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QUANTITATION IN HIGH-PERFORMANCE MICRO-THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Silica gel 60 F_{254} pre-coated plates for nanogram-scale thin-layer chromatography were developed for high-performance thin-layer chromatography (HPTLC) on the basis of silica gel of pore size 60 Å. Under the usual chromatographic conditions, it was possible to achieve a plate height of 12 μm. The application and evaluation techniques were matched to the reduced scale and the higher performance of the HPTLC plate, so that in the visible and UV spectral ranges absorbing substances were determined quantitatively between 100 pg and 100 ng and substances that can be excited to produce fluorescence were determined between 10 pg and 100 ng. The standard deviations for the individual values were between 1 and 10%, depending on concentration. The regression lines mainly passed through the origin and exhibited correlation coefficients between 0.997 and 0.9999. It was ascertained that, for a resolution $R_s > 1.5$, measurement of the chromatograms at right-angles to the solvent flow results in considerable advantages such as increased sensitivity of detection by optimization of the wavelength, shorter measuring times, more measurement data and higher statistical certainty of the results.

INTRODUCTION

Quantitative determinations of chemical substances on thin-layer plates have been carried out for more than 10 years[1–3]. In the mid-1960s, chromatogram spectrophotometers were brought on to the market that showed great technical improvements and with their aid it was possible to carry out quantitative determinations directly on the thin-layer plate of substances that absorb light of wavelength approximately 200–800 nm in the visible and UV spectral ranges or that emit visible light on excitation with UV light. For the possibilities of quantitative evaluation, see refs. 13–15, 20, 21, 23–25.

Optimization of the procedure for substance application, the development of the plate and subsequent optical evaluation finally led to the detection of absorbing substances of average absorptivity at levels down to 100 ng and for fluorescing substances down to about 10 ng, a relative standard deviation of $S_{rel} = 2–10\%$ being achieved[4–7]. Some inadequacies of the method could be improved by adopting special procedures. Variations in the thickness of the layer on the plate affect the quantitative result, particularly with transmittance measurements[4,8,9].

Of the two procedures available for measurements in the visible range, *i.e.*, transmittance and reflectance, it is reflectance that is preferred, or a combination of both (simultaneous measurement)[10–12].

Because of the absorption edge of the glass plate, the only method available for the UV region is that of reflectance. A further possibility for reducing errors resulting from the layer thickness is afforded by the double-beam process at two loci and the double wavelength process at one locus. It can be shown, however, that irregularities in the layer thickness on a good plate need to be considered only at distances in excess of several centimetres. Reducing the scale of thin-layer chromatography (TLC) should therefore lead to improvements, with the advantages of a considerable reduction in running time at almost the same velocity coefficient of the mobile phase, lower diffusion of the substances, smaller spots and thus higher sensitivity of detection and a linear dependence of the measuring signal on concentration. The measuring area could be reduced and the signal-to-noise ratio, or at least the noise component caused by the thin layer, improved. In the linear range of fluorescence and reflectance, the latter corrected by the Kubelka-Munk function[16],

$$f(R) = \frac{(1 - R)^2}{2R}$$

where R = degree of relative reflectance, detection at levels below 100 ng could be achieved.

When attempting to scale down TLC, the separating properties of the thin-layer plate have to be improved and the quantitative determination of substance mixtures is possible only at adequate resolution. The resolution, R_s, is inversely proportional to the square root of the height equivalent to a theoretical plate, H:

$$R_s \approx \frac{1}{w} \approx \frac{1}{\sqrt{H}}$$

where

$$R_s = 2\left(\frac{z_{x2} - z_{x1}}{w_1 + w_2}\right)$$

and

$$H = \frac{w^2}{16\, z_x}$$

It was first necessary to optimise the highly time-consuming and inaccurate determination of H. The analogue output of a chromatogram spectrophotometer (Zeiss PMQ II) was linked to a process control computer (IBM 1800), which is capable of recording the photometer signal directly or via a TE converter and correcting this as required by using the Kubelka–Munk function.

