THIN-LAYER CHROMATOGRAPHY

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THIN-LAYER CHROMATOGRAPHY

Proceedings of the Symposium held at the Istituto Superiore di Sanità, Rome, 2-3 May, 1963

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PREFACE

The Istituto Superiore di Sanità decided to organise this symposium on thin-layer chromatography because past experience has shown how useful extended discussion of new and rapidly developing research techniques can be.

Thin-layer chromatography was hardly known and only infrequently employed ten years ago. It owes its present popularity to recent improvements of the technique and though several monographs have appeared of late, it was felt that a meeting of the main research groups would contribute further to its development, as well as permitting a large number of younger research workers to profit from discussions with the experts.

The present volume consists of a series of plenary lectures covering all the principal aspects of thin-layer chromatography and a number of original papers both from the Istituto Superiore di Sanità and from other sources which were represented at the symposium. They serve to illustrate yet again the variety of possible applications of this technique.

In publishing this symposium as a separate volume, the Istituto Superiore di Sanità has departed from its previous practice of publishing such work in the "Scientific Reports of the Istituto Superiore di Sanità" which has now been discontinued. Since the symposia range from pure chemistry to specialised medical topics it was felt that a greater service to the reader would be performed by publishing them separately. This volume is thus the first of a series which will replace the "Scientific Reports". The various volumes will appear as soon as possible after each symposium and will therefore not be bound to particular publication dates, as would a journal.

G. B. MARINI-BETTÒLO

The present Volume constitutes the Proceedings of the

SYMPOSIUM ON THIN-LAYER CHROMATOGRAPHY

Rome, 2–3 May, 1963

organised

by

Istituto Superiore di Sanità

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INTRODUCTORY SPEECH

by

Prof. GIORDANO GIACOMELLO

Director, Istituto Superiore di Sanità

It is a great pleasure to me, personally and on behalf of the Istituto Superiore di Sanità, to welcome here today Prof. Cramarossa, who is representing the Minister, and all those taking part in this International Symposium on Thin-Layer Chromatography — the speakers, the various department heads, the many representatives of Italian and foreign universities, the directors of our provincial laboratories and their assistants, and all who are gathered here today to discuss a new analytical technique of great value in basic research and above all in applied research, and which closely concerns several aspects of the activity of this Institute.

On several occasions in the past, the Institute has organized meetings directly or indirectly concerned with problems of health and hygiene.

Last October we held a Symposium on Food Additives to study the orientation and conclusions of scientists of the various European countries concerning the use of chemical additives in foodstuffs. This Symposium was of considerable importance in clarifying many aspects of the subject and in supplying our legislators with a basis upon which to formulate the Law on alimentary substances.

This was followed by a Symposium on Food Microbiology, called to seek the opinion of the most eminent scholars of the subject on one of the points of the Law regarding the hygiene of food substances.

To these we now add today's Symposium on Thin-Layer Chromatography.

The Law of 30 April, 1962, No. 283, provides, in fact, for the elaboration of analytical methods for the identification and the determination of additives in alimentary substances. Thin-layer chromatography today represents one of the most up-to-date techniques of analytical chemistry, combining a remarkable simplicity and rapidity of execution with a specificity and sensitivity greater than can be expected of other chromatographic methods.

This technique, in rapid evolution, today not only offers a refined instrument for the identification of additives and the evaluation of their purity, but also constitutes a highly efficient means for the detection of alimentary frauds.

Considering solely the field of food substances, in the literature already existing on the subject of thin-layer chromatography we have methods for establishing the purity of food dyes — methods which we owe principally to Dr. Wollenweber, here with us today.

We have, furthermore, a series of methods which facilitate the separation and recognition of the main antioxidants and antifermentatives, and I am happy to note that Prof. Covello is to speak to us of his work on food additives.

However, not only additives can be identified with this technique: many substances used as plasticizers can also be isolated, and their identification in very small quantities is of utmost importance in the evaluation of food containers.

Still in the field of alimentation, we must also consider in particular the use of this technique for the separation and identification of lipids and fats, which, in conjunction with gas chromatography, offers the possibility of a complete study of fatty substances.

