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STUDY OF THE PERFORMANCES OF THIN-LAYER CHROMATOGRAPHY

VII. SPOT CAPACITY IN TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

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SUMMARY

In two-dimensional thin-layer chromatography the spot capacity is the number of spots, resolved with a resolution unity, that can be placed on the plate between the two solvent fronts and the parallels to these fronts through the center of the original sample spot. This is difficult to calculate because the plate height in thin-layer chromatography (TLC) is a complex function of the characteristics of the solvents and the plate, since during development in one direction the spots spread in both directions and since calculation of the density of the most dense spot packing requires topological information that is not available. Some simplifying assumptions are made and an iteration method is used.

The results show that it is very easy to achieve a spot capacity between 100 and 250, but difficult to reach 400 and nearly impossible to exceed 500, except in very favourable circumstances. As for one-dimensional TLC, the spot capacity in two-dimensional TLC increases with decreasing diffusion coefficients and with increasing plate quality (*i.e.*, packing homogeneity) and kinetic coefficients of the solvents. For a given solvent and development length there is an optimum particle size which provides the maximum spot capacity.

The development time for a capacity of 300 spots is around 30 min but it is very difficult to obtain accurate quantitative results if the analysis is fast.

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INTRODUCTION

Thin-layer chromatography (TLC) can easily be carried out in two dimensions, successively. Only one sample spot is developed on a square (or rectangular) plate. The sample is placed at a corner of the plate, and the two developments are carried out successively, parallel to the two sides of the plate, using two different chromatographic systems, for example two different solvents. It is more difficult to spread the components of a mixture evenly over the entire plate than to spread them over the one dimension of standard TLC or column liquid chromatography; this requires far more ingenuity from the analyst in combining the different retention mechanisms.

Two-dimensional TLC was first reported by Consden *et al.*[1]. They used a 45 × 55 cm paper sheet to separate proteinic amino acids. The first development using collidine–water lasted 72 h. After drying, this was followed by a development using phenol–water in an atmosphere containing a small amount of ammonia, that lasted from 27 to 48 h. At least 15 of the 22 amino acids were separated[2]. Detection was carried out using ninhydrin. The sensitivity was of the order of 1 μg allowing the analysis of 200-μg samples of protein hydrolyzates.

Later this technique was used by Munier and co-workers[3] to separate a variety of acids important in biochemistry (malonic, lactic, citric, malic, tartric, etc.) and by Nordmann *et al.*[4,5] to separate 21 organic acids in urine. The spots on the chromatogram published differ widely in size, reflecting not only variations from spot to spot in both development directions, but also differences in concentration[4,5]. It is well known that spot shapes drawn after visual inspection have a size depending markedly on the amount of the corresponding compound[6]. Nevertheless, taking the average surface area of a spot on the chromatogram (8×10^{-3} R_F^2) we derive a spot capacity of 126 which is remarkably large in view of the crude technique used.

Two-dimensional TLC has been used for a large number of difficult separations[7]. For example, excellent separations of amino acids have been reported by Von Arx and Neher[8] and very impressive separations of carbohydrates by Lato and co-workers[9–11]. This technique has had an important impact on the development of several important fields of biochemistry, such as the elucidation of the reduction cycle of carbon in photosynthesis and its connection to other metabolic pathways[12,13] and the unravelling of other biochemical pathways[14].

This method is also related to other techniques used in biochemical analysis. For example, the separation of oligonucleotides can be carried out by ionophoresis on a two-dimensional system using cellulose acetate in one dimension and DEAE-paper in the other[15]. Similarly, large numbers of proteins are separated by two-dimensional electrophoresis[15].

Two basic techniques have been used. In the first the same chromatographic bed is developed successively with two different solvent mixtures along the two directions. In the second method a plate is coated with a strip of a sorbent along one edge and a large layer of a second sorbent, and two successive developments are carried out, with two different solvents. The preparation of such plates is difficult[7,17].

The main advantage of the technique is its high resolution power, already exemplified above, associated with the simplicity of TLC. The drawbacks are the necessity of selecting two different retention mechanisms, the possible interference between the solvent used for the first development and the second retention mecha-

nism and particularly the detection of the separated compounds for quantitative analysis.

Already TLC is plagued by the lack of a good measuring device. The human eye is a wonderful instrument to detect a pattern of spots but is unable to perform any quantitative measurement[6,18]. A scanning photometer, although not very practical and rather slow[19], can be used to scan a one-dimensional TLC chromatogram. To obtain quantitative results several minutes are required to scan a conventional TLC plate. It would be almost impossible to scan a complete plate for a two-dimensional chromatogram. This would require several hundred parallel scans and would take many hours, since we know from column chromatography that at least ten data points are required per standard deviation[19]. For the same reason, although seemingly attractive, the use of a Vidicon tube[20] raises a difficult problem of optical resolution. Equipment able to handle 10 × 10 cm plates with a spot capacity of 400 (spot diameter *ca.* 5 mm) should have a resolution of 0.13 mm, *i.e.*, 800 points should be distinguished along one side of the plate. This largely exceeds the specifications for the screen of commercial TV sets or video display monitors (512 × 512 pixels).

Up to now the problem has been solved satisfactorily only for the analysis of radioactive samples[16,21], using photographic techniques and autoradiography.

The purpose of this work is to calculate the performance expected from two-dimensional TLC and the range of spot capacity attainable in practice. The specifications for a detection system could then be derived.

THEORETICAL

The peak capacity in one-dimensional TLC can be calculated using an approach developed recently[22]. As both the spot diameter and the height equivalent to a theoretical plate (HETP) corresponding to each spot vary along the distance on the plate between the sample spot and the solvent front, an iteration method is used.