The first and second derivatives for each point on the signal curve are calculated by approximation (Fig. 1). The points at which the second derivative crosses the x-axis indicate the points of inflection of the sections of the curve on either side of the peak. The maximum and minimum for the first derivative and the signal height at the points of inflection show the positions of the tangents through the points of inflection.

The position of the peak maximum with respect to time, in other words the migration distance, z_x, of a spot, is determined by the point at which the first derivative

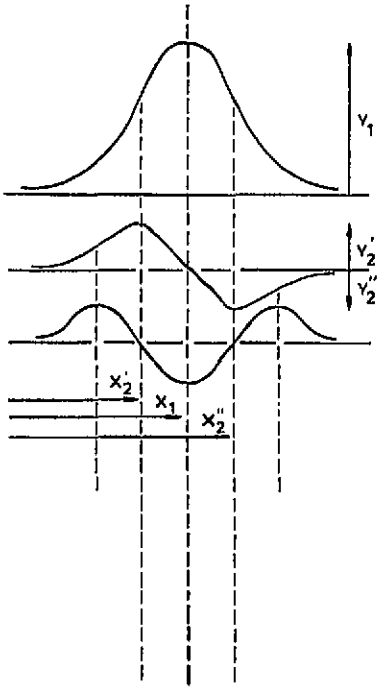


Fig. 1. Original signal and first and second derivatives of a chromatogram peak. X_1 is the point at which the first derivative crosses the x-axis and corresponds to the peak maximum with respect to time of the original curve. X_2' and X_2'' are the points at which the second derivative crosses the x-axis and correspond to the points of inflection. Y_2' and Y_2'' are the maximum and minimum values of the first derivative and correspond to the gradient of the tangents through the points of inflection.

crosses the x-axis. The base-line is determined according to customary chromatographic criteria. The peak width, w, is given by the difference with respect to time between the two points of intersection of the tangents through the points of inflection with the baseline. H is then calculated in micrometres from the equation

$$H = \frac{w^2}{16\, z_x}$$

It thus became possible to determine H for almost any number of spots on a chromatogram in less than 2 min. The standard deviation for this determination of H of 10 chromatographic bands on one plate is better than $S_{rel} = \pm 1.5\%$. The corresponding manual evaluation would take many hours and would yield a standard deviation of $S_{rel} > 10\%$.

This equipment made it possible for us to include the determination of the separating efficiency in the form of H values and other parameters such as R_s, R_F values, k' values and velocity coefficients (κ) in the assessment of the quality of separated substances both during the development of these high-performance pre-coated plates and also in the quality control of the conventional pre-coated plates. (The same programme can be applied in modified form in the quality control of supports and adsorbents for gas chromatography and high-performance liquid chromatography.)

SPECIFICATION

	TFO =	10.000 SEC				
	TF =	210.000 SEC				
TOTAL MIGRATION TIME	T =	220.000 SEC				
TRANSPORT VELOCITY	U =	0.125 MM/SEC				
MIGRATION DISTANCE	ZF =	30.663 MM				
TOTAL DISTANCE	Z =	35.663 MM				
VELOCITY COEFFICIENT	KAPPA =	5.781 MM**2/SEC				
	PEAK NO.:	2	3	4	5	6
HRF-VALUE	HRF =	11.526	16.375	35.294	43.799	61.605
PEAK-BASE WIDTH IN MM	W =	1.738	1.681	2.059	2.135	2.167
NUMBER OF PLATES	N =	573.74	871.09	1252.06	1444.46	1971.86
NUMBER OF PLATES/SEC	NS =	2.607	3.959	5.691	6.565	8.963
PLATE HEIGHT IN µM	H =	53.444	35.201	24.490	21.228	15.550
CORR. PLATE HEIGHT IN µM	H50 =	12.320	11.528	17.287	18.596	19.160
RESOLUTION	R =		0.869	3.101	1.243	2.537

Fig. 2. Computer print-out.

