytical work. In all assays reported on cellulose, the mobile phases in both directions were water–organic mixtures[105–111]. Munier *et al.*[109,110] achieved the separation of 22 polar and non-polar amino acids, among which certain pairs that were previously unresolved such as leucine isoleucine, arginine–lysine and serine–glycine. A high degree of resolution is afforded owing to differences between alcohol–solute interactions in the first direction and ketone–solute and tertiary amine–solute interactions in the second, and to the ionic equilibria created by using 2-methylpyridine together with formic acid in the second eluent. The water content of the mobile phase, by affecting solute partition, plays a determining role in these assays (Fig. 13). Munier *et al.*[110] found a 20% water content in both directions to be optimal. Smaller percentages did not ensure the elution of the most polar amino acids and higher percentages led to decreased resolution.

Kraffczyk *et al.*[107] included a 2 cm wide layer of a strongly acidic ion exchanger at the bottom of the cellulose plate for the desalting of urine samples when analysing

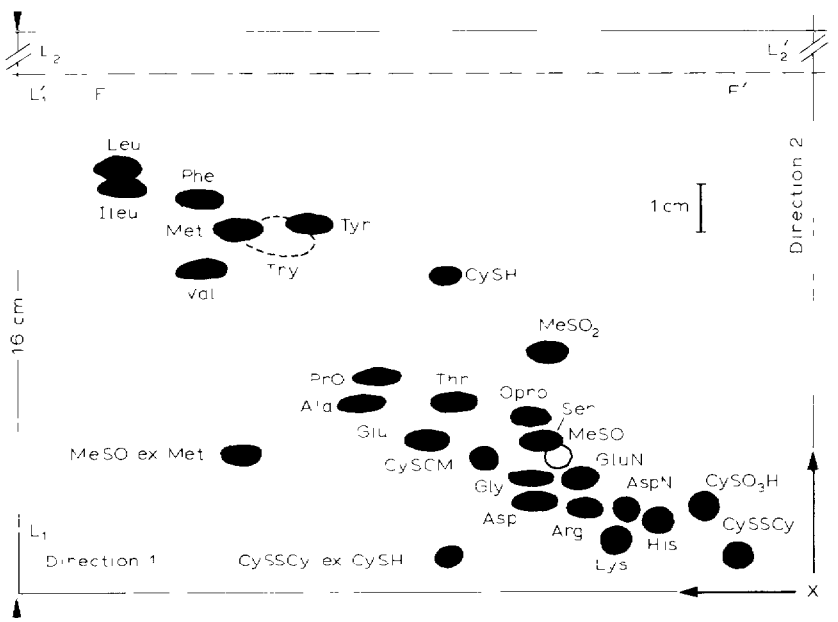


Fig 13 Two-dimensional separation of amino acids on short-fibre cellulose. Solvent used in the first direction, propanol formic acid–water (75 5 20), and in the second direction, methyl ethyl ketone–2-methylpyridine–formic acid–water (31 47.2 20) Reproduced from ref 110 with permission

TABLE 9

SEPARATION OF Dns- AND DNP-AMINO ACIDS

Column headings as in Table 1

N	*Compounds*	*Stationary phase*	*SSP (cm)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref*
18	Dns-amino acids	Polyamide sheet, 15 × 15 cm		Water 90% formic acid (200 3)			Benzene acetic acid (9 1)		117
22	Dns-amino acids (renal tubular fluid sample)	Polyamide TLC, Schleicher & Schull, 3 × 3 cm		4% Formic acid			(a) Benzene glacial acetic acid (8 2) (b) Ethyl acetate methanol glacial acetic acid (20.1.1)		118 (Fig. 14)
39	Dns-amino acids + others (serum and urine samples)	Micropolyamide plates, F1700, Schleicher + Schull, 4.2 × 4 2 cm	0 5	Formic acid water (1.5 98 5)	5 min	Dry, hot air, cool to room temp	Benzene–acetic acid (4.5 1)	5 min	119
19	Dns-amino acids	Kieselgel G, 0 25 mm		Ethyl acetate chloroform ethanol acetic acid (70 45 4 4)			Chloroform acetic acid–water (50·45·5)		120
19	Dns-amino acids	HPTLC Fertigplatten RP-8 F 254, 10 × 10 cm		*n*-Heptane ethyl acetate acetic acid (65 33·2)	7 cm		Methanol 0 01 *M* Na_2HPO_4 (75 25)	7 cm	121
20	DNP-amino acids	RP-18		Hexane–ethyl acetate acetic acid (80 18.2)	6 cm		1 *M* ammonia + 3% KCl in 60% methanol	6 cm	34 (Fig 2)
17	DNP-amino acids	Polyamide, 15 × 15 cm, 100°C, 15 min	2.0	*n*-Butanol–glacial acetic acid (90 10)	10 cm, 200 min	Dry, hot air, 15 min	90% formic acid water (50 50)	11 cm, 60 min	122
18 + 23	DNP- + Dns-amino acids	Cellulose powder, Macherey, Nagel & Co , 30 × 20 cm, 0 11 mm		Toluene–ethylene glycol chlorhydrin–pyridine 0 8 *M* ammonia (150·90:45.90) + a few drops of *n*-octanol	30 cm		Water water satd with ammonium sulphate sodium dodecyl sulphate (700 ml.100 ml 567 mg)	20 cm	124

for urinary amino acids. Other separations on silica gel were reported[112,113]. An organic–basic eluent was used in the first direction followed by an organic–acidic mixture in the second Solute retention depended on the types of functional groups and their degree of ionization. Ionophoresis followed by chromatography on silica gel has also been applied to the separation of amino acids[114].

4.4 2 Dimethylaminonaphthalenesulphonyl (Dns) and dinitrophenyl (DNP) amino acids (Table 9)

Seiler[116] has reviewed the separations of various Dns-derivatives, among which Dns-amino acids Two-directional TLC has been achieved on a polyamide stationary phase[117–119] (Fig. 14) and has recently been applied to the identification of human acidopathies in serum and urine[119] Although Dns-amino acids have also been analysed on silica[120], assays on polyamide are preferred as they are less time consuming, do not result in spot tailing and do not require previous deionization of urine samples[119] An aqueous eluent is generally used in the first direction followed by an organic eluent in the second. This change in the nature of the mobile phase causes alterations in the sorbent properties such that the mechanisms for solute retention are different in each direction. Polyamide is thought to act as a reversed phase in the presence of the aqueous eluent, and it behaves as a hydrophilic sorbent there-

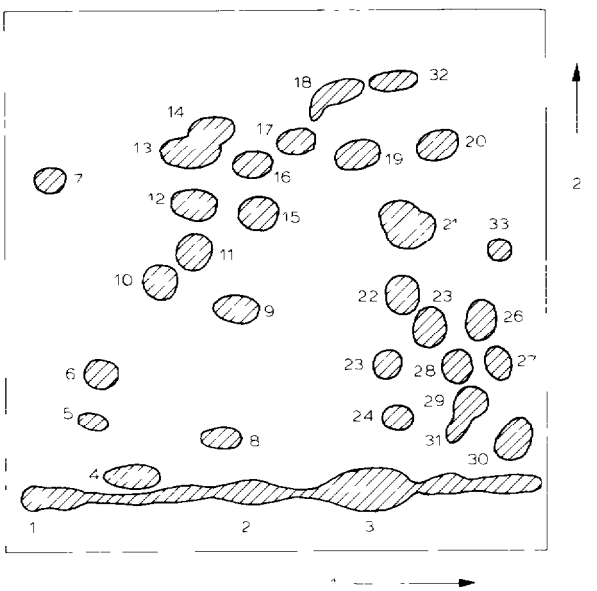


Fig 14 Two-dimensional chromatogram of some Dns-amino acids on polyamide after autoradiography 1 = Starting point, 2 = taurine, 3 = Dns-OH, 4 = cystine, 5 = *n*-serotonin, 6 = tryptophan, 7 = bis-tyrosine, 8 = *n*-tyrosine, 9 = ornithine, 10 = bis-lysine, 11 = phenylglycine; 12 = phenylalanine, 13 = leucine and histidine, 14 = isoleucine, 15 = methionine, 16 = norvaline, 17 = valine, 18 = proline, 19 = GABA, 20 = δ-aminolevulinic acid; 21 = alanine and Dns-NH_2, 22 = glycine, 23 = glutamic acid, 24 = aspartic acid, 25 = hydroxyproline, 26 = glutamine, 27 = asparagine, 28 = threonine, 29 = serine, 30 = arginine, ε-lysine and α-lysine, 31 33 = unknown dansylation products Solvent used in the first direction, 4% formic acid, and in the second direction, benzene glacial acetic acid (8 2) followed by ethyl acetate methanol glacial acetic acid (20 1 1) Reproduced from ref 118 with permission.

TABLE 10

SEPARATION OF PEPTIDES

Column headings as in Table 1

N	*Compounds*	*Stationary phase*	*Solvent 1*	*t/L*	*Solvent 2*	*t/L*	*Ref*
11	Peptides	Cellulose MN 300, 20 × 20 cm, 0.25 mm	*tert*-Amyl alcohol–eucalyptol 2-chloroethanol pyridine 98% formic acid (150 150 200 280 43 200)	Top, 5 h	*n*-Amyl alcohol pyridine H_2O (4 4 2)	5 h	125
8	Oligopeptides	Cellulose MN 300, 0 25 mm	Isoamyl alco-hol ethanol glacial acetic acid pyridine water (17 5·5·1 3·17 5·15) (pH 5 75)		Isopropanol ethanol–ammonia (20.20:15) (pH 12 3)		126
19	Peptides	Cellulose MN 300, 40 × 20 cm, 0.25 mm	Electrophoresis, 1 04 *N* formic acid, 427 V/40 cm, 8 mA/20 cm × 0 25 mm	3.5 h	*n*-Butanol pyridine 98% formic acid–water (479 278 43 200)	To top, 3 h	125
19	Peptides	Cellulose MN 30, 40 × 40 cm, 0 25 mm	Pyridine ethanol water (4·4·2)	Top, 4 h	Electrophoresis, 0 025 *M* aq borax soln., 380 V/ 40 cm, 4 5 mA/20 cm × 0 25 mm	4 h	125
	Polypeptides/proteins with acidic or neutral p*I*	(1) Tube gel, polyacrylamide	(1) 9 *M* urea, Nonidet P-40, pH 3 5 10 Ampholines, 400–700 V	20 h	(2) Electrophoresis in presence of sodium dodecyl sulphate	3 h	128, 129
		(2) Slab gel, acrylamide gradient					
	Basic proteins	(1) Tube gel, polyacrylamide	(1) Dipalmitoyl-L-α-phosphatidylcholine + urea, pH 3 5–10 Ampholines, 800 V	2 6 h	(2) Unchanged, 3200 V/h		133
		(2) Slab gel, acrylamide gradient					

after[121]. Thus, whereas the R_F values for arginine and aspartic acid are relatively large in the first direction, they are much smaller in the second[117–119].

Exploiting similar solvent changes with a reversed-phase plate, Macek *et al.*[121] succeeded in resolving 19 Dns-amino acids. The RP-8 TLC plate behaved as a normal phase in presence of the non-aqueous eluent used in the first direction and as a true reversed phase when developed with an aqueous solvent in the second direction. Exact reversal of the retention order of the Dns-glycine, -alanine, -valine and -leucine could be observed in the second direction with respect to the first elution. In addition, the aqueous eluent was basic enough to reduce the amino group ionization and hence to increase solute retention on the reversed-phase plate

DNP-amino acids were separated under similar conditions on an RP-18 thin--layer plate[34]. It is interesting that the addition of a salt to the non-aqueous solvent did not modify the solute behaviour, whereas it increased the retention when present in the aqueous eluent, a true expression of reversed-phase solvophobic behaviour. Separations of DNP-amino acids were also accomplished on polyamide[122] and cellulose[123,124] by development with an organic solvent in the first direction and an aqueous solvent orthogonally. The efficiency of the chromatographic system required judicious selection of the mobile phase components Munier and Drapier[124] included a few drops of *n*-octanol in the first eluent in order to suppress the chromatography of the solvent itself In addition, as impregnation of the plate with the organic mobile phase caused irregularities in the perpendicular progression of the second aqueous solvent, spot tailing and diffusion, sodium dodecyl sulphate was added to this aqueous phase and helped to achieve small and round spots

4.4.3 Peptides and oligopeptides (Table 10)

Peptides[125] and oligopeptides[126] have been separated on cellulose with an acidic solvent in one direction and a basic solvent in the other In addition to the differences in solute ionization which originate from the use of eluents at different pH values, differences in molecular weight also affect the retention and the partition process In a given category of compounds, the dipeptides for instance, solutes with a large number of hydrophobic carbon centres, more soluble in the organic portion of the eluent, exhibit larger R_F values[126]

Other peptide assays on cellulose[125] have combined electrophoresis in one direction and chromatography with an organic–aqueous mobile phase in the other The electrophoresis step necessitated the use of binder-free cellulose, sufficiently large plates, a weak gradient potential and an adequate electrolyte solution. Peptides migrated according to their charge, the solute pK_a values and the pH of the electrolyte.

