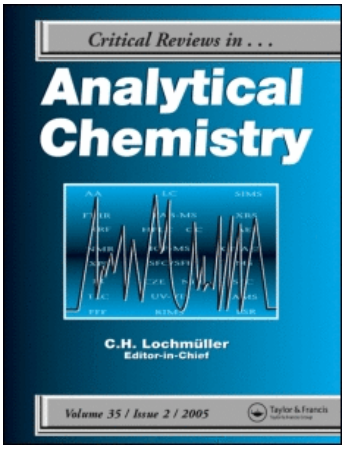

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THE PRESENT STATE OF QUALITATIVE THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

The history of thin-layer chromatography (TLC) commenced in 1938 when Izmailov and Shraiber[1] reported on the use of layers of adsorbents on glass plates instead of columns in the analysis of pharmaceutical tinctures. These workers used alumina and other adsorbents on microscope slides and called the technique "spot chromatography." It took about ten years before the method attracted more attention. Then, Williams,[2] Meinhard and Hall,[3] Kirchner, Miller and Keller[4-6] and Reitsema[7 8] applied several modifications of TLC, mainly in the analysis of lipophilic substances.

However, the extremely rapid development of TLC into an indispensable analytical tool in almost every laboratory started after the work of Stahl[9-13] (1956-1959), who paid special attention to the following points:

1. Standardization of the technique, including preparation of the adsorbent layers, layer thickness, and the sizes of the glass plates and the development chamber.
2. Construction of suitable apparatus.
3. Production of appropriate adsorbents and standardization of their properties.
4. Applicability of the method.

Soon the technique gained recognition as a very powerful separation technique and became widely accepted, first in Europe and then in the other continents. Today TLC is used in almost every research or routine laboratory. The advantages of thin-layer chromatography are its high resolution power, its speed, and its simplicity and low cost. Moreover, the technique can be carried out on every laboratory bench without requiring much space, and it gains versatility from the fact that a variety of sorbents can be used. After chromatography, detection of the separated substances is, in general, performed within a few minutes, either by using fluorescent indicators incorporated in the sorbent and irradiating with UV light, or by spraying with various reagents. It should also be noted that the majority of sorbents allow the use of drastic spray reagents. The limits of detection in TLC usually lie in the order of 0.1 -1 μg.

In the beginning the technique was most often applied to lipophilic substances. This was undoubtedly of advantage because paper chromatography, being very popular at that time, was chiefly successful in the hydrophilic field.

However, further investigations on the applicability of TLC clearly revealed that the technique could also be used in separations of hydrophilic character, and that, in many instances, TLC was even more effective than paper chromatography. The separation capacity, the sharpness of the spots, the speed, and the detection limits of TLC were generally superior to those of paper chromatography, so that during the last ten years TLC has superseded paper chromatography to a considerable extent. It should not be thought, however, that paper chromatography has no further possibilities.

The wide range of applicability of TLC and the progress in results are best illustrated by the various reviews that are devoted to the separation possibilities within the various classes of substances and that have appeared in the literature during recent years. The excellent material in the series *Advances in Chromatography*[14] and *Chromatographic Reviews*[15] may be mentioned here as an example.

Besides these results obtained in the various fields of application, a second type of progress in TLC can be noticed, namely, the progress in the understanding of the actual separation process. The question: "What happens on the plate during development and which factors are involved in the separation?" has had growing attention, especially during the last few years. Obviously, this has led to a much better insight into the various factors involved in TLC; and many of the laws from the early days of TLC, derived from empirical observations or based on incorrect conclusions, have since been modified or sometimes completely rejected. On the other hand, the growing understanding of the principles of TLC has been an important factor in improving TLC results, and, moreover, it has been responsible for the development of newer TLC techniques yielding even better separation possibilities than the conventional method. These new techniques are not always as simple as the parent technique, but their increased resolution power offers new possibilities in those cases which cannot be resolved with conventional methods.

It is the aim of this chapter to highlight the developments which have been of chief impor-

tance for the accretion of understanding in practical TLC, to evaluate the newer techniques and trends, and to discuss their possible role in the future development in TLC.

The practice of TLC can be divided into three distinct stages, namely: processes prior to development, the actual development, and processes after development.

In the next paragraphs this scheme will be followed.

BEFORE DEVELOPMENT

Sorbents

Perhaps the most important feature which has brought TLC to so many different fields of application is the availability of a great many sorbents* which may serve as supporting medium. Nowadays, however, adsorption processes are far less important in the general practice of TLC, so that the use of the name "sorbent" seems more correct. Silica gel, alumina, cellulose, modified celluloses such as DEAE-, ECTEOLA, CM- and PEI-celluloses, polyamides, dextran gels, etc. are available in various types and sorts (see for review[16-18]). Differences between the types may be due to differences of particle size, pore volume, active sites, pH, the presence of a binder, the presence of a fluorescent indicator, or the grade of purity. This has the advantage that the analyst can choose the most appropriate sorbent or use an impregnated sorbent for his problem; and, if he cannot find what he wants today, he may find it tomorrow as the number of available supports is still increasing.

On the other hand, however, the great variety of sorbents also carries severe disadvantages. Standardization of sorbents becomes almost impossible, and this will have its influence on comparative investigations. Furthermore, one should carefully distinguish among sorbents of different origins. Identically named sorbents from different sources are by no means comparable. The properties of the sorbents vary from manufacturer to manufacturer, chiefly due to different preparation procedures. It has even been observed that a supplier of TLC sorbents sold materials obtained from different sources under the same name.[19]

How far different production batches of the same sorbent remain comparable to one another is a somewhat difficult question. It is true that the various manufacturers have understood that the properties of their products should be as reproducible as possible, and usually this requirement is satisfactorily fulfilled. However, over a period of years, identically named products may give significantly different results because even the manufacturers are not always acquainted with all the factors influencing the properties of their products. It will be clear that this situation has rather dangerous aspects with regard to the publishing of R_F-values.

A new and completely different type of sorbent was introduced very recently by Cremer et al.[20] In this new type of solid-liquid chromatography, which was called thin-film chromatography, the support consists of a vacuum-evaporated layer of metal oxides with a thickness of about 1 um. Separations were obtained ten times faster than by conventional TLC, and a number of theoretical plates up to 4000/cm was claimed. Nanogram quantities of the materials separated could be detected by means of a specially devised conductivity detector. Oxides of indium and bismuth have been tested with dyes and amino acids.

This technique looks interesting and may become a useful method beside the existing TLC techniques. It may also be expected that this report will initiate research for other types of sorbents which differ basically from those presently in use in TLC.

Preparation of the Plates

Handmade Plates

The standard size plates in TLC are 20 x 20 cm and 10 x 10 cm, but microscope slides are also recommended by various authors. The former plates are best spread with one of the commercially available products.** Spreading is, in general, preferable to methods such as pouring, dipping, or spraying the layers. Slurries can be made in many different ways. (For review see reference 18.) One of the major problems in spreading is caused by the fact that the glass

*In earlier years the name "adsorbent" has been frequently used for the layer material because of the fact that many TLC separations, carried out in the lipophilic field, could be described as adsorption chromatography.

**Loose layers are no longer used.

plates for TLC have an allowed tolerance of thickness of ± 0.1 mm. This problem has been solved by recently designed apparatus in which the plates are pressed up to two metal channels, one on each side of the plate, so that the surfaces of all plates are level irrespective of their thickness. Apparatus designed on the above principle can be obtained by Shandon Ltd., London, or Quickfitt and Quartz, London. In the opinion of the author an almost ideal apparatus would be obtained if the above apparatus could be combined with the Desaga slurry container according to Stahl. The latter spreader, of the tip-over type, can be more precisely adjusted and allows the analyst more time after pouring the slurry into the container. The combination of these two items should be advantageous in the practice of TLC but is probably difficult to realize in view of patent and license rights. Semiautomatic spreaders for large-scale production, as well as applicators for preparing gradient layers, are now also available.

Ready-to-use Plates

During recent years a variety of prefabricated ready-to-use plates or sheets has appeared on the market. These plates are more expensive than homemade layers but are directly available to the analyst, and they overcome the difficulties that unskilled workers sometimes encounter in spreading the plates. Furthermore, it is claimed that ready-to-use plates give more reproducible R_F-values than can be obtained with the conventional homemade layers. Investigations of Shellard[21] and Jork[22] have revealed that better reproducibility is only partially obtained. Within the same ready-to-use plate the R_F-values of the same substance show less variation than on handmade layers, but R_F-values obtained on different ready-to-use plates, even from the same pack, show considerable variations. The latter may be due to differences in layer thickness. The problem of a change in properties over a period of years, which sometimes occurs with normal sorbents, may also hold for prefabricated plates.

The use of prefabricated layers on plastic sheets or aluminum foils has the advantage that the sheets can be cut in the desired dimension. It is sometimes noticed, however, that the substances on these sheets do not move vertically but towards the center of the plate and that tailing is more severe than on normal plates.[21 23] Prefabricated layers are now also obtainable on reels.

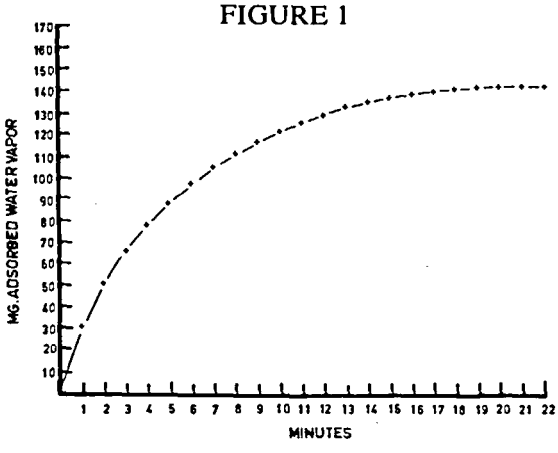
Activation of the Plates

It has been common practice in TLC with inorganic sorbents, such as silica gel and alumina, to heat the plates after spreading and air-drying the spread slurry to obtain layers with suitable "activity." During the heating period the excess of water used to prepare the plate and physically bound water will be removed. There is no doubt that this activation procedure has brought about much confusion, which is reflected in an abundance of different drying procedures; these range in time from 30 minutes to 36 hours and in temperature from room temperature to 240°C. Furthermore, it is claimed that many of the ready-to-use plates need no activation at all. Fortunately, the factors involved in the activation process recently have been studied in more detail, and the better understanding will probably make things less complicated.

In the following we will examine these factors in more detail with silica gel as the model, but the principles are applicable to other sorbents as well.

The adsorption properties of silica gel are determined by the number of surface hydroxyl groups (silanol groups).[24] The siloxane bonds also present at the surface are much less capable of adsorption. With water, strong hydrogen bonding will occur at the silanol groups, and this physisorbed water prevents the adsorption of other less polar components. Physisorbed water can be reversibly removed by heating at temperatures up to 150°C, whereas irreversible dehydration will occur at temperatures above 200°C.[25 26] Thus, heating the plate at about 150°C would give the highest activity. After heating, the plates are usually cooled and stored in a desiccator over a drying agent and then spotted. However, most activated plates are spotted while in close contact with the ambient laboratory atmosphere, i.e., *in contact with moisture*, and this means that within a few minutes the plate has lost most of its activity. The speed of adsorption of water vapor has often been underestimated. Figure 1 shows the adsorption rate of water vapor at a relative hu-

FIGURE 1



Adsorption of water vapor by a TLC silica gel plate at a relative humidity of 50%. Plate dried for 30 min at 110°C, cooled and stored over calcium oxide, then exposed to the air. Plate size 20 x 20 cm; layer thickness when spread 0.25 mm.[27]

midity of 50%.[27] Similar results were obtained by Dallas[28] and Geiss et al.[29] The use of a cover over the active plate in order to decrease adsorption of water vapor should not be recommended, for this does not actually prevent the adsorption but merely causes its extent to be irreproducible. When working with stable substances, reactivation of spotted plates by a further heating for, e.g., 30 minutes has also been advocated to obtain active plates,[30] but it still remains a problem that these plates must be transferred from the oven to the desiccator and thence to the development chamber. During these manipulations, water vapor will be immediately adsorbed, and even small amounts of water may have great influence on the chromatographic behavior of the spots.[19 31]

It will thus be clear that TLC requires facilities which allow working under controlled humidity conditions, especially to obtain reproducible results. As it is almost impossible to exclude any influence of water vapor, it will be necessary to work under standardized humidity conditions so that the sorbent always takes up a fixed amount of water. Suitable solutions for this problem have been designed. Geiss et al.[29 32] used their KS-Vario-chamber, in which the plate can be developed over saturated salt solutions of appropriate concentrations, thus giving a fixed relative humidity. Drost and Reith[33] made use of a box of the incubator type, in which air of constant relative humidity is introduced continuously and in which plastic gloves, attached to the wall, allow manipulations from outside with the plate in the box. After activation, the plate is cooled, stored, spotted, and developed in this box.

In our opinion, however, the optimal solution for accurate work in TLC is the use of a room with temperature- and humidity-controls that can be set at any desirable value. During spotting, the plates are allowed to equilibrate with the existing relative humidity and temperature, which will result in the uptake of fixed amounts of water vapor. Obviously, such a room has great advantages for standardized reproducible work in TLC,[34] but it is somewhat expensive to set up.

If one does not have a variable humidity- and temperature-controlled room available, the reliability of R_F-values found can be tested in the following way. Each day the humidity and the temperature must be registered; and by chromatographing a suitable reference mixture, one can establish the range in which reproducibility is observed. If work is performed outside this range, R_F-values should be considered with care. For work done within the range, R_F-values will be fairly reliable. In general the chromatography of lipophilic substances is easily affected by humidity changes, and here the conditions will have to be controlled within narrow ranges to obtain satisfactory reproducibility. With more polar systems the permissible range of conditions is usually wider. These phenomena will be discussed in more detail under the heading "Reproducibility."

Some sorbents, e.g., cellulose plates, are often air-dried overnight without further heating. This means that the water content of such plates will reach equilibrium with the ambient relative humidity. If the latter can be kept constant, the water content of the plates will be constant too. If not, the water content will vary as the relative humidity changes. Again, the effect of different water contents is usually more pronounced in non-polar systems.

Recapitulating, it should be emphasized that the "activation" procedure can only be considered to be a rapid drying method. It does not guarantee any actual activity during the chromatographic process as a consequence of the fast readsorption of water during the handling of the plate after the drying procedure. It would be fortunate if this could be mentioned

in the brochures and folders of the sorbent manufacturers. Also, the frequent assertion that ready-to-use plates are of high activity and would not need further activation is very misleading because, here also, the plates readily adsorb water vapor from the ambient atmosphere. Their actual activities are thus determined by the humidity and temperature in the laboratory and *not* by any drying procedure of the manufacturer.

Hence, if accurate TLC work with reproducible R_F-values is required, work must be done under controlled relative humidity and temperature.

Application of the Sample

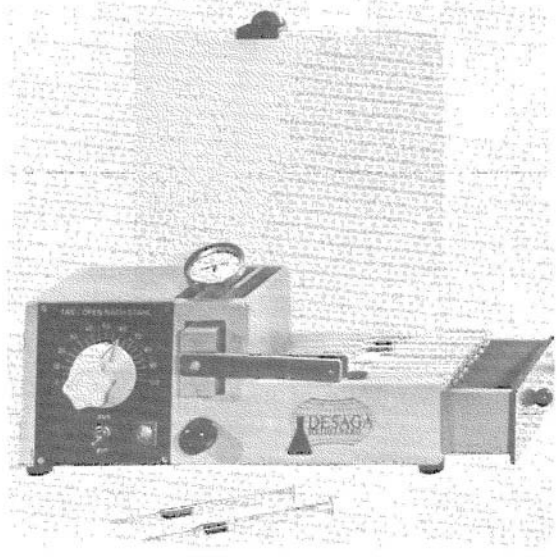
For qualitative work samples are best applied with micropipets or with disposable microcaps. Melting-point capillaries can also be used, but they lack any indication of the applied volume. Most layers adhere well enough to be touched so that a micropipet can be placed vertically on the layer, but one should avoid injuring the layer. This has the advantage that diffusion of the spots during application can be kept to a minimum as the outflow can be controlled just as in volumetric analysis. Simultaneous drying during application, e.g., with a cold hair drier, is useful, particularly with less volatile solutions and if the sample is not affected by air.

When unstable substances are under investigation, they should be handled with special care. Spotting should be done immediately after dissolution, light should be excluded as much as possible, and it is recommended to spot the samples in an inert atmosphere, e.g., CO_2 or N_2. Furthermore, one should be aware that decomposition of the samples may occur during development. This can be detected by two-dimensional development, using the same solvent twice. If no decompositions or artifacts occur, the spots should lie on the diagonal.

If a reasonable quantity of the sample is available, it may be advantageous to apply the sample as a small streak. Because there is less diffusion in the direction of solvent flow, the separations may become sharper.

A new application technique was recently developed by Stahl[35] for substances which are volatile at higher temperatures or which can be steam-distilled. He designed a TAS-oven (=thermomicroseparation, transfer, and application according to Stahl) which enables its user to separate and to apply volatile substances directly from mixtures or natural products onto the TLC plate without recourse to time consuming extraction, cleanup, etc. Its temperature can be set at any desired value up to 200°C, and it is thus very suitable for the analysis of volatile substances in drugs, natural products, etc. The only disadvantage is, however, that until now, the TAS-oven could not be used for quantitative work. The method works with a glass cartridge open at one end containing a solid with crystal water, e.g., silica gel, and the sample. The cartridge is placed in an oven with the open end protruding a few mm out of the oven. The oven is then heated electrically, and the emerging vapors are released at the end of the open tip and directly deposited as a spot on the TLC plate. If necessary, a stream of gas or air can be passed through the cartridge to speed up the transport of volatile substances towards the plate. The apparatus can be seen in Figure 2. Pyrolyses of thermally unstable compounds can also be studied with this apparatus.