But the application of thin-layer chromatography is not limited to alimentary analysis. There are many other analytical problems of great interest which it can help us to solve.

Sufficient to mention that it allows a rapid and accurate analysis of insecticides, particularly in agricultural products. In the development of this particular application much has been contributed by Dr. Petrowitz, whom I am happy to be able to welcome among us today. This is a field of traditional interest to the Institute, which devotes to it a great deal of attention under the guidance of Prof. Alessandrini.

Furthermore, thin-layer chromatography permits not only the identification of individual drugs in mixtures, but also the products of decomposition. It is of valuable assistance in the study of antibiotics, and in research for new representatives of this type of compound, particularly when used in conjunction with microbiological techniques.

In the field of toxicology, it permits the separation of alkaloid mixtures and the study of drug metabolism, in that it allows the separation and recognition of the metabolic products of these substances in blood and urine.

Not to be underestimated, furthermore, is its contribution in simplifying the evaluation of the stability of many drugs which are particularly subject to alteration, and it is hardly necessary to mention its value in the examination of vitamin and hormone preparations, etc.

Yet another sphere in which thin-layer chromatography can be of value to us is in the study of atmospheric contamination, when it is necessary to establish the nature of the filter-separated hydrocarbons to identify those which are harmful. Prof. D'Ambrosio and Dr. Pavelka have availed themselves of this technique in their study of atmospheric contamination in Milan.

One might say that there is no field of research to which thin-layer chromatography has not contributed. Even in nuclear studies it has proved its worth, in the separation of radioelements as in the study of the products of radiolysis of the organic moderators of reactors.

However, to remain in the field of major interest to the chemist and biological chemist, mention should be made of the contribution this technique has made and will make to the study of natural substances of animal and vegetable origin, as is also shown by the research carried out in this very Institute under the direction of Prof. Marini-Bettòlo.

Modern pharmacognosy, too — as demonstrated by the research work of Prof. Stahl — has benefited from its use, as has the chemistry of aromatics and essential oils, as Dr. Demole has shown.

In biological chemistry its application to the separation of nucleic acids, of sugars, of amino acids, of steroids, has led to important results, owed particularly to the work of Dr. Neher, which demonstrate, among other things, the possibility of following the metabolic processes of the various constituents. In this same category are Prof. Opieńska-Blauth's studies on urinary amino acids, and those of Prof. Siliprandi and his colleagues on mitochondria.

To conclude this brief summing-up, I would like to emphasize that thin-layer chromatography, initiated with a layer of silica gel, is undergoing constant evolution, as did column chromatography.

Thus we have thin-layer alumina chromatography developed especially by the Czechoslovakian authors, Dr. Lábler among them; the techniques based on the use of cellulose paste; of exchange resins; of polyamides; and finally of dextran derivatives which constitute a novelty of this Symposium, presented by the School of Biological Chemistry of the University of Rome.

To the aspect of qualitative analysis must be added the preparatory aspect, to be illustrated by Drs. Seiler and Fétizon.

In conclusion, I once again extend to each of you here present a very sincere welcome, and would like to move a vote of thanks to Dr. M. Lederer, who, with Prof. Marini-Bettòlo, arranged this very pleasant gathering. It has already proved worthwhile by bringing together in collaboration so many eminent scholars from every country, and I trust that it will prove equally fruitful from every point of view.

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DEVELOPMENT AND APPLICATION OF THIN-LAYER CHROMATOGRAPHY*

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1. INTRODUCTION

A few years ago the concept of thin-layer chromatography was unknown and only a few "outsiders" were using thin, inorganic sorption layers for separating lipids. Now, tens of thousands of mixtures are analysed daily this way and two books have recently appeared on this subject in Germany. Compared to other methods, which are coming into increasing use and which often demand considerable apparatus and servicing personnel, the method to be described here is basically simple and easy to learn.

Before discussing the general principle of the experimental technique, its applications, and possibilities for future development, here is a "curriculum vitae" of thin-layer chromatography.