4 4.4 Proteins

Although not related to TLC, the high-resolution, two-dimensional electrophoretic method developed by O'Farrel[127] and later by Anderson and co-workers[128–136] should also be mentioned. Nearly 1000 spots were obtained from such an analysis of lymphocyte proteins using autoradiography (Fig 15) More sensitive detection techniques are expected to afford the resolution of about 5000 components.

In the first dimension, proteins or polypeptides were separated according to their charge by isoelectric focusing in a narrow gel tube. Next, the gel was extruded and positioned on a polyacrylamide gradient slab, and electrophoresis was performed

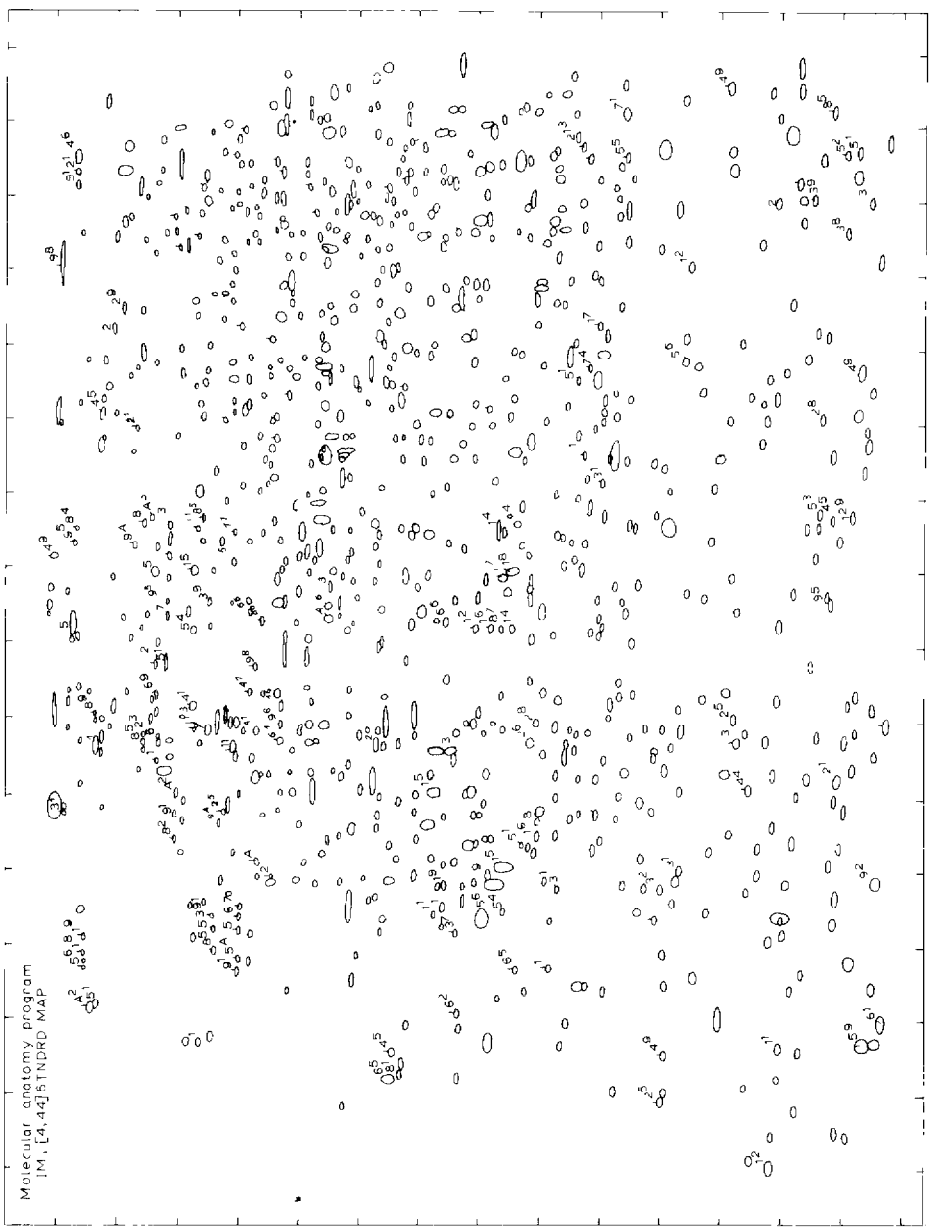


Fig. 15. Diagram showing the spots resulting from the two-dimensional gel electrophoresis of lymphocyte proteins Numbers are the key to information about the respective spots in the lymphocyte protein index data base developed by Anderson *et al* For further details see ref 48 Reproduced from ref 48 with permission

in the presence of sodium dodecyl sulphate. In this second dimension, compounds were resolved according to their molecular weight, as sodium dodecyl sulphate binds electrostatically to the solutes, thus masking charge differences (Table 10).

The method described was applied successfully to a study of the protein constituents of physiological fluids and tissues in normal and diseased individual [49,50, 129–131,134,135,137–140]

Because of the considerable number of proteins that can be separated in one analysis, data handling becomes very complex as huge files have to be stored and monitored. The corresponding software has been developed by Anderson *et al.*[48] and Lester *et al.*[49]. A discussion of the problems of spot detection, segmentation, integration and pairing is beyond the scope of this review

4.5. Carbohydrates (Table 11)

Sugars have been separated on cellulose[141] and Kieselgel G[142], developed with different water–organic solvents. Hotta and Kurokawa[143] modified a silica gel plate by dipping it in 0.2 *M* sodium dihydrogen phosphate solution, a procedure known to enhance sugar retention in proportion to the number of hydroxyl substituents[144]. This selective stationary phase was developed with an eluent containing 10% of water in the first direction and 25% in the second.

The effect of water on chromatographic mechanisms can be illustrated by the behaviour of rhamnose and fucose in each eluent. These sugars differ in the steric arrangement of their functional groups: rhamnose has one pair of *cis*-hydroxyl groups in a β-position with respect to a bulky methyl group, compared with two pairs of *cis*-hydroxyl groups in fucose, one β- and one α- to the methyl group. With the first eluent, the adsorption of rhamnose may be more sterically hindered than that of fucose and thus rhamnose migrates faster. These structural differences are no longer important with the second eluent, as solute adsorption on to silica is reduced owing to plate deactivation by water present in the mobile phase. The solutes then are believed to partition between the mobile and the modified stationary phases, which results in similar R_F values for rhamnose and fucose (Fig. 16).

Carbohydrates have also been separated on silica gel impregnated with sodium tetraborate, sodium tungstate[145] and boric acid[144]. In the first direction, a neutral solvent was used and sugars were retained according to their capacity to form borate complexes. In the second elution, the impregnating agent was completely deactivated by the acidic solvent. Solute partition was in effect and the heavier disaccharides were retained the longest. Phenylboronic and boric acids have more recently been used in the mobile phase to resolve carbohydrates on silica[146]. Other separations have been accomplished on cellulose developed in different mobile phases[147].

4.6 Glycopeptides

Moczar[148] reported the separation of some glycopeptides from a glycoprotein enzymatic digest on silica. Electrophoresis in presence of acetic acid–pyridine–water (10:1:89) (pH 3.8) was performed for 2–3 h at 10–20 V/cm on a 20 × 20 cm plate. This operation was followed by a continuous development with ethanol–nitromethane–acetic acid–water (5:3:3:3) for 18–30 h.

TABLE 11

SEPARATION OF CARBOHYDRATES

Column headings as in Table 1

N	*Compounds*	*Stationary phase*	*SSP (cm)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref*
12	Sugars	Cellulose MN 300 G, 110°C, 10 min	2	*n*-Butanol–acetone–diethylamine–water (10.10 2 5)		80°C, 30 min	Vapour phase equilibrated with 25% ammonia, phenol–water (3·1)		141
4	Man, Gal, Glc-NAc, Man, 1 6-Man, Gal-β1 4-Glc-NAc	Kieselgel G (Merck)		*n*-Butanol acetic acid-water (12 3 5)	16 h		*n*-Propanol-ethyl acetate–water (7 2 1)	6–8 h	142
8	NANA, Gal, Glc, Man, Gal-NAc, Glc-NAc, Fuc, Rham	Si gel F_{254} dipped in 0 2 *M* sodium dihydrogen phosphate, 110–120°C, 30 min, 20 × 20 cm, 0 25 mm		Butanol acetone-water (4 5 1) (2 ×)	15 cm		Phenol water (3 1) (2 ×)		143 (Fig. 16)
40	CHO_s including deoxy sugars and methylglycosides	Si gel (1) 4 2 cm, impregnated with sodium tetraborate boric acid sodium tungstate (2) 14 5 cm, impregnated with boric acid		On (1) methyl acetate-isopropanol-water (2 2 1)		550°C < 3 h	On (2) ethyl acetate acetic acid-methanol water (60 15 15 10)		144
16	CHO_s	Sodium tetraborate sodium tungstate impregnated silica, 20 × 20 cm, 0 3 mm, 90°C, 30 min		Ethyl acetate isopropanol–water (2 2·1)	2 h	70°C, 4–5 h	Ethyl acetate-acetic acid–methanol–water (60 15 15 10)	50 min	145
13	Sugars	Si gel 60 (Merck)		(a) Ethyl methyl ketone diisopropyl ether 2-propanol–pyridine water–phenylboronic acid (40 10 30·10·10·1 2)	10 cm		(b) Diisopropyl ether methanol–acetic acid–water (88 12.2 2) (c) Ethyl methyl ketone–2-propanol–acetonitrile 0 5 *M* boric acid + 0 25 *M* isopropylamine-acetic acid (40 30 20 15 0 4)	10 cm	146
15	Sugars	Cellulose, 20 × 20 cm, 0 1 mm		2-Propanol–90% formic acid–water (80 4 20)	5 6 h		Lutidine (2,6-dimethylpyridine) water (65.35)	6 h	147

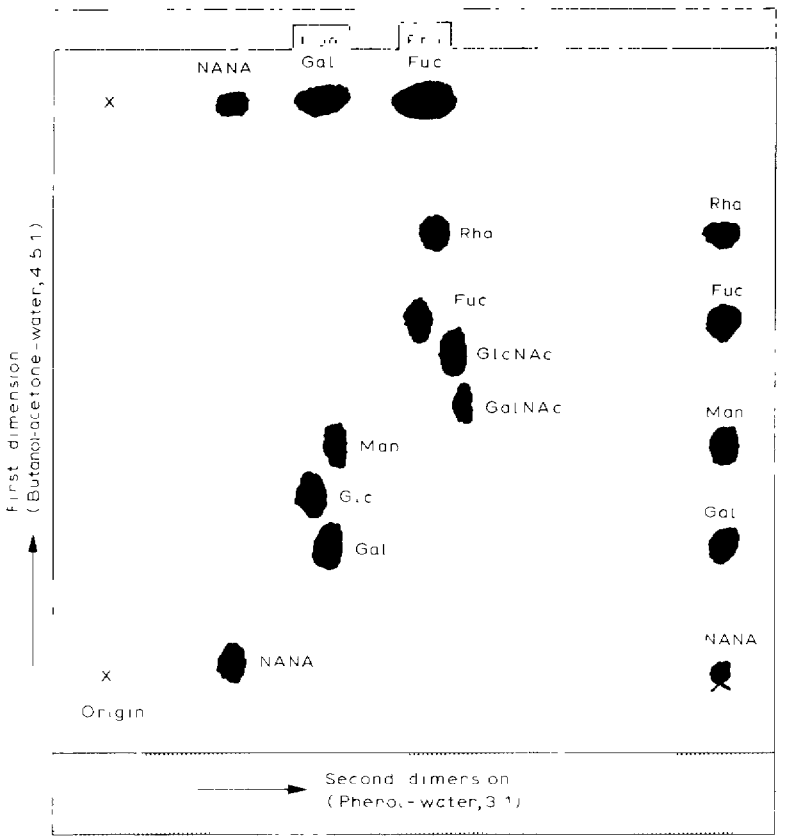


Fig 16 Two-dimensional TLC of sugar mixture on silica gel dipped in 0 2 *M* sodium dihydrogen phosphate Rha = rhamnose, Fuc = fucose, GlcNAc = acetylglucosamine, GalNAc = acetylgalactosamine, Man = mannose, Glc = glucose, Gal = galactose, NANA = N-acetylneuraminic acid First direction two successive developments in butanol-acetone water (4·5 1) Second direction two successive developments in phenol water (3 1) Reproduced from ref. 143 with permission

More recently, the soluble inhibitory factor (SIF), a molecule constituted of both protein and glycolipid moieties, has been isolated from serum and purified on a silica plate using chloroform–methanol–water in the proportions 60:38:8 in the first dimension and 50:50·5 in the second[149].