Silica gel 60 F_{254} pre-coated plates for nanogram-scale TLC specially developed for high-performance TLC (HPTLC) are optimal as regards particle-size range, layer thickness, solvent running distance and amount of substance applied. The silica gel type is kept constant, as are the binder and indicator additives, the suspending process, the coating process, the lipophilic and hydrophilic chromatography systems, the chamber type, the pre-saturation, the temperature and the applicator system. The resulting data, compiled in the form of a computer print-out (Fig. 2), made it possible either to achieve or to confirm the following results.

(1) The optimum separation is achieved at various running distances depending on the particle size range of the adsorbent. This optimum separation occurs at small running distances for small particles and higher running distances for larger particles.

(2) The plate height and thus the resolution are highly influenced by the amount of substance applied; the smaller the amount applied, the more favourable is the resolution.

(3) Very thin layers do not lead to an improvement but, in fact, to a considerable deterioration of the separation.

On the basis of these criteria, it was possible, by using sophisticated technology and exacting control analysis, to prepare the silica gel pre-coated HPTLC plate for nanogram-scale TLC on the basis of silica gel of pore size 60 Å as used with conventional pre-coated TLC plates[22]. Whereas the most favourable separations are achieved on a conventional TLC plate at a running distance of about 100 mm, the smallest plate heights are achieved on the HPTLC plates at running distances of 30–60 mm.

The velocity coefficients of the high-performance layer are about one third less than those on conventional TLC layers. Nevertheless, considerably shorter running times are achieved with the optimum running distance of the HPTLC plates.

Table I illustrates the separating power of the silica gel 60 F_{254} pre-coated HPTLC plate for amounts of lipophilic dyestuffs of 100 ng in 100 nl or of 20 ng in 20 nl when chromatographed in a normal chamber with chamber saturation, using benzene as the eluent.

TABLE I

SEPARATION OF DYESTUFFS ON SILICA GEL 60 F_{254} PRE-COATED HPTLC PLATES

Solvent migration distance, z_f (mm)	*Amount applied (ng)*	*Mean of the plate height H (μm) referred to $hR_F = 50$*	*Number of plates (N') available for TLC assuming a max. hR_F of 80*
20	100	24	665
	20	17	940
30	100	19	1265
	20	13	1845
40	100	18	1780
	20	12	2665
50	100	17	2355
	20	13	3075
60	100	18	2665
	20	14	3430

SAMPLE APPLICATION

Whereas the application of substances in amounts up to about 0.1 μl can still be carried out by using a simple applicator such as that supplied by Merck (Darmstadt, G.F.R.; Cat. No. 10226) with suitable microcapillaries (such as those supplied by Karl Hilgenberg, Glaswarenfabrik, Malsfeld, G.F.R.), the device that has proved best for the application of even smaller amounts (down to about 5 nl) is the Hamilton Type 7001 N 1-μl syringe, head type 3, canula length 70 mm, in conjunction with an Agla micrometer (Shardlow Micrometer, Sheffield, Great Britain) (Fig. 3). With slight pressure on the plate, the required amount of sample is applied with a slightly bent canula to a high degree of accuracy. With a little practice, about 10 spots per minute can be applied for quantitative examination. Automatic applicators have proved best for streak-wise application. Using such a TLC applicator (supplied by ICN, Eschwege, G.F.R.) it was possible to apply a 1-μl volume evenly over a line 80 mm long.

The application of such small amounts of substances produces spots that are less than 1 mm in diameter prior to development and, depending on the running distance, about 2–3 mm in diameter after development.

EVALUATION PROCEDURES

Using a few substance groups as examples, we shall illustrate the methods of measuring reflectance in the visible and UV spectral ranges and fluorescence, together with their optimization and limitations.