It is assumed that the distance between two successive spots which are separated with a resolution of unity is equal to the diameter of the first of these two spots. The migration distance, z_{p-1}, of the spot number $p + 1$ is thus related to the migration distance of spot p and the width of that spot by

$$z_{p-1} = z_p + 4\sigma_p \tag{1}$$

where σ_p is the standard deviation of the concentration distribution of spot p along the development direction, assuming a Gaussian profile. The spot capacity, n, is such that:

$$\sum_{p=0}^{n} 4\sigma_p < L - z_0 < \sum_{p=0}^{n-1} 4\sigma_p \tag{2}$$

where L is the migration distance of the solvent front and z_0 is the distance between the solvent level in the tank and the original sample spot. The calculations are carried out using a HP 67 calculator. The retention ratio is:

$$R_F = z/L \tag{3}$$

In this calculation we neglect the variation of the density of the solvent near its front but assume a piston flow of the mobile phase. This is in part compensated by rounding off n to the lower integer, and assuming that the non-retained solute has a circular spot whereas it actually has a semi-circular or crescent-shaped one. Also the less strongly retained spots are also the longer ones in the direction of the development so that it is rare that the second spot has an R_F larger than 0.85–0.90 (ref. 22).

The spot diameter is obtained using the addition of variances

$$\sigma^2 = \sigma_i^2 + zH \tag{4}$$

where σ_i is the standard deviation of the sample spot deposited on the plate and H is the average HETP corresponding to the spot compound[23]. H is obtained by integrating the Knox empirical equation for the reduced plate height[23]

$$h = \frac{B}{v} + Av^{1/3} + Cv \tag{5}$$

with

$$h = H/d_p \text{ and } v = ud_p/D_m \tag{6}$$

where d_p is the diameter of the particles used to make the chromatographic bed, u is the solvent velocity and D_m is the diffusion coefficient of the compound in the solvent. The integration is carried out to account for the variation of the solvent velocity during the development, since the movement of the solvent front obeys the quadratic law

$$L^2 = kt \tag{7}$$

where t is the time, L the migration distance of the solvent above its level in the solvent tank and k the kinetic coefficient of the solvent:

$$k = \theta d_p \tag{8}$$

θ is a function of the nature of the solvent[24]. Integration of eqn. 5 using eqns. 6–8 gives[23]

$$H = b(L + z_0) + \frac{a}{L - z_0}(L^{2/3} - z_0^{2/3}) + \frac{c}{L - z_0} \ln \frac{L}{z_0} \tag{9}$$

with

$$a = 3Ad_p^{5/3}\theta^{1/3}/2(2D_m)^{1/3} \tag{10}$$

$$b = B/\theta d_p \tag{11}$$

$$c = C\theta d_p^3/2D_m \tag{12}$$

while B is related to the diffusion coefficients by[23]:

$$B = 2\left(\gamma_m D_m + \frac{1 - R_F}{R_F} \cdot \gamma_s D_s\right) \tag{13}$$

γ is the tortuosity and D the diffusion coefficient, while the subscripts m and s refer to the mobile and stationary phases respectively. As a first approximation, $\gamma_m D_m$ and $\gamma_s D_s$ are similar and we assume them to be equal. Hence:

$$B = 2\gamma D/R_F \tag{14}$$

Combination of eqns. 3, 4, 9 and 14 gives

$$\sigma^2 = \sigma_i^2 + L\left[\frac{2\gamma D}{\theta d_p}(L + z_0) + R_F H_0\right] \tag{15}$$

with:

$$H_0 = \frac{a}{L - z_0}(L^{2/3} - z_0^{2/3}) + \frac{c}{L - z_0} \ln \frac{L}{z_0} \tag{16}$$

σ_0, σ_1, ... σ_p are calculated using eqns. 1, 3, 15 and 16 and summed until n is obtained.

The calculation of the spot number in two-dimensional TLC is slightly more complicated, since all spots spread during the two successive developments, unequally in the direction of development and in the perpendicular direction. This is illustrated in Fig. 1. Let n_1 and n_2 be the spot capacities obtained in one-dimensional TLC along the two different development directions with a sample spot standard deviation of σ_i and ^2n be the spot capacity achieved in two-dimensional TLC. Obviously ^2n is smaller than the product $n_1 n_2$, for two reasons. First, when the second development starts the spots have a dimension (length along the second direction, *i.e.*, width perpendicular to the first direction) which is larger than σ_i. Accordingly the spot capacity for this second development is smaller than n_2. The spot capacity in the second direction should be calculated for an original spot dimension σ_0, such that

$$\sigma_0^2 = \sigma_i^2 + 2\gamma D t_1 = \sigma_i^2 + \frac{2\gamma D_1 L_1^2}{k_1} \tag{17}$$

where the subscript 1 refers to the first solvent. This gives n_2', the spot capacity along the second direction in two-dimensional TLC. Secondly, during the second development, the spots also spread laterally, so they must be separated with a resolution higher than unity at the beginning of this second development if they are to have a resolution of 1 at the end. Some of the resolution provided by the first development is lost during the second one.

Accordingly, in two-dimensional TLC the standard deviation to use in eqn. 1 to calculate n_1 is given by:

$$\sigma_p^2 = \sigma_i^2 + z_p H + \frac{2\gamma_2 D_2 L_2^2}{k_2} \tag{18}$$

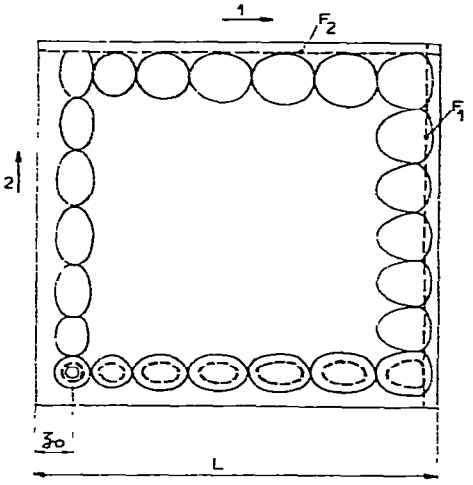

Fig. 1. Scheme of spot distribution on a two-dimensional TLC plate, after the two developments are completed. 1,2 = The two development directions; F_1, F_2 = solvent fronts. In this case $n'_1 = n'_2 = 7$; n_1 is between 9 and 10. The spots corresponding to compounds which do not move during the second development ($R_{F,2} = 0$) spread to some extent (*cf.*, dotted profiles).

Then the spot capacity in two-dimensional TLC is given by

$$^2n = n'_1 n'_2 \tag{19}$$

where n'_1 and n'_2 are calculated using eqns. 1–3, 9–[illegible]16 and 18. These calculations have been made for a number of combinations of [illegible]nd solvent characteristics to investigate the performances which are accessible.