4.7. Nucleic acids and their constituents

In cells, nucleic acids are carriers of genetic information and their constituents, the free bases, nucleosides and nucleotides, actively participate in cellular metabolism Detailed conditions for the two-dimensional separation of these compounds are given in Tables 12 and 13.

4.7 1 Bases, nucleosides and nucleotides (Table 12)

Randerath and Randerath[150] achieved the first rapid, bi-directional separation of 23 ribonucleotides on PEI-cellulose. Several mono-, di- and trinucleotides were

TABLE 12

SEPARATION OF NUCLEOTIDES, NUCLEOSIDES AND BASES

Column headings as in Table 1.

N	*Compounds*	*Stationary phase*	*SSP (cm)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref.*
23	Ribonucleotides	PEI, cellulose, 20 × 20 cm, 0.5 mm		(a) 0.2 *M* LiCl (b) 1.0 *M* LiCl (c) 1.6 *M* LiCl	2 min 6 min 13 cm or 75 min	Air, < 50°C, wash LiCl in CH_3OH	(d) 0.5 *M* formate buffer (pH 3.4) (e) 2.0 *M* formate buffer (f) 4.0 *M* formate buffer	30 sec 2 min 15 cm or 60 min	150 (Fig. 17)
8	2′-, 3′-G, A, U, CMP	PEI, cellulose MN 300	3	1.0 or 0.1 *N* formic acid (pH 3.6)	10 15 cm	Air	1 *M* LiCl (pH 7.0)	8 cm	151
9	Adenine + pyridine phosphates	PEI, cellulose (Brinkmann) 20 × 20 cm, 0.1 mm	3	0.5 *M* formate buffer (pH 3.6)		Dry, wash in CH_3OH, dry	0.2 + 1.0 *M* LiCl		152
14	Phosphate esters	Cellulose MN 300, 20 × 17.5 cm, 0.25 mm		*n*-Propanol–ammonia–water (6:3:1) (2×)	3 h		*n*-Propyl acetate–90% formic acid–water (11:5:3) (2×) or electrophoresis with 0.28 *M* acetate buffer (pH 3.6), 1000 V, 35 mA	80 min 16 min	153
22	Nucleosides, nucleotides	Cellulose MN 300		Isobutyric acid conc. ammonia–water (57:4:39)			Methanol–1 *M* ammonium acetate (7:3) (2×)		154
8	2′-, 3′-G, A, U, CMP	Cellulose MN 300, 100°C, 30 min, 20 × 20 cm, 0.5 mm	3	*n*-Butyric acid ammonia–water (57:4:39)	5–6 h, 12–15 cm	Overnight in hood	Sat. ammonium sulphate–1 *M* sodium acetate–isopropanol (79:19:2)	3–4 h, top	155

8	5′-O-Methyl-Nucleotides	Cellulose		Isobutyric acid 0.5 *N* ammonia (5:3)			Isopropanol–conc. HCl water (17:15:15)		156
5	Bases: A, C, G, T, bromo-U	Cellulose (Eastman 6065), 3.5 × 20 cm, 20 × 20 cm (2 layers)	2	Isopropanol water-HCl (65:18.4:16.6)	14 cm 6 h		1 *M* disodium hydrogen phosphate 1.2 *M* ammonium sulphate isopropanol (123:74:3)	12 cm 1 h	36
37	Purine and pyrimidine nucleosides and bases (not all resolved) in urine	Cellulose DC-Alufolien, 10 × 10 cm, 0.1 mm	1.5	Isopropanol 5% ammonia (8:2) (2×)	90 min		Butanol acetic acid H_2O (8:2:2) (2 ×)		157
44	Bases, nucleosides, nucleotides (not all resolved)	Cellulose MN 300, 20 × 20 cm, 0.5 mm	3	*n*-Propanol–25% ammonia water (6:3:1)	3 h, 18 cm	Cold air, 20 min	Isopropanol sat. ammonium sulphate–water (2:79:19)	3 h	158 (Fig. 18)
19	Bases, nucleosides, nucleotides	Polygram CEL 400, UV 254 nm, 10 × 10 cm		(a) Methanol (b) Methanol isopropanol 25% (w/w) ammonia 1% (w/v) EDTA (3:9:2:6) (c) Solvent (b)	Middle Top		(d) Isobutyric acid 25% (w/w) ammonia 1% (w/v) EDTA (200:9:114)	Top	159
22	Methylated tRNA bases	Si–cellulose (40:60), 20 × 20 cm, 0.4 mm, desiccated	2-3	Ethyl acetate methanol–water 88% formic acid (100:25:20:1)	150 min	60 min	Acetonitrile ethyl acetate–2-propanol 1-butanol–58% ammonia–water (40:30:20:10:22:5)	150 min	162
13	Bases, nucleosides, nucleotides	Si gel (Merck) HPTLC F_{254}, 10 × 10 cm	2	1-Butanol acetone-acetic acid–5% ammonia water (10:5:2:3:2)	7 cm	Thorough drying	1-Propanol methanol ammonia water (10:1:5:2)		163

resolved, as well as some nucleotide sugars Increasing concentrations of lithium chloride solutions and formate buffer (pH 3.4) were used stepwise in the first and second directions, respectively Compared with elution at constant concentration, step-gradient elution resulted in sharper circular spots. Solutes were resolved according to their charge: the most negatively charged trinucleotide species migrated the least on the PEI anion exchanger (Fig 17). Similar conditions were later applied by Santini and Ulrich[151] to the separation of 2'- and 3'-adenine, guanine, uridine and cytosine monophosphates, and by Barton *et al*[152] to that of nine pyridine and adenine nucleotides

Various phosphate esters[153,154], 2'- and 3'-monophosphates[155], 2',O-methyl nucleotides[156], purine and pyrimidine bases[36] and nucleosides[157] have also been chromatographed on cellulose. The pH of the eluent, which affects the extent of solute dissociation, plays an important role in these assays. Thus, in most instances, development was accomplished with an acidic solvent in one direction and a basic solvent in the other

The use of a basic solvent has been coupled with that of a salt in order specifically to affect nucleotide retention[158,159]. A mixture of 44 bases, nucleosides, deoxynucleosides, 2'-, 3'- and 5'-nucleoside monophosphates, nucleoside di- and triphosphates and nucleotide sugars was resolved by Pataki[158] on cellulose. The eluents were *n*-propanol–25% ammonia–water (5 3·1) in the first direction and isopropanol–saturated ammonium sulphate–water (2·79·19) in the second. Triphosphates were retained the most with the first eluent The second buffer system enhanced their migration whereas it hardly affected the position of nucleosides and bases (Fig. 18) Randerath and Randerath[160] had previously observed these ionic strength effects on the chromatographic behaviour of nucleic acid constituents.

Using a silica gel–cellulose plate with acidic and basic solvents, Munns and co-workers[161,162] resolved 22 methylated tRNA purine and pyrimidine bases Solutes with primary amine groups such as adenine, guanine and cytosine exhibited lower R_F values in a formic acid than in an ammonia system These differences were found to arise from interactions with silica, the adsorption of the primary amines also being decreased by their methylation. At a basic pH, both the bases and the silica phase are negatively charged, which weakens adsorbent solute interactions.

Finally, Das[163] separated 13 bases, nucleosides and nucleotides on pure silica by varying the solvent pH and composition in the second direction

4 7.2. Oligonucleotides and nucleic acids (Table 13)

Miller and Burgess[164] resolved five oligonucleotides on PEI-cellulose by means of lithium chloride–EDTA solution at pH 6 5 in the first direction and at pH 2.0 in the other. The charges on both the phosphate and base moieties affected the separation Also by varying the pH of the eluent, Mirzabekov and Griffin[165] were able to separate 18 oligonucleotides on PEI-cellulose. The first direction involved successive developments with 1 4 and 1.8 *M* solutions of lithium formate at pH 3.4, the lower concentration ensured the resolution of smaller oligonucleotides and the higher one that of the larger solutes. The second eluent was 0.8 *M* lithium chloride solution in Tris base at pH 8.0 The chromatographic assay was carried out at 60°C for sharper spot formation. Migration in the first direction was according to charge and nucleotide composition whereas in the second direction it was more a function of molecular

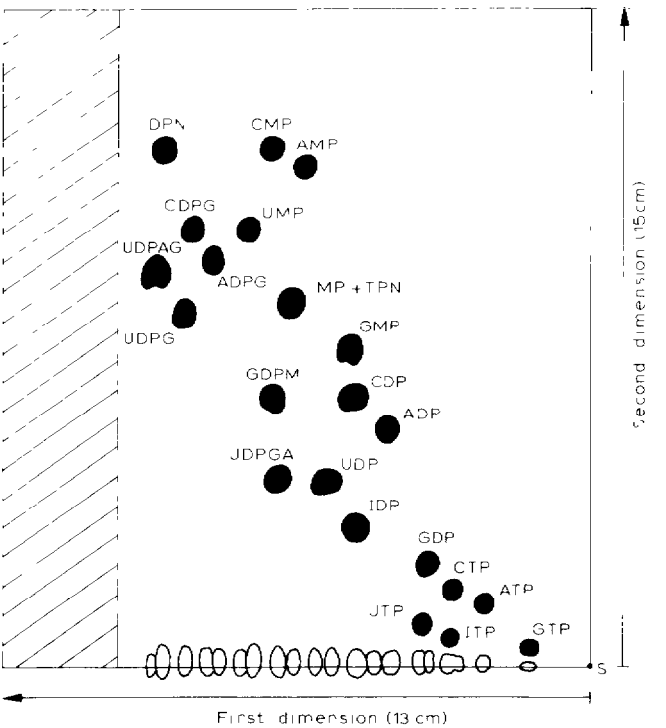


Fig 17 Two-dimensional anion-exchange separation of nucleotides on PEI-cellulose First dimension: successive developments in (a) 0 2 *M* LiCl, (b) 1 0 *M* LiCl, (c) 1 6 *M* LiCl Second dimension successive developments in (d) 0 5 *M* formate buffer (pH 3 4), (e) 2.0 *M* formate buffer, (f) 4 0 *M* formate buffer CMP = cytosine 5′-monophosphate, AMP = adenosine 5′-monophosphate, CDPG = cytidine diphosphate glucose, UMP = uridine 5′-monophosphate, UDPAG = uridine diphosphate-N-acetyl-glucosamine, ADPG = adenosine diphosphate glucose, IMP = inosine 5′-monophosphate, GMP = guanosine 5′-monophosphate, GDPM = guanosine diphosphate mannose, CDP = cytosine 5′-diphosphate, ADP = adenosine 5′-diphosphate, UDPGA = uridine diphosphate glucuronic acid, UDP = uridine 5′-diphosphate, IDP = inosine 5′-diphosphate, GDP = guanosine 5′-diphosphate, CTP = cytosine 5′-triphosphate, ATP = adenosine 5′-triphosphate, UTP = uridine 5′-triphosphate; ITP = inosine 5′-triphosphate, GTP = guanosine 5′-triphosphate, DPN = nicotinamide adenine dinucleotide, TPN = nicotinamide adenine dinucleotide phosphate Reproduced from ref 150 with permission

weight. More complex mixtures of half or whole 5S-RNA molecules were resolved by continuous gradient elution. The concentrations of the salt in the eluents depended on the fragments to be separated, the chromatography of a whole 5S-RNA molecule requiring 4.5 *M* lithium formate.

The separation of more than seven methylated mRNA 5′-termini, achieved by Gross *et al.*[166] on cellulose, again involved different ionic equilibria in each direction and their effect on solute migration patterns. Faster assays on cellulose have been reported by Bergquist[167], who separated 17 oligo- and mononucleotides by coupling electrophoresis with TLC. The system described is extremely powerful, capable of resolving tetra- or oligonucleotides of similar charge and chain length.