Wavelength optimization

The quantitative evaluation of the components of a mixture of substances according to the order of their k' values is a familiar method and is essential in elution chromatographic methods such as gas and liquid chromatography. Even in TLC it is always advisable to evaluate in the direction of the solvent flow when closely neighbouring or non-resolved substances also exhibit similar absorption or fluorescence

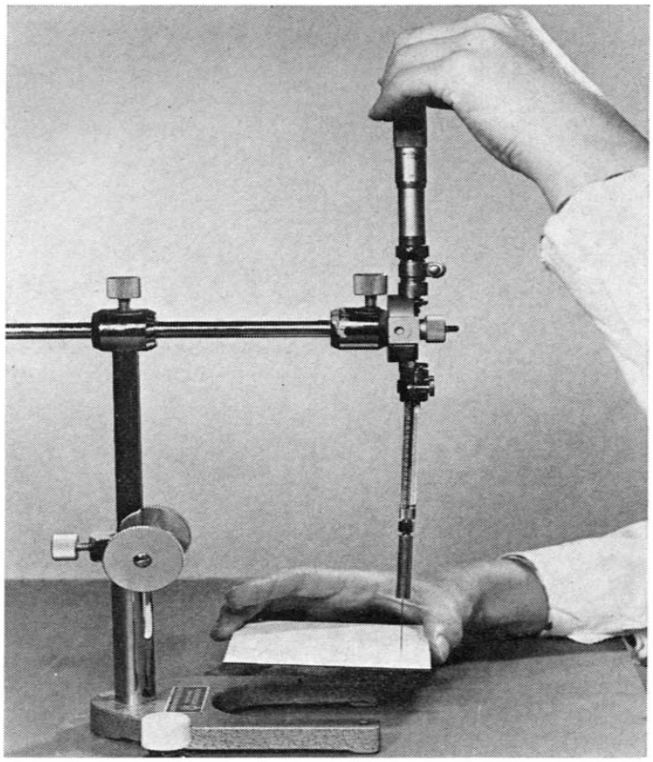


Fig. 3. Device for applying volumes at the nanolitre level.

maxima. If the resolution between the separated substances is sufficiently large, *i.e.*, $R_s > 1.5$, which should be achievable with an efficient TLC plate, then measurement at right-angles to the solvent flow has some advantages.

In order to demonstrate a possible optimization of the wavelength, a mixture of seven lipophilic dyestuffs was separated on an HPTLC plate (Fig. 4).

In many instances, a substance exhibits its absorption maximum at a particular wavelength at which the other substances do not absorb. For measurements in the direction of the solvent flow, it is always necessary to achieve a compromise, because for technical reasons connected with the equipment it is not possible to change the wavelength during the measurement.

In measurements at right-angles to the solvent flow, the optimum wavelength is set for each substance and thus maximum sensitivity is achieved (Fig. 5).

The second advantage is the possibility of referring to the background reflectance, R_0, in the immediate vicinity of the spot at one point that is guaranteed to be free of substance as it lies next to the chromatography band. This is extremely important for the accurate determination of the base-line. The only other method available for solving this problem is the double-beam procedure in the direction of the solvent flow, but without optimization of the wavelength.

The third advantage is that of the measuring speed. Up to 35 bands can be measured on a 10-cm wide plate with only one equipment setting (see Fig. 5). Most

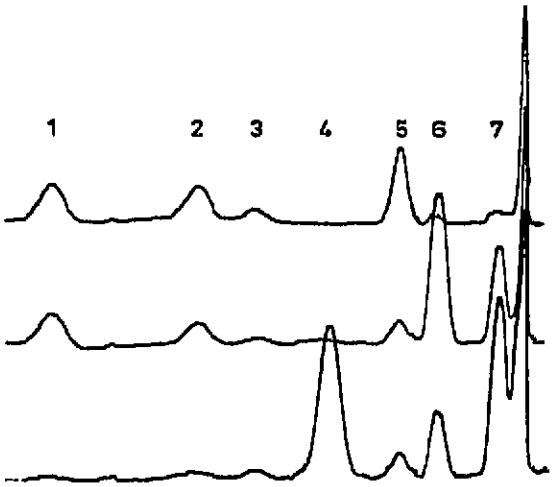


Fig. 4. Mixture of seven lipophilic dyestuffs (10 ng each) developed with benzene and measured a 420 nm (lower curve), 500 nm (centre curve) and 580 nm (upper curve). 1, Ceresviolett BRN; 2, Ceresschwarz G; 3, accompanying substance with 2; 4, Fettgelb 3G; 5, Bleu VIF Organol; 6, Ceres rot G; 7, Ceresbraun BRN.