These derivations assume that the thin-layer bed is homogeneous and isotropic, so that there is no coupling between the two developments. We have neglected the compression effect at the beginning of the second development; the solvent front reaches the lower side of the spots first and moves it towards the centre of the spots, so actually it reduces the effective spot width by a factor $(1 - R_F)$. This phenomenon was also neglected when it acts on the original sample. It may result, however, in a significant increase in the spot capacity.

We have considered it implicit that the spot capacity is equal to the product $n'_1 \times n'_2$ and the spots are arranged in rows and columns after a regular square pattern, the numbers of spots n'_1 and n'_2 being calculated along the axes 1 and 2 through the centre of the sample spot. The spot capacities along these directions would be slightly smaller if calculated at the other end of the plate, along the solvent front F_1 for direction 2 and along solvent front F_2 for direction 1, since the corresponding spots have moved over a longer distance. The difference is not great in most cases however, as molecular diffusion tends to control spot broadening in TLC[23]. This effect, which would result in a decrease in spot capacity, is approximatively compensated by the fact that the spots could be packed more densely than in a square-based tessellation: a regular hexagonal tessellation could accommodate $2/\sqrt{3}$ or 15% more spots.

Also neglected in eqns. 17 and 18 is the contribution to radial or lateral band

broadening due to the anastomosis of the flow stream pattern. It is at most equal to $0.15\gamma d_p$ and thus negligible compared to $2\gamma Dt$.

Finally the limiting spot capacity, reached after an infinitely long development time in both directions, so that the sample spot size becomes negligible compared to the final spot size, is

$$^2n_T = \left[\frac{L}{4\sqrt{2\gamma\left(\frac{D_1}{k_1} + \frac{D_2}{k_2}\right)L^2}}\right]^2 = \frac{d_p}{32\gamma\left(\frac{D_1}{\theta_1} + \frac{D_2}{\theta_2}\right)} \quad (20)$$

as derived from eqns. 4, 7 and 8 of ref. 22.

RESULTS AND DISCUSSION

We have carried out calculations using the model developed above to assess the effects on spot capacity of the various characteristics of the chromatographic systems used, and of the parameters of the TLC bed.

We first studied the effect of the sample spot size and of the distance of this spot above the solvent level, then the most important parameters, the plate size (it is assumed to be square) and the average particle size. We assume that the TLC bed is thin enough so that the plate efficiency is not affected by vertical segregation of particles of different sizes during preparation of the bed. Then we calculated the effect of the quality of the TLC bed (parameters A and C of eqn. 5) and of the parameters of the chromatographic system: the diffusion coefficient of the solute and kinetic parameter of the solvent. The diffusion coefficient is assumed to be the same for all solutes. Another assumption (such as a relationship between D and R_F) would be equally arbitrary and would lead to extremely complicated calculations. Thus the reduced velocity is also taken to be the same for all solutes.

Throughout this work we have taken the bed tortuosity to be 0.7, a value often employed[22]. Except when the effects of these parameters is studied, the bed characteristics A and C are equal to 1 and 0.01 respectively, in agreement with experimental results[24,25].

Although it is quite reasonable to assume that the plate characteristics (A,C,γ,d_p) are the same in both directions, this is less acceptable for the solvent characteristics. The kinetic coefficient is quite different from one solvent to another and so is the diffusion coefficient. The latter can be approximated by the Wilke–Chang equation[26]

$$D_m = 7.4 \times 10^{-10}\sqrt{\varphi_1 M_1}\cdot\frac{T}{\eta_1 V_2^{0.6}} \quad (21)$$

where M_1 and η_1 are the molecular weight and viscosity of the solvent, respectively. T the temperature (°K) and V_2 the molar volume of the solute. φ is an association constant (2.6 for water, 1.9 for methanol, 1.5 for ethanol, 1 for non-associated liquids). A correlation exists between θ and the diffusion coefficient of any given solute, at least in normal chromatography, as in this case the cosine of the wetting

angle is unity for all solvents: θ is inversely proportional to the solvent viscosity as is D_m and the surface tension increases gradually with the molecular weight, except for light, very polar solvents like acetonitrile and acetone. Accordingly, most of the calculations have been made using values of θ and D_m which are both smaller in one direction than in the other one. We have chosen for D_1 and D_2 values of 5×10^{-6} and 2×10^{-6} cm^2/sec respectively, which are typical of medium size molecules constituting most of the complex mixtures of current interest, and for θ_1 and θ_2, values of 120 and 60 corresponding respectively to fast and rather slowly moving TLC solvents (*cf.* eqns. 7 and 8). In a separate section, the influence of these two parameters is studied and calculations are made using different combinations of values for θ_1, D_1 and θ_2, D_2.

As very little experimental work has yet been done in two-dimensional TLC, it is not useful at this stage to make a thorough investigation of the whole situation; it is sufficient to obtain enough data to give a flavour of the potential of the technique.

Theoretical limit of the performance

The theoretical spot capacity, achieved with either a sample spot diameter of zero or an infinitely long development time, unrealistic conditions in both cases, has been calculated for a variety of experimental conditions, using eqn. 20. The results are reported in Table I, together with the corresponding values for one-dimensional TLC. The spot capacity in two-dimensional TLC exceeds that in conventional TLC, using the same plate characteristics and solvent systems, by about one order of magnitude, although it is markedly smaller than the product of the spot capacities in both directions, as expected from the radial diffusion of the spots.

The theoretical performance cannot be reached, as usual in chromatography, but we can expect to be able to achieve rather easily half the theoretical limit, since it has already been demonstrated that the development time required for a similar effect in one-dimensional TLC is very reasonable[22].

This means that, in spite of the limits of the TLC technique, two-dimensional TLC could be comparable to column liquid chromatography in terms of resolution power, provided two independent retention mechanisms can be found.