FIGURE 2



TAS-oven according to Stahl.
Courtesy: C. Desaga, GmbH, Heidelberg.

DEVELOPMENT OF THE PLATE

Introduction

Originally, the technique devised by Stahl

made use of rectangular glass development chambers, about 25 x 21 x 10 cm. These are now known as N-chambers. All chromatograms were run after saturating the chamber atmosphere with solvent vapors, just as in paper chromatography. Rapid saturation was obtained by lining the chamber walls with filter paper. It was soon observed that the reproducibility of the technique could be improved. This has led to the introduction of the Sandwich-chamber or S-chamber.[36] In this chamber the TLC plate is covered by a clean glass plate, kept together by spring clips but separated by means of U-shaped spacers at the side and the top edges. This of course provides a very narrow chamber which can be rapidly saturated, and troubles originating from the gas phase were thus reduced to a minimum. Indeed, the reproducibility could be increased. These N- and S-chambers are by far the most commonly used for normal ascending development and, in many cases, give satisfactory results for routine work. It is surprising, however, that despite the exhaustive attention that has been given to reproducibility, activation, sorbent, quality of the solvent, and many other factors, very little has been done to evaluate the influence of solvent vapor during the run. Recent investigations have revealed that solvent vapor plays a major role in the separation, particularly when multicomponent solvents are used. The essential details will be discussed below, again with silica gel as the model.

Influence of Solvent Vapor

It is not to be expected that the adsorptive properties of the solvent would be restricted to water vapor alone, and, in fact, all solvent vapors appear to be adsorbed to different extents, their degrees of adsorption being dependent on their abilities to undergo hydrogen bonding. Geiss et al.[29] were the first to show that the commonly used TLC sorbents were capable of adsorbing significant amounts of benzene vapor, with silica gel up to 50% of its own weight and alumina up to 11%. These workers related the benzene-vapor adsorption to the water-vapor-adsorption equilibrium, and it was found that benzene vapors could displace water vapor from the sorbent, as a consequence of the adsorption competition between benzene and water. The amount of adsorbed benzene also has its influence on the development because the preloaded plate needed smaller amounts of benzene solvent for development.

Then a number of investigations, carried out in the laboratory of the present author, revealed the general importance of solvent vapor for TLC.[27 37 38] These investigations, primarily done in order to find an explanation for the fact that unsaturated chambers* yielded a better separation of barbiturates than saturated chambers, showed that each vapor had its own adsorption affinity for the sorbent, its magnitude being dependent on the ability to undergo hydrogen bonding. This is depicted in Figure 3, in which adsorption data are given for three solvent vapors in both saturated and unsaturated chambers. Due to the fact that in the beginning more vapor is available in the saturated chamber, these curves reach their maxima earlier than those in unsaturated chambers. When a TLC plate is brought into an atmosphere with two vapors, e.g., chloroform and acetone, it is clear that there will be an adsorption competition between chloroform and acetone, the final rates of adsorption being dependent on the adsorption affinities, vapor concentrations, and temperature. Moreover, it should be remembered that water vapor will always be present and that it will also take part in the adsorption competition.

The investigations also revealed that the amount of vapor adsorbed by the plate plays a very important role because it affects the migration rates of the substances to be chromatographed. If, for example, we take a binary mixture of chloroform and ether as the solvent, we may conceive of the following processes:

1. By capillary action of the porous adsorbent, the solvent ascends and will be demixed by a process comparable to frontal analysis; a certain zone of chloroform is followed by the binary mixture. Because of the stronger hydrogen bonding between ether and the sorbent, the ether is adsorbed from the solvent until equilibrium is obtained.

2. In the dry part of the plate adsorption

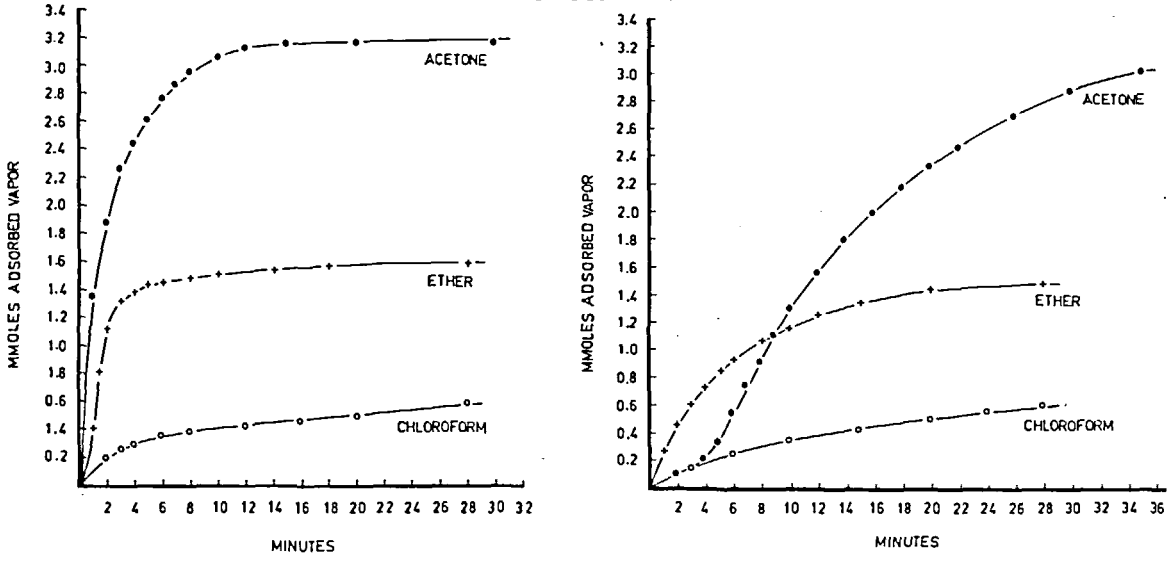
*An unsaturated chamber is one into which the plate is placed immediately after introduction of the solvent. During development the chamber is closed with a lid.

of solvent vapor will occur; and, ether, being more strongly adsorbed than chloroform, the adsorbate will consist mainly of ether.

3. In the wet part of the plate absorption of vapor and evaporation of solvent take place.

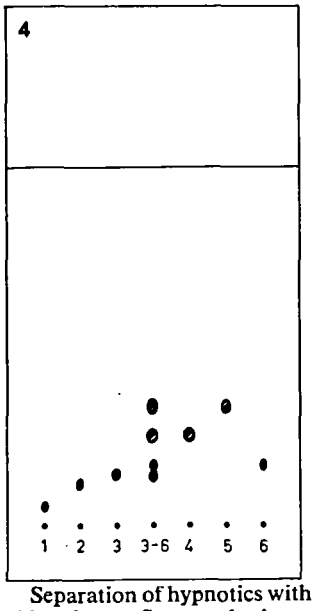
As a consequence of processes (2) and (3), the leading edge will not be pure chloroform in normal TLC. Furthermore, the extent to which the processes (2) and (3) will affect development will depend on many factors such as the vapor pressure and the relative affinity of the solvent components for the sorbent, the geometry of the chambers, and the degree of chamber saturation. This is demonstrated in the following figures. Figure 4 shows chromatograms of heptobarbital (1), phenobarbital (2), allobarbital (3), hexobarbital (4), methylphenobarbital (5), bromisoval (6), and a mixture (3-6) of the latter four components on silica gel, developed with chloroform, whereas Figure 5 shows chromatograms of the same substances with ether as the solvent. Two differences can be observed when comparing these figures: with ether as solvent, allobarbital (3) runs faster than hexobarbital (4) and bromisoval (6) runs slower than spots 3, 2, and 1. When using chloroform-ether (75 + 25) as solvent, Figure 6 shows a combined effect of chloroform and ether: the spots run higher than with chloroform alone, and allobarbital and hexobarbital show about the same migration rate. In unsaturated chambers, however, and again with chloroform-ether (75 + 25) as solvent, a different picture is obtained, as shown in Figure 7. First, the spots run higher than in the saturated chamber due to increased evaporation of solvent from the plate during the run so that more solvent is required to develop the plate to the same height. Second, and much more important, allobarbital runs distinctly slower than hexobarbital—which is reminiscent of the separation in Figure 4 with chloroform as solvent—and, accordingly, the mixture 3-6 is clearly separated. Furthermore, bromisoval has the same migration rate as heptobarbital. Thus, Figure 7 shows the influence of both ether and chloroform, but, in comparison to Figure 6 (saturated chamber), the separation sequence reveals a much smaller ether effect. This can be explained by Figure 3, showing that in unsaturated chambers the amount of adsorbed ether vapor will be less than in saturated chambers, particularly at the beginning of the run. Obviously, the amount of adsorbed ether greatly affects the separation, with the amount of ether present in the solvent being of less influence since this was not changed. Additional and perhaps even stronger evidence for this fact can be seen in Figure 8, which was obtained by developing a plate in an unsaturated chamber with chloroform only, but

FIGURE 3



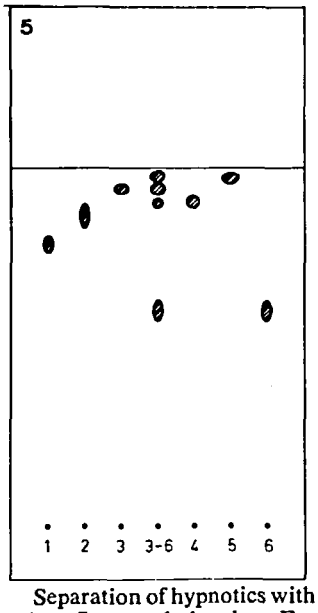
Vapor adsorption of TLC solvent components on a dry silica gel plate in saturated (left) and unsaturated (right) atmospheres. From *Anal. Chem*, 40, 915, 1968. Copyright 1968 by the American Chemical Society and reprinted by permission of the copyright owner.

FIGURE 4



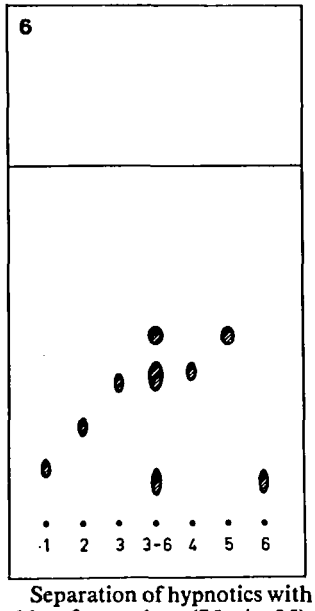
Separation of hypnotics with chloroform. Saturated chamber. 1 = heptobarbital, 2 = phenobarbital, 3 = allobarbital, 4 = hexobarbital, 5 = methylphenobarbital, 6 = bromisoval, 3 - 6 = mixture of 3 + 4 + 5 + 6.

FIGURE 5



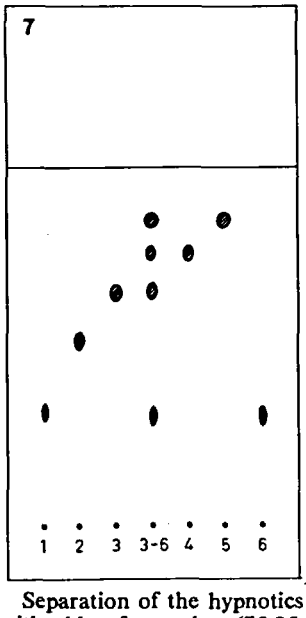
Separation of hypnotics with ether. Saturated chamber. For numbering see Figure 4.

FIGURE 6



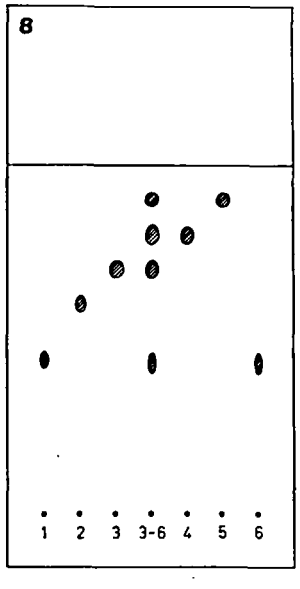
Separation of hypnotics with chloroform-ether (75 + 25). Saturated chamber. For numbering see Figure 4.

FIGURE 7



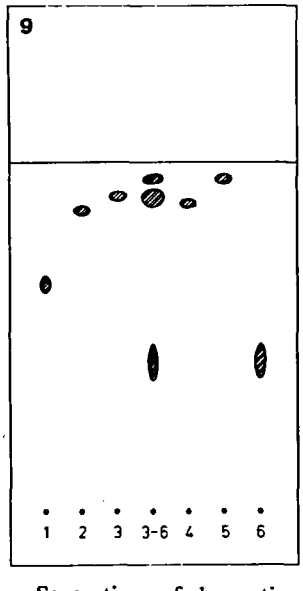
Separation of the hypnotics with chloroform-ether (75:25, v/v). Unsaturated chamber. For numbering see Figure 4.

FIGURE 8



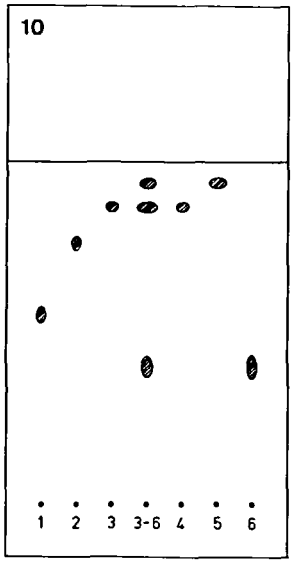
Separation of hypnotics with chloroform in the presence of a trough containing ether. Unsaturated chamber. For numbering see Figure 4.

FIGURE 9



Separation of hypnotics. Presaturation of the plate with vapor by a trough containing ether for 5 min., followed by development with chloroform. For numbering see Figure 4.

FIGURE 10



Separation of hypnotics. Presaturation of the plate with vapor by a trough with ether for 30 min, followed by development with chloroform in an S-chamber. For numbering see Figure 4. Figures 4–10, *J. Chromatogr.*, 32, 43, 1968, reproduced with permission of Elsevier Publishing Co.

a trough containing 10 ml of ether was present at the bottom of the chamber during development. As no ether is present in the solvent, any influence of ether on the separation can be due only to its vapor. This influence is evident if one compares Figure 8 with Figure 4 (solvent chloroform, no trough). It can also be observed that the separation in Figure 8 is almost identical with that in Figure 7, using chloroform-ether (75 + 25) as the solvent.

With higher amounts of adsorbed ether vapor, obtained by putting the plate and the trough into the chamber for a five-minute presaturation time and followed by the solvent chloroform, a correspondingly increased effect of ether on the separation is obtained, as can be seen in Figure 9, spots run higher than in Figure 8, allobarbital and hexobarbital now have identical migration rates, and bromisoval runs slower than heptobarbital. Presaturation with ether vapor for 30 minutes, followed by development in an S-chamber with chloroform, results in the separation of Figure 10. The influence of ether here becomes apparently so large that the separation is comparable to that in Figure 5 when we used ether only as solvent. The S-chamber was preferred here for three reasons:

1. Decreased evaporation of adsorbed ether from the plate.
2. Decreased removal of adsorbed ether by chloroform vapor.
3. Decreased ether adsorption by the solvent.

It thus becomes clear from these experiments that quite small amounts of adsorbed vapor can have significant effects on the separation. Calculations showed that with chloroform-ether (75 + 25) as solvent, 3.10 g is needed to wet 11.5 cm of the plate, in which about 420 mg of ether is found. However, the influence—if any—of these 420 mg, which should be responsible for the separation in Figure 7, is far less than the influence of the adsorbed 112 mg of ether vapor which caused the separation in Figure 10. These data underline the fact that the amount of adsorbed vapor is of chief importance for TLC and that the composition of the initial solvent is of minor importance.

Independently of our experiments, Van Dijk and Mys[39] showed similar results in separations of phenols. When benzene was used alone as solvent, but in combination with a trough containing benzene-methanol (90 + 10), the same separation was obtained as with benzene-methanol (90 + 10) as the solvent.

It should be remembered that the influence of vapor is not restricted to vapor adsorption on the dry plate. In addition, there is also absorption of vapor by the solvent on the wet part of the plate. In our opinion, however, the influence of the latter process will be rather small.

The question now arises: what will happen with the solvent vapors after being adsorbed by the sorbent, in particular when the ascending solvent reaches the vapor-impregnated areas? With single-component solvents the answer can be simple since the solvent and the vapor impregnation have the same identity. Part of the solvent, together with part of the vapor impregnation, will remain adsorbed by the sorbent, depending on the affinity for this sorbent. With multi-component solvents, however, the situation is much more complex. The vapor impregnation will consist of the more polar solvent component(s) whereas, due to the demixing process during development, the top solvent

front will consist of the less polar solvent component. It may be expected that the adsorbed vapor will be washed off by the ascending solvent, but it has been shown by Van Dijk,[39 40] using his specially designed pear-shaped TLC apparatus with trapping device at the top,[41] that the top solvent front in TLC with a multicomponent mixture does not contain peak concentrations of the more polar solvent components, i.e., a considerable amount of the vapor impregnation remains adsorbed and is not washed away. In order to see what the effects may be of this remaining vapor impregnation, it will be useful to examine the total number of processes involved in TLC so that a better insight can be obtained. A scheme of those processes which can be influenced by the analyst has been given recently[27] and may be summarized here.