The combination of gel electrophoresis and TLC has more recently been applied to RNA sequencing studies[168] First, 3′-terminal RNA half-molecules are sep-

TABLE 13

SEPARATION OF OLIGONUCLEOTIDES AND NUCLEIC ACIDS

Column headings as in Table 1

N	*Compounds*	*Stationary phase*	*SSP (in)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref*
5	5′-RNA terminal oligonucleotides	PEI, cellulose MN 300, Brinkmann, 20 × 20 cm	1	2 0 *M* LiCl + 0 01 *M* EDTA (pH 6.5)	Top	Wash in CH_3OH, dry	3 *M* LiCl + 0 01 *M* EDTA–3 6 *M* formic acid (pH 2 0, adjusted with solid LiOH)		164
18	Oligonucleotides (T1 RNase digest of 5S RNA)	PEI, cellulose MN 300, 20 × 20 cm		(a) 1 4 *M* lithium formate in 7 *M* urea (pH 3 4), 60°C (b) Above mixture, 1 8 *M* (pH 3 4), 60°C	10 cm, top	Wash in CH_3OH, dry	(c) 0 8 *M* LiCl in 0 02 *M* TMS base and 7 *M* urea (pH 8)		165
	Partial fragments of 5S RNA	PEI, cellulose MN 300, 20 × 20 cm		4 0 *M* lithium formate in 7 *M* urea, gradually introduce 7 5 *M* above solution (pH 3 4)			1 0 *M* LiCl in 7 *M* urea; gradually introduce 3 2 *M* LiCl (pH 8), 60°C		165
>7	Methylated mRNA 5′-termini	Cellulose (Merck), 20 × 20 cm		Isobutyric acid–ammonia–water (pH 23.7) (66 1 33)	8 h	Hood, overnight	Sat ammonium sulphide–1 *M* sodium acetate (pH 5 5)–propan-2-ol (40 9 1)	6 7 h	166
17	Oligonucleotides + mononucleotides	Cellulose MN 300, 20 × 20 cm, 0 25 mm		Electrophoresis with 0 1 *M* ammonium formate (pH 2 45) containing 0 001 *M* EDTA, 50V/cm, 16–20 mA	20–25 min	Dry under IR lamp	*tert*-Butanol–0 08 *M* formic acid–isoamyl alcohol (50 50 2)	10 cm, 4 h	167
	(1) Oligonucleotides (2) Nucleotide sequencing	(1) Gel electrophoresis (2) DEAE-cellulose		(1) Electrophoresis			(2) 15 m*M* sodium citrate buffer (pH 3 1)		168
17	Oligonucleotides	(1) Cellulose acetate paper strips (2) DEAE-cellulose plates		(1) Electrophoresis (pH 3 5)			(2) Homochromatography		169

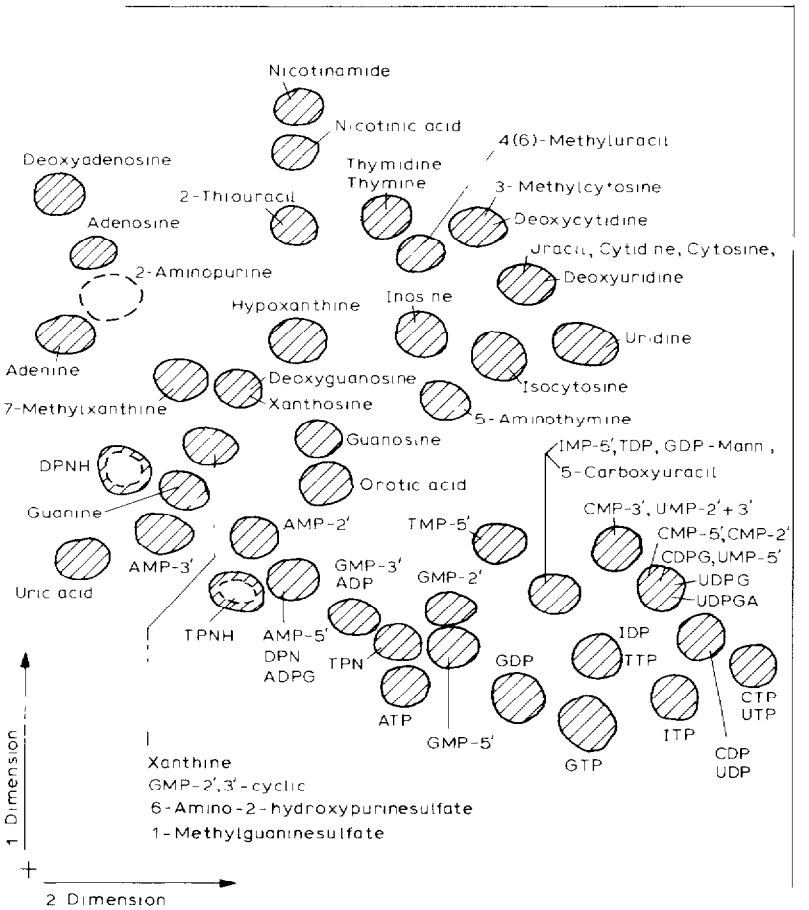


Fig 18 Two-dimensional separation of nucleo-derivatives on purified cellulose layers. First dimension, *n*-propanol 25% ammonia water (6 3 1), second dimension, isopropanol saturated ammonium sulphate water (2:79 19) Absorption spots are hatched, fluorescence spots are surrounded by broken lines Reproduced from ref 158 with permission

arated by gel electrophoresis. Then, the entire ladder of bands is excised from the gel, transferred to a thin layer of DEAE-cellulose by blotting and hydrolysed *in situ* with takadiastase RNAase The thin layer is developed in a direction perpendicular to gel electrophoresis with 15 m*M* sodium citrate buffer (pH 3.1) The enzyme is supposed to hydrolyse one nucleotide at a time and thus the resulting pNp spots correspond to the sequence of nucleotides in the original RNA molecule

4.8. Environmental pollutants (Table 14)

It is of interest for the environmental chemist to identify and quantify poly-aromatic hydrocarbons and aza heterocycles in air samples from different sources. This was achieved by Sawicki and co-workers[170–176], who analysed by two-dimensional TLC extracts obtained directly or after column fractionation of airborne particulate and coal-tar pitch fume samples.

TABLE 14

SEPARATION OF ENVIRONMENTAL POLLUTANTS: AZAARENES AND POLYNUCLEAR AROMATIC HYDROCARBONS (PAHs)

Column headings as in Table 1.

N	*Compounds*	*Stationary phase*	*SSP*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref.*
8	Azaarenes + others	Al_2O_3		Cyclohexane ethyl acetate (1:1)	15 cm		Toluene	15 cm	170
33	Benzene-soluble fraction of airborne particulate sample; acridine, pyrene, benz[*c*]acridine identified	Alumina cellulose (2:1), 20 × 20 cm, 0.25 mm		Pentane	15 cm		DMF water (35:65)	15 cm?	173
41	Benzene-soluble airborne particulate fraction with anthracene + other PAHs	Alumina cellulose acetate (2:1)		Pentane	15 cm		Ethanol–toluene water (17:4:4)	15 cm	173
65	Coal-tar pitch basic fraction with various azaarenes	Si gel-cellulose (2:1), 20 × 20 cm	1.5 cm from base	Pentane–diethyl ether (9:1)	13 cm, 3 h		DMF–water (35:65)	13 cm, 4.5 h	177 (Fig. 19)
67 or 15	Tokyo air with various PAHs; PAH reference solutions	(1) Alumina, 4 × 20 cm (2) 26% acetylated cellulose, 16 × 20 cm, 0.3 mm. 1 h, 110°C, store at 20% humidity		On (1): *n*-hexane diethyl ether (19:1) with sat. potassium acetate, 2% humidity	15 cm, 35 min	Air, 5 min	Methanol–diethyl ether–water (4:4:1) (3 × for Tokyo air sample)	10 cm, 55 min	178
Numerous	Benzene-enriched air-particulate extract; no spot identification	Chemically bonded phase, 5 × 5 cm		Methanol water (97:3)	2 min		Methanol acetonitrile	4 min	184

Of all of the compounds in an airborne particulate, benzene-soluble, "oxy" fraction, compounds with an aza nitrogen such as 9-acridone and 6(5H)-phenanthridone exhibited the longest retention on an alumina plate Substitution of the aza nitrogen by an oxygen atom or sulphur weakened adsorption, xanthen-9-one and thioxanthen-9-one migrating faster than 9-acridone[170]

In their assay on an alumina–cellulose (2 1) mixed sorbent plate, Sawicki *et al.*[173] used an organic eluent for the first elution. Compounds were primarily retained by adsorption; acridine had the smallest and pyrene the largest R_F values In the orthogonal direction, the plate was developed with a solvent containing 65% of water Water interfered with solute adsorption on alumina and also impregnated the cellulose fibres, creating a partitioning medium. Under these conditions, acridine migrated further than pyrene.

Similar mechanisms were effective in the two-dimensional separation of coal-tar pitch aza heterocyclic on a silica–cellulose (2·1) plate Adsorption on to silica prevailed on development with the organic eluent· owing to the steric hindrance of its aza nitrogen, benzo(*h*)quinoline was less retained than benzo(*f*)quinoline (Fig 19) In the orthogonal direction, compounds were separated according to their partition coefficients the bulky dibenz(*a,j*)acridine was retained the most whereas acridine, benzo(*h*)- and benzo(*f*)quinoline had the largest R_F values, in general, retention increased with the number of additional aromatic substituents[177].

Matsushita and Suzuki[178] succeeded in resolving the polyaromatic hydrocarbons anthracene, phenanthrene, pyrene, benz(*c*)anthracene, chrysene, perylene, coronene, benz(*a*)- and benz(*e*)pyrene, benzophenylene, dibenzanthracene and dibenzopyrene The first development was carried out on an alumina strip with an organic eluent Adsorption increased with aromaticity and was affected by steric factors. The second development was performed on the acetylated cellulose part of the plate with a polar eluent. Compounds were separated by partition, those with the smallest number of rings migrating further than the others

Polyaromatic hydrocarbons have more recently been separated on acetylated cellulose[179] and on an acetylated cellulose–silica gel alumina sorbent mixture[180]. Other methods had involved the use of GC and TLC in combination[181,182]. Janák[182] and Kaiser[183] reviewed in depth both the instrumentation and the applications of this technique.

4.9 Pesticides

Various pesticides, including aldrin and dieldrin[185], DDE and DDT analogues[186], organophosphorus[187] and other compounds[188–190], have been separated on silica plates developed with different solvents in two directions (Table 15).

From the chromatographic behaviour of Planavin and its substituted 4-methylsulphonylaniline derivatives, it could be seen that the propylation of a primary amine weakens its adsorption on to silica, whereas its substitution by a hydroxyl group increases the retention[188]. The chlorination of phenol or benzoquinone also decreases their retention on a silica plate[190].

Yasuda's[189] separation of various substituted benzenes, phenols and anilines on a silica gel–zinc dust plate show that retention generally decreases in the order phenol > aniline > N-methylaniline > benzene > chlorobenzene (Fig 20). In the

TABLE 15

SEPARATION OF PESTICIDES

Column headings as in Table 1

N	*Compounds*	*Stationary phase*	*SSP (cm)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref*
3	Dieldrin, *p,p'*-TDE, aldrin from interferences	Alumina, 10 × 20 cm	3	*n*-Heptane	10 cm	Air, 15 min	Acetonitrile saturated with *n*-heptane		185
10	*p,p'*-DDE + DDT analogues from PCBs	Kieselgel G-HR HN + $AgNO_3$ (chromogenic agent), 20 × 20 cm, 0.25 mm, 80°C, 20 min	3	*n*-Heptane	10 cm, 15 20 min	Air, 5-10 min	*n*-Heptane acetone (98 2)	10 cm	186
16	Group I organophosphorus pesticides	Si gel G, 20 × 20 cm, 0 25 mm, no activation if <60% humidity, if >60%, 110°C, 1 h	3 8 cm, bottom	Toluene	10 min	30 sec exposure to bromine vapours, chromatography of oxidation products in other directions	25% heptane in ethyl acetate	5 min	187
10	Group II organophosphorus pesticides	idem		25% Heptane in ethyl acetate	10 min	idem	Ethyl acetate	5 min	187
12	Planavin herbicide + related compounds	Si gel F_{254} (Merck), 20 × 20 cm, 0.25 mm		Hexane–ethyl acetate THF (66:30:4) (2×)			Benzene–acetonitrile (3.2)		188
23	Tetryl and related compounds	Si gel G–Zn (30·0 5, w/w), 110°C, 2 h		Chloroform	55 min	Air, 10 min	Ethyl acetate–light petroleum (1 3)	55 min	189 (Fig 20)
9	Chloranil + chlorinated derivatives of *p*-benzoquinone, hydroquinone + phenol	Reflex Si gel foils (Kavalier), UV 254 nm, 12 × 12 cm, 0 3 mm, 120°C, 60 min	1 5	Chloroform–benzene (1 6 78)	10 cm		*n*-Heptane–carbon tetrachloride (1 11 56)	10 cm	190