Influence of sample spot size

The results are given in Table II. The calculations have been made for square plates having sides from 1 to 5 cm. The sample spot is placed on the plate diagonal at

TABLE I

THEORETICAL LIMIT OF THE SPOT CAPACITY IN TWO-DIMENSIONAL TLC (*cf.* EQN. 20)

$\gamma = 0.70$; $n_T = \sqrt{\theta d_a / 32\gamma D}$

d_a	3	5	7	10	15	20	7	7	7	7
$D_1 \times 10^6$	5	5	5	5	5	5	1	2	10	10
θ_1	120	120	120	120	120	120	30	60	120	120
$D_2 \times 10^6$	2	2	2	2	2	2	1	1	5	10
θ_2	60	60	60	60	60	60	30	30	100	120
$n_{T,1}$	18	23	27	32	40	46	30	30	19	19
$n_{T,2}$	20	25	30	36	44	51	30	30	25	19
^2n_T	178	297	416	595	892	1190	468	468	234	187

TABLE II

INFLUENCE OF THE SAMPLE SPOT SIZE ON THE SPOT CAPACITY

$A = 1$; $C = 0.01$; $\gamma = 0.70$; $d_p = 5\ \mu m$; $D_1 = 5 \times 10^{-6}$ cm²/sec; $D_2 = 2 \times 10^{-6}$ cm²/sec; $\theta_1 = 120$ cm/sec; $\theta_2 = 60$ cm/sec. σ_i is the standard deviation of the sample spot, in cm. The theoretical limit, ${}^2n_1$, is 297 (*cf.*, Table I).

L(cm)	$\sigma_i = 0$			$\sigma_i = 0.02$			$\sigma_i = 0.04$			$\sigma_i = 0.06$			$\sigma_i = 0.10$			$\sigma_i = 0.20$		
	n_1'	n_2'	${}^2n$	n_1'	n_2'	${}^2n$	n_1'	n_2'	${}^2n$	n_1'	n_2'	${}^2n$	n_1'	n_2'	${}^2n$	n_1'	n_2'	${}^2n$
1*	10	10	100	7	7	49	5	5	25		–			–			–	
2*	13	12	156	11	11	121	9	9	81	7	7	49	5	5	25		–	
5*	15	15	225	15	15	225	14	14	196	12	12	144	10	10	100		–	
10	16	16	256	16	16	256	16	16	256	15	15	225	14	14	196	10	10	100
15	17	17	289	17	17	289	16	16	256	16	16	256	15	15	225	13	13	169

* $z_0 = 0.2$ cm; otherwise $z_0 = 0.5$ cm.

TABLE III

ANALYSIS TIME (MIN) IN TWO-DIMENSIONAL TLC

$t_A = t_1 + t_2$; $\theta_1 = 120$ cm/sec; $\theta_2 = 60$ cm/sec.

L(cm)	$d_p = 3\ \mu m$			$d_p = 5\ \mu m$			$d_p = 7\ \mu m$			$d_p = 10\ \mu m$			$d_p = 15\ \mu m$			$d_p = 20\ \mu m$		
	t_1	t_2	t_A	t_1	t_2	t_A	t_1	t_2	t_A	t_1	t_2	t_A	t_1	t_2	t_A	t_1	t_2	t_A
1	0.46	0.92	1.40	0.28	0.56	0.83	0.20	0.40	0.60									
2	1.8	3.70	5.6	1.11	2.22	3.33	0.79	1.59	2.38	0.56	1.1	1.7	0.4	0.7	1.1			
4	7	15	22	4.4	9	13.3	3.2	6.3	9.5	2.2	4.4	6.6	1.5	3.0	4.5			
																5 min		
6	17	33	50	10	20	30	7	14	21	5	10	15	3.33	6.66	10	2.5	5	7.5
10	1 h 46	93	139	28	55	83	20	40	60	14	28	42	9	19	28	7	14	21
15	3 h 104	208	312	62	125	187	44.6	89	134	31	62	94	21	42	63	15.6	31	47
20	185	370	555	111	222	333	79	159	238	55	111	167	37	74	111	27.8	55	83
25				173.6	347	521	124	248	372	87	174	260	58	116	174	43	87	130
30							179	357	536	125	250	375	83	167	250	62.5	125	187.5
40													148	296	444	111	222	333
50																174	347	520

a distance z_0 from each side and development is carried out successively in both directions, until the solvent front reaches the opposite edge of the plate in both cases. Only 5-μm particles are considered here, as it has already been shown that the effect of the sample spot size is most important on short plates made from small particles[22].

As expected the spot capacity falls dramatically for sample spot sizes larger than 0.1–0.2 mm with the small plates. With larger plates it becomes easy to achieve half the theoretical spot capacity of the plate with acceptable sample size: with a sample spot diameter of 2 mm and a 5-cm plate it is still possible to resolve 144 spots, close to half the limit of 297 (Table I, column 2). The total development time is only 21 min, to which some time should be added to allow for an intermediate, drying step between the two developments.

It will be possible to achieve more than half the theoretical performance in most cases with quite reasonable specifications, except for small plates, which are very fast to develop but conversely require very small samples[27].

Plates larger than 15 cm have not been considered because of an excessive development time. Development times calculated for a number of combinations of plate size and average particle diameter are reported in Table III. The total analysis time is the sum of the two development times and the time necessary to dry the plate

TABLE IV

INFLUENCE OF z_0 ON THE SPOT CAPACITY

$A = 1$; $C = 0.01$; $\gamma = 0.70$; $D_1 = 5 \times 10^{-6}$ cm^2 sec; $D_2 = 2 \times 10^{-6}$ cm^2 sec; $\theta_1 = 120$ cm sec; $\theta_2 = 60$ cm sec.

L (cm)	d_p (μm)	z_0 (cm)	σ_z (cm)	n'_1	n'_2	^2n	^2n_T
1	5	0.1	0	10	10	100	297
		0.2	0	10	10	100	
		0.5	0	7	7	49	
2	3	0.1	0	12	12	144	178
			0.04	9	9	81	
		0.2	0	12	12	144	
			0.04	8	8	64	
		0.5	0	10	10	100	
			0.04	7	7	49	
	5	0.1	0	13	13	169	297
			0.04	9	9	81	
		0.2	0	13	12	156	
			0.04	9	9	81	
		0.5	0	11	11	121	
			0.04	8	7	56	
	7	0.1	0	12	12	144	416
			0.04	9	9	81	
		0.2	0	12	12	144	
			0.04	9	8	72	
		0.5	0	11	11	121	
			0.04	7	7	49	
4	5	0.1	0	15	15	225	297
		0.2	0	15	15	225	
		0.5	0	15	14	210	

between the two developments. This drying should be made very carefully[18] as the reproducibility of the retention data during the second development is strongly influenced by the presence of minor amounts of the first solvent sorbed on the stationary phase. The last step cannot be undertaken in less than 10 min.