The chromatographically important processes start at moment 0 when the liquid solvent (L) is introduced in the chamber, and vapor (V) is originated

$$O^1.\quad L \rightarrow V_O$$

When the plate is introduced, the dry sorbent (A) will be vapor-impregnated (iA)

$$O^2.\quad V_O + A \rightarrow iA_O$$

Development then starts at moment I with the ascending of the solvent into the vapor impregnated sorbent. This will cause a partial adsorption of the solvent by the sorbent similar to frontal analysis. This adsorbed part of the solvent, *together* with the underlying sorbent, will be called the stationary phase S, and the remaining, non-adsorbed, part of the solvent will be called the mobile phase M (*cf.* [42]).

$$I^1.\quad L + iA_O \rightarrow S_I + M_I$$

At the same time vapor equilibria, which tend to form between the solvent, the vapor phase, the adsorbent, etc., play a role:

$$I^2.\quad M_I, V_O \text{ and } L \rightarrow V_I$$

$$I^3.\quad V_I + iA_O \rightarrow iA_I$$

$$I^4.\quad V_I + M_I + S_I \rightarrow M_I^+ + S_I^+$$

(M+ S+ represent the vapor-induced mobile and stationary phase)

Further ascending of solvent is represented by

$$I^5.\quad M_I^+ + iA_I \rightarrow S_{II} + M_{II}$$

At the same time, a new supply of solvent is required, new vapor equilibria will tend to form (II^1 - II^4), the ascending of the solvent will continue (II^5), etc.

$$II^1.\quad L + S_I^+ \rightarrow S_I^1 + M_I^1$$

$$II^2.\quad M_{II}, M_I^1, V_I \text{ and } L \rightarrow V_{II}$$

$$II^3.\quad V_{II} + iA_I \rightarrow iA_{II}$$

$$II^4.\quad V_{II} + M_{II} + S_{II} \rightarrow M_{II}^+ + S_{II}^+$$

$$V_{II}^+ M_I^1 + S_I^1 \rightarrow M_I^{1+} + S_I^{1+}$$

$$II^5.\quad M_{II}^+ + iA_{II} \rightarrow M_{III} + S_{III}$$

$$S_{III}^+ + M_I^{1+} \rightarrow S_{II}^1 + M_{II}^1$$

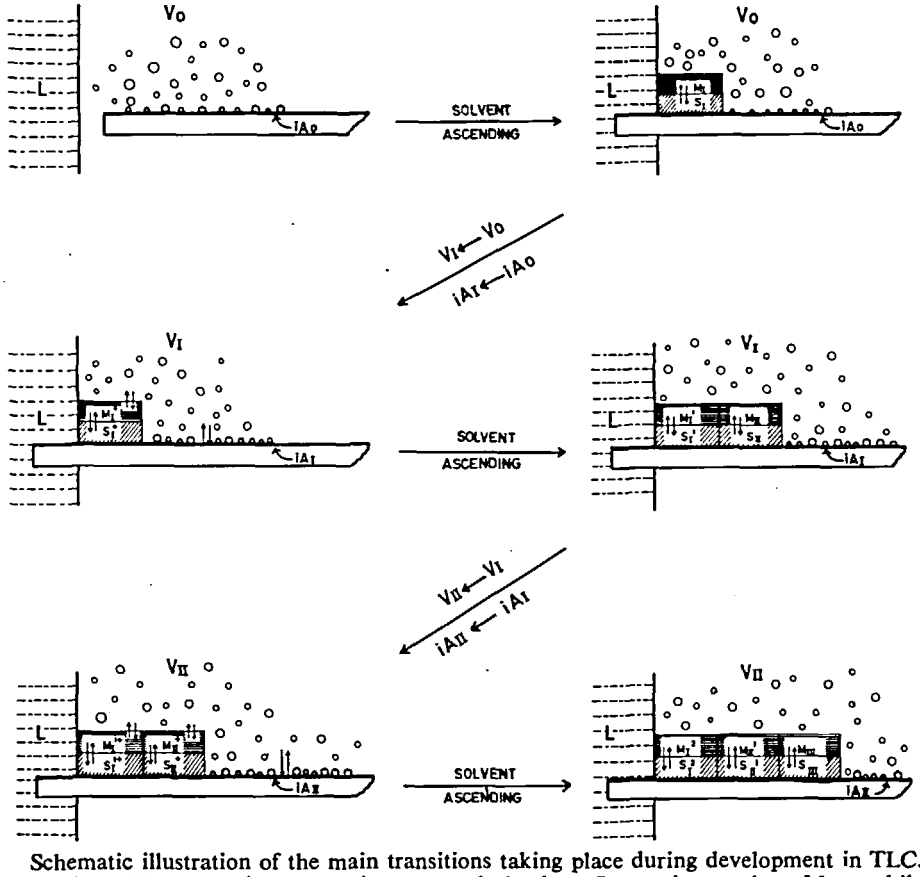
$$III^1.\quad L + S_I^{1+} \rightarrow S_I^2 + M_I^2, \text{ etc.}$$

The illustrations in Figure 11 may help in elucidating this scheme. Now, the impact of the vapor adsorption by the dry plate is that it can be considered as a kind of precursor of the stationary phase, formed when the solvent reaches the vapor-impregnated areas because of the more polar character of the vapor impregnation. Solute separation will be very dependent on the stationary phase, and, as is shown in the above experiments, this phase can be significantly affected by vapor adsorption.

It was also shown that the best separations are not *a priori* obtained in saturated chambers, but that unsaturated chambers can also be valuable. We will discuss this phenomenon in one of the next sections.

The effects of adsorbed vapor are, of course, greatly dependent on the ability to give hydrogen bonding. Thus, with rather non-polar solvent components like hexane, cyclohexane, and benzene, vapor adsorption will be small; and the stationary phase S will also be of minor importance due to the low affinity between these components and the commonly used sorbents. With solvents of medium polarity like chloroform, ether, acetone, and ethyl acetate, vapor

FIGURE 11



Schematic illustration of the main transitions taking place during development in TLC. L = solvent, V = vapor, iA = vapor impregnated adsorbent, S = stationary phase, M = mobile phase.[27]

component and single-component systems. With the latter the molecules of S and M are of the same nature, but the adsorbed part S will have different properties than the non-adsorbed part M. Consequently, the presence of a stationary and a mobile phase will allow partition adsorption will take place to a considerable extent, as can be seen in Figure 3; and hence the role of the stationary phase will also increase, while for highly polar components such as ethanol, methanol, ammonia, and water both the adsorption and the stationary phase will be of even greater importance.

Directly related to these phenomena is the character of TLC. Until now TLC has often been considered to be adsorption chromatography, probably because of the earlier experiments in the lipophilic field, done with rather non-polar solvents. However, with more polar solvents a stationary phase of adsorbed molecules will be present. This holds for both multi-processes to occur. Of course these partition processes may be influenced by adsorption processes within the stationary phase, so that it seems most suitable to describe TLC processes as a mixture of adsorption and partition chromatography. The extent to which one of these will predominate will be dependent on the polarity of the solvent components and on the sorbent. With cellulose, for example, it can be expected that the extents of adsorption processes will be rather small.

The occurrence of partition chromatography would be in agreement with the observation of round spots in various TLC systems, particularly with polar solvents, and with the linearity over a wide range of many "adsorption" isotherms in TLC. Moreover, Brenner et al.[43] have shown that the additivity rule of Martin[44] holds for various other polar solvent systems. This includes a linear relation between the R_M value, [$R_M = \log\left(\frac{1}{R_F} - 1\right)$] and the number CH_2

of CH_2 groups in homologous series in separations based on partition chromatography.

Finally, a few words may be added on the comparison between TLC and column chromatography. TLC is sometimes identified as "open column chromatography" and, moreover, many attempts have been made to transfer TLC separations to columns in order to obtain a separation on a preparative scale (*cf.* [39]). However, the above results make the assumed similarity between TLC and column chromatography with multicomponent solvents very doubtful because vapor impregnation in column chromatography cannot take place. This explains why many TLC separations with multicomponent solvents cannot be obtained on columns. Also, dry columns[45] [46] are different from TLC, although, in some instances, preliminary equilibration of the dry sorbent with the vapor components may be helpful.

Degree of Saturation of the Chamber Atmosphere

From the beginning of TLC it has been common practice to develop the plate in a vapor-saturated atmosphere. This was obtained by saturation of the development chamber prior to development. Most often the plate was left outside the chamber during saturation. This working procedure was already known in paper chromatography, and it should help to reach a type of ideal chromatography with a mobile phase of constant composition. Moreover, it was observed that chamber saturation increased the reproducibility and that it prevented the occurrence of edge phenomena.

During recent years, however, some confusion appeared to exist, which became most obvious during a symposium devoted to reproducibility problems in TLC and paper chromatography.[47–49] What is the degree of saturation in the normal practice of TLC, and is it optimal to affect suitable separation? The answer to the first part of the question can be found very clearly in a paper by Vanhaelen,[50] who observed the following phenomena.

1. Complete saturation of the chamber takes very long and is dependent on the diffusion velocity of the vapors under consideration. For example, with N,N-dimethylformamide, having a diffusion coefficient of 0.0844 cm^2/sec which is neither unusually large nor unusually small, it took over 10 hours to obtain complete saturation of a normal tank chamber.

2. When a TLC plate is introduced into a completely saturated chamber, significant desaturation occurs by vapor adsorption, thus fully disturbing the vapor equilibria.

3. If disturbance of the vapor equilibria is not desired, one can equilibrate the plate in the chamber during the saturation period. This method requires that the samples be spotted prior to equilibration. However, as spots tend to diffuse during saturation, this gives rise to poorer separations.

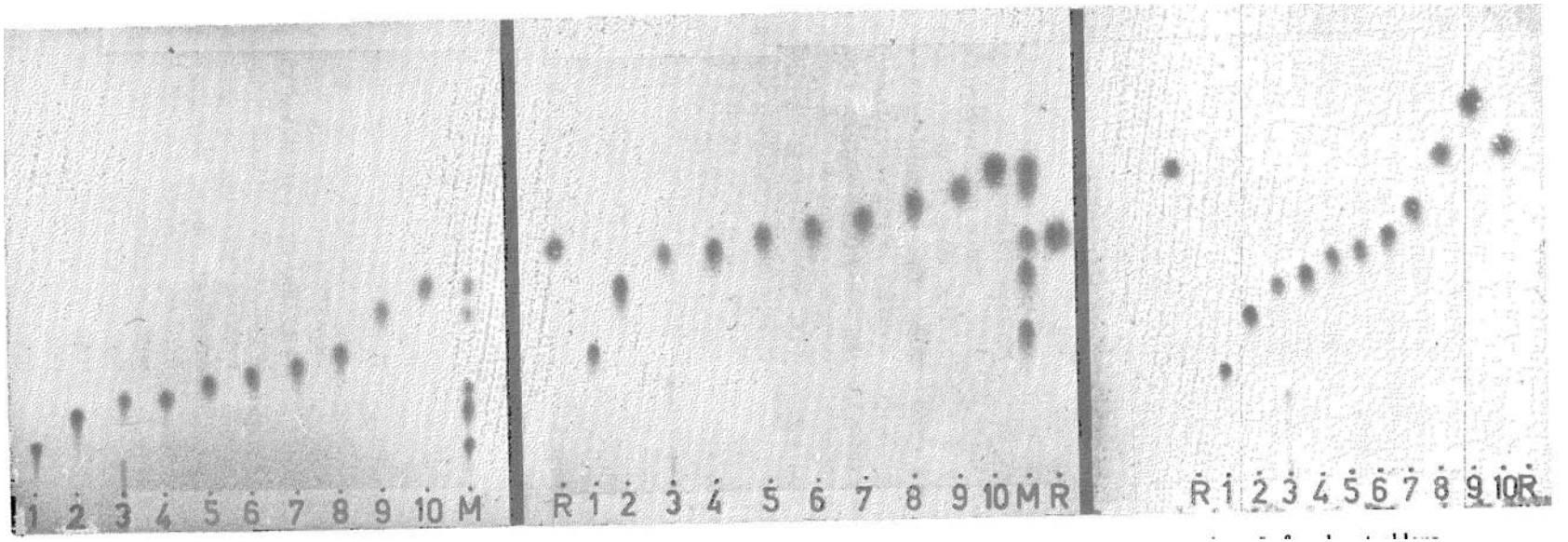
4. S-chambers give suitable saturation *during* development, and the spots remain of small size; but the resolution often compares unfavorably with normal chambers.

Thus, it can be concluded that saturation and optimal separation do not go hand in hand in TLC. The commonly used saturation procedure in TLC does *not* give saturation at all, and it can only be considered as an attempt to standardize the vapor conditions so that reproducibility may increase. This standardization is certainly valuable; but when working with TLC, the analyst should be aware of the actual conditions governing his separation in either an N-, an S-, a BN-, or any other chamber. The papers of Vanhaelen and those of Brenner[48] and Geiss[49] will be of great help.

Unsaturated Chambers

Beside the critical evaluation of the processes in saturated chambers, a new trend has been advocated during recent years, particularly in view of its increased resolution power: the use of unsaturated chambers. Here, development of the plate is started immediately after the solvent is introduced without any prior saturation or pre-equilibration. Thus the chamber atmosphere is almost devoid of solvent vapor at the beginning but becomes gradually more and more saturated during development, which, in fact, takes place under conditions even further from equilibrium than in "saturated" chambers. Increased resolution power has been reported in a variety of papers dealing with several classes of substances and solvents. Solvents were all two- or multi-component systems. A possible explanation which is closely related to vapor influence was recently developed in the author's laboratory.[53] An example

FIGURE 12



Improved separation of barbiturates on silica gel in unsaturated chambers as compared to separations in saturated chambers. Left: solvent, chloroform-acetone (90 + 10); saturated chamber. Center: solvent, chloroform-acetone (80 + 20); saturated chamber. Right: solvent, chloroform-acetone (90 + 10); unsaturated chamber. 1 = heptobarbital, 2 = phenobarbital, 3 = cyclobarbital, 4 = allobarbital, 5 = butobarbital, 6 = pentobarbital, 7 = itobarbital, 8 = secobarbital, 9 = hexobarbital, 10 = methylphenobarbital, M = mixture of 1 + 2 + 4 + 9 + 10, R = reference substance 4-nitroaniline.

of the improved separation in unsaturated chambers can be seen in Figure 12. When considering the vapor-adsorption curves of Figure 3, it becomes clear that with chloroform–acetone as solvent the vapor uptake will chiefly consist of acetone. In saturated chambers the acetone-vapor uptake takes place very rapidly, and, in a few minutes, the plate has reached its maximum adsorption. In unsaturated chambers, however, acetone vapor becomes only gradually available; and as the solvent will ascend at once and will gradually cover more and more of the dry adsorbent, it will prevent the lower parts of the plate from adsorbing more acetone vapor. On the other hand, the upper parts of the plate will continue to adsorb increasing amounts of acetone, and, accordingly, a concentration gradient of adsorbed acetone will exist before the solvent covers the vapor-impregnated areas. Such a gradient will be very suitable for the resolution, for the faster running spots will gradually meet areas with more of the polar vapor component, and this will result in an acceleration of the migration of these spots. The lower spots will only meet areas with a relatively small amount of polar vapor, and their migration rates will be affected to a much smaller extent. Thus, with multi-component solvents a better separation can be obtained in unsaturated chambers. The rate of separation improvement will be dependent on the steepness of the vapor gradient, the volatilities and diffusion rates of the vapor components, the chamber volume, and the temperature so that a distinctly improved separation cannot be guaranteed for every case. Moreover, it should be remembered that in unsaturated chambers solvent evaporation during the run will take place to a higher extent than in saturated chambers, thus requiring extra solvent supply from the solvent reservoir. As a consequence of this extra supply, more solvent will be transported across the lower parts of the plate than across the higher parts, and this in turn will result in a higher migration of the lower spots. This "pushing up" will hence have an adverse effect on the spread of the spots, but, as can be seen from the literature, this effect is often of minor importance in comparison to the positive effect of the improving vapor gradient.

A rather surprising result with regard to unsaturated chambers should also be mentioned here. During a recent interlaboratory investigation in The Netherlands, primarily carried out to evaluate the reproducibility of R_F-values and the usefulness of R_F-correction methods, it was found that with a *single-component* solvent a significantly improved separation was obtained[51] (color dyes developed with benzene). A possible explanation for this phenomenon is not yet available. It may be that the adsorbed benzene vapor carries certain properties which are not yet fully understood. Further investigations are being carried out to see whether similar observations can be made with other single-component systems.

It has been remarked that unsaturated chambers should involve some disadvantages, including less reproducible results and the appearance of edge effects. However, if the procedure in unsaturated chambers is standardized, i.e., if the plate is always introduced immediately after the solvent, and if other factors (temperature, humidity, chamber volume) can be kept constant as well, the same reproducibility can be obtained as in saturated chambers. Edge effects have not been reported by the recent users of unsaturated chambers so that the earlier observations may have been caused by other artifacts. The explanation given at that time also does not seem very reasonable.[27] The only difficulty with unsaturated chambers may be the occurrence of vapor convection as a consequence of different specific gravities of the vapor components, but this has been observed in only a few cases.

Thus the use of unsaturated chambers, especially with multicomponent solvents looks very promising, and it may be expected that it will be very helpful in the increasing demand for improved resolution power. The time required for development is somewhat longer; but since no saturation is needed, there remains a considerable gain in time, which is another practical advantage.