2. HISTORY OF DEVELOPMENT

In the thirties there was a persistent search in organic chemistry for an adsorption-chromatographic micro-separation process. Tswett-columns, 2–5 or more cm thick, were replaced by thin capillary tubes. The problem does not lay so much in the separation as in the detection of the substances separated. The decisive step forward was, in my opinion, the change to "open" separation columns, which avoided many of the earlier difficulties and which was first described in 1938 by IZMAILOV AND SCHRAIBER. Swiss colleagues have informed me that Professor IZMAILOV (Fig. 1) died in 1961 while Director of the Pharmaceutical Institute of the University of Kharkov and that, although more than 200 publications came from his pen, he did no more on "drop chromatography" as he then called his method. He took specimen plates on which was spread a 2-mm thick, firmly adhesive layer of alumina, introduced 1 drop of alcoholic plant extract to the layer and gradually dropped alcohol on to the centre of this spot. By this means he obtained small round chromatograms. At the side of Fig. 2, which is taken from his first report, will be seen a series of illustrations. At that time he used the method to investigate all the tinctures of the Russian pharma-

* 11th Communication.

Fig. 1. N. A. IZMAILOV,
born June 26th, 1907, died October 2nd, 1961.

copeia and demonstrated the advantages of this procedure over column chromatography. A short while later, CROWE in the U.S.A., referring to this work, reported that he had used loose aluminium oxide layers in the same way as IZMAILOV AND SCHRAIBER, but only, however, as a preliminary test for column chromatography. WILLIAMS also investigated the technique and protected the loose layer by covering with a second glass plate. Similarly in England the CONSDEN, GORDON AND MARTIN group was confronted with the problem of repeating their partition-chromatographic separations of amino acids, first carried out in columns, on a micro-scale. This group also went over to "open" columns, in this case filter paper. As a result of the as-

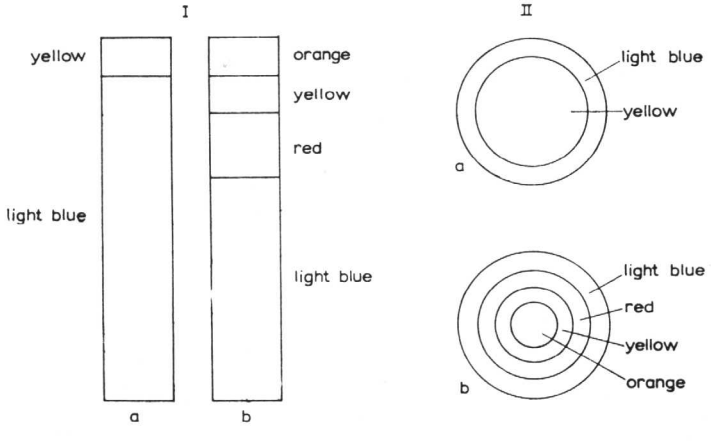

Fig. 2.
Comparison between the fluorescence colours of an alumina column chromatogram (*I*) and a "drop" chromatogram (*II*) of Belladonna extract. *a* before developing, *b* after developing with alcohol.

tounding success of this method, all kinds of separation problems were then investigated by this means. New solvents were continually tried and, if no progress was achieved, attempts were made to change the characteristics of the cellulose by impregnation or by chemical transformations. In order to carry out adsorption-chromatographic separations on cellulose- or glass fibre paper in the same way as had been done with columns, the papers were impregnated with alumina or silicic acid. KIRCHNER was one of the first to recommend this method. It is therefore not clear why he, in collaboration with MILLER in 1951, referred back to the *Surface Chromatography* of MEINHARD AND HALL which appeared in 1948. It is, however, certain that this technique was also based on IZMAILOV's separation layers, except that longer strips of glass were used and the name "Chromatostrip Technique" was employed. Despite a series of excellent papers from the group of MILLER, KIRCHNER AND KELLER in the U.S.A., the method was only used occasionally in a few laboratories dealing with terpene derivatives. Perhaps the reason was that the technique was not yet satisfactorily perfected. It is a general rule that a method evolves from the problem, *i.e.* the goal of the investigation, and so it was in our own team. Eleven years ago, and still to-day, we wanted to separate the contents of individual plant and animal cells. This was not possible with any of the existing methods, so we first turned our attention to the structure of the separation layers. The coarse fibre structure of the papers, and also the commercial adsorption media for column chromatography made the very small amounts of substance used (less than 0.1 μg) disappear. So we went on increasingly to finer and thinner sorption layers until we finally managed to separate chromatographically the contents of a few plant-glands, these being practically invisible to the naked eye, on a silica-gel layer 20 μ thick. In this way we showed the products contained therein could in no way be identified with the isolated products. The advantages of such fine grain separation layers became increasingly clear to us during the subsequent years, and we learned how to separate more and more new classes of compounds. Our first publication on the subject, in 1956, passed with as little notice as that of IZMAILOV AND SCHRAIBER and their successors, and we began to wonder why the method was not being generally adopted. At first we had to contend with the objection that it was another of the current chromatographic gimmicks, of the sort we have seen in recent years even in thin-layer chromatography. But, giving up trying to solve individual problems, we devoted ourselves for five years to investigating the method and have tried to evolve the best possible procedures.