Calculations have been made for combinations of plate length and particle size which result in a total development time not exceeding 10 h. In spite of the work of the pioneers in this field[1–5], it seems that longer times are not realistic and we do not consider further conditions which require development times in excess of a few hours.

Influence of z_0

This influence is particularly significant on small plates, so it has been studied on plates having sides from 1 to 4 cm, made from 3–7 μm particles. The data are reported in Table IV. They show that z_0 has little influence as long as it is less than 20% of the plate side and no influence at all if it is 10% or less. However, with a small plate it is not possible to achieve a large fraction of the theoretical limit. This is discussed in the next section.

In the following we have used values of $z_0 = 0.2$ cm for plates smaller than 5 cm square, and 0.5 cm for larger plates. This is reasonable and meeting these specifications does not seem to raise any significant experimental problem. It is worth noting also that retention data are reproducible only if the migration distance of the solvent front is large compared to z_0, at least three times and preferably ten times larger[18].

Influence of plate length and particle size

These are the most important characteristics of a plate, together with the homogeneity of the packing which is considered in the next section. Performances have been calculated for various combinations of plate length and particle size and the results are reported in Table V, together with the theoretical maximum spot capacity as calculated by eqn. 20. The original spot size used ($\sigma_i = 0.4$ mm), although quite realistic for most TLC applications, may appear somewhat large in view of the progress which may be expected in the near future. The data in Table II show that with such a spot size there is a marked decrease in the performances of short plates. To allow further comparison, other data are given in Table VI, calculated for a much smaller sample size, close to the technical minimum with present technology ($\sigma_i = 0.1$ mm). The results in Table VI agree with those in Table II showing that the sample spot size has a significant effect only for plates smaller than 5 cm. For 2-cm plates, for example, the improvement obtained with a four-fold decrease in sample spot size is very important.

For plates made from small particles it does not seem too difficult to reach a spot capacity close to the theoretical limit within an acceptable analysis time. Analysis times are given in Table III and calculations have been carried out only for combinations of L and d_p which lead to analysis times shorter than about 3 h, already a long time by present day standards. In 2 h and 20 min it is possible to achieve 98% of the maximum spot capacity using a 10 cm long plate made from 3-μm particles if the sample spot standard deviation is 0.4 mm, while the same performance is achieved within 30 min with $\sigma_i = 0.1$ mm. In a similar time, only about half of the theoretical spot capacity is achieved with a 30 cm long plate made from 20-μm particles.

TABLE V

INFLUENCE OF PLATE DIMENSIONS AND PARTICLE SIZE ON SPOT CAPACITY

$A = 1$; $C = 0.01$; $\gamma = 0.70$; $D_1 = 5 \times 10^{-6}$ cm^2/sec; $D_2 = 2 \times 10^{-6}$ cm^2/sec; $\theta_1 = 120$ cm/sec; $\theta_2 = 60$ cm/sec; $\sigma_t = 0.04$ cm; $z_0 = 0.2$ cm for $L < 5$ cm, 0.5 cm for $L > 5$ cm.

L(cm)	$d_p = 3\ \mu m$			$d_p = 5\ \mu m$			$d_p = 7\ \mu m$			$d_p = 10\ \mu m$			$d_p = 15\ \mu m$			$d_p = 20\ \mu m$		
	n_1'	n_2'	^2n	n_1'	n_2'	^2n	n_1'	n_2'	^2n	n_1'	n_2'	^2n	n_1'	n_2'	^2n	n_1'	n_2'	^2n
1	5	5	25	5	5	25												
2	8	8	64	9	9	81	9	8	72									
3	10	10	100	12	12	144	11	11	121	11	10	110	9	9	81			
4	12	12	144	14	14	196	14	14	196	14	14	196	13	12	156	11	11	121
5	12	12	144	15	15	225	16	16	256	16	16	256	15	15	225	13	13	169
7	13	13	169	16	16	256	17	17	289	18	18	324	18	17	306	16	16	256
10				16	16	256	19	19	361	20	20	400	21	20	420	19	19	361
15										21	21	441	22	22	484	22	21	462
20																25	25	625
30																		
$^2n_\Gamma$		172			297			416			595			892			1190	

TABLE VI

INFLUENCE OF PLATE DIMENSIONS AND PARTICLE SIZE ON SPOT CAPACITY

As for Table V except $\sigma_i = 0.01$ cm.

L(cm)	$d_p = 3\ \mu m$			$d_p = 5\ \mu m$			$d_p = 7\ \mu m$			$d_p = 10\ \mu m$		
	n_1'	n_2'	^2n	n_1'	n_2'	^2n	n_1'	n_2'	^2n	n_1'	n_2'	^2n
2	11	11	121	12	12	144	12	12	144	11	10	110
3	12	12	144	14	14	196	14	14	196	13	13	169
5	13	13	169	15	15	225	16	16	256	16	15	240
7	13	13	169	15	15	225	17	17	289	17	17	289
^2n_T		172			297			416			595	

Although the theoretical spot capacity is much larger, development is much slower and the time required to reach 90% of the spot capacity would be prohibitively long.