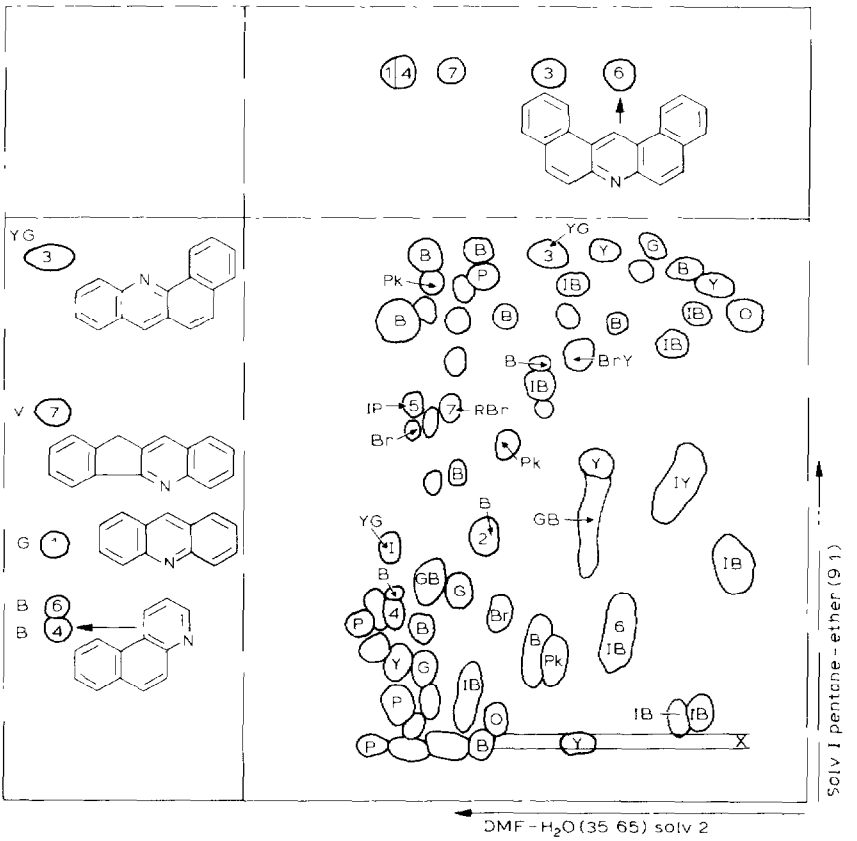


Fig 19 Two-dimensional thin-layer chromatographic separation on silica gel-cellulose (2 1) of basic fraction equivalent to 2 mg of coal-tar pitch and the characterization of seven spots Standards: (1) acridine, (2) benz[*a*]acridine, (3) benz[*c*]acridine, (4) benzo[*f*]quinoline, (5) benzo[*h*]quinoline; (6) dibenz[*a,j*]acridine, (7) 11H-indeno[1,2-*b*]quinoline The plate was sprayed with trifluoroacetic acid fumes and the fluorescence colours were marked B = blue; Br = brown, G = green, l = light, P = purple, Pk = pink; R = red, Y = yellow, O = orange, V = violet, = no fluorescence, so located by the quenching of the fluorescent plate Reproduced from ref 177 with permission

second direction, differentiation was further made between *meta-*, *ortho-* and *para-*isomers. The elution order may appear surprising at first, as *m*-nitroaniline, a 35-fold stronger base than *p*-nitroaniline, is less retained on the acidic silica phase. Similarly, 3,5-dinitroaniline is less retained than 2,4-dinitroaniline. This may be attributed to the effect of zinc dust in the sorbent mixture.

Gardner's[187] assay of organophosphorus pesticides is one of the few that uses solute derivatization prior to the second plate development This was accomplished in order to obtain, in the second direction, a widely different migration pattern from the first. The resulting spots are then more separated, which allows better identification of multiple pesticide residues. Thus, after the first development, the plate was exposed to bromine vapour, which converted the thionate and dithioate pesticides into their corresponding oxygen analogues The plate was then chromatographed with a more polar solvent in a direction perpendicular to the first one.

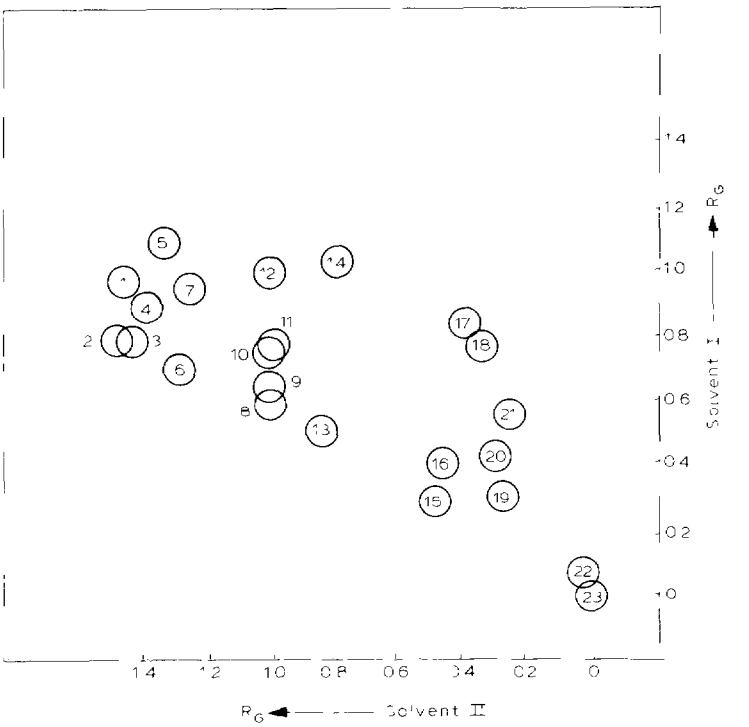


Fig 20 Two-dimensional separation of tetryl and related compounds on a silica gel G zinc plate (30 0 5, w/w) 1 = 1-Chloro-2,4,6-trinitrobenzene (picryl chloride), 2 = N,N-dimethylaniline, 3 = 1,3,5-trinitrobenzene (TNB), 4 = 2,4,6-trinitroanisole, 5 = 1-chloro-2,4-dinitrobenzene, 6 = N-methylaniline, 7 = N-methyl-2,6-dinitroaniline, 8 = 2,4,6-trinitroaniline (picramide), 9 = N,2,4,6-tetranitroaniline, 10 = N-methyl-2,4,6-trinitroaniline, 11 = *o*-nitroaniline, 12 = 1,3-dinitrobenzene (reference compound), 13 = N-methyl-N,2,4,6-tetranitroaniline (tetryl), 14 = 1-chloro-2,6-dinitrobenzene; 15 = 3,5-dinitroaniline, 16 = *m*-nitroaniline, 17 = 2,4-dinitroanisole, 18 = N-methyl-2,4-dinitroaniline, 19 = 2,4-dinitroaniline, 20 = *p*-nitroaniline, 21 = N-methyl-N,2,4-trinitroaniline, 22 = 2,4-dinitrophenol, 23 = 2,4,6-trinitrophenol (picric acid) Solvent I, chloroform, solvent II, ethyl acetate light petroleum (1 3) Reproduced from ref 189 with permission

4.10. Inorganic compounds

Perhaps the most important application of the two-dimensional separation of inorganic compounds is shown in Fig. 21, where polyphosphates are separated according to both their type and their chain length. This is achieved by combining an alkaline and an acidic solvent[191]

In the separation of metal ions, two possibilities have been explored, as follows.

(1) The separation is effected using different kinds of complex formation in two directions. This is only feasible, however, if complexant I is volatile or does not interfere with complexant II. A typical example of the latter is shown in Fig 22. The first solvent (methanol ethanol 2 *N* hydrochloric acid) separates metal ions mainly according to hydration differences and complexation with hydrochloric acid at low acid concentrations. The second solvent separates according to the extraction of complexes at high hydrochloric acid concentrations as well as forming complexes with hydrofluoric acid [mainly for Zr(IV) and Nb(V)][192].

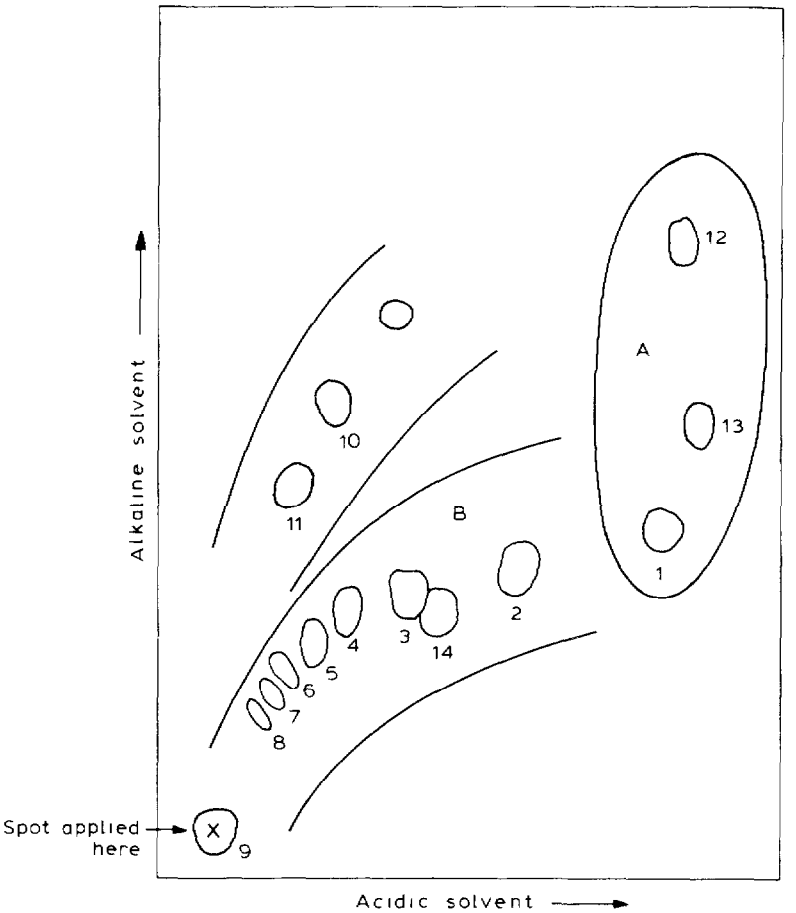


Fig 21 Two-dimensional separation of oxyacids of phosphorus on paper Alkalin solvent 1 (Ebel) acidic solvent 3 (Grunze and Thilo) 1, orthophosphate, 2, pyrophosphate, 3, triphosphate, 4, tetraphosphate, 5, pentaphosphate, 6, hexaphosphate, 7, heptaphosphate, 8, octaphosphate, 9, Graham's salt, 10, trimetaphosphate, 11, tetrametaphosphate; 12, hypophosphite, 13, phosphite, 14, hypophosphate Reproduced from ref 191 with permission

(2) It is also possible to separate in one direction by partition chromatography (butanol 2 *N* hydrochloric acid) and in the other by ion exchange (1 *N* aqueous magnesium chloride) on phosphorylated cellulose paper (Fig. 23). In the presence of hydrochloric acid (in the first dimension) the ion-exchange groups remain practically unionized and the metal ions move as on ordinary cellulose paper The butanol hydrochloric acid solvent cannot be removed entirely by evaporation and the remaining hydrochloric acid is neutralized with ammonia vapour[193]

4.11 Miscellaneous

Several reports have discussed the two-dimensional chromatography of organic acids on paper[5–7,194] and cellulose[195], of indole derivatives[196,197] and catechola-

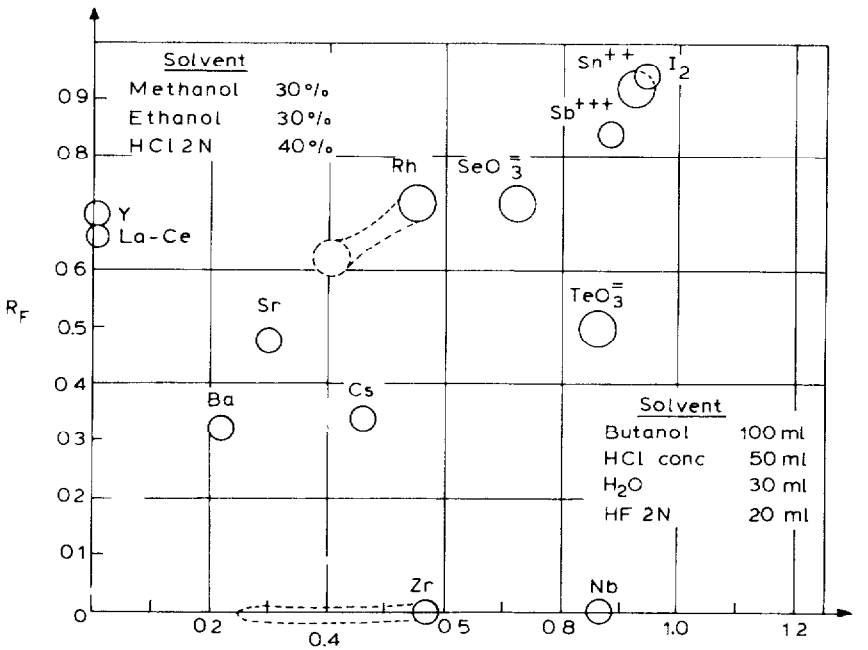


Fig 22. A two-dimensional chart showing the distribution of the major fission products when chromatographed first with methanol–ethanol 2 *N* HCl (30 30 40) and then with butanol–conc HCl water–conc HF (100 50:48 2). Reproduced from ref 192 with permission