Choice of Chamber Type

Two types of chambers, the N-chamber with an inner diameter of about 10 cm and the S-chamber with an inner diameter of 1-2 mm, are the most often used in normal qualitative TLC. The S-chamber has been devised to minimize the difficulties in irreproducible vapor condi-

tions, and it has been advocated that the S-chamber be used as the standard chamber for TLC. This may be useful when using single-component solvents, but with multicomponent systems there are severe objections,[52-54] also based on the influence of solvent vapor. The processes involved in TLC with multicomponent solvents in N-chambers were described in an earlier section and can be summarized as follows:

1. The solvent ascends by capillary action and is demixed on the plate by a process comparable to frontal analysis.

2. In the dry part of the plate adsorption of vapor will take place, the more polar components being preferentially adsorbed.

3. In the wet part of the plate absorption of vapor, as well as evaporation of solvent, takes place.

As a consequence of processes (2) and (3) in N-chambers, the top solvent front will not contain the least polar compound alone but will also contain the other, more polar solvent components. In S-chambers, however, the situation is much different. The demixing process will also take place here, but due to the very small diameter of the S-chamber, diffusion of solvent vapor will be strongly limited within the time given. Saturation of the chamber volume will be achieved by solvent evaporation from the solvent front; and because this front will only consist of the least polar component, no polar vapors will be available for adsorption on the dry plate. Hence processes (2) and (3) in the S-chamber have no influence on the composition of the top solvent front which remains to be composed of the least polar component only. This will give rise to the occurrence of sharp β, γ, δ,. . . , etc., fronts in the S-chambers. Because the R_F-values are dependent on the amount and on the character of adsorbed vapor on the dry plate, it thus will be obvious that the separations in the N-chamber and the S-chamber come out quite differently because in the S-chamber little or no polar vapors can be adsorbed by the dry plate. Schweda[52] and De Zeeuw[53] showed that several separations even became impossible in the S-chamber.

Thus S-chambers and N-chambers cannot be compared because the vapor-adsorption conditions are totally different. In fact, S-chambers show more resemblance to column chromatography because on columns vapor diffusion and impregnation are also limited. On the other hand, it becomes obvious that the very advantage of TLC in N-chambers is caused by the vapor adsorption processes, and this is probably the reason that various TLC separations with multicomponent systems are superior to those on columns or in S-chambers.

It will be clear that with regard to reproducibility problems the S-chamber is advantageous only with single-component solvents. It has been recommended to use S-chambers throughout as a fundamental step towards improved reproducibility and to avoid solvent mixtures as well. It will be obvious from the above discussion that by such procedures TLC would be considerably deprived of its resolution power. The writer fully agrees with Brenner[54] when he says: "Do not let us indulge l'art pour l'art. Reproducible R_F-values do not improve TLC, though they make it considerably more complicated." Obviously, many separations are only possible with multi-component solvents in N-chambers or even unsaturated N-chambers. It is important to remember practical TLC is primarily concerned with the feasibility of the separation. In order to achieve this, it is not always possible to work under optimum reproducibility conditions.

Some of the disadvantages of the S-chamber can be overcome to a certain extent by preadsorption of vapor on the plate prior to development or by the use of a counterplate soaked with the solvent. These techniques are somewhat complicated, however, and they also involve various factors which remain different from those in N-chambers.

Some confusion seems to exist about the saturation in S-chambers. Several authors[55,56] have used the name "saturation chamber," but we should like to warn against its use. Normal S-chambers are completely unsaturated at the beginning of development, and vapor impregnation does not take place except in a small zone just above the ascending solvent front. During development, saturation of the small chamber volume takes place very rapidly, but this is not important for the separation process.[27,49]

A special type of S-chamber is the horizontal BN-chamber, which should be distinguished from the normal S-chamber and for which the

above conclusions do not apply. The BN-chamber, a horizontal S-chamber which can be thermostated and used for continuous development, has been especially devised to make profitable use of the demixing process with multicomponent solvents so that "polyzonal" TLC becomes possible. In order to prevent vapor convection, development is carried out horizontally, and a cooling device is present to avoid temperature changes due to liberation of heat of adsorption. The BN-chamber has proved to be useful in separations of substances belonging to different polarity classes.[57 58]

Reproducibility

There is no denying that reproducibility in TLC is a rather disputed point; some people doubt the usefulness of stating R_F-values, whereas others claim the reproducibility to be quite satisfactory. In the early years of TLC the factors affecting reproducibility were not always fully understood, and hence satisfactorily reproducible results were not often obtained. During recent years, however, much more understanding has been obtained of the factors involved in reproducibility, and it has been found that, with suitable standardization, satisfactory reproducibility can indeed be obtained. This reproducibility is comparable to that of paper chromatography. A more detailed discussion of the reproducibility factors can be found elsewhere.[34 47] It should be emphasized that even under maximum standardization R_F-values should not be regarded as definite values or as definite proofs of identity but be considered as *guide values*, e.g., like melting points. Particularly in association with color reactions or with reference substances, these guide values can give reliable information about the identity of the unknown compound.

A factor requiring special attention is the relative humidity under which the work is done. We have learned from the previous paragraphs that water vapor, due to its presence in the laboratory atmosphere, always plays a role in TLC separations. If work is done under standardized humidity and with standardized procedures, there are no problems, of course, as the influence of water vapor will always be the same. If work is done under varying humidity conditions, then irreproducible results may be expected. This will be most noticeable when working with non-polar solvents and substances. Small differences in the amount of water present will be of great influence due to the high polarity of water. When working with more polar solutes and solvents, the effect of various amounts of water will decrease because of the fact that the whole system is of greater polarity; furthermore, the more polar the solvent, the more adsorbed water vapor will be expelled from the sorbent because of the high concentration of solvent vapor. Hence, it can be observed that in polar systems reproducibility is obtained over 40—80% relative humidity, whereas in a non-polar system the range will be as small as 5—10%. It is therefore very important that authors give the relative humidity under which their work was done. It has too often happened that clear separations in one laboratory could not be confirmed in others. After much confusion it often turned out that a difference of humidity was the critical factor. Obviously, all other conditions under which results were obtained should be mentioned, together with the divergences, the means, and the standard deviations of the R_F-values.

Finally, we should like to emphasize that multicomponent solvents should only be used once for development. For reasons of economy it has often been common practice to re-use multicomponent solvents for successive developments. Due to preferential adsorption of the more polar components to the plate, however, re-used solvents will suffer gradual decreases in the concentrations or their more polar solvent components and will consequently give irreproducible results. Moreover, partial evaporation of solvent components during the opening of the chamber will also affect the composition of the solvent. In order to avoid the latter process, it has been recently proposed to make use of azeotropic solvent mixtures.[59] However, preferential adsorption onto the plate will still occur, and hence azeotropic mixtures should also be used only once.

Reaction Chromatography

The inert character of many TLC sorbents makes them very suitable for carrying out chemical reactions directly on the plate. Using this technique, the sample can be spotted and then covered with a reagent, or the reagent can be incorporated in the solvent so that reactions

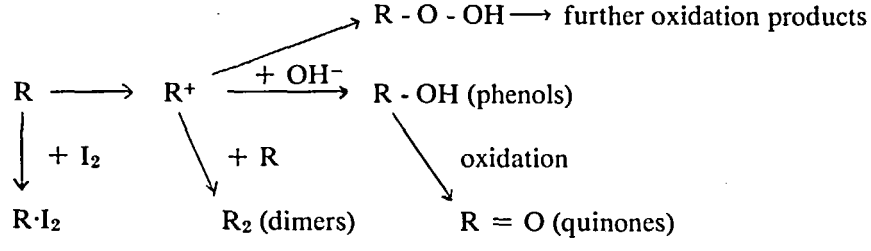
take place during development. With the former technique it remains possible to follow the migration of an untreated spot on the same plate which makes comparisons much easier. Already proposed by Miller and Kirchner[60] the method has gained increased recognition during the last few years. It can be applied to many classes of substances and various types of reactions have already been described, as can be seen from the following examples.[18]

1. Oxidations. Various reagents are available.
2. Reductions. Often with metallic reductors.
3. Dehydrations. With sulfuric acid or phosphorus oxychloride.
4. Bromination. With bromine solutions in water or chloroform.
5. Enzymatic action.
6. Esterification. With trifluoroacetic acid.
7. Preparation of derivatives, e.g., phenylhydrazones, dinitrophenyl amino acids, nitroso estrogens.
8. Reactions with vapors, e.g., iodine and bromine.

Besides many other reactions can be carried out as well.

Reaction chromatography can be carried out in one-dimensional chromatography, but it can also be valuable in two-dimensional TLC with the reaction occurring after the first development. This Separation-Reaction-Separation technique[61] considerably increases the identification possibilities.

One of the main advantages in reaction chromatography is the speed of the reaction. Here the sorbent represents a rather active medium so that a great many reactions will take place which cannot be obtained in the same time and with the same simplicity in solution. In many cases the reactions on the plate are chain reactions or may go into different directions or will not go to completion. Hence, reaction chromatography will give a number of different spots, often accompanied by the parent compound. This may result in a characteristic pattern of spots which enables easier identification. An example of the reactions which may occur in reaction chromatography can be found in the scheme of Wilk and Brill who studied the reaction of iodine on alkaloids with silica gel as adsorbent.[62]



iodine complexes

Thus reaction chromatography may become a very suitable and versatile tool in the increasing demand for specific identification techniques. However, it will be necessary to standardize the various techniques because the results are not always sufficiently reproducible and reliable.

Special Techniques

The possibilities of TLC can often be considerably enlarged by using special techniques which have already found wide application to many different types of separations. The most important procedures will be summarized here and may serve to remind the reader of the many alternatives that can be used. (See for details[16-18 30]).

1. *Continuous development.* Applicable in ascending, descending, and horizontal development. Solvent is allowed to evaporate or to drip off at the end of the plate. Because of the longer run thus obtained, substances with small differences in R_F-values may be separated.

2. *Multiple development.* Development is repeated several times with evaporation of solvent after each run. The method is time-con-

suming but has the advantage over (1) that the diffusion of the spots usually remains smaller.

3. *Stepwise development.* The chromatogram is developed with different solvents and to different heights. Solvents show increasing polarity. This technique can be used for mixtures consisting of groups of substances of different polarity. Thus, with solvent I and a length of run of 15 cm, substances of the nonpolar class A may be separated, while in a second development, over 7 cm with solvent II, substances of the polar class B are resolved.

4. *Two-dimensional development.* After the first development a second development is applied perpendicular to the first direction. A versatile technique for many complex and difficult separations, especially when the development in the second direction is fundamentally different from that in the first direction (e.g., neutral-acid, neutral-alkaline, acid-alkaline). The two-dimensional combination of TLC with thin-layer electrophoresis is very suitable for charged substances. Artifacts or decomposition of the substances during chromatography may be detected by using the same solvent twice.

5. *Multidimensional development.* This technique is a variation of (4). A number of plates are developed identically in the first direction, but in the second direction each plate is developed alone in a different solvent. After chromatography various spray reagents can be used for the different plates.

6. *Wedge-shaped development.* The sample first moves through a narrow strip of sorbent, and after 1 or 2 cm it is allowed to expand into a wider section. Spots finally appear as narrow bands so that a more effective separation is obtained. The effect is similar to that obtained in the next technique.

7. *Radial chromatography.* Better separations may be obtained because spots appear in a narrow band instead of spots with a considerable diameter.

8. *Centrifugal chromatography.* Useful to increase the solvent flow but requiring a complicated technique.

Gradient Techniques

Gradient techniques are becoming more and more important in TLC because they have several advantages over normal TLC. They are capable of separating substances of various polarity classes in a single run, they may give sharper spots, and they can be very useful in the search for optimal chromatographic conditions. In a series of recent papers Niederwieser has discussed the various advantages and the new developments.[63] It is rather difficult to give a suitable definition of gradient TLC. Niederwieser[63] defined the technique as TLC with locally different separation conditions in the separation area; but as we know from the foregoing discussion, this definition also applies to any normal separation with multicomponent solvents. Perhaps the definition should be extended as follows: gradient TLC is a technique with locally different separation conditions in the separation area as a consequence of intentional changes in one or more of the following parameters: layer thickness, composition of the sorbent, sorbent impregnation, sorbent activity, composition of the solvent, composition of the vapors in the chamber, or solvent flux. However, even this definition does not cover all possibilities nor does it seem to exclude normal TLC with multicomponent solvents.

Niederwieser also proposed a very useful general nomenclature for gradient TLC. A full description of a gradient needs, besides length, strength, shape, and the direction in which it operates. This can be obtained by using arrows indicating the result of the gradient effect on the separation. Accordingly, the arrow of the gradient will point into the direction where the majority of the investigated substances show their greatest mobilities. For example, the arrow will point in the direction of the sorbent with lowest sorptive action or, in the case of a solvent gradient, in the direction of the mobile phase with greatest elution power. Furthermore, each type of gradient chromatography is based on a combination of two vectors: that of the gradient and that of the solvent flow. The possibilities thus arising are:

1. *parallel:* the direction of the gradient and that of the solvent flow are identical.

2. *antiparallel:* the direction of the gradient and that of the solvent flow are opposed.

3. *diagonal:* the direction of the gradient and that of the solvent flow are oblique but work in the same direction.

4. *antidiagonal:* as under (3) but working in opposite directions.

5. *orthogonal:* the direction of the gradi-

ent is perpendicular to that of the solvent flow.

If a gradient direction goes from left to right, it is indicated as a (+) gradient; if it goes from right to left it is indicated as a (−) gradient.

The various possibilities are illustrated in Figure 13; there is no doubt that these gradient notations are very useful for giving adequate description of the gradient used.

To date antiparallel (ap) gradients are the ones most often used in TLC, either as elution gradients or as sorbent gradients. The character of the (ap) gradient enables the separation of a great many substances of different polarities in the same run, and it has proved to be useful in separations of natural products, serum lipids, etc. By using an (ap) gradient the spots will continuously move into a more retarding medium so that the chromatogram becomes more or less "compressed." Diffusion of the spots and/or tailing are reduced so that sharp spots are obtained. The (ap) gradient is not suitable for closely related substances with small differences in R_F-values.

Orthogonal (o) gradients are very suitable in establishing optimum conditions for the separation or in two-dimensional TLC. Antidiagonal (ad) gradients may be applied in the second direction of two-dimensional TLC. After fractionating the substances in the first direction with an (ap) gradient development in the second direction can also take place in an appropriate (ap) gradient so that maximum information can be obtained.

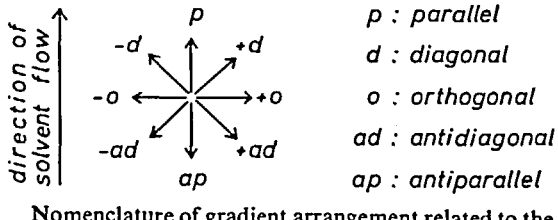
Sorbent Gradients

The first type of discontinuous sorbent gradient was introduced by Berger et al.[64] and other suitable devices were described by Abott and Thomson[65] and Leibmann and Schumann.[66] A device for continuous gradients was developed by Stahl.[67] The gradient may be obtained by using different sorbents or by using different solutions for sorbent impregnation, e.g., pH gradients, buffer gradients, salt gradients, and complexing agent gradients. These types of gradients seem to be more successful than pure sorbent gradients.[63] Dumont[68] recently showed the various possibilities of (o) pH gradients in separations of acids, bases, and amphoteric substances. When the substances are applied in bands, plots of R_F against the pH of the layer often give curves which are reminiscent of titration curves. Unfortunately, the correlation between normal pK-values and "chromatographic" pK-values is not as yet always satisfactory, whereas with indicators it was observed that in most cases the color change of the indicator did not coincide with the inflection point of the plotted curve. However, the (o) pH gradients are very suitable for establishing optimal layer conditions, and in the cases in which rather specific curves are obtained the technique can be useful for identification purposes.

(ap) Sorbent gradients were found to be successful in two-dimensional chromatography, but little information is yet available.[63]

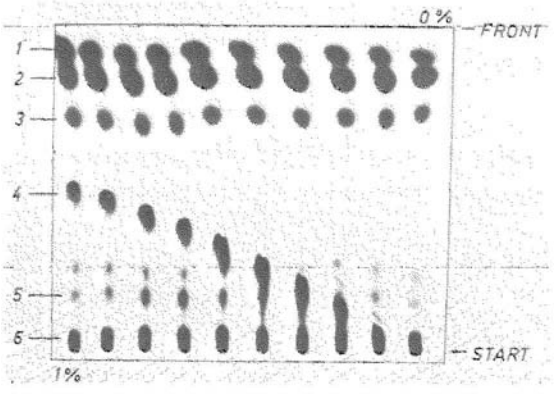
The preparation of (ad) gradients can be performed according to Niederwieser.[63] Triangular pieces of sorbent should be removed from the plate at the four corners so that a new smaller square of sorbent is obtained. The gradient spread parallel to one of the borders of the plate now stands diagonally on the remaining sorbent square. Using thin layers on plastic sheets or aluminum foil, one can simply cut off the corner pieces. If a glass plate is used, the solvent can be brought onto the plate by using a small elongated solvent trough packed with solvent-soaked cotton. The trough is pressed onto the layer so that the cotton material touches the sorbent, and the plate is covered with a counter plate as in an S-chamber. A capillary solvent distributor[42] may also be used. An example of the usefulness of an (ad) gradient is illustrated in Figure 14. The method certainly has advantages when searching for optimal (ap) gradients, but the technique becomes rather complicated. It thus may be questioned whether satisfactorily reproducible results can always be obtained.