By 1958 our work was sufficiently advanced for us to present the method to a large number of scientists in the "ACHEMA 1958" (Frankfurt) and the "ILMAC 1959" (Basle). At first, thin-layer chromatography was taken up by the industrial laboratories of South Germany and Switzerland and from there it has spread to the whole western world. Even in Eastern Europe and the Soviet Union the method is known, but there they work mainly with loose layers, as had been done from 1951 to 1956 particularly by MOTTIER and his colleagues in Switzerland.

3. EXPERIMENTAL TECHNIQUE

The following points seemed important to us:

(*i*) Rational preparation of uniform thin layers.
(*ii*) Arranging the necessary equipment into a single basic apparatus suitable for immediate use.
(*iii*) Finding the most universally applicable sorption media.
(*iv*) Ascertaining the factors influencing the process, and establishing standards, *i.e.* creating a starting basis.
(*v*) Deciding the range of application of the method.

Preparation of thin separation layers

Thin layers may be prepared in several ways and there is little need to scratch one's head over the matter, as such techniques are already known in the film, varnish, and manufacturing industries. A suspension of sorbent may be applied to a fixed carrier-plate, *e.g.* of glass or metal in the following ways:

(*a*) Spreading
(*b*) Pouring
(*c*) Spraying
(*d*) Dipping.

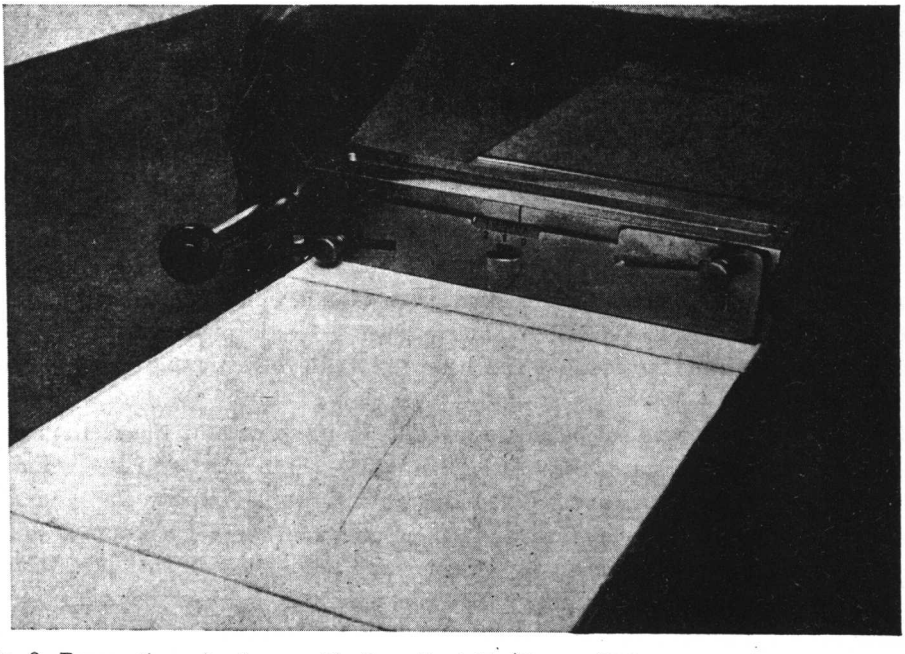

Fig. 3. Preparation of a layer with the adjustable Desaga-TLC-spreader. (Manufactured by Fa. C. Desaga, Heidelberg, W.-Germany.)