As in conventional TLC, the spot capacity increases monotonously towards the theoretical limit (eqn. 20) with increasing development length, while at constant length there is an optimum particle size (*cf.*, Fig. 2). For smaller particle sizes the spot capacity decreases with decreasing d_p because the development is too slow and diffusion becomes more and more important, while for larger particle sizes the spot capacity decreases with increasing particle size because of increasing flow velocity and band broadening due to packing heterogeneity. Nevertheless, the large spot capacities which can be achieved in rather moderate analysis times are striking. They are com-

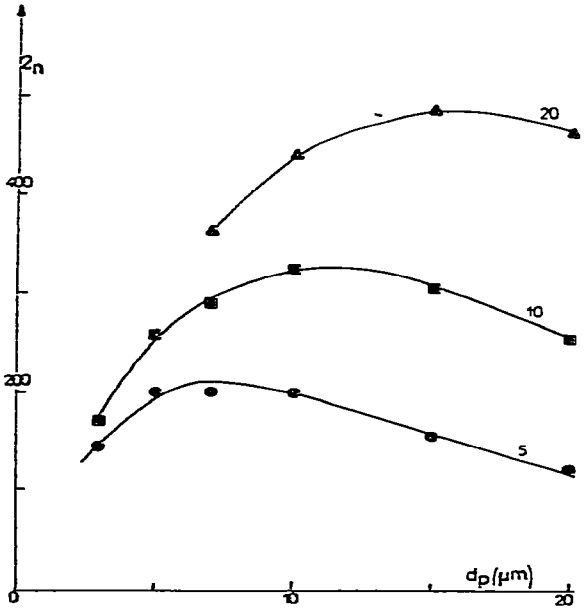

Fig. 2. Plot of the spot capacity in two-dimensional TLC *versus* the particle size for three different development lengths as indicated on the corresponding curves (L in cm). Conditions as in Table V.

TABLE VII

INFLUENCE OF THE DIFFUSION COEFFICIENT AND KINETIC PARAMETER ON THE SPOT CAPACITY

$A = 1$; $C = 0.01$; $\gamma = 0.70$; $L = 10$ cm; $d_p = 10$ μm; $z_0 = 0.5$ cm; $\sigma_i = 0.04$ cm.

$D_1 \times 10^6$ (cm² sec)	θ_1* (cm sec)	$D_2 \times 10^6$ (cm²/sec)	θ_2* (cm/sec)	n'_1	n'_2	^2n	^2n_T**
2	60	2	60	19	19	361	669
2	120	2	60	19	20	380	892
2	120	2	120	19	19	361	1339
5	120	2	60	18	18	324	595
–	–	2	120	19	18	342	765
–	–	5	20	11	12	132	153
–	–	5	60	15	16	240	357
–	–	5	100	17	17	289	487
–		5	120	18	18	324	535
–	–	5	140	18	18	324	576
–	–	7	60	14	15	210	281
–	–	7	120	17	17	289	446
–	–	10	60	13	13	169	214
–	–	10	120	15	16	240	357
10	60	10	60	11	11	121	133
10	120	10	60	12	12	144	178
10	120	10	120	14	14	196	267

* Development time: 14 min for $\theta = 120$ cm/sec, 28 min for $\theta = 60$ cm/sec.
** From eqn. 20.

parable to or larger than the peak capacities which can be obtained with the best columns available in high-performance liquid chromatography (HPLC).

A peak capacity of 100 requires a 40,000-theoretical plate column, which is more than most HPLC columns can produce: it requires at least a 40 cm long column packed with 5-μm particles and the analysis time at $r = 3$ would be 2 h 45 min for $k' = 6.4$. This is certainly possible to achieve with current technology, but it becomes increasingly difficult to do better, while spot capacities in the range 200–300 and more do not seem terribly difficult to achieve in TLC (Tables III, V and VI). Fairly large values of the sample spot standard deviation can be tolerated for practical applications, and dilution does not greatly exceed one order of magnitude, which still permits sensitive detection. A peak capacity of 300 requires a 360,000-plate column, which is more than half the world record[28] and more than almost anybody has yet been able to achieve. Nevertheless, data from Tables III and V show that it can be achieved in two-dimensional TLC in an hour or so. For example, a 10-cm square plate coated with a layer of 10-μm particles has a spot capacity of 324 with $\sigma_i = 0.4$ mm and its two developments take a total of 42 min. The ultimate performance achievable in two-dimensional TLC, in terms of spot capacity, is of the order of 500, which exceeds that which can be obtained in column chromatography with reasonable experimental conditions[29].

Finally it should be noted that the procedure of calculation resulting from the application of the law of variance addition ensures that the spot capacity is independent of the order in which the two developments are carried out. We also

TABLE VIII

INFLUENCE OF PLATE CHARACTERISTICS ON SPOT CAPACITY

$D_1 = 5 \times 10^{-6}$ cm^2/sec; $D_2 = 2 \times 10^{-6}$ cm^2/sec; $\theta_1 = 120$ cm sec; $\theta_2 = 60$ cm sec; $L = 10$ cm; $d_p = 10$ μm; $\sigma_t = 0.04$ cm; $\gamma = 0.70$; $^2n_T = 595$.

A	C	n'_1	n'_2	^2n
3	0.01	15	15	225
1	0.01	18	18	324
0.8	0.01	19	19	361
0.5	0.01	20	20	400
1	0.03	18	18	324
1	0.10	18	17	306
1	0.30	17	16	272

observe from Tables V and VI that with the solvent characteristics chosen (D_m, θ) the spot capacities in the two directions are almost always identical. This would not be true for more dissimilar solvents, but this is an improbable situation.

Influence of the solvent characteristics

There are two important parameters which depend on the solvent used: the diffusion coefficient, which for a given solute can vary by a factor of 2 to 5, and the kinetic coefficient which is usually between 60 and 120 and can vary between 20 and 140 at most[24]. Calculations have been made using different set of values for both solvents and are reported in Table VII. We have chosen a plate with good potential performance for these calculations, a 10 × 10 cm square coated with 10-μm particles.

The spot capacity which can be achieved in a reasonable time (total development time about 45 min) increases markedly with decreasing diffusion coefficient in and increasing velocity coefficient of the two solvents. TLC is not well suited to the analysis of low-molecular-weight compounds because the average reduced velocity during a development carried out under the usual conditions is too low and spot broadening by molecular diffusion is too important.

We observe also that the performances achieved with the plate considered are markedly lower than the theoretical performances and increase much more slowly. In fact it is extremely difficult to find conditions in which the spot capacity would reach 400 without drastic requirements, especially regarding analysis time.

Influence of the plate characteristics

Besides the plate dimensions and the particle size already discussed, other characteristics to be considered are the coefficients of the theoretical plate height equation (eqn. 5), the bed tortuosity, γ, the packing homogeneity coefficient, A, and the coefficient of resistance to mass transfer, C.