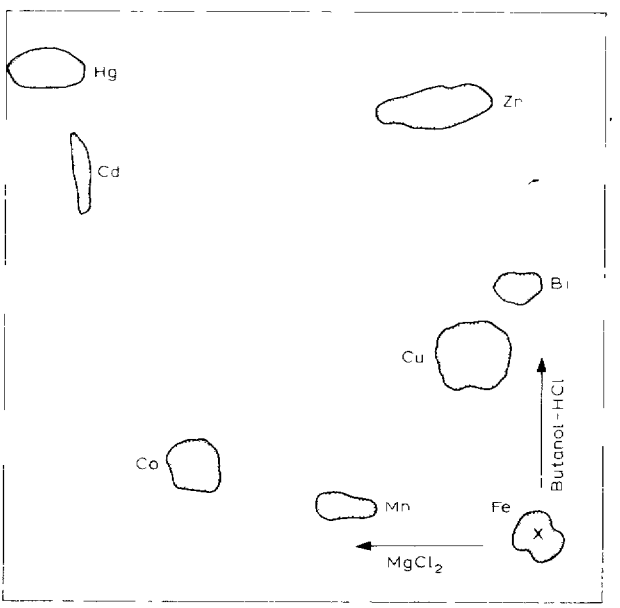


Fig 23 Two-dimensional separation of metal ions on a 14-in square of phosphorylated cellulose paper containing dihydrogen phosphate groups in the monoammonium salt form Development is by the downward flow of the organic phase of a mixture of *n*-butanol saturated with 2 *N* HCl (partition chromatography) followed by 1 *N* magnesium chloride (ion-exchange chromatography) After the first run, the sheet is dried over ammonia to neutralize excess acid and to convert the paper to the original form Reproduced from ref. 193 with permission

TABLE 16

SEPARATION OF MISCELLANEOUS COMPOUNDS

Column headings as in Table 1

N	*Compounds*	*Stationary phase*	*SSP (in.)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref*
12	Organic acids	Paper (Durieux 122), 45 × 45 cm		Isopropanol-ammonia water (70 5 25)	12 h, 35-40 cm	Hood	1-Propanol eucalyptol-formic acid (50 50 20) + water until turbid	5 h, 35 40 cm	5
21	Organic acids	Paper (Whatman No 1), 40 × 50 cm		Ethanol-ammonia water (80 5 5)	14 h	8 h	1-Propanol eucalyptol formic acid (50 50 20) + water until turbid	14 h	6,7
14	Organic acids	Paper (Whatman 3MM)	3	*sec*-Butanol *n*-propanol 90% formic acid water (75 25 100 48), after standing, use upper layer for development while lower layer saturates tank for 48 h prior to run	7 h	Dry, 12 h, wash with acetic acid, dry	*n*-Propanol-ammonia water (1000 1 25 2 00)	9 h	194
8	Oxy acids	Cellulose MN 300 HR, 20 × 20 cm, 0 35 mm		Isopropanol ethyl acetate water (23 5 65 11 5)	14 5 cm		Formic acid-ethyl acetate water (1 3 1)	14 5 cm	195
6	Indole derivatives	Si gel G 22, 20 × 10 cm, 0 3 mm, 20°C, 12 h		Isopropanol 25%-ammonia-water (20·1 2)			Butanol acetic acid water (15.3.5)		196
2	Indoleacetic and indolecarboxylic acids	Si gel G (Merck), 20 × 20 cm 0 3 mm 105°C, 20 min		Methyl acetate isopropanol-ammonia (45 35 20)			Chloroform acetic acid (95·5)		197

(*Continued on p. 184*)

TABLE 16 (*continued*)

SEPARATION OF MISCELLANEOUS COMPOUNDS

Column headings as in Table 1

N	*Compounds*	*Stationary phase*	*SSP (m)*	*Solvent 1*	*t/L*	*Drying*	*Solvent 2*	*t/L*	*Ref*
5	Catecholamine metabolites	18% cellulose, 10 × 10 cm, 0 4 mm		Isopropanol 5% ammonia (4 1)	2 h		Benzene–acetic acid water (146 73 2)	45 min	198
11	Dansylated derivatives of brain catecholamines and their derivatives	Kieselgel G		(a) Diisopropyl ether (b) Butyl acetate triethylamine (100 20) (2 ×)			Triethylamine diisopropyl ether (100 20) (2 ×)		120
11	N-Acetylated derivatives of biogenic amines	Kiesel G		(a) Ethyl acetate–butyl acetate (100 20) (b) Benzene–methanol (90 10) (2 ×)			Chloroform–triethylamine (100 20)	13 cm	120
20	Hypnotics (15 barbiturates, 3 bromoureides, phenytoin + glutethimide)	Si gel GF_{254}, 20 × 20 cm, 0 25 mm, 105°C, 30 min		Chloroform diethyl ether (75·25)	10 cm		Isopropanol chloroform–25% ammonia (45 45 10)	10 cm	199
3	Streptomycins	Si gel (Merck), 0 03 mm, 110°C, 60 min	5 0 cm away from cathode	2% Piperidine + 2% *p*-toluenesulphonic acid in water-saturated *n*-butanol	16 cm, 3 5 h		Electrophoresis with 1% $Na_2B_4O_7$ solution (pH 9 6), 300 V, 23–28°C	3 h	200
11	1,4-Benzodiazepine drugs (blood, urine)	Si gel G, 20 × 20 cm, 0 25 mm, 110°C, 30 min	1 5	Chloroform benzene–diethyl ether (20.10 70)	10 cm		Benzene–acetone (90.10)		201, 202
15	Chlorpromazine and metabolites	Si gel (Brinkmann 254F), 0.25 mm, 100°C, 4 h	2	Acetone–methanol methanolamine (100 30 1)	12 cm	42°C, 10 min	Isopropanol–ethanol ammonia (2 1 1)	12 cm	203
14	Phthalodinitrites and related metabolites	(1) Si gel HF–cellulose (1 1) on 4 × 20 cm strip (2) Acetylated cellulose F layer, 15 × 20 cm		On (1) Carbon tetrachloride–ethyl acetate–acetic acid (7 2 0 5)	15 cm	Air, 20 min	On (2) Methanol–diethyl ether water (7·2·0 5)	10 cm	204

4	Aflatoxins b_1, b_2, g_1, g_2 (figs)	Si gel 60, 105°C, 1 h		Toluene–ethyl acetate–90% formic acid (5 4.1)	17 cm	Dry, dark, room temp., 15 min	Acetone–chloroform (1 9)	17 cm	205
4	Aflatoxins (corn)	SILG-HR-25 (Brinkmann), 10 × 5 cm		Acetone–chloroform (12 88)	8 cm	Air stream	95% Denatured ethanol (Mallinckrodt 7006)	4 5 cm	206
37	Milk constituents from which aflatoxin M_1 isolated	Si gel 60 (Merck), 20 × 20 cm, 0.2 mm		Chloroform–acetone–2-propanol (80·15·15)	10 cm		Toluene–ethyl acetate–90% formic acid (60.30.10)	10 cm	207
12	Carbonyl dinitrophenylhydrazones	MgO Microcell T38 (1 1), 60°C, 1 h, 20 × 20 cm		Light petroleum–chloroform (85 35)	12 cm		(a) Chloroform SVR (80 20) (2 ×) (b) 20% Carbowax 400 in chloroform (impregnates plates) (c) Rotate plate 180° and develop in PE		208 (Fig 24)
5	Mixture of Ni(II) bis-diethyl- and bisdihexyl-dithiocarbamate complexes	Silufol Si gel (Kavalier), 15 × 15 cm, 120°C, 1 h		Chloroform–cyclohexane (3·4)	20 min		Chloroform–cyclohexane (3 4)		209
7	B_6 vitamers	Si gel H, 10 × 15 cm, 0 25 mm		Isoamyl alcohol–acetone–water–diethylamine (24.18.6.8)		Warm air	2-Butanol–ethanol 1 5 *N* ammonia–isoamyl alcohol–diethylamine (20 6 7 2 2)		210
12	1,3,5-Triamino-2,4,6-trinitrobenzene and its impurities	30 g Si gel G + 1 g Zn dust, 20 × 20 cm, 110°C, 2 h	1 in	1,2-Dichloroethane	1 h	10 min	Acetone–light petroleum (1 9)	1	211

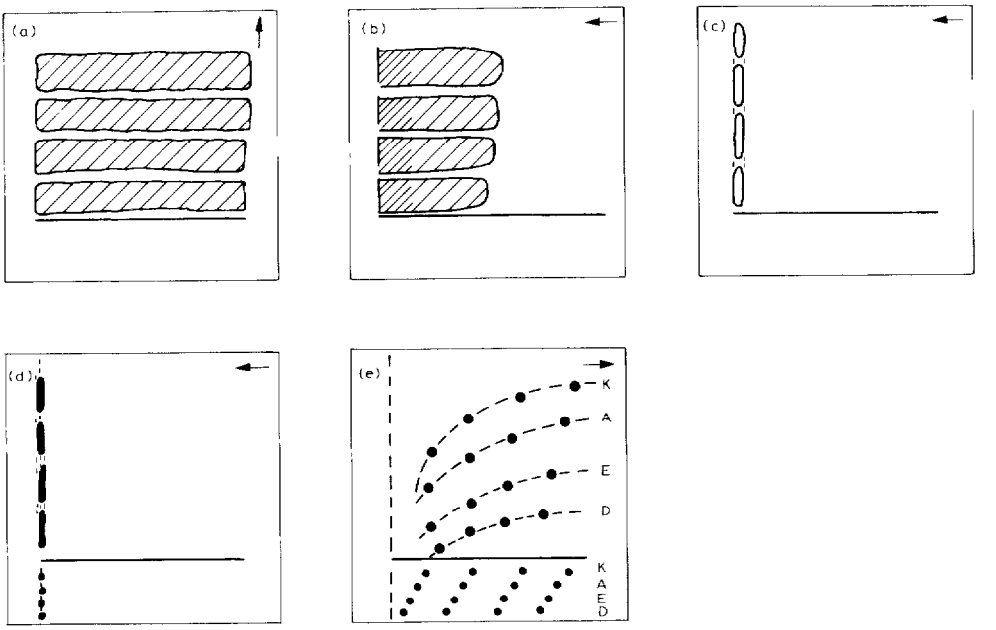


Fig 24 Diagrammatic representation of the two-dimensional separation of dinitrophenylhydrazones (direction of solvent travel indicated by arrow) (a) Stage 1, class separation, (b) stage 2, first concentration, (c) stage 3, second concentration, (d) stages 4 and 5, impregnation and marker application, (e) stage 6, chain length separation K = ketones, A = anals, E = enals, D = dienals Reproduced from ref 208 with permission

mine metabolites[120 198] on silica and cellulose, of barbiturates[199], streptomycins[200], benzodiazepine[201,202] and chlorpromazine[203] drugs on silica and of phthalodinitriles[204] on a mixed plate, where a silica–cellulose strip had been coated at the side of an acetylated cellulose layer. In addition, aflatoxins[205–207], carbonyldinitrophenylhydrazones[208], dithiocarbamate complexes[209] and some B_6 vitamers[210] have also been resolved by two-dimensional TLC. Detailed chromatographic conditions for these separations are given in Table 16.

Craske and Edwards[208] developed an original two-dimensional technique for the separation of carbonyl dinitrophenylhydrazones (Fig. 24). Solutes were first resolved into different classes (alkanones, alkanals, alk-2-enals and alka-2,4-dienals) by adsorption, and next on the basis of chain length by partition. Whereas initial class separation required a small sample to prevent overloading, secondary chain length separation following Carbowax 400 impregnation required a large sample to facilitate solute detection. This problem was solved by streaking the sample across the width of the plate, developing in the first solvent and subsequently performing a chromatographic concentration step Orthogonal elution in chloroform–SVR (80:20) concentrated the spots on one side of the plate. The layer was then impregnated with 20% Carbowax 400 in chloroform The plate was rotated through 180° and chain resolution was effected by development in light petroleum.

5 CONCLUSION

In summary, two-dimensional TLC applications may be achieved using:

(1) a one-sorbent layer and solvents with different selectivity for the various solutes;

(2) a mixed-sorbent layer and eluents chosen such that one particular sorbent dictates retention in a given direction;

(3) coupled layers where two different sorbents are coated side-by-side, each effecting a separation according to a different retention mechanism;

(4) modification of the plate (impregnation) prior to the second development;

(5) derivatization of the solute prior to the second development;

(6) coupled separation techniques (TLC–GC, TLC–electrophoresis).