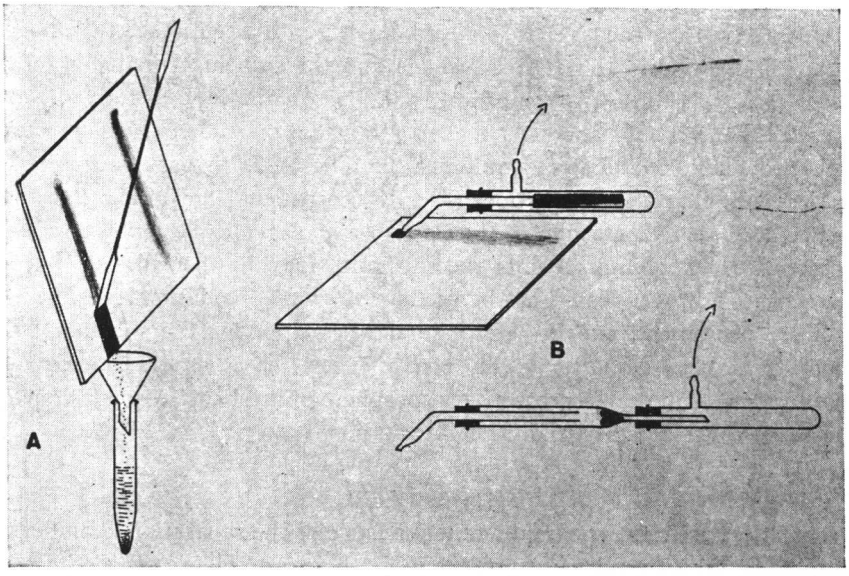

Fig. 4. Isolation of thin-layer chromatographically-separated substances. (A) scratching off, (B) with a simple "vacuum cleaner".

All these methods have been tested by us, insofar as they affect the practical needs of a medium-sized laboratory. In accordance with the stipulation for a rational preparation of layers of defined thickness, the method should also correspond, as regards safety and cleanliness, with the laboratory facilities. The last few years have shown that the following suggested standardisation of the method may be taken as a good basis. Most analytical separation problems may be resolved on a square 20 × 20 cm plate to which is applied a 250-μ thick, adhesive layer. It is also being increasingly recognised that thin-layer chromatography can be an excellent tool for micro-preparative separation of mixtures. For this, it is better to use thicker layers of 0.5 to 1 mm and glass plates 40 cm wide. These allow easy separation of mixtures in amounts of 10 mg up to a maximum of 500 mg, giving sufficient material for determination of chemical composition or of physical constants. The problem of adjusting the layer thickness is easily overcome by use of the thin-layer applicator we have developed (Fig. 3). For micro-preparative separation, however, it has hitherto been extremely difficult to apply a starting band of 1 ml of a solution on to the layer with sufficient uniformity to obtain the optimal separation effect. A small device — recently also described by RITTER AND MEYER — can help us here. The substances are not applied drop by drop to form the starting band, but are sprayed on evenly in a line (see Fig. 11 in *Dünnschicht-Chromatographie, ein Laboratoriumshandbuch*, Springer, Berlin). There is still some difficulty in determining the exact dosage, but we hope that this problem may also be overcome.

Both in micro-preparative work, and in quantitative evaluations of thin-layer chromatograms, there is the task of recovering the substances quantitatively from

the plate and of extracting them. Fig. 4 sketches the possibilities available so far. With a sharp spatula, the layer is removed directly into a centrifuge tube filled with the extraction medium; this is then shaken and the sorbent centrifuged off. From the clear residual solution the substance may be obtained or directly determined photometrically. For substances not sensitive to oxidation a micro-"vacuum cleaner", arranged from simple laboratory equipment may be used. We use two types of these micro-"vacuum cleaners". The upper (Fig. 4B) corresponds in principle to that described by RITTER AND MEYER, in the lower a percolation tube serves to gather up the material. By applying solvents the substance may be then directly extracted. This procedure however should not be used for the many oxidation-sensitive natural substances. One should also be very careful when applying the substances to the sorption layers which are often highly active. It is often advisable to carry out application of the samples in a protective atmosphere of inert gas. A small preparation box has been constructed specially for this purpose.