There is little one can do about γ. The axial diffusion term has not been studied intensively since the classical work by Knox[30]. Recent data by Theumneum and Hawkes[31] show that in gas chromatography it is not constant but increases slightly with increasing gas velocity. Whether the same is true in liquid chromatography and to what extent is still unknown. In all our calculations γ is taken as constant and equal to 0.7. Significant changes of γ, however, much larger than the range of variations

reported by Hawkes, would be required to affect markedly the spot capacity. For example, under the conditions given in Table VIII, for $A = 1$ and $C = 0.01$, $n'_1 = 16$ for $\gamma = 0.50$, 18 for $\gamma = 0.70$ and 20 for $\gamma = 1.0$. In practice we can consider that γ is between 0.65 and 0.75 which leads to a value of n'_1 of either 19 or 18, hardly a significant variation.

The influence of A and C has been studied and results are reported in Table VIII. The influence of A has been studied in the range from a value of 0.5 which corresponds to an extremely homogeneous bed to one of 3 which corresponds to a fairly poor bed. In column chromatography it is very difficult to achieve values of A less than 1, but making an homogeneous thin packing as in TLC seems an easier task and values of A smaller than 1 have been obtained for commercial plates[24]. Reducing A seems to be the easiest way to improve the plate performance, since this does not change the analysis time nor does it require any adjustment of the chromatographic system properties. However, there is as yet little information on how to do it, and the improvement, although major when performances of plates of high and low packing

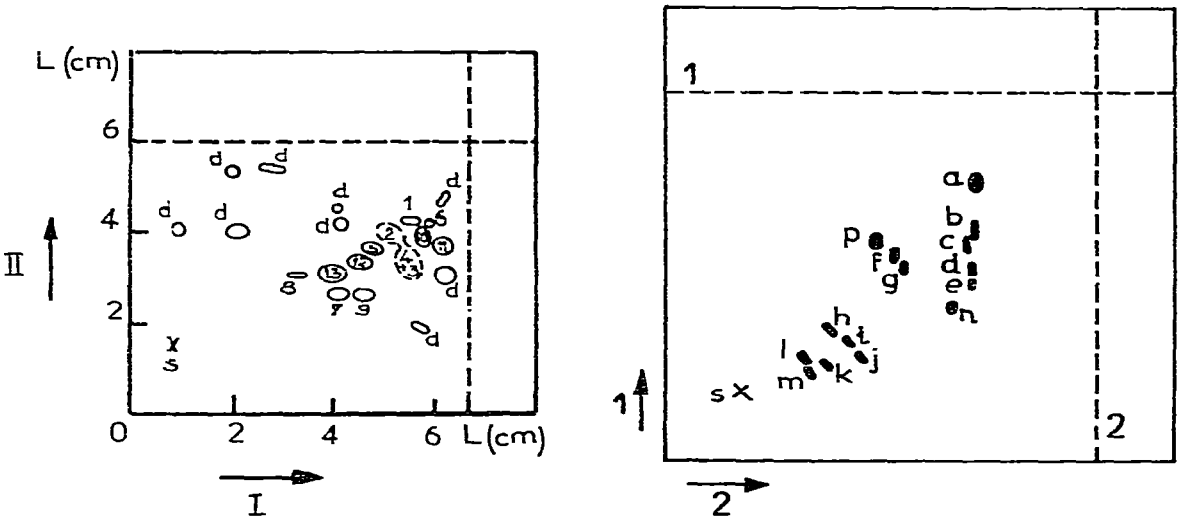

Fig. 3. Separation of azaarenes by two-dimensional TLC. Stationary phase: RP-18 (Merck). Development I: ethanol–water (20:1). After drying the plate is briefly dipped in a solution of ethanol–water–ammonia – 1 *M* $Cu(NO_3)_2$ (13:1.6) carefully avoiding wetting the strip where are the compounds separated by the first development. Development II: after drying, ethanol–water + 1 *M* $Cu(NO_3)_2$ (15:2). Plate size: 10 × 10 cm. Spots: 1 = indenopyridine; 2 = benzo-5,6-quinoline; 3 = benzo-3,4-quinoline; 4 = benzo-7,8-quinoline; 5 = 7-azafluoranthene; 6 = 2-tolyl-3-methylquinoline; 7 = 9-methylbenzo-5,6-acridine; 8 = 5-ethyl-9-methylbenzo-1,2-actidine; 9 = 2,2'-biquinoline; 10 = acridine; 11 = phenazine; 12 = benz[*a*]acridine; 13 = 4-azapyrene; d = unknown; s = sample.

Fig. 4. Separation of a mixture of nucleic acid components by two-dimensional TLC. Stationary phase: silica gel $60F_{254}$, 5-μm particles. Plate size: 10 × 10 cm. No activation prior to analysis. Development I: 1-butanol–acetic acid–water (12:3:5); drying for 5 min at 110°C and 2 h at ambient temperature. Development II: 1-propanol–ammonia–water (50:5:10). Spots: a = thymidine; b = adenosine; c = hypoxanthine; d = guanine; e = cytosine; f = xanthosine; g = guanosine; h = 5'-thymidine monophosphate; i = 5'-uridine monophosphate; j = 5'-adenosine monophosphate; k = 5'-inosine monophosphate; l = 5'-cytidine monophosphate; m = 5'-guanosine monophosphate; n = cyclic adenosine monophosphate; p = uridine; s = sample. The mechanisms of these separations will be discussed elsewhere.

qualities are compared, is not very important for values of A lower than 1. This is because during a TLC development the solvent velocity is so low most of the time that the contribution to spot broadening of the A term of the plate height equation is minor[23].

A marked reduction of this contribution is accordingly not very significant, even so the conditions selected for Table VII are such that the first term of the plate height equation (axial diffusion) is not as predominant as it is in many TLC analyses.

All this discussion applies as well to the influence of the parameter C. The third term of the plate height equation usually gives a very minor contribution to band broadening in TLC[23]. A thirty-fold increase of C only reduces n'_1 by 1, which is hardly significant. Any kind of packing material which gives fair results in column chromatography, as far as resistance to mass transfer is concerned, will be useful in TLC and will not contribute significantly to band broadening.