FIGURE 13



Nomenclature of gradient arrangement related to the direction of solvent flow, according to Niederwieser.[63] Reproduced with permission of Fried. Vieweg & Sohn GmbH.

FIGURE 14



Chromatography in an (ad) gradient as a means of finding out a useful (ap) gradient, 0% linear 1% g/g tartaric acid on silica gel G. Solvent chloroform-ether (25+75). 1 = Dimethylaminoazobenzene, 2 = Cibacetred, 3 = Artisilblue, 4 = Eosine, 5 = Artisilbluegreen, 6 = Cresylviolet and Fuchsine. The gradient is effective only for eosine at its impurities. After Niederwieser,[63] reproduced with permission of Fried. Vieweg & Sohn, GmbH.

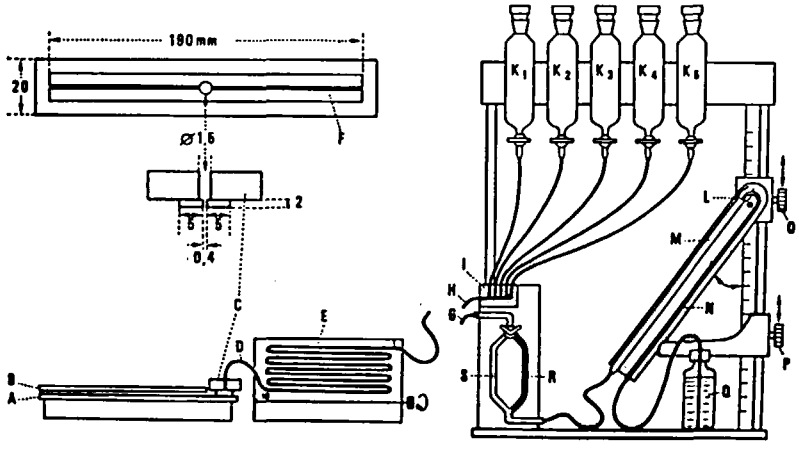
Solvent Gradients

In fact, any normal TLC development with a multicomponent solvent represents a sort of solvent gradient TLC in itself, but we will not go into details here as gradients are not intentionally wanted in normal TLC.

In polyzonal TLC,[57 58] however, the situation is different, and this technique can be considered as the simplest type of solvent gradient TLC. The intentionally wanted demixing of the multicomponent solvent system will give a discontinuous antiparallel gradient. The height and steepness of the gradient will depend on the composition of the solvent and on the character of the single components.

Solvent-gradient TLC, in which the solvent in the solvent reservoir is changed continuously or discontinuously, has already been used for a few years, the techniques being closely related to those in column and paper chromatography. (For a review see Niederwieser and Honegger.[42]) These authors have pointed out, how-

FIGURE 15



b

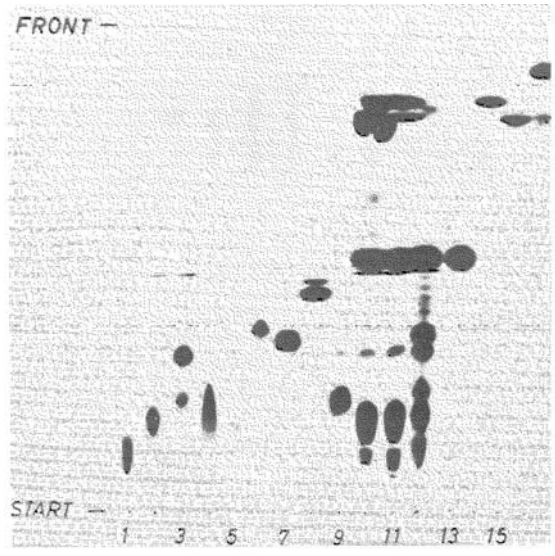
Device for solvent gradient TLC, according to Niederwieser et al.[63] Basic equipment (Figure 15a) consisting of thin-layer plate A, cover plate B, all-glass distributor C bearing two fused-on glass strips F, Teflon tubing ("loop") D mounted wave-like on a table E with adjustable slope. Accessory for routine work (Figure 15b): 1. Solvent filling station: Reservoir flasks for solvents K_1 to K_5 provided with tightly fitting Teflon valves, Teflon lines leading to a Teflon multiway piece I, ending in a stainless-steel capillary (tap) H. 2. Hydraulic solvent pump: The pressure tube M (volume 6 ml, length about 50 cm, fitting to a meander volume of 6.5 ml, inner diameter of meander tubing 1 mm) with adjustable height of upper end (with knob 0) and of lower end (with knob P) is joined with a very thin glass capillary R (80 mm long thick-wall thermometer capillary of inner diameter 0.28 mm, inner volume 5 µl) and a wide glass tubing S (inner diameter about 3 mm), which combine through a three-way stopcock to tap G (stainless steel capillary, outer diameter 1.5 mm); the plastic reservoir flask Q for pumping liquid (e.g., 0.1% eosine in water/methanol 9:1) is joined to the inlet capillary L through the line N. Reproduced with permission of Fried. Vieweg & Sohn, GmbH.

ever, that with the commonly used system only one single type of gradient shape is possible and that magnetic mixing and a considerable excess of solvent are required. Moreover, the proper use of a variable gradient in TLC requires a rate of supply of solvent to the layer which is determined by the migration rate of the solvent flow, otherwise deformation of the desired gradient shape will occur. In order to overcome these difficulties, a new type of apparatus has been devised for solvent-gradient TLC and has already proved its usefulness. This apparatus is shown in Figure 15. Its most important feature is a capillary tubing solvent reservoir which is filled in succession with a definite volume of each of a number of solvents. The tubing is connected with a special solvent distributor which transforms the outflowing solvent in a band of capillary thickness, touching the layer along the whole length of the plate. Hydrostatic pressure or capillary suction regulates the solvent flow into the sorbent in such a way that all the liquid leaving the tubing is picked up by the sorbent. This system allows free choice of gradient shape and involves reproducible partial mixing of the solvent components by laminar flow in the tubing. Since practically no solvent is lost during chromatography, this will ensure no distortion of the desired gradient. The capillary tubing can be connected with a mixing battery.

The technique has been very successful with (ap) gradients in separations of substances with a wide variety in polarity. This is demonstrated in Figure 16, showing the separation of lipids into substance classes.[69] The advantages of this technique are obvious, and the (ap) gradients thus obtained promise a wide field of application.

(o) Solvent gradients can also be applied to TLC following a technique described by Niederwieser.[63 70] A small elongated solvent trough is filled with a tight cylinder of cotton or cellulose with 1-3 mm protruding over the trough opening. The desired solvent gradient is mixed in a mixing battery and then filled into the cotton cylinder by moving the battery outlet from one end of the cylinder to the other. After filling, the solvent trough is immediately placed upside down on the TLC plate. Development takes place in a sandwich arrangement. The results obtained so far are not very satisfying, probably due to too much solvent diffusion on the plate. Solvent diffusion in the cotton cylin-

FIGURE 16



(ap) Solvent gradient TLC on Silica gel/Kieselguhr (80 + 20, w/w), according to Niederwieser.[63] Samples: Sphingomyelin 1, lecithin 2, phosphatidyl ethanolamine 3, phosphatidyl serine 4, phosphatidyl inositol 5, cardiolipin 6, cerebrosides 7, ceramides 8, sulphatides 9, hyperlipemic serum lipids 10, normal serum lipids 11, bovine spinal marrow lipids 12, cholesterol 13, cholesteryl stearate 14, triglycerides 15, squalene 16. Solvent gradient [24] : 6 ml loop (1 mm inner diameter) filled in succession with I: 1 ml heptane/dichloromethane/diethylether (37 + 58 + 5); II: 1 ml heptane/dichloromethane / diethylether / methanol / glacial acetic acid (19 + 48 + 24 + 7 + 1.5); III: 1 ml heptane/dichloromethane / diethylether / methanol / acetic acid / water (5 + 35 + 35 + 20 + 3 + 2); IV: 1 ml chloroform/methanol/water (69 + 26.5 + 4.5) and V: 2 ml chloroform/methanol/water (60 + 32.5 + 7.5). Thin-layer activated before use (20 min at 110°). Distance between immersion line (slit of all-glass distributor C, see Figure 15) and starting line 10 mm. Migration distance of solvent front 15 cm; running time 50 min. Detection with 10% phosphomolybdic acid in ethanol. Reproduced with permission of Fried. Vieweg & Sohn, GmbH.

der can also play a role if this is not uniformly packed.

An (ad) solvent gradient can be obtained in a similar way as described for (o) gradients by moving the elongated solvent trough along the starting line during development.[63]

Although the techniques described above certainly have their value to the field, it cannot be denied that the handling becomes rather complicated, thus detracting from the simplicity of TLC in its original form. A second important factor is the degree of reproducibility which can be obtained with these techniques, also with regard to investigations in different laboratories. In the opinion of the author these comments will apply in particular for (o) and (ad) gradients. (ap) Gradients are easier to handle and will have a wider field of application.

Vapor Impregnation Gradients

The newer insight into the influence of solvent vapor discussed in the previous paragraphs has revealed that vapor-impregnation gradients already occur in normal TLC with multicomponent solvents[53] and that vapor impregnation can be used very effectively to influence the migration rates of the substances.[38 39] This has led to a search for new techniques by which vapor impregnation can be suitably obtained. Geiss and Schlitt[71] modified their previously described chamber for humidity control,[39] which is now introduced as KS-Vario-chamber (Camag, Muttenz, Switzerland). In this horizontal chamber the plate faces an insert tray which can be divided in several ways by partition walls. The distance between the sorbent layer (layer thickness 0.25 mm) and the tray walls is about 1 mm. The various compartments of the tray can be filled with different liquids, the vapors of which, in turn, will be adsorbed by the plate. Solvent is brought upon the plate as in the BN-chamber, by means of a filter-paper bridge. The KS-Vario-chamber can be seen in Figure 17, together with the different insert trays. Suitable results have been obtained with discontinuous (ap) gradients, the effects being similar to those obtained with (ap) solvent gradients. By using salt solutions of different concentrations and compositions, (ap) water-vapor gradients can also be applied. The method has also been used with (o) gradients, but the results are not very satisfactory. The distances between the plate and the tray walls are obviously too large, and anomalous oblique spots result from vapor diffusion among the various compartments. Accordingly, good reproducibility is questionable. Therefore, in the opinion of the author, satisfactory use of the KS-Vario-chamber will be restricted to the field of (ap) gradients. As the procedures are relatively easy, the chamber may compare favorably to (ap) solvent gradients, provided that (ap) gradients of sufficient strength can be applied with the KS-Vario-chamber. This, again, is dependent on the rate of vapor diffusion between the various tray compartments. Comparative material is not yet available.

The reader will have noticed that parallel or diagonal gradients have not been discussed so far. The reason is that these types of gradients are difficult to use, particularly with solvent gradients and vapor-impregnation gradients. With the former, the solutes will migrate rather fast and thus cannot be influenced by the following less polar solvents; with the latter, severe tailing of the spots will occur as a consequence of the stepwise character of the vapor impregnation gradient.

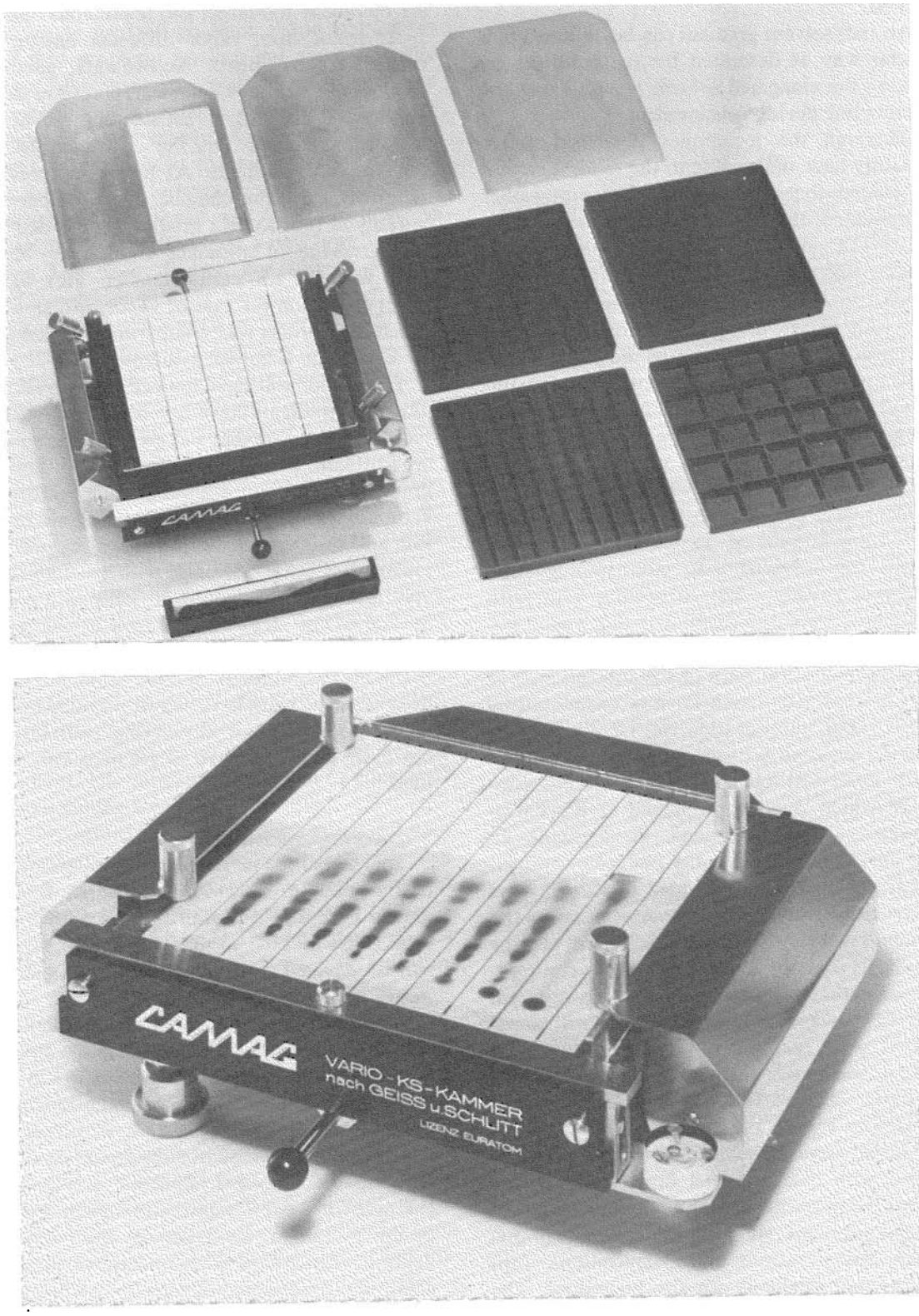
Parallel continuous sorbent gradients have been described and have shown a suitable effect in the separation of close-lying substances,[72] but these gradients have as yet been very little used.

Vapor-Programmed TLC

This technique is, in fact, a vapor-impregnation technique. However, its design is completely new, and it promises to become a powerful trend in TLC so that a special paragraph will be devoted to its properties and possibilities.

After becoming aware of the influence of solvent vapor in TLC, it was felt in the author's laboratory that optimal separations would only be obtained if optimum vapor conditions could be established. The separation improvements which can be obtained in unsaturated chambers with multicomponent solvents are a good example of better vapor conditions, but no doubt it cannot be expected that *optimum* vapor conditions are always obtained simply by using an unsaturated chamber. As we have seen, the improvements are caused by a parallel concentration gradient of adsorbed vapor on

FIGURE 17



KS-Vario-Chamber with insert trays and shutters, according to Geiss and Schlitt.[71] Courtesy: Camag A.G., Muttenz.

the dry part of the plate, with the gradient being mainly dependent on the rate of evaporation and diffusion of the solvent components and on their affinity for the adsorbent used. It will be clear that little or no control can be exercised on the extent to which the gradient develops on the plate in the unsaturated chamber. With low-polar solvent components the gradient may be too flat or will not reach its maximum within the time of development, whereas with high-polar components the gradient may become too steep. Moreover, it cannot be expected that every separation problem will need the type of gradient obtained in the unsaturated chamber. It may well occur that a parallel gradient is desired on the upper part of the plate only so that the slower running spots are not affected or that the steepness of the gradient on the upper and lower part of the plate should be different to give complete separation.

Therefore, we have searched for development techniques providing a more efficient control of the vapor processes before and during development so that full benefit of the influence of vapor can be obtained. This has led to the apparatus described below, which allows full vapor programming over the entire plate, thus making it possible to affect and to guide the migration of each individual spot.[73]

The Vapor-Programming chamber or VP-chamber (obtainable from C. Desaga, Heidelberg, West Germany) is shown in Figure 18. It consists of three parts, all of chromium-plated brass: the solvent reservoir (A), 20 x 1 x 2 cm; the ground plate (B), 20 x 20 x 1 cm; with a tube at the upper end (D) to pass warm water and insulated from the ground plate by 2 mm of asbestos. The inner part of the ground plate contains a tube system for water-thermostating; the outlet and inlet of this system are visible at F. Fixation clamps are visible at E. The most important part is the trough chamber (C), containing 21 troughs of i.d. 6 mm and depth 12 mm. Partition walls are 2 mm and side walls 5 mm. A space of about 1 mm is needed between the solvent reservoir and the trough chamber to prevent disappearance of solvent by capillary action between the walls.