Separation chambers for thin-layer chromatography

At first little attention was paid to the influence of the separation chamber. Even today there is often a tendency to ignore it, as there is to ignore other intermediate influencing factors, and R_F-values are published which cannot be reproduced. In many cases this is certainly not intended, for as soon as one comes from paper chromatography, one is astounded at the smallness of the spots and the much better separation effects and one overlooks the fact that there is still much to be considered. Thus it may be observed that, if solvent mixtures and ordinary aquarium tanks are used, an undesirable edge effect is obtained, as described by DEMOLE and by us in 1958. This stems, we showed, from inadequate saturation of the chamber with the solvent vapour. Fig. 5 demonstrates this. This unsatisfactory saturation of the chamber may however be countered simply by lining the tank completely with filter paper saturated with the solvent. The running times are thereby shortened, and, only if this so-called chamber saturation is employed, can one genuinely speak of R_F-values. It is also possible to reduce the chamber volume to improve saturation.

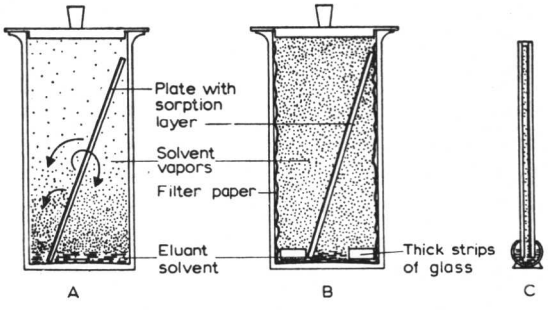

Fig. 5. Separation chambers and saturation. (A) chamber with normal saturation (= NS), (B) chamber saturated with solvent vapours by lining the jar completely with filter paper (= CS), (C) S-chamber.

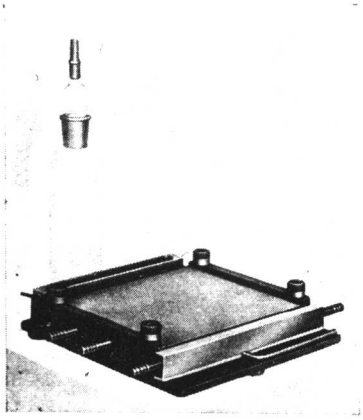

Fig. 6. BN-chamber for continuous-flow technique. (Manufactured by Fa. C. Desaga, Heidelberg, W.-Germany).

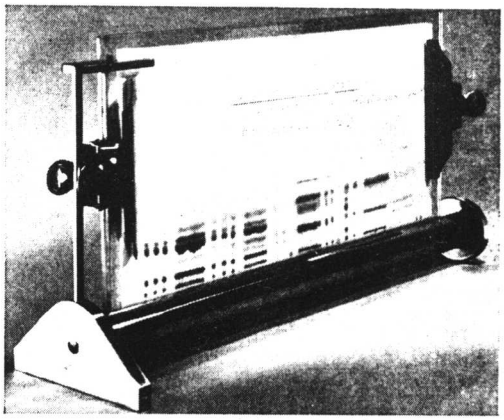

Fig. 7. S-chamber system. The plate is 40 cm wide. (Manufactured by Fa. C. Desaga, Heidelberg, W.-Germany).