Sample purification may be achieved in one direction and separation in the other. The main advantage of the technique lies in its efficiency, as a large number of spots may be resolved on the chromatographic plate[21], provided that the initial spot size is small and the sample is not overloaded. In addition, widely different components may be resolved from a given mixture.

Perhaps the main disadvantages of a two-dimensional system are the serious difficulties encountered in quantitating a significant number of spots, the difficulties of predicting R_F values obtained from the second plate development, as these are generally affected by the previous elution, and the possible irregular ascent of the second solvent, leading to spot distortion. It is possible to correct for the latter problem, if not by selecting more "miscible" solvent systems, then by grafting two thin layers and transferring spots from one to the other prior to the second development[212]. As for the prediction of R_F values, a blank plate could be developed in the first eluent prior to spotting the various reference solutions and chromatography in the second direction.

Thus the problem of measuring compound concentrations remains the most critical, at least when working with non-radioactively labelled material or with compounds that cannot all react with a fluorescent or similar tag.

Nevertheless, this method is extremely powerful and rapid. With a total analysis time of about 1 h, a spot capacity well exceeding those currently achieved in conventional TLC or even HPLC can easily be obtained. The spot capacity of the figures reproduced above is in the range 150–300 (except in Fig. 15). We are of the opinion that this method or more generally bi- and tri-directional chromatographic techniques have considerable potential for the separation of complex mixtures, when there are a few ten to a hundred components to resolve, their use may save a considerable time in the selection of a convenient solvent mixture and/or chromatographic system allowing the complete separation in one HPLC run. If the mixture is more complex (several hundred components) they offer the only possibility of a simple, total analysis.

Accordingly, instrumentation for two-dimensional chromatography will be the subject of intense development work in the near future, and solutions will eventually be found for the problems discussed above.

6 SUMMARY

In view of the large spot capacity available, much larger than in conventional thin-layer chromatography (TLC), two-dimensional thin-layer chromatography is an extremely powerful and rapid separation technique. This paper presents a review of the literature up to early 1982 on the analytical applications of two-dimensional TLC classified according to the chemical nature of the compounds separated. Technical aspects of the method (single, mixed or coupled sorbent layers, plate impregnation, solute derivatization, coupling with other analytical techniques), together with effective mechanisms of solute retention are discussed Available methods for spot detection and quantitation are reported. Future trends of two-dimensional TLC are commented. Over 200 references are cited.

REFERENCES

1 I M Hais, *J Chromatogr*, 187 (1980) 466
2 G Guiochon, A Siouffi, H Engelhardt and I Halasz, *J Chromatogr Sci*, 16 (1978) 152
3 R. Consden, A H Gordon and A J. P Martin, *J Biochem*, 38 (1944) 224
4 B T Overell, *Austr J. Sci*, (1952) 28
5 R -I Cheftel, R Munier and M Macheboeuf, *Bull Soc. Chim Biol*, 34 (1952) 380
6 R Nordmann, O Gauchery, J -P du Ruisseau, Y Thomas and J Nordmann, *Bull Soc Chim Biol.*, 36 (1954) 1461
7 R Nordmann, O Gauchery, J -P du Ruisseau, Y Thomas and J Nordmann, *Bull. Soc Chim Biol*, 36 (1954) 1641.
8 F Pocchiari and C Rossi, *Chromatogr Rev*, 4 (1961) 1
9 M Calvin, *Harvey Lect*, 46 (1950 51) 218
10 M Calvin, *Proc 3rd Int Congr Biochem., Brussels, 1955*, p 211
11 A Beloff-Chain, R. Catanzaro, E B Chain, I Masi. F Pocchiari and C Rossi, *Proc Roy. Soc (London), Ser B*, 143 (1955) 481.
12 A Beloff-Chain, R Catanzaro, E B Chain, I Masi and F Pocchiari, *Sel Sci. Pap Ist Super Sanità*, 2 (1959) 109
13 E B Chain, A Beloff-Chain and F Pocchiari, *Sel Sci Pap Ist Super Sanità*, 1 (1956) 389
14 E B Chain, *Brit. Med J.*, 2 (1959) 709.
15 H. J Issaq and E W Barr, *Anal Chem*, 49 (1977) 83A
16 E Stahl, *J Chromatogr*, 165 (1979) 59
17 J A Perry, *J Chromatogr*, 165 (1979) 117
18 G Zweig and J Sherma, *Anal Chem*, 52 (1980) 276R
19 H J Issaq, *Separ Purif Methods*, 10 (1981) 73
20 D C. Fenimore and C M Davis, *Anal Chem*, 53 (1981) 252A
21 G Guiochon, M F Gonnord, A. Siouffi and M Zakaria, *J Chromatogr*, 250 (1982) 1.
22 G Guiochon, L A Beaver, M. F. Gonnord, A Siouffi and M Zakaria, *J Chromatogr*, 255 (1983) 415
23 U A Th Brinkman, G Devries and R Cuperus, *J Chromatogr*, 198 (1980) 421
24 R Delley and G Szekely, *Chimia*, 32 (1978) 261.
25 L R Snyder, in E Heftman (Editor), *Chromatography A Laboratory Handbook of Chromatographic and Electrophoresis Methods*, Van Nostrand Reinhold, New York, 3rd ed, 1975, pp 46 76
26 O. Motl and L. Novotny, in O Mikěs (Editor), *Laboratory Handbook of Chromatographic and Allied Methods*, Ellis Horwood, Chichester, 1979, pp 150 217
27 K Randerath, *Chromatographie sur Couches Minces*, Gauthier-Villars, Paris, 2nd ed., 1971, p 22
28 R K Gilpin and W R. Sisco, *J Chromatogr*, 124 (1976) 257
29 M Ericsson and L G Blomberg, *J High Resolut Chromatogr Chromatogr Commun*, 3 (1980) 345
30 I D Wilson, S Scalia and E D Morgan, *J Chromatogr*, 212 (1981) 211
31 H Halpaap, K.-F Krebs and H E Hauck, *J. High Resolut Chromatogr Chromatogr Commun.*, 3 (1980) 215

32 O Mikěs, J Štamberg, M Hejtmánek and K Šebesta, in O Mikěs (Editor), *Laboratory Handbook of Chromatographic and Allied Methods*, Ellis Horwood, Chichester, 1979, pp 218 327
33 O Motl and L Novotny, in O Mikěs (Editor), *Laboratory Handbook of Chromatographic and Allied Methods*, Ellis Horwood, Chichester, 1979, pp. 462 523
34 L Lepri, P G Desideri and D Heimler, *J Chromatogr*, 235 (1982) 411
35 K Randerath and E Randerath, *J Chromatogr*, 16 (1964) 111.
36 P S. Bond, *J Chromatogr*, 34 (1968) 554
37 J Pore, J. P Houis and I Rasori, *Rev Fr Corps Gras*, 72 (1981) 164
38 A. R Ritchie, S. R B Souy and M H Clear, *J Chromatogr*, 150 (1978) 557
39 Y. V Zanevskit, A B Ivanov, V D Peshekhonov, I A Tyapkin, S P. Chernenko, L B Kaminir, E P Senchenkov and V N Kalinin, *Report 1977, JINR-R-14-10934, C.A*, 90 (1979) 114557a
40 R. W. Frei, *Progr Thin-Layer Chromatogr Relat Methods*, 2 (1971) 1.
41 V Pollak, *J Chromatogr.*, 152 (1978) 201.
42 J Goldman and R R Goodall, *J Chromatogr*, 32 (1968) 24
43 J C Touchstone, S. S Levin and T. Murawec, in J C Touchstone (Editor), *Quantitative Thin-Layer Chromatography*, Wiley-Interscience, New York, 1979, pp. 1 22
44 P. Knapstein, J C Touchstone, P Menzel and G W Oertel, *J. Chromatogr*, 44 (1969) 190
45 V Pollak, *J Liq Chromatogr.*, 3 (1980) 1881
46 G Kerenyi, T. Patakı and T. Devenyi, *Hung Pat*, 170-287 (1976)
47 T Devenyi and S Pungor, *Hung Sci. Instrum*, 50 (1980) 3
48 N. L. Anderson, J Taylor, A. E. Scandora, B P Coulter and N. G. Anderson, *Clin. Chem.*, 27 (1981) 1807
49 E P Lester, P F Lemkin and L E. Lipkin, *Anal Chem*, 53 (1981) 390A
50 A K Thorsrud, H F Haugen and E Jellum, *Electrophoresis '79, Advan. Methods Biochem. Clin Appl, Proc Int Conf Electrophoresis*, Walter de Gruyter, Berlin, 1980, pp. 426 435
51 M. L. Guanelli, I B Callis, N. H. Anderson and G D Christian, *Anal. Chem.*, 53 (1981) 1357
52 L A Beaver and G Guiochon, U S patent pending.
53 G Guiochon, L A Beaver, M F Gonnord, A M Siouffi and M Zakaria, *Chromatographia*, 17 (1983) 21
54 C V Viswanathan, F Phillips and W O Lundberg, *J Chromatogr*, 38 (1968) 267.
55 J. M. Bailey, R W Bryant, S J Feinmark and A. N. Makheja, *Prostaglandins*, 13 (1977) 479.
56 D C Palmer, J. A Kintzios and N. M Papadopoulos, *J Chromatogr Sci*, 10 (1972) 107.
57 T M Lee and A der Marderosian, *J Pharm Sci.*, 70 (1981) 89
58 B A. El-Zeany and A -K -S Ahmed, *Egypt J Food Sci*, 1 2 (1977) 1
59 H H O. Schmid, W J Baumann, J M Cubero and H. K Mangold, *Biochim Biophys Acta*, 125 (1966) 189
60 T Takatori and A Yamaoka, *Nippon Hoigaku Zasshi (Jap J Legal Med)*, 30 (1976) 368
61 C Bouillé, J. Y Daniel, J Boissin and I Assenmacher, *Steroids*, 14 (1969) 7
62 D C Bicknell and D B Gower, *J Chromatogr*, 61 (1971) 358
63 T Taylor, *J Chromatogr*, 66 (1972) 177
64 G Cavina, G Moretti and M Petrella, *J. Chromatogr*, 103 (1975) 368
65 H L Issaq, *J. Liq Chromatogr*, 3 (1980) 841.
66 R E Levitt and J C. Touchstone, *J High Resolut Chromatogr Chromatogr. Commun*, 2 (1979) 587
67 D W Vidrine and H. J Nicholas, *J Chromatogr.*, 89 (1974) 92
68 H -Ch Curtius and M Muller, *J Chromatogr*, 32 (1968) 222
69 M. I. Whittle, A. I. Wilson, C. R. Whitfield, R. D. Paton and R. W. Logan, *Brit. Med. J.*, 282 (1981) 428.
70 T. L. Gross, M. V. Wilson, P. M. Kuhnert and R. I. Sokol, *Clin. Chem.*, 27 (1981) 486.
71 G Rouser, S. Fleischer and A Yamamoto, *Lipids*, 5 (1970) 494
72 R M Broekhuyse, *Clin. Chim Acta*, 23 (1969) 457.
73 G S Getz, S Jakovcic, J. Heywood, J Frank and M. Rabinowitz, *Biochim Biophys Acta*, 218 (1970) 441
74 S K Jain and D Subrahmanyam, *Ital J Biochem*, 27 (1978) 11
75 B J H M Poorthuis, P J Yazaki and K Y. Hostetler, *J Lipid Res*, 17 (1976) 433
76 K G Blass, R L Briand, D S Ng and S Harold, *J Chromatogr.*, 182 (1980) 311
77 S Pollet, S Ermidou, F Le Saux, M Monge and N Baumann, *J Lipid Res*, 19 (1978) 916