Normal 20 x 20 cm TLC plates are used. Plates should be of sufficient stoutness; sagging plates or sheets cannot be used. Samples are spotted 2—2.5 cm from the bottom edge of the plate. The side edges and the bottom edge of the sorbent are stripped 0.5 cm wide. The troughs are filled with a series of liquids of appropriate composition to give suitable vapors. For example, if a parallel gradient is desired, mixtures of two or three solvent components can be used with the mixtures having an increased proportion of the more polar components. The empty solvent reservoir is placed in the holder and fitted with a folded strip of filter paper, 18.5 x 2 cm, with 1 cm folded over the inner wall. The side walls of the chamber are fitted with thin spacers. The TLC plate is then placed on the chamber, the sorbent downward facing the troughs, the stripped side edges resting on the side spacers, and the bottom edge just covering the solvent reservoir. The side spacers should prevent the adsorbent layer from touching the walls, and the thickness should be adapted to that of the layer. The solvent reservoir is then filled with about 25 ml of the appropriate solvent, which is brought upon the plate by means of the filter-paper strip. It should be noted that the strip contacts the plate below the starting points. The solvent reservoir and the filter-paper strip are pressed smoothly to the plate by means of two springy strips underneath the solvent reservoir. This ensures good contact between the sorbent and the filter.

Thus, the use of the VP-chamber permits vapor adsorption from the underlying troughs, and by filling the troughs with suitable liquid mixtures, the vapor conditions can be programmed over the entire plate. Optimal vapor conditions can then be obtained because every desired polarity can be applied to the various parts of the plate via the vapor phase.

The principles of the VP-chamber are rather simple, but there are some important factors to which special attention must be paid.[73] [74] The thickness of the side strips is of great importance in the applicability of a certain vapor program. Due to the differences in the liquid compositions in the troughs, strong vapor diffusion will occur if the space between the plate and the trough walls is too large. On the other hand, the sorbent should not touch the walls. If it does development stops immediately because the mobile phase disappears into the troughs. Furthermore, most sorbents swell more or less when wetted by the solvent, the rate of swelling increasing with increased polarity of the sol-

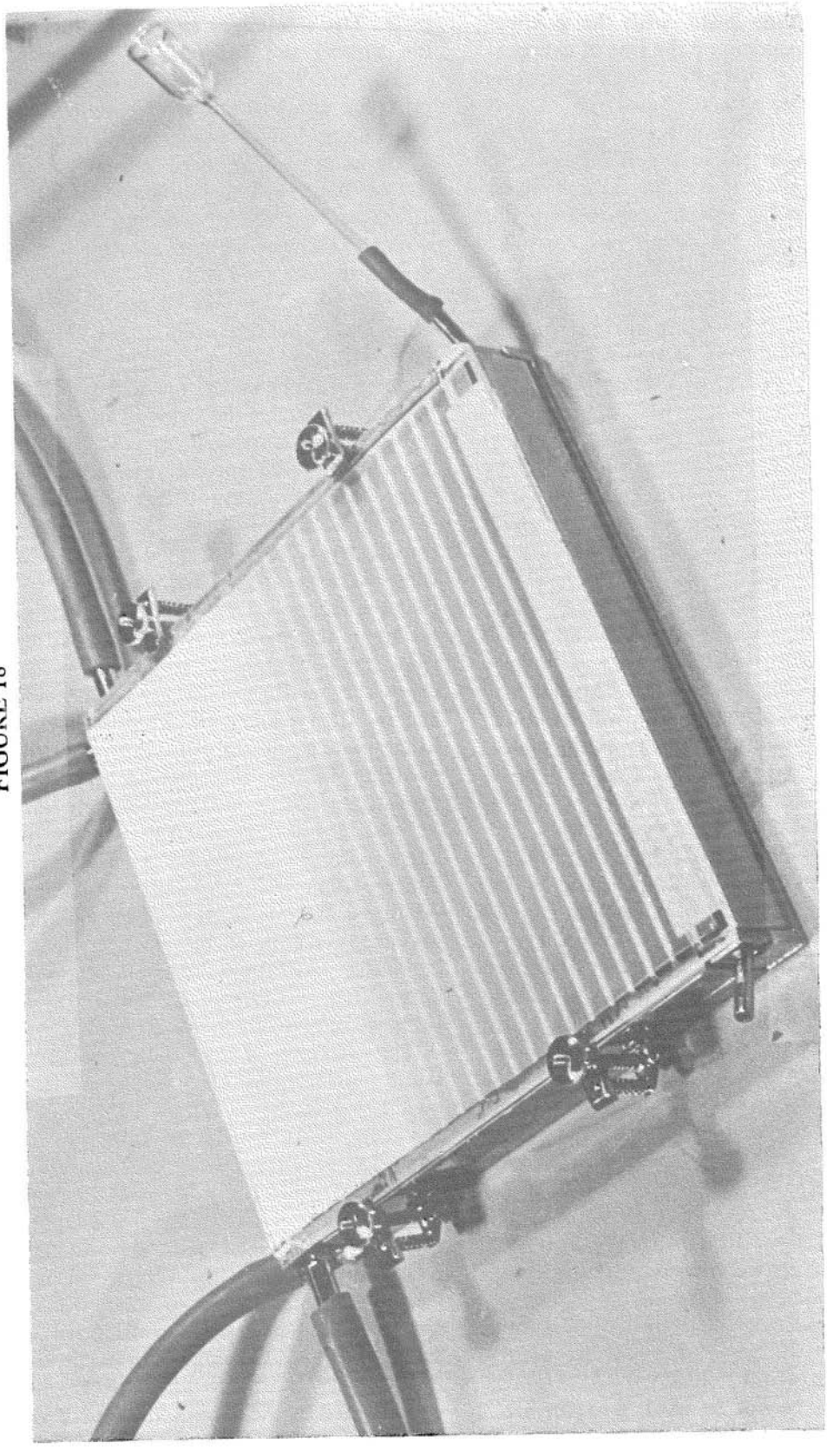
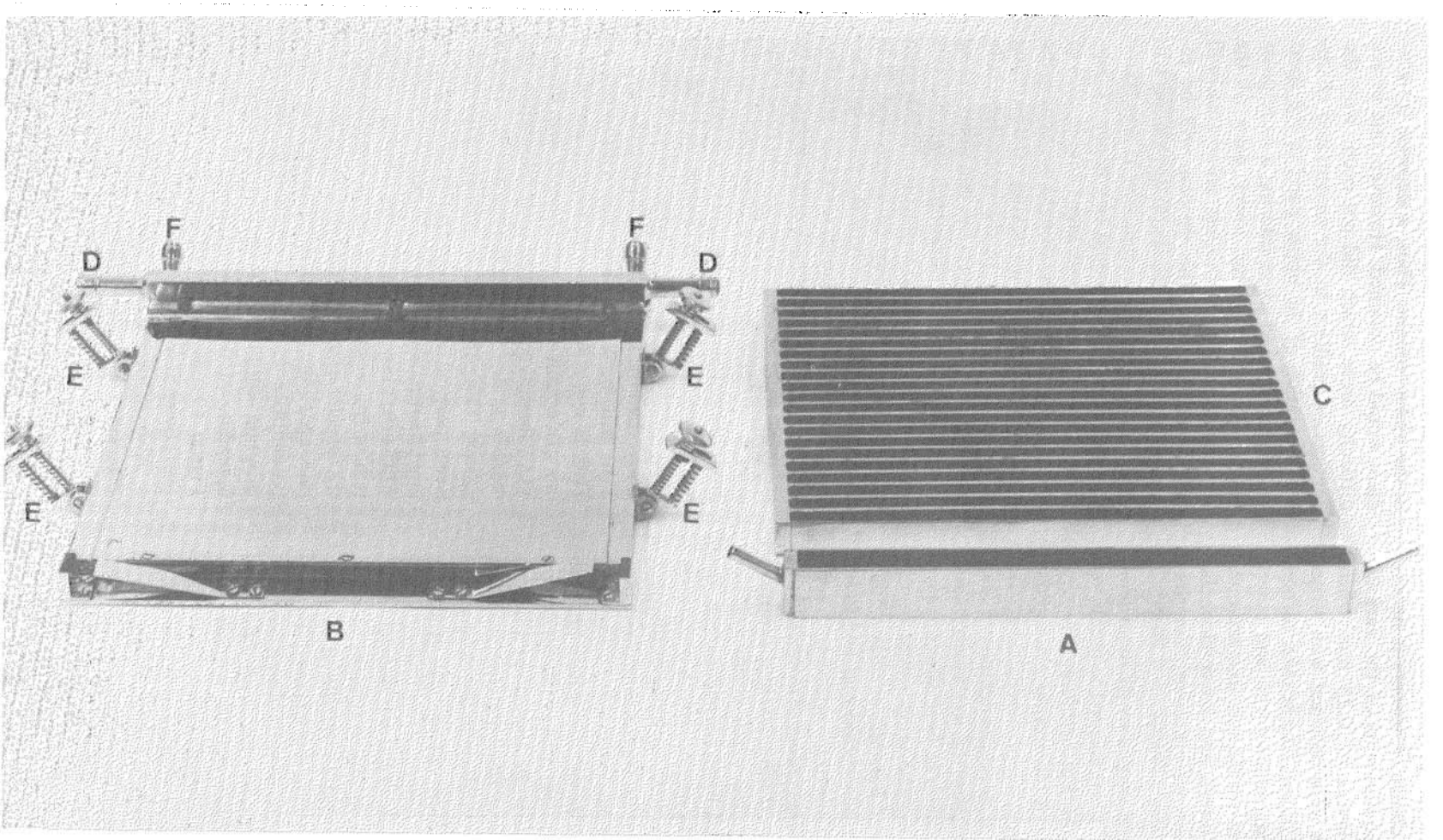


FIGURE 18

FIGURE 18



The Vapor-Programming chamber according to De Zeeuw.[73] A = solvent reservoir, B = ground plate, C = trough chamber. The ground plate is equipped with a warm water tube (D), fixation clamps (E) and an internal tube system for water thermostating, with the inlet and outlet visible at F. *Anal. Chem.*, 40, 2134, 1968. Copyright 1968 by the American Chemical Society and reproduced with permission of the copyright owner.

vent. Thus, careful choice of spacers is necessary to meet the requirements of the various phenomena. With layers of 0.25 mm when spread, good results were obtained with strips of 0.3—0.5 mm for liquids like hexane, carbon tetrachloride, benzene, chloroform, and ether, and of 0.5—0.8 mm for liquids like acetone, acetic acid and its esters, and lower alcohols, whereas 1-mm spacers were needed when ammonia was involved.

It will be clear that constancy of room temperature within ± 1 °C is required to obtain reproducible separations. Furthermore, it proved to be necessary to cool the ground plate about 1—3 °C below room temperature; otherwise the migration rates of the spots decrease markedly, presumably because of too much vapor adsorption on the plate. The heat of adsorption may also play a role. The temperature of the cooling system, if kept constant, enables a good control of the vapor-adsorption rate and is indispensable for establishing adequate vapor programs. Draughts or other air circulation over the VP-chamber during development must be avoided. The critical vapor conditions become easily disturbed by these phenomena, resulting in anomalous migrations. A Plexiglass hood, about 35 x 25 x 20 cm, open at the back for the water-circulation connections, will provide suitable protection.

Continuous development can easily be done in the VP-chamber by means of the warm water tube underneath the top of the plate so that the mobile phase reaching this area can rapidly evaporate. Continuous development is often needed in difficult separations of closely related substances.

Before development is started, the plate must be equilibrated over the filled troughs in order to be sure that reproducible quantities of vapor will be adsorbed. During this period—10 minutes is sufficient—the small volume over the troughs becomes almost saturated. Not until after this saturation period is the solvent reservoir filled and development started.

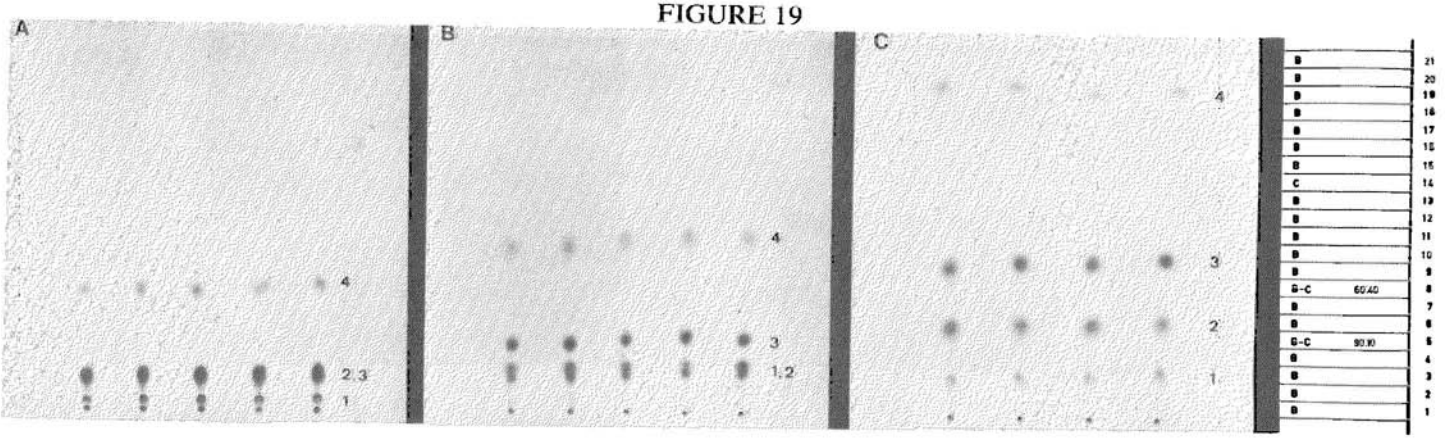
The VP-chamber was primarily devised to establish parallel gradients similar to those in unsaturated chambers with multicomponent solvents. This can be done, for example, by using mixtures of two liquids, one polar and the other non-polar, with the mixtures having an increased percentage of the polar components in the upper troughs. Thus, a stepwise (p)-gradient would be obtained. However, one severe difficulty arose which did not occur with the continuous gradient in the unsaturated chamber: migration of the upper parts of the spots will be accelerated when entering a more polar vapor area, whereas the lower parts of the spots, still moving in the less polar area, will not be accelerated. This process will be repeated at each partition wall, and tailing will consequently result. Fortunately, it was found that this disadvantage could be completely suppressed by interspersing troughs with liquids of low polarity between the troughs containing the more polar mixtures, thus having a decelerating effect on the migration of the spots, especially on the upper parts. In general, two decelerating troughs follow one accelerating trough, but this ratio may be changed at will to 1:1, 3:1 or even 4:1 if severe tailing must be suppressed. It is not yet fully understood how the process of acceleration and deceleration works in actuality, but there is no doubt that compact spots can be obtained without decreasing the improved separation capacity. It may be presumed that the improvement is caused not only by acceleration but also by the deceleration forces, with the less polar substances being decelerated to a higher extent than the more polar components.

It will be clear that Vapor-Programmed TLC is not a very easy technique; and that if one can obtain a satisfactory result with normal TLC, there is certainly no reason to switch. But there is no denying that the resolution power of normal TLC often leaves something to be desired, particularly in separations of closely related substances. Here Vapor-Programmed TLC offers unique possibilities because the system of (p) + (ap) gradients, as represented by the acceleration and deceleration forces, will pull apart the various substances. The many possibilities in the composition of the vapor program will thus allow optimal separation and spreading of the problem under investigation. Figure 19 and Figure 20 illustrate the resolution power of VP-TLC.

Also in preparative and quantitative work (application of the substance in a streak) VP-TLC is very valuable as can be seen from Figure 21, but we will not go into details here.