Independently and in the contexts of different problems, technical difficulties have been overcome by my colleagues BRENNER AND NIEDERWIESER, using the horizontally arranged "BN-chamber" and by ourselves with the "S-chamber". The next two illustrations will show you these separation-chamber systems specially devised for thin-layer chromatography (Figs. 6 and 7). The BN-chamber, demanding a little more in the way of construction, enables one to use the continuous flow technique, which is of particular importance for the separation of many amino-acid mixtures. Moreover it has the advantage that it is possible to cool it, hence permitting low-temperature chromatography; it is also capable of being used for the ascending technique. In our own laboratory, if we wish to carry out micro-preparations or comparative investigations, *e.g.* if we wish to develop 30 or 40 samples under exactly similar conditions, we use 40-cm wide plates and the S-chamber. In fact these experiments have shown that in many instances the separation effect of the S-chamber is notably better than that obtained from the tank chambers used hitherto. For example, we found that resins and balsams which are very difficult to analyse, spread out into narrow bands, enabling us to recognise many more substances than was possible with the methods used earlier. This finding was confirmed with other mixtures. The central problem of thin-layer chromatography now arises, namely the sorption media.

Sorption media for thin-layer chromatography

In all chromatographic experiments an optimal separation is reached only when the correct *stationary phase* and the appropriate *mobile phase* are used. In paper chromatography one is more or less restricted to cellulose. In column chromatography the dominant adsorbent, in the past as at present, has been aluminium oxide. Usually one attempts to attain separation by varying the mobile phase, that is, by altering the solvent. In thin-layer chromatography we now have the possibility of investigating the most varied kinds of separation layers in a short time and of quickly

seeing the separation results. The value of the method is not appreciated if one simply degrades it to a "silica-gel thin-layer chromatography", imagining it to be only a question of taking the coarse-grained sorption media of column chromatography and applying it layer-wise on to a plate for subsequent separation. The progressive step lies in the use of fine-grained material, may it be inorganic or organic. Layers consisting of small particles, 1–25 μ in size, allow many separation problems to be solved in an astonishingly short time. We can only have thin layers of very fine-grained material. Because of this we have taken the step of not calling the method "drop chromatography" or "surface chromatography" or "chromatostrip" or "chromatoplate" or "thin-film chromatography"; rather we name this advance "thin-layer chromatography". In my opinion the most important advance resulting from the method is its ability to render visible uniform separations at an ultra-micro level. This is possible because the concentration of the separated components in each spot area is higher than it is on the fibrous or coarse sorption layers used up to now. Perhaps this can be clarified by the next illustration. In Fig. 8 the zone sizes of a paper chromatogram are compared with those of a thin-layer chromatogram for increasing amounts of substance. Particularly impressive are the comparisons in the work of BRENNER and his associates (Chapter : "Amino Acids and Derivatives" in *Dünnschicht-Chromatographie, ein Laboratoriumshandbuch*, Springer Verlag, Berlin, Göttingen, Heidelberg).

These most important findings can be explained if we consider the grain size and

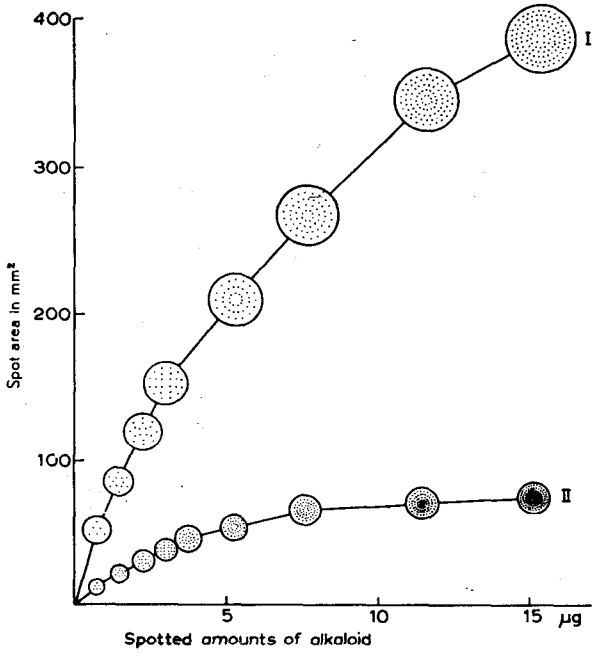

Fig. 8. Comparison between the spot area of separated alkaloids (spotted in increasing amounts) on paper impregnated with formamide (I) and on a silica gel layer (II). Dots symbolise the number of molecules.