78 S S. Radwan, *J Chromatogr Sci*, 16 (1978) 538
79 S Ikawa and M Goto, *J Chromatogr*, 114 (1975) 237
80 F -H Hubmann, *J Chromatogr*, 86 (1973) 197
81 M Ohashi, *Lipids*, 14 (1979) 52
82 R W Ledeen, J E Haley and J A Skrivanek, *Anal Biochem*, 112 (1981) 135
83 G. D. Hunter, V M Wiegant and A J Dunn, *J Neurochem*, 37 (1981) 1025
84 C J Clarke and P H Cobb, *J Chromatogr*, 168 (1979) 541
85 J Metivier and A M Viana, *J Exp Bot.*, 30 (1979) 805
86 R E Knowles and A L Livingston, *J Chromatogr.*, 61 (1971) 133
87 A S. L Tirimanna, *Mikrochim Acta*, 2 (1981) 11
88 J Sherma and G Zweig, *J. Chromatogr*, 31 (1967) 589
89 G H Elder, *J Chromatogr*, 59 (1971) 234
90 K Saitoh, M Kobayashi and N Suzuki, *Anal Chem*, 53 (1981) 2309
91 Y. Onoue, *Bull Chem Soc Jap*, 54 (1981) 1972
92 D B Mullick, *J Chromatogr*, 39 (1969) 291
93 J Aurenge, G Barbe-Richaud and L Gelpi, *J Chromatogr*, 22 (1966) 369
94 G Nadeau, G Sobolewski, L Fiset and C G Farmilo, *J Chromatogr*, 1 (1958) 327
95 G B. Marini-Bettòlo and G C Casinovi, *J Chromatogr*, 1 (1958) 411
96 R. L Munier and S Meunier, *Chromatographia*, 13 (1980) 259
97 A. Viala and M Estadieu, *J. Chromatogr*, 72 (1972) 127.
98 P Le Xuan, R L Munier and S. Meunier, *Chromatographia*, 13 (1980) 693
99 S Agurell, *Acta Pharm. Suecia*, 2 (1965) 357
100 A. J P Martin and R L M Synge, *Anal Chem Proteins*, 1 (1945) 1
101 W E Cohn, *J Amer Chem Soc.*, 72 (1950) 1471
102 C S Knight, *Nature (London)*, 188 (1960) 739
103 M C Corfield and E C Simpson, *J Chromatogr*, 17 (1965) 420
104 J H Buchanan and M C Corfield, *J Chromatogr*, 31 (1967) 274
105 D Horton, A. Tanimura and M L Wolfram, *J Chromatogr*, 23 (1966) 309
106 J Maugenet and M -L Dalmeyda, *Ann Technol Agr*, 18 (1969) 237
107 F Kraffczyk, R Helger and H Lang, *Clin Chem*, 16 (1970) 662
108 R L Munier, B Faivre and A Lebreau, *Chromatographia*, 6 (1973) 71
109 R. L Munier, C Gervais and A -M Drapier, *C R. Acad. Sci, Ser D*, 281 (1975) 1513.
110 R. L Munier, C Gervais and A -M Drapier, *C R Acad. Sci, Ser D*, 281 (1975) 1757.
111 J J. Harper and G H G Davis, *Int J Syst Bacteriol*, 29 (1979) 56.
112 M M Frodyma and R W Frei, *J Chromatogr*, 17 (1965) 131
113 K Chmel, *Collect Czech Chem Commun*, 37 (1972) 3034
114 C G Honegger, *Helv Chim. Acta*, 44 (1961) 173
115 E Von Arx and R Neher, *J Chromatogr*, 12 (1963) 329
116 N Seiler, *Methods Biochem Anal*, 18 (1970) 259
117 P Fernlund and L Joseffson, *Biochim Biophys Acta*, 158 (1968) 262
118 M Weise and D E Oken, *J Chromatogr*, 152 (1978) 175
119 D Biou, N Queyrel, M N Visseaux, I Collignon and M Pays, *J Chromatogr, Biomed Appl*, 226 (1981) 477
120 N Seiler and M Wiechmann, *J Chromatogr*, 28 (1967) 351
121 K Macek, Z Deyl and M Smrž, *J Chromatogr*, 193 (1980) 421
122 K -T Wang, J M K Huang and I. S Y Wang, *J Chromatogr*, 22 (1966) 362
123 R L Munier and G Sarrazin, *J Chromatogr.*, 22 (1966) 347
124 R L Munier and A M Drapier, *Chromatographia*, 5 (1972) 306.
125 R L Munier, B Faivre and A Lebreau, *Chromatographia*, 6 (1973) 14
126 I. M. Wainwright and P Shapshak, *J Chromatogr Sci*, 17 (1979) 535
127 P H O'Farrel, *J Biol Chem*, 250 (1975) 4007
128 N G Anderson and N. L Anderson, *Anal Biochem*, 85 (1978) 331
129 N L Anderson and N G Anderson, *Anal Biochem*, 85 (1978) 341
130 N L Anderson and N G Anderson, *Biochem Biophys. Res Commun*, 88 (1979) 258.
131 N G. Anderson, N L. Anderson, S L Tollaksen, H Hahn, F Giere and J Edwards, *Anal Biochem*, 95 (1979) 48.
132 N L Anderson and B J Hickman, *Anal Biochem*, 93 (1979) 312

133 K E Willard, C S Giometti, N L Anderson, T E O'Connor and N. G Anderson, *Anal Biochem*, 100 (1979) 289
134 B J Hickman, N L Anderson, K E. Willard and N G Anderson, *Electrophoresis '79, Advan Methods Biochem Clin Appl, Proc Int Conf Electrophoresis*, Walter de Gruyter, Berlin, 1980, p 341
135 N L Anderson, *Immunol Lett*, 2 (1981) 195.
136 T Pearson and L Anderson, *Anal Biochem*, 101 (1980) 377.
137 C S Giometti, N G Anderson and N L. Anderson, *Clin Chem*, 25 (1979) 1877.
138 C S Giometti, N G. Anderson, S L Tollaksen, J J Edwards and N L Anderson, *Anal Biochem.*, 102 (1980) 47
139 G Volden, A. K Thorsrud, I Bjørson and E Jellum, *J. Invest Dermatol*, 75 (1980) 421
140 E Jellum, *J Chromatogr*, 239 (1982) 29
141 S. M Petrović and V D Canić, *Mikrochim Acta*, (1969) 599
142 E Moczar and M Moczar, *Bull Soc Chim. Biol*, 49 (1967) 1159
143 K Hotta and M Kurokawa, *Anal Biochem*, 26 (1968) 472
144 T Mezzetti, M Ghebregziabher, S Rufini, G Ciuffini and M Lato, *J Chromatogr*, 74 (1972) 273
145 M Ghebregzabher, S Rufini, G Ciuffini and M Lato, *J Chromatogr*, 95 (1974) 51
146 M Ghebregzabher, S Rufini, G M Sapia and M Lato, *J Chromatogr*, 180 (1979) 1
147 E A Ryan and A M Kropinski, *J. Chromatogr*, 195 (1980) 127
148 E Moczar, *J Chromatogr., Biomed Appl*, 146 (1978) 337
149 R L Wolf and J Andreoni, *Cell Immunol*, 67 (1982) 299
150 E Randerath and K Randerath, *J Chromatogr.*, 16 (1964) 126
151 G Santini and V Ulrich, *J Chromatogr*, 49 (1970) 560
152 R A Barton, A Schulman, E L Jacobson and M K. Jacobson, *J. Chromatogr*, 130 (1977) 145
153 R L Bieleski, *Anal Biochem*, 12 (1965) 230
154 C V Cole and C. Ross, *Anal Biochem*, 17 (1966) 526
155 D S S Kumar, L Pinck and L Hirth, *Anal Biochem*, 48 (1972) 497
156 S Hashimoto, W S M Wold, K. H Brackmann and M Green, *Biochemistry*, 20 (1981) 6640.
157 A H van Gennip, D Y van Noordenburg-Huistra, P K de Bree and S K Wadman, *Clin Chim Acta*, 86 (1978) 7
158 G Pataki, *J Chromatogr*, 29 (1967) 126
159 S Seipel and U Reichert, *J Chromatogr*, 135 (1977) 485.
160 E Randerath and K Randerath, *J Chromatogr*, 16 (1964) 111
161 T W Munns, K C Podratz and P A. Katzman, *J Chromatogr*, 76 (1973) 401
162 T W Munns and H F Sims, *J Chromatogr*, 111 (1975) 403
163 I Das, *Biochem Soc Transactions, 576th Meet, London*, 6 (1978) 1047
164 J S Miller and R R Burgess, *Biochemistry*, 17 (1978) 2054
165 A D Mirzabekov and B E Griffin, *J Mol Biol*, 72 (1972) 633
166 H J Gross, H. Kroath, H -G Janda and C Jungwirth, *Mol Biol Rep*, 4 (1978) 105.
167 P L Bergquist, *J Chromatogr*, 19 (1965) 615
168 L Diels and R DeWachter, *Arch Int Physiol Biochim*, 88 (1980) B26
169 S Hashimoto, W. S M. Wold, K H Brackmann and M. Green, *Biochemistry*, 20 (1981) 6640
170 E Sawicki, T W Stanley and W C Elbert, *Mikrochim Acta*, (1965) 1110
171 E Sawicki, T W Stanley, W C Elbert and M Morgan, *Talanta*, 2 (1965) 605
172 E Sawicki and H Johnson, *J Chromatogr*, 23 (1966) 142
173 E Sawicki, T W Stanley, S McPherson and M Morgan, *Talanta*, 13 (1966) 619
174 E Sawicki, C. R Engel and W C Elbert, *Talanta*, 14 (1967) 1169
175 E Sawicki, T W Stanley, W C. Elbert, J Meeker and S McPherson, *Atmos Environ*, 1 (1967) 131
176 E Sawicki, M Guyer, R Schumaker, W C Elbert and C R Engel, *Mikrochim Acta*, (1968) 1025
177 C R Engel and E Sawicki, *J Chromatogr*, 31 (1967) 109
178 H. Matsushita and Y Suzuki, *Bull Chem Soc Jap*, 42 (1969) 460
179 J Kraft, A Hartung, K -H Lies and J Schulze, *J High Resolut Chromatogr Chromatogr Commun*, 5 (1982) 482
180 D Srzić and L Klasinc, *Recent Dev Mass Spectrom Biochem Med Environ Res*, 7 (1981) 195
181 J Janák, *J Gas Chromatogr*, 1 (1963) 20
182 J Janák, *Progr Thin-Layer Chromatogr Relat Methods*, 2 (1971) 63
183 R Kaiser, *Chromatographie in der Gasphase*, Bibliographisches Institut, Mannheim, 1969

184 R E Kaiser and R. Rieder, *J Chromatogr*, 142 (1977) 411
185 J A Kawatski and D L Frasch, *J Ass Offic Anal Chem*, 52 (1969) 1108.
186 N V Fehringer and J E Westfall, *J Chromatogr*, 57 (1971) 397
187 A. M. Gardner, *J Ass Offic Anal Chem.*, 54 (1971) 517
188 W B Burton, *J Chromatogr*, 53 (1970) 397
189 S K Yuda, *J Chromatogr.*, 50 (1970) 453
190 P Švec and M Zbiasrovský, *Sb Vys Sk Chem.-Technol. Praze, Org Chem Technol*, 17 (1972) 95.
191 J P Ebel, *Metodi di Separazione nella Chimica Inorganica*, Vol 1, CNR, Rome, 1963
192 A. Grand-Clement, Z Jakovac, M Lederer and E. Pluchet, *Proceedings of International Symposium on Microchemistry, Birmingham, 1958*, Pergamon Press, Oxford, 1960, p 231
193 C S Knight, *Nature (London)*, 183 (1959) 165
194 F Stern, G Grumet, F Trabal, A Mennis and H H Zinsser, *J Chromatogr*, 19 (1965) 130
195 D Baraldi, *J Chromatogr*, 42 (1969) 125
196 J Opieńska-Blauth, H Kraczkowski, H Brzusz-Kiewicz and Z Zagórski, *J. Chromatogr.*, 17 (1965) 288
197 H Takaki, *Miyazaki Daigaku Nogakubu, Kenkyu Hokoku*, 27 (1980) 211
198 K Breebart, A M Haan and S K Wadman, *Clin Chim Acta*, 37 (1972) 157.
199 R A de Zeeuw, *Chromatogr., Electrophor Symp. Int, 4th*, (1968) 537.
200 T Katayama and H Ikeda, *Sci Pap Inst Phys Chem Res*, 63 (1969) 49
201 S N Tewari and S K Shukla, *Pharmazie*, 32 (1977) 536.
202 S N Tewari and S K Shukla, *J Indian Acad Forensic Sci*, 17 (1978) 80
203 P Turano and W J Turner, *J Chromatogr*, 64 (1972) 347
204 H Matsushita and K Arashidani, *Bunseki Kagaku (Jap. Anal.)*, 20 (1971) 78
205 L Allen, *J Ass Offic Anal Chem*, 57 (1974) 1398
206 R J Alexander and M C Baur, *Cereal Chem*, 54 (1977) 699
207 D S P. Patterson, E M Glancy and B A Roberts, *Food Cosmet Toxicol*, 16 (1978) 49
208 J. D Craske and R A Edwards, *J. Chromatogr*, 51 (1970) 237
209 O Liška, J Lehotay, E Brandsteterová and G Guiochon, *J Chromatogr.*, 171 (1979) 153
210 J. D. Mahuren and S P. Coburn, *Anal Biochem*, 82 (1977) 246
211 S K Yasuda, *J Chromatogr*, 71 (1972) 481
212 A M Pumphrey, *Biochem J*, 102 (1964) 30P