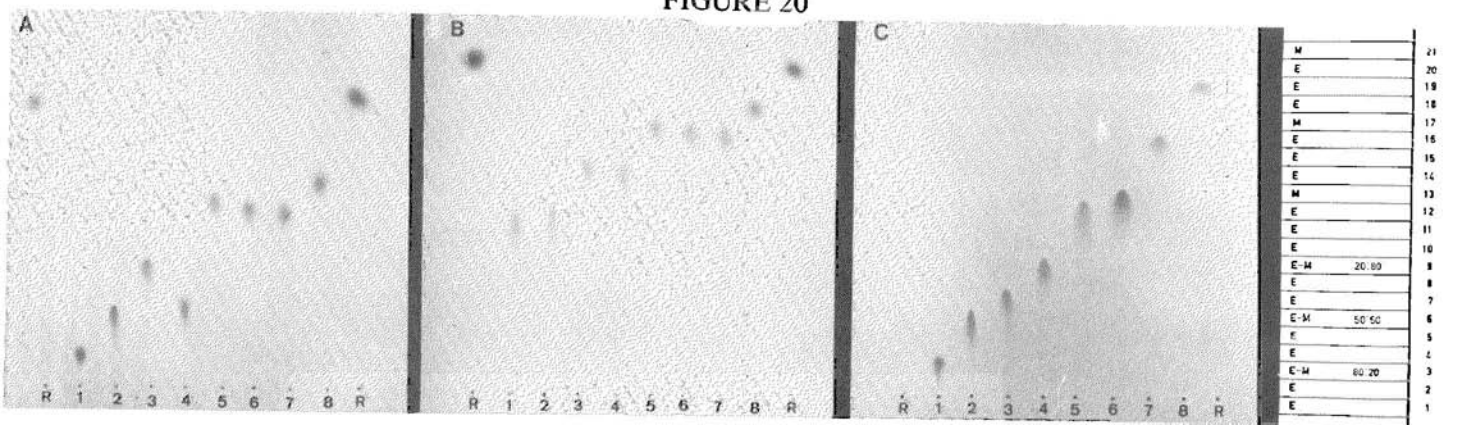
Finally, it should be remembered that, although it was not primarily designed for this

FIGURE 19



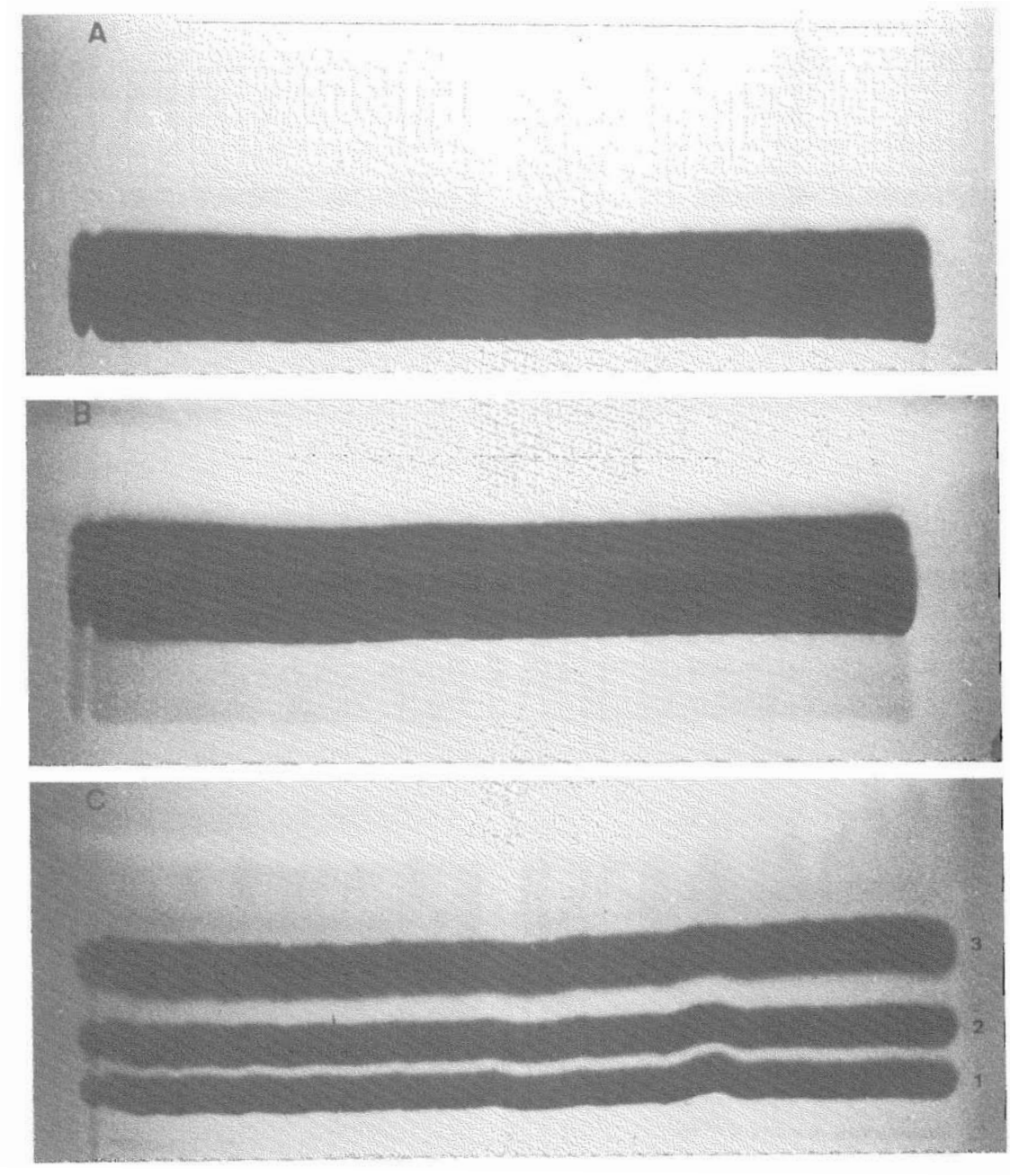
Improved separation of color dyes in the vapor-programming chamber as compared to classical development in saturated N-chambers.[73] A: N-chamber, solvent benzene, temp 21.0°C, relative humidity 26%, development 36 min, saturation 45 min; B: N-chamber, solvent benzene-chloroform 80:20, temp 22.0°C, relative humidity 30%, development 39 min, saturation 45 min; C: VP-chamber, solvent benzene, temp 21.7°C, relative humidity 29%, development 110 min, saturation 10 min, strips 0.3 mm, cooling 19°C. Code. B = benzene, C = chloroform. The position of the troughs and the liquid composition herein are given at the right. 1 = indophenol, 2 = 4-nitroaniline, 3 = sudanred G, 4 = butter yellow. Plates: Silica gel GF 254; load: 20 μg.

FIGURE 20



Improved separation of sulfonamides in the vapor-programming chamber as compared to classical development in saturated N-chambers. A: N-chamber, solvent ether-methanol 90 + 10, temp 21.5°C, relative humidity 35%, development 38 min, saturation 45 min; B: N-chamber, solvent ether-methanol 80 + 20, temp 21.4°C, relative humidity 33%, development 39 min, saturation 45 min; C: VP-chamber, solvent ether-methanol 95 + 5, temp 21.5°C, relative humidity 35%, development 63 min, saturation 10 min, strips 0.5 mm, cooling 19°C. Code: E = ether, M = methanol. The position of the troughs and the liquid composition herein is shown at the right. 1 = sulfaguanidine, 2 = sulfisomidine, 3 = sulfathiazole, 4 = sulfacetamide, 5 = sulfadimidine, 6 = sulfapyridine, 7 = sulfisoxazole, 8 = sulfanilamide. Plates: Silica gel GF 254, load 3 μg.

FIGURE 21



Improved preparative separation of sulfonamides in the VP-chamber as compared to conventional development. A: conventional development in a saturated chamber, solvent chloroform-methanol-25% ammonia (50 + 40 + 10), temperature 20.9°C, relative humidity 29%, saturation 60 min, development 67 min. B: As under A, development repeated two times. C: VP-chamber, solvent chloroform-methanol-25% ammonia (75 + 20 + 5), temp. 20.9°C, relative humidity 27%, saturation 10 min, development 180 min, strips 1.5 mm, cooling 19°C. Vapor program: troughs 1, 3, 4, 5, 7, 8, 9, 11, 12, 13, 15, 16, 17, 19, 20 and 21: chloroform, saturated with 25% ammonia, trough 2: chloroform-methanol-ammonia (50 + 40 + 10), trough 6: chloroform-methanol-ammonia (20 + 70 + 10), trough 10: acetone-methanol-ammonia (20 + 70 + 10), trough 14: acetone-methanol-ammonia (20 + 70 + 10). 1 = sulfadiazine, 2 = sulfamerazine, 3 = sulfamethazine. Plates: Silica gel PF 254 + 366 (Merck), layer thickness 1 mm when spread, load 30 mg. (De Zeeuw and Wijsbeek, to be published).

technique, the VP-chamber can also be used very effectively with (ap) gradients simply by filling the troughs with liquid mixtures showing decreased polarity in the upper troughs. The small distances between the sorbent and the trough walls enable rather strong gradients to be established. As was said before, (ap) gradients are most useful for separating substances belonging to different polarity classes on the same plate. An illustration of the properties of the VP-chamber with the same mixture separated with a (p) gradient and with an (ap) gradient can be seen in Figure 22. With the former all spots are nicely separated and spread over the entire plate; with the latter the separation of all spots can be maintained, but the mixture is now spread over about one-fifth of the plate so that substances with different polarity can be guided to other non-occupied areas.

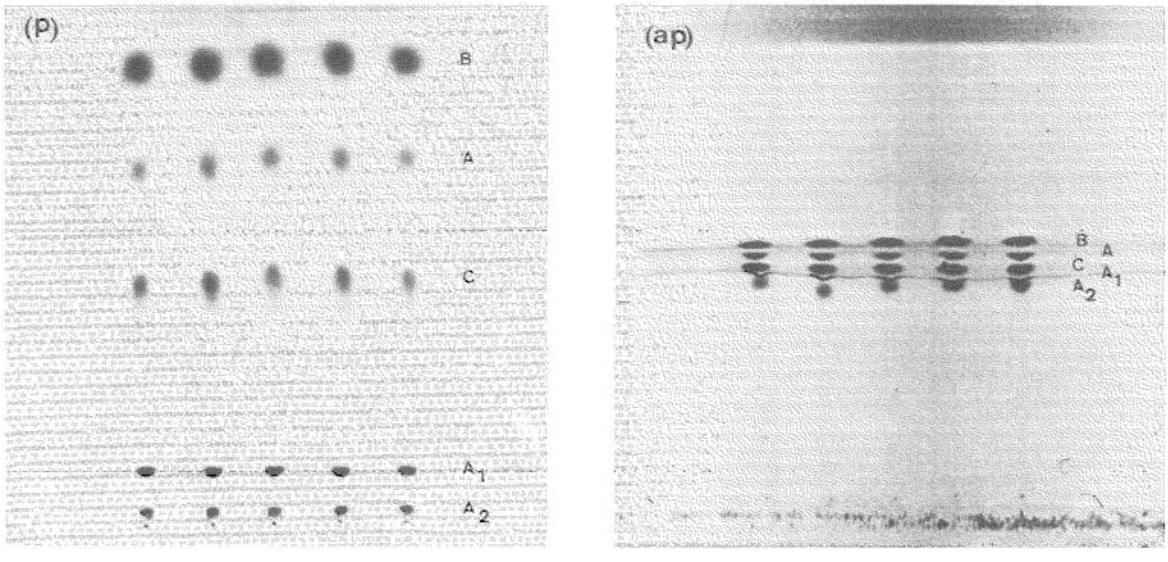
Thus, Vapor-Programmed TLC promises a wide field of application, particularly in those cases where normal TLC leaves something to be desired. The technique is more complicated, requires carefully controlled conditions, and may take some more time, but the gain in resolution is well worth these inconveniences.

It should be mentioned that the previously discussed KS-Vario-chamber cannot be compared with the VP-chamber.[63] The former provides insufficient temperature control, the distances between the sorbent and the trough walls may be too large, and the number of troughs is too small so that optimal vapor conditions cannot be established.

Flux-Gradient TLC

This technique represents the most recent development in TLC and has been devised by Niederwieser.[63] During development a controlled proportion of the mobile phase is removed from the wet layer either by means of evaporation or by diffusion through a membrane.[75] The partial removal of mobile phase will give extra solvent from the solvent reser-

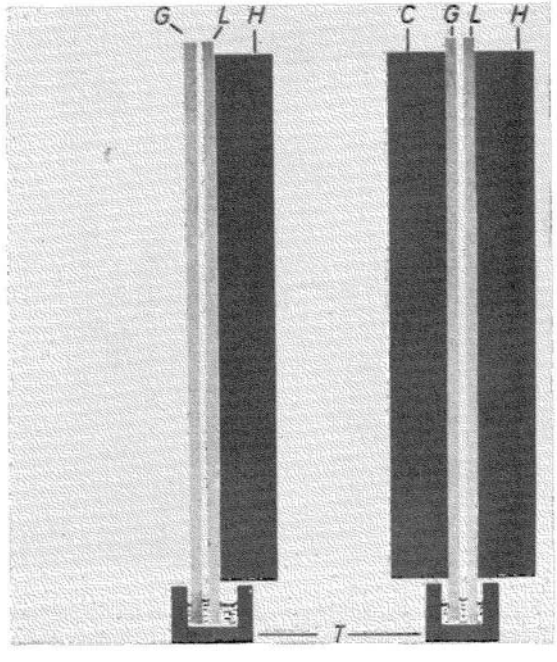
FIGURE 22



Properties of the VP-chamber with a (p) gradient and an (ap) gradient. A, A_1 and A_2 = components of Artisilblue BSQ, B = Butter Yellow, C = Cibacetred B3. (ap): solvent carbontetrachloride, temp 21.0°C, relative humidity 45%, saturation 10 min, development 85 min, strips 0.5 mm, cooling 20°C. Vapor program: troughs 2, 3, 4, 6, 7, 8, 10, 11, 12, 14, 15, 16, 18, 19, 20 and 21: carbontetrachloride, trough 1: carbontetrachloride-methanol (95 + 5), trough 5: carbontetrachloride-methanol (80 + 20), trough 9: carbontetrachloride-methanol (60 + 40), trough 13: carbontetrachloride-methanol (40 + 60), trough 17: carbontetrachloride-methanol (20 + 80). (p): solvent carbontetrachloride-benzene (80 + 20), other conditions as stated above. Vapor program: trough 1: carbontetrachloride-methanol (20 + 80), trough 2: carbontetrachloride-methanol (40 + 60), trough 3: carbontetrachloride-methanol (60 + 40), trough 4: carbontetrachloride-methanol (80 + 20), trough 5: carbontetrachloride-methanol (97.5 + 2.5), troughs 6-21: carbontetrachloride. Plates: Silica gel GF 254 (Merck), layer thickness 0.25 mm when spread, load about 3 μ g of each component (De Zeeuw and Wijsbeek, unpublished).

voir to replenish the evaporated proportions, and this will result in an antiparallel flux gradient of mobile phase. "Flux" is defined as the volume of mobile phase traversing a unit of cross-sectional area per unit time. When using multicomponent solvents, an additional (ap) elution gradient will exist beside the flux gradient because, due to preferential removal of one solvent component, the local composition of the developing system will change. It will be clear that it is necessary to obtain fully controllable and reproducible evaporation conditions, and this can be performed in the apparatus devised by Niederwieser[63] which is illustrated in Figure 23. Two suitable arrangements can be seen. A vertical sandwich-chamber with thin-layer plate L and cover plate G is mounted on a thermostated heating block H. The distance between the plates L and G is about 1 mm, and the plates are immersed in a solvent tank T. If necessary, the cover plate G can be cooled by a cooling block C. During development, mobile

FIGURE 23



Two arrangements for solvent flux GLTC, with thin-layer plate L, cover plate G, heating block H, cooling block C and solvent trough T. The right-hand side arrangement allows full thermostating to establish a reproducible temperature gradient between thin layer and cooling plate. After Niederwieser,[63] reproduced with permission of Fried. Vieweg & Sohn, GmbH.

phase evaporates from L and is removed by condensation on the cooler cover plate. The rate of evaporation can be controlled by adjusting the temperatures of the heating and cooling blocks.

With single-component solvents the effects will be comparable to multiple development TLC or continuous development TLC, but the separations in flux-gradient TLC will be obtained much faster.

With multicomponent solvents, the solvent components are selected in such a way that the most non-polar component has the lowest vapor pressure, and the most polar component has the highest vapor pressure. During development the low-boiling polar component evaporates from the layer, condenses on the cooling layer, and flows back into the solvent tank. The polarity of the solvent in the tank thus increases. On the other hand, the high boiling non-polar components accumulate on the layer. An antiparallel concentration gradient is thus obtained which can be locally stable in spite of the intense solvent flux. Niederwieser has characterized this technique as a combination of TLC with fractional distillation. Substances migrating in this system will accumulate at a position where the mobile phase has become so non-polar that it is unable to transport the substance any farther. Figure 24 illustrates a separation obtained with this technique.

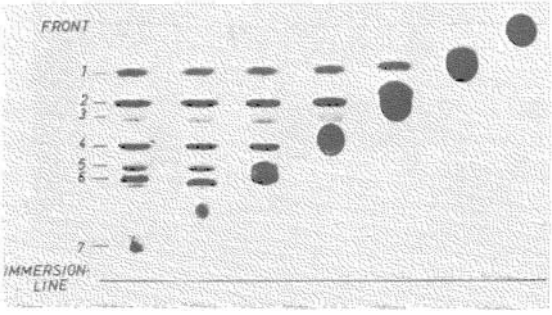
With this technique very strong (ap) gradients can be obtained, and the handling seems rather simple. Although it may be difficult to always find suitable solvent mixtures with the non-polar components having the highest boiling point, we are looking with great interest to further application possibilities.

AFTER DEVELOPMENT

Detection

In general, detection of substances after TLC development does not cause many problems. Colored substances are usually directly visible, while for colorless compounds a great many non-selective and/or selective spray reagents are available. Moreover, many colorless compounds can be visualized under U.V. light when chromatographed on sorbents with a fluorescent indicator. Two types of fluorescent indicators are commonly used: manganese-acti-

FIGURE 24



Effect of an (ap) flux gradient on TLC of dyes using a multicomponent solvent. For detection of the gradient effect the substance mixture was applied several times on an oblique starting line. On account of the pronounced, stable (ap) elution gradient in addition to the (ap) flux gradient the substances concentrate on horizontal lines, independent of their place and area of application. The relatively large starting area is still noticeable in original size on the right upper corner. Solvent: Fraction of alkanes bp. 130-155°/benzene/methyl formiate/solution III/solution V (3 + 4 + 5 + 5 + 2). Temperature difference 40°/24°. Silica gel g/Kieselguhr (80 + 20 w/w). Running time 27 min. 1 = Dimethylaminoazobenzene, 2 = Cibacetred B, 3 = Artisilblue BSQ, 4 = Eosine, 5 = Artisilbluegreen, 6 = Cresylviolet, 7 = Fuchsine. After Niederwieser,[63] reproduced with permission of Fried. Vieweg & Sohn, GmbH.

vated zinc silicate, giving a bright green fluorescence when irradiated by U.V. light of 254 nm and various organic compounds having a blue or orange fluorescence at about 350 nm. If compounds are present that are capable of adsorbing the U.V. light being used, they appear as dark spots on a fluorescent background. Substances having their absorption maxima near 254 or 350 nm have very low limits of detection (0.01—0.1 μg), but other substances that show only weak absorbance in these regions can often be detected as well at levels of 1—10 μg. A disadvantage of some of the 350-nm organic fluorescent indicators is that they are attacked by alkaline solvent systems of high pH, for example with ammonia.