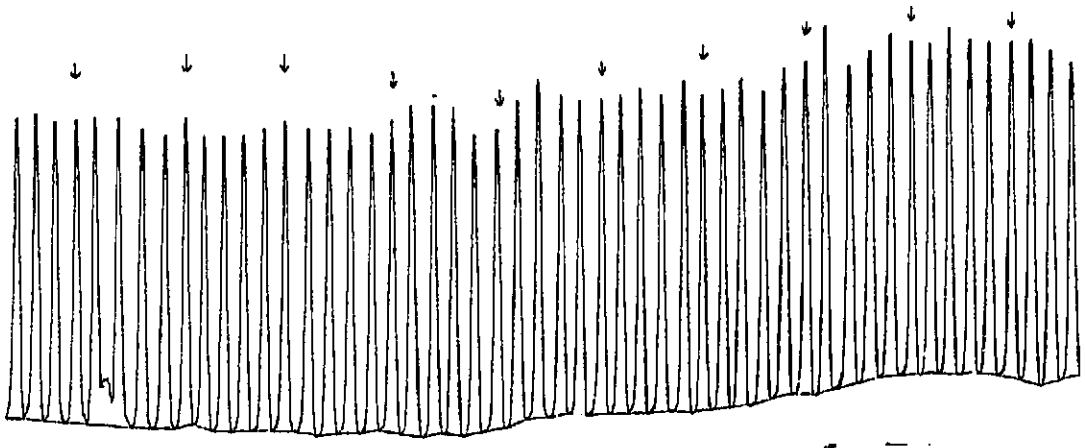


Fig. 5. Section from the measurement of blue dyestuff at right-angles to the solvent flow across about 70 chromatography bands. Distance separating bands = 2.5 mm. Measuring conditions: λ = 420 nm; slit area, 1.8 × 0.7 mm; measuring rate, 30 mm/min.

of the chromatograms on one plate of 20-cm width encompassed 70 bands. The band separation distance was 2.5 mm.

At a migration distance of 30 mm, the spot diameters are so small that it is only just possible to measure the background reflectance, R_0. The minimum separation distance of the bands is determined by the properties of the substance and the eluent and by the migration distance. For a migration distance of 50 mm, the recommended separation distance between bands safely to exclude the possibility of overlapping is 5 mm.

The time required for a measurement at right-angles to the solvent flow is the more favourable, compared with measurement in the direction of the solvent flow, the more bands there are on the plate.

Internal standard

The method used to eliminate dosing errors involves the use of an internal standard, in this instance, red dyestuff. Dividing the integral of the standard by the integral of the corresponding dyestuff peak gives the factor, f, that is equivalent to the amount present of the dye in question.

The computer print-out of a statistics programme (Fig. 6) gives individual values, Nalimov runaways, mean, standard deviations and confidence limit (standard deviation of the mean), in this case for the substance-specific factor of the blue dyestuff, for which the standard deviation covering all individual data, without runaways, is 3.5%.