Comparison with experimental data

Few quantitative data are available in two-dimensional TLC. This technique is hardly amenable to scanning because of the difficulty in localizing the exact centre of a spot and the fact that a number of parallel profiles (about 40–50) should be obtained for each spot. We have attempted such scans on spots obtained on various plates, but it takes a very long time to scan a small part of a plate and it was not possible to achieve illustrative results; so we show two chromatograms in a conventional way, the spots being drawn as the contours of the luminous spots seen when the developed plate is placed under an UV lamp.

Fig. 3 shows the separation of thirteen different azaarenes and nine unidentified impurities, probably other azaarenes. The separation compares favourably to those obtained by Engel and Sawicki[32]. From the measurements of the spot dimensions in the x and y directions it appears that the spot capacities in these two directions are 12 and 15 respectively, hence the total spot capacity of the plate is 180. Theory predicts 19 for one single TLC development[22], 14 for each development in two-dimensional TLC and a total of 196 (*cf.*, Table V). The agreement is excellent. It will be noted, however, that the spot capacity is markedly larger in the direction of the second development. This results from the concentration effect at the beginning of the second development as the lower edge of the spots starts moving upward before the upper end. Account of this effect could be taken by multiplying the second term of the right-hand side of eqn. 17 by R_F.

A similar effect is observed in Fig. 4 which shows a separation of fifteen nucleic acid components. Although, as in the chromatogram of Fig. 3, the same adsorbent is used with two different chromatographic systems, the spots are much narrower in the second direction (16.6×10^{-3} R_F instead of 31×10^{-3} R_F). Accordingly the spot capacities along the two directions are 60 and 31 respectively, with a total two-dimensional TLC capacity of 1860, whereas theory would predict about only 320, because of the low values of the diffusion coefficients. Part of the considerable difference probably results from the low sensitivity of the detection and the necessity to draw spot shapes in dim light. In such a case there can be little relationship between spot width and zone standard deviation[6]. Nevertheless, chromatograms such as this one attest to the power of the technique.

TABLE IX

COMPARISON BETWEEN ANALYSIS TIMES IN TLC, TWO-DIMENSIONAL TLC AND COLUMN CHROMATOGRAPHY

Conditions for all systems: $A = 1$; $C = 0.01$; $\gamma = 0.70$; $D_{m,1} = 5 \times 10^{-6}$ cm²/sec.

Spot or peak capacity required, n	*Conditions in TLC**			*Conditions in two-dimensional TLC***			*Conditions in column chromatography****			
	L(cm)	d_p*(μm)*	t_A*(min)*	*L(cm)*	d_p*(μm)*	t_A*(min)*	*L(cm)*	d_p*(μm)*	*ΔP(atm)*	t_A*(min)*
10	3	5	2.5	Practically impossible because it is *too easy*			Practically impossible because it is *too easy*			
15	5	7	5							
20	10	12	12							
30	30	20	62				4.9	5	18	4.0
50	Practically impossible because it is *too difficult*						13.6	5	49	11.1
100				3.5	5	10	54	5	195	45
150	–			4.5	7	12	122	5	439	100
							73	3	1220	36
200	–			6	10	15	217	5	780	178
300	–			10	15	28	Practically impossible because it is *too difficult*			
500	–			22.5	20	105				

* $z_0 = 0.2$ cm for $L < 5$ cm; $\theta_1 = 120$ cm/sec; $\sigma_i = 0.5$ mm. Analysis time = development time.

** $\theta_1 = 120$ cm/sec; $\theta_2 = 60$ cm/sec; $D_2 = 2 \times 10^{-6}$ cm²/sec; $\sigma_i = 0.5$ mm. Analysis time = sum of the two development times.

*** $v_0 = 2.8$; $h = 1.94$; $\eta = 0.6$ cP; $k_0 = 1 \times 10^{-4}$. Analysis time = elution time for $k' = 6.4$.

CONCLUSIONS

Whereas TLC offers a resolution power quite lower than column chromatography, with an analysis time which increases much faster than the necessary plate number, in contrast to what happens in column chromatography[21], the situation in two-dimensional TLC is quite different (*cf.*, Table IX). The resolution power available is much larger than anything attainable in column chromatography and the analysis time remains quite reasonable, although again it increases rapidly with increasing spot capacity. This makes two-dimensional TLC very attractive in principle for the separation of complex mixtures, much more powerful, in theory at least, than column chromatography (*cf.*, Table IX).

However, two major practical problems remain to be solved, one of which seems to be much more difficult than the other one, as discussed in the Introduction.

First, whereas in TLC or column chromatography only one retention mechanism, or chromatographic system, has to be selected, in two-dimensional TLC we need two such mechanisms or systems which are compatible and which are independent or orthogonal, *i.e.*, there should be little correlation between the retention patterns in both systems, otherwise the spots tend to agglomerate along the bisector of the plate and the spot capacity is merely multiplied by $\sqrt{2}$. True, neither system needs to separate all the constituents of the mixture, but the interferences must be different with the two systems. Thus the spots corresponding to the different components will be spread over the entire plate and advantage can be taken of the large spot capacity. Advances in the understanding of retention mechanisms and of the physico-chemical basis of selectivity in column chromatography could certainly be used to select such combinations of mechanisms as normal phase LC, reversed-phase LC, size exclusion LC, affinity chromatography, etc. Nevertheless two-dimensional TLC has been used with success in the past as explained in the Introduction and continues to be applied[1–5,7–18]. There are thus many ways to solve this difficult problem.

However, data acquisition remains the real bottleneck of the technique. Neither spectrophotodensitometers, definitively too slow for this application, nor Vidicon cameras, which lack the optical resolution, offer even the hope of a satisfactory solution. Our calculations have shown that two-dimensional TLC offers spot capacities between 100 and 400 which are easy to achieve with current equipment. Only the use of diode arrays could be helpful in this situation, or advanced image analyzers[33].

Thus, our calculations demonstrated that whereas two-dimensional TLC offers an extremely high resolution power, it also presents a great challenge to the equipment designer and will certainly require a sophisticated and expensive system for data acquisition and handling.

It seems to us that, in the quest for an extremely high resolution power, a chromatographic system simpler than a multi-million-plate column[28,29] but less crude than a TLC system[1] should be used. There seems to be a way to combine the resolution power of two-dimensional TLC and the flexibility and efficiency of column chromatography[19,34,35].

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