With regard to spray reagents, a variety of new and more selective systems has been developed through recent years, but it would be beyond the purpose of this chapter to discuss them here. Excellent information on this subject can be obtained in Kirchner's recent review.[18]

Identification of Substances

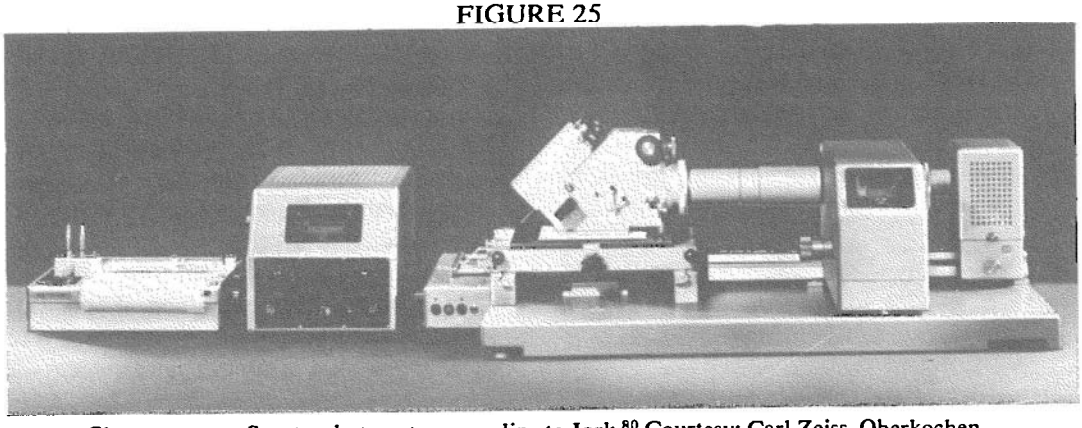
It should always be remembered that TLC is a separation technique. Although the separation parameters, the R_F-values, can give a reliable indication, they should never be regarded as definite proofs of identity. Even if one has established the R_F-values in a number of other systems so that a "chromatographic spectrum" is obtained, if fluorescence or quenching in U.V. light has been examined, if specific spray reagents have been used, or if known reference substances were available, unequivocal identification is not always achieved. Thus, additional identification techniques become necessary, and fortunately, TLC can be coupled with a variety of suitable methods:[76]

1. Spectroscopy: U.V., visible, I.R., NMR, fluorescence.
2. Physical methods: polarography, melting point, sublimation, crystal form, gas chromatography, mass spectrometry.
3. Chemical methods: color reactions, derivatives, pyrolysis.
4. Biological methods: bioautography (microbiological tests on the plate), enzymatic action, etc.
5. Isotopes.

For most of these methods, the substance must be removed from the layer by elution. After careful and complete removal of the sorbent, the material can then be tested with the most appropriate method. As these methods belong to other fields of analytical chemistry, they will not be discussed here. The reader will find the latest progress about the various identification techniques in the proceedings of a recent symposium devoted to this subject.[77]

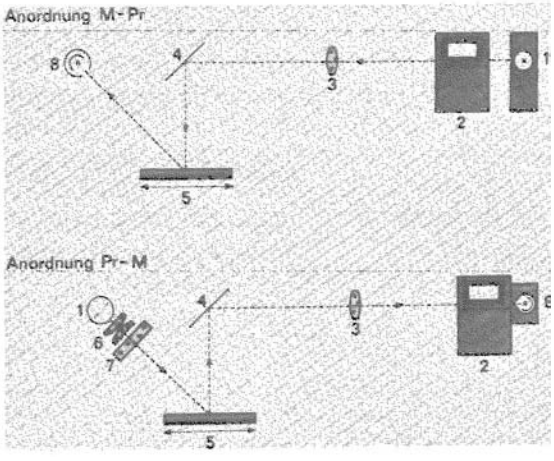
However, special attention is required for a technique for *in situ* identification on the plate, namely, reflectance spectroscopy. Frodyma, Frei, and Williams[78] were among the first who used reflectance spectroscopy in connection with TLC; and, recently, Jork has developed a special instrument, the Chromatogram-Spectrophotometer (C. Zeiss, Oberkochen, W. Germany), which allows the direct measurement of reflectance spectra on the plate as well as *in situ* quantitative analysis.[79 80] The apparatus consists of the well known Zeiss PMQ-II spectrophotometer in combination with a measuring head and a suitable plate holder and can be seen in Figure 25. The monochromator is capa-

FIGURE 25



Chromatogram-Spectrophotometer according to Jork.[80] Courtesy: Carl Zeiss, Oberkochen.

FIGURE 26

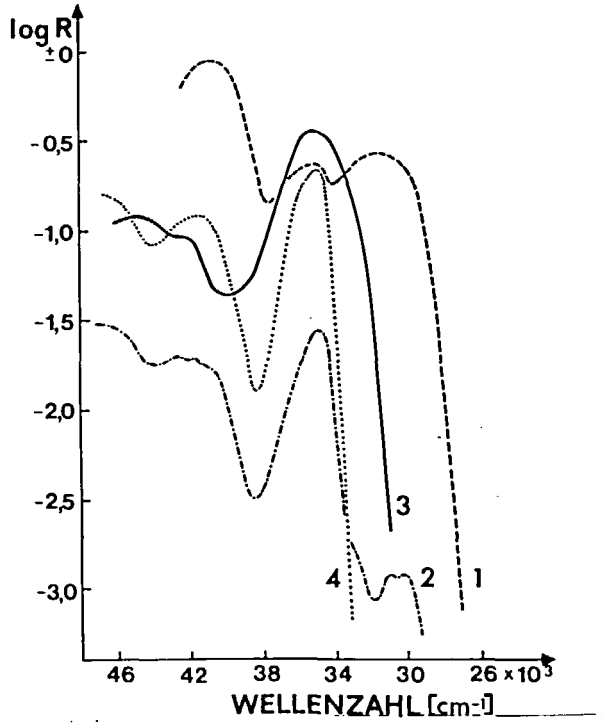


Possible light paths in the Chromatogram-Spectrophotometer. Upper diagram for reflectance spectra (M-Pr), lower diagram for fluorescence spectra (Pr-M). 1 = Light source; 2 = Monochromator; 3 = Focusing lenses; 4 = Mirror; 5 = TLC plate on measurement table; 6 = Lenses; 7 = Filter; 8 = Photomultiplier.[80]

ble of giving any wavelength from 200 to 2500 nm. The measurement can take place in two different ways as is illustrated in Figure 26. The upper one is to be used for reflectance, the lower one for measuring fluorescence spectra. An example of a reflectance spectrum is depicted in Figure 27.

The Chromatogram-Spectrophotometer represents a very versatile identification technique, at the same time being capable of giving quantitative information. Although rather expensive, it will prove to be indispensable to those laboratories commonly involved in the identification of unknown material.

FIGURE 27



Reflectance spectra of some Opium alkaloids. 1 = Papaverine; 2 = Morphine; 3 = Thebaine; 4 = Codeine; Sorbent = Silica gel H. Wave number (cm^{-1}) versus log R.[80]

Following this development, it may be expected that an apparatus for *in situ* reflectance I.R.-spectroscopy will also be devised and no doubt will be a suitable technique either alone or in combination with the Chromatogram-Spectrophotometer.

Reflectance spectroscopy can also be done with the recently introduced Camag-Z-Scanner (Camag, Muttenz, Switzerland).

Publication of Data

TLC data have been published in a great variety of ways, and, unfortunately, this has caused a number of problems. Therefore, it will be good to consider the various aspects.

Firstly, consider the listing of the experimental conditions. Numerous authors have already suggested that these should be specified as completely as possible. In the foregoing we have discussed the most important parameters influencing the separation; and it will be obvious that, if the experimental conditions are not given or are only partially given, a publication can cause great confusion in other laboratories. We would by no means advocate a too rigid standardization or recommend that another worker should use absolutely the same conditions; but when all experimental conditions are given, a skilled worker will know which factors in his laboratory differ from those described in the publication, and he will be able to adapt his conditions in such a way that a satisfactory separation can be obtained. Thus, preparation and handling of the plates, description and application of the sample, saturation or unsaturation, height of the starting points, running distance, development chamber, quality and composition of the solvent system, temperature, relative humidity, detection technique, etc. must be described.

Secondly, the question of R_F-values should be recorded. The latest edition of the AOAC methods has avoided R_F-values and suggests comparison with reference substances. Lederer[81] disagreed with this and has pointed out that R_F-values are certainly advantageous if one realizes their true natures especially with regard to accuracy and reproducibility. We fully agree with this and would like to emphasize that, in addition to R_F-values, photographs should be published to illustrate the actual separation possibilities, the effects of tailing, the shapes of the spots, and the contrast obtained with the detection method. A certain difference in R_F-values may give a good separation in one case, whereas in another instance there will be no separation at all. Drawings usually are insufficient to provide a good idea of the separation. Thus, publication of both R_F-values and photographs will be indispensable. R_F-values should be given to not more than two decimals as the accuracy of the measurement does not allow a third decimal.[81] It should also be specified whether single R_F-values or mean values are recorded, and, in the latter case, their standard deviations should be given.

Publishing of data also requires standardization of terminology to avoid the possible consequence of procedural errors and misinterpretations. Stahl has recently proposed suggestions for such a standardization of procedures and terminology in three languages.[82] Although it seems that several terms and descriptions need to be modified (for example, the terms "stationary phase = sorbent" and "mobile phase = solvent") and that additional terminology has to be taken up, the suggestions can be a good starting point. It will be necessary, however, that further international cooperation and agreement be obtained on this subject. Perhaps a IUPAC Commission can be formed so that the rules can be internationally adopted.

SUMMARY

The present state of qualitative thin-layer chromatography has been outlined. This has been done especially with regard to the technique as such; the progress in the various fields of application has not been discussed.

The preparation of the plates for TLC will, in general, not cause many difficulties. Adequate equipment is available, or ready to use plates can be obtained. The great variety of sorbents ensures the analyst a suitable choice, and newer types of sorbents will undoubtedly become available in the future, either general types or types to be used in special fields of application. In addition, vacuum-evaporated metal-oxide layers may be expected to expand the versatility of TLC and to encourage the development of other sorbent-application techniques. The standardization of the properties of the sorbents remains a problem, however, particularly when this is considered over a couple of years.

The "activation" process must be considered as a rapid drying method only. The actual activity of the sorbent during development is determined by the relative humidity and the temperature of the room atmosphere in which the plate is handled. The perfect reversibility of the water vapor adsorption by the sorbent is now clearly understood.

The application of the sample requires some skill but remains simple. The TAS-technique looks promising for the rapid qualitative analysis of natural products.

A topic of basic importance for the separation process is the influence of solvent vapor, a phenomenon which has had extensive attention during the last two years. The much better understanding now available allows a more rational approach to the various separation problems while several of the TLC "laws" from the early years have undergone revision. It can be expected that the future will see this better theoretical understanding increased.

Normal tank chambers and sandwich chambers have to be clearly distinguished, whereas unsaturated chambers have proved to be capable of giving improved resolution. The accretion of understanding has also been responsible for better reproducibility.

Although TLC has shown to provide excellent separations in many cases, the continuing demand for newer techniques with increased resolution power has indicated that variations in sorbents, solvent systems, and detection reactions are not enough to resolve the more difficult separation problems. Special techniques and refinements have thus been introduced, including continuous, multiple, and two-or-more-dimensional developments, centrifugal chromatography and gradient techniques. These methods have become more and more polished, and various suitable apparatus are now available. Their use is steadily increasing, and no doubt the interest in these techniques, as well as the development of improved methods, will continue.

The introduction of vapor-programmed TLC, using vapor impregnation as a controllable variable, has opened a new dimension in TLC because it allows closely related substances to be pulled apart, which can be compared with the effect of a parallel gradient. Moreover, other gradient effects can be obtained as well, and the technique provides "guided chromatography" of the individual spots. Its strikingly increased resolution power promises a wide field of application where conventional TLC methods are unable to yield satisfactory results.

The use of temperature as a controllable variable will also be among the techniques of tomorrow. Flux-gradient TLC is a first example, providing steep (ap) gradients.

There is no doubt that these newer development techniques are more complicated than classical TLC. Of course this is regrettable, but even with these newer methods the technique remains fairly simple and cheap by comparison with other separation techniques. We would by no means suggest, however, that classical TLC has no future at all. The classical technique certainly remains very valuable for a great many purposes, but the growing number of difficult separation problems will obviously require more sophisticated techniques providing optimal separation conditions. Therefore, the author is confident that the newer techniques will be welcomed by many analysts and that additional development in this area is to be expected.

With regard to the techniques after development, the use of spray reagents and fluorescent indicators makes detection very easy. Identification of unknown substances is a more difficult problem. Here, the compatibility of TLC with other analytical techniques has already proved to be valuable. *In situ* identification on the plate can be performed by reflectance spectroscopy, for which suitable instruments are now available.

Publishing of data should be done with special attention to the experimental conditions and the R_F-values. Furthermore, it is recommended to illustrate the separation possibilities with photographs and to come to a standard terminology.

ACKNOWLEDGMENT

The author thanks Professor E. Stahl, Professor M. Wilk, Dr. H. Jork, Dr. A. Niederwieser, and the publishers of *Analytical Chemistry*, *Archiv der Pharmazie*, *Chromatographia*, and *Journal of Chromatography* for their permission to reproduce material from previous publications. He is also grateful to the firms C. Desaga, GmbH, Carl Zeiss, and Camag A.G. for photographs of chromatographic apparatus, and to Dr. E. Dumont for a copy of his thesis.

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CRC CRITICAL REVIEWS in ANALYTICAL CHEMISTRY

RECENT DEVELOPMENTS IN THE ANALYTICAL CHEMISTRY OF RHENIUM AND TECHNETIUM

by Charles L. Rulfs
University of Michigan
Ann Arbor, Michigan

Developments in the chemistry of rhenium, least abundant of natural elements, and technetium, highly abundant artificial element, have become very numerous within the past decade. Several of the more analytically-significant portions of this chemistry are selected for detailed critical examination, with some attention being given to other, less-studied but promising, areas.

The many oxidation levels and species possible in solution for both elements give rise to a complex redox and electrolytic behavior. This important aspect of these elements' chemistry is especially reflected in a number of polarographic, and related, studies.

The chemistry and properties of the most common (VII)-state solution species is discussed. This includes the spectrophotometry of the normal tetrahedral MO_4^- ions, the meso-forms of these and the HMO_4 acids. There exist a large number of (V)-state chelates and complexes for both elements. While this state is not a common one for aqueous media of simple composition, these compounds are very useful for measurement and separation purposes. The 8-quinolinol chelates provide unusually interesting representatives of this class of compound. There appear to be some unique features in the liquid-liquid extraction behavior of this system.

Some applicaons of infrared, mass spectroscopic, X-ray and radiochemical techniques are also described.

PHASE SOLUBILITY ANALYSIS

by W.J. Mader
Drug Standards Laboratory
Washington, D.C.

Phase solubility analysis has been established on the sound theoretical principles of the Gibbs Phase Rule and is the application of precise solubility measurements to the deter-

mination of the purity of a substance. The great merit of the phase solubility is that the state of a system is defined entirely by the relation existing between the number of the components and the phase present since it is a univariant system, no account being taken of the molecular complexity of the substance being determined. No theories of kinetics are involved. The equipment is quite simple and usually available in most laboratories. The procedure is simple in application, however; at times the data may easily be, and are, misinterpreted. A relatively small sample is required and all or a pure fraction can be recovered. The method is applicable to all species where the impurity is present as a distinct phase except in the quite unusual instance where the ratio of the components coincides with the ratio of their solubilities. This indicates that D and L isomers may be determined except when they are present in a 1:1 ratio.

Phase solubility analysis has been in use for over 20 years and the use of the method as a means of determining absolute purity of complex organic compounds is gaining favor, both in research and control. Since the method is so simple in application, instances of misapplication and misinterpretation are occurring. Precautions to follow and useful modifications will be discussed.

A CRITICAL COMPARISON OF ATOMIC EMISSION, ATOMIC ABSORPTION AND ATOMIC FLUORESCENCE FLAME SPECTROMETRY

by J.D. Winefordner
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Atomic emission (AE), atomic absorption (AA), and atomic (AF) fluorescence flame spectrometry will be compared with respect to: function of the flame and interferences resulting from use of flames; similarities in the basic expressions relating the intensities emitted, absorbed, and fluoresced to the concentration of the atomic species; use of the basic expressions for estimation of fundamental parameters, such as quantum efficiencies, free atom fractions (degree of atomization), dissociation energies of diatomic molecules, damping constants, etc.; spectral interferences in the three methods; signal levels of AA and AF using line and continuum sources with respect to signal levels in AE; sensitivity (limits of detection) of AA and AF using line and continuum sources with respect to sensitivity in AE; and instrumental requirements of AE, AA, and AF. On the basis of the comparison, predictions will be made concerning the optimum methods and instrumentation for trace metal analysis. The use of non-flame cells for AE, AA, and AF will also be considered and compared with flame cells.