DATE 2.5.75

TIME 19.47

MEASURED VALUES (** = NALIMOV RUNAWAY)

LIPOPH. 7TH BLUE 580 NM RESP. RED

1.089	1.051	1.045	1.041	1.137
1.108	1.052	1.091	1.109	1.104
1.056	1.128	1.134	1.159	1.125
1.132	1.102	1.093	1.144	1.126
1.131	1.120	1.146	1.151	1.175
1.226**	1.169	1.170	1.207**	1.100
1.073	1.078	1.022**	1.014**	

RUNAWAYS (P = 95) 11. %

RESULTS = MEAN +/− S (+/−T;P%;N) (S_{rel})

1.1112 +/− 0.0386 (+/− 0.0791;95; 30) (3.48%)

Fig. 6. Computer print-out of statistics programme. S = Absolute standard deviation; S_{rel} = relative standard deviation; I = confidence limit; P = confidence interval in per cents; and N = number of measured values without significant runaways.

The error is reduced considerably by averaging the results of five adjacent bands (Fig. 7), which should suffice to produce a reliable result for the determination of the concentration. For the blue and green dyestuffs, standard deviations between 0.5 and 3% are obtained for the individual values and between 0.2 and 1.4% for the means. The standard deviations for the individual values and means for the violet dyestuffs are between 2 and 7% and 0.8 and 3%, respectively.

We attribute the slight increase in the errors and the deviation from the

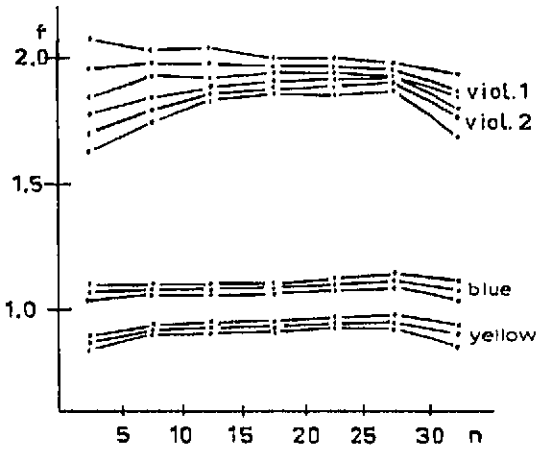


Fig. 7. Lipophilic dyestuffs, 35 bands; means of the specific substance factors for five adjacent bands in each case, referred to the red dyestuff as internal standard. The upper and lower lines show the course of the absolute standard deviation.

specified value in the edge zones of the plate to inadequacies in the chromatographic procedure, for example temperature gradients in the chromatography chamber.

Hydrocortisone is determined alongside oestrogens (Fig. 8). The maximum absorption occurs at 242 nm and that of the oestrogens at 225 and 280 nm; the absorptivity $A^{1\%}_{1cm} = 445$ and the molar absorptivity $\varepsilon \approx 15{,}000$.

With these substances of medium absorbance, the sensitivity of the detection can be increased by optimization of the wavelength and measurement at right-angles to the solvent flow. If all values from the two concentrations of 50 and 20 ng are included in the calculation of the regression line, a correlation coefficient of $r = 0.987$ is obtained. If the runaways present at the edge of the plate are rejected, the correlation coefficient then becomes $r = 0.9986$ and the line passes almost through the origin. On the chromatogram, it is possible to recognize a distinct migration of the substances in the edge bands towards the centre of the plate and the resulting falsification of the measured results.

As amino acids cannot be detected photometrically, they have to be converted into absorbing and fluorescing substances prior to or following the chromatography. We use ninhydrin to convert amino acids into substances that absorb in the visible spectral range and *o*-phthalaldehyde and mercaptoethanol in buffer solution[17,18] to convert amino acids into substances that can be excited to produce fluorescence in the UV region. Both types of conversion product show similar limits of detection (about 0.1 ng) and satisfactory results from 1 ng upwards.

Measurement of the fluorescence offers the advantages that the measured result is independent of the measuring area and the area of the spot and, further, the measured signal is a linear function of the concentration. This relationship and the sensitivity of fluorimetric measurements permit a high-grade analysis with even considerably lower quantities of substance.

The aflatoxins, for example, are some of the most potent naturally occurring poisons and carcinogens. They are metabolic products of the mould *Aspergillus flavus*,

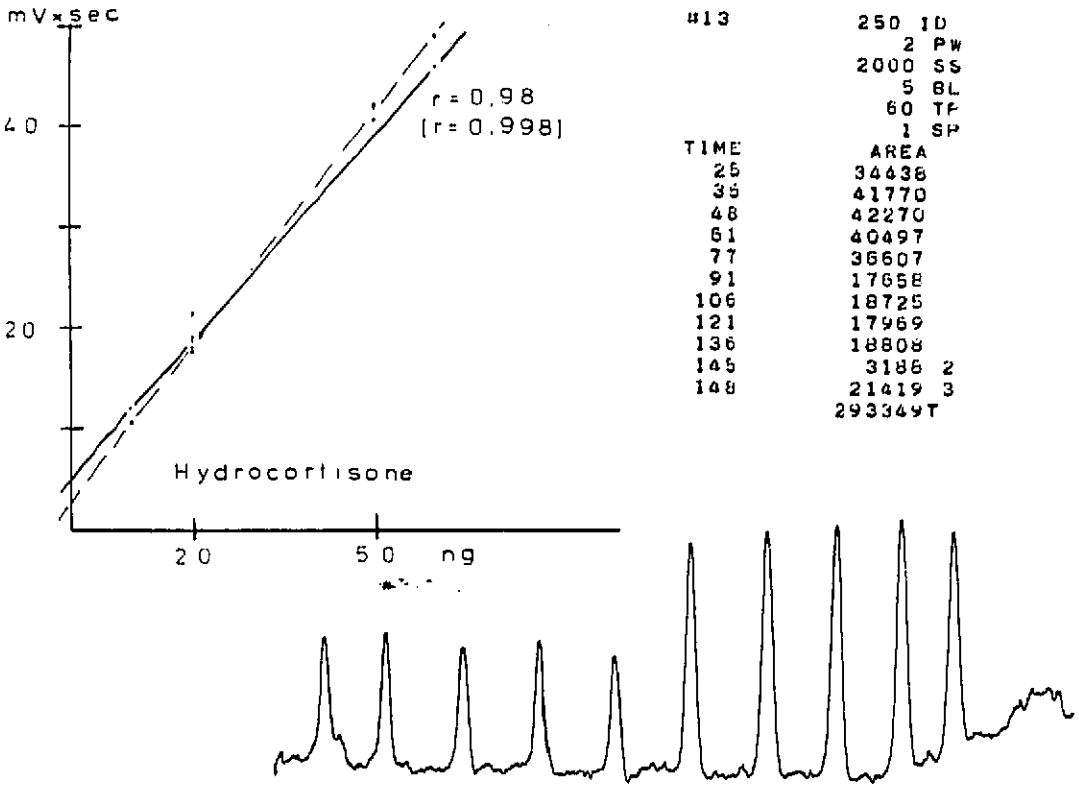


Fig. 8. Chromatogram recorded at right-angles to the solvent flow of 20 and 50 ng of hydrocortisone; integrator print-out and regression lines of all values and of values excluding significant runaways. Measuring conditions: $\lambda = 242$ nm; slit area 2.0 × 0.7 mm; measuring rate 30 mm/min; Autolab minigrator (Spectra Physics).

which occurs predominantly in cereal products. The quantitative detection of aflatoxins in the range 400–3000 ng in extracts of Aspergillus cultures using HPLC was described recently[19].

Aflatoxins B_1, B_2, G_1 and G_2 are separated on the HPTLC plate after two-fold development with chloroform–acetone (9:1). Alternating amounts of 200, 500 and 1000 pg are applied for the determination of the correction curve. Twenty four bands could be accommodated on a 10-cm wide plate, which made it possible to perform an 8-fold determination for each concentration (Fig. 9).

The standard deviation of the individual values for 1000 pg was 1.5–3.6%, for 500 pg it was 3.0–4.5% and for 200 pg it was 3.5–12.5% (Table II).

The regression lines (Fig. 10) pass through the origin and their regression coefficients are better than 0.9987 in all instances. In this region, there is a linear relationship between the fluorescence signal and the concentration. The limit of detection for aflatoxins B_1, G_1 and G_2 is about 10 pg and that for B_2 is even lower. Application, development, measurement and calculation for this plate require about 1-h work.

These measurements were carried out with a spectrophotometer with a tube-

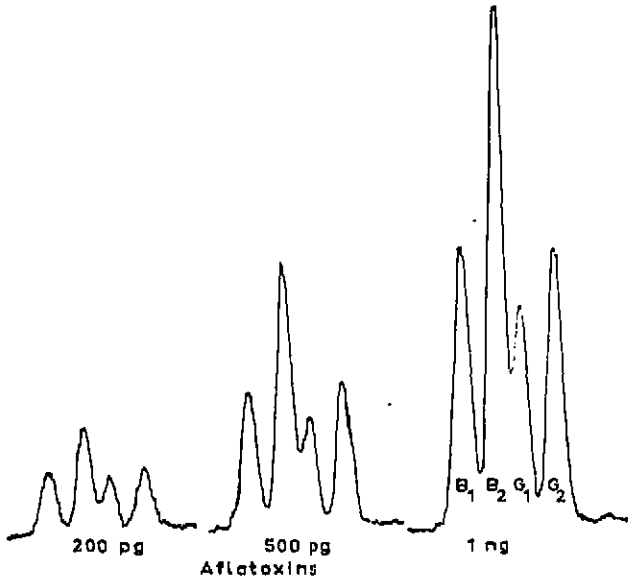


Fig. 9. Section of the evaluation of aflatoxins on a plate with 24 bands. Excitation wavelength, 366 nm; measuring wavelength, 460 nm; slit area, 3.5 × 0.7 mm; measuring rate, 30 mm/min. Chromatography conditions: chloroform–acetone (9:1); two-fold development to a height of 70 mm; normal chamber with chamber saturation.

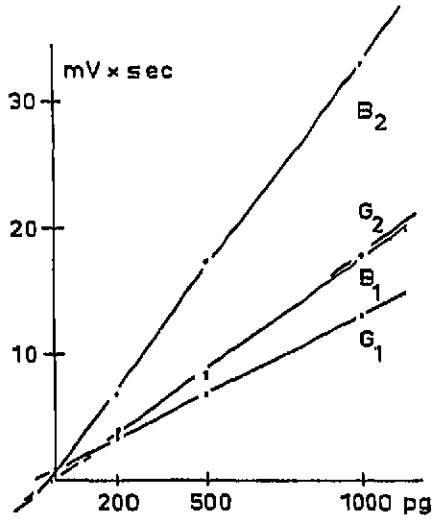


Fig. 10. Regression lines for the aflatoxins in the range 100–1000 pg (see also Table II).

TABLE II

STANDARD DEVIATIONS FOR THE DETERMINATION OF AFLATOXINS

The standard deviations, S_{rel}, are valid for n', *i.e.* for all values on the plate. r', a' and b' are the correlation coefficient, gradient and coordinate section, respectively, of the regression line and were calculated for the averaged concentrations. r, a and b are the corresponding means for six neighbouring individual measurements.

Substance	S_{rel} (%)			n'	r'	a'	b'	n	r	a	b
	1000 pg	*500 pg*	*200 pg*								
Aflatoxin B_1	±2.5	±3.0	±12.5	22	0.9987	1.740	+0.04	6	0.997	1.781	−0.27
Aflatoxin B_2	±1.5	±3.2	± 3.5	24	0.9995	3.274	+0.65	6	0.998	3.385	+0.13
Aflatoxin G_1	±3.6	±4.1	± 9.1	24	0.9998	1.286	+0.27	6	0.998	1.283	+0.38
Aflatoxin G_2	±2.4	±4.5	± 7.0	23	0.9998	1.795	−0.29	6	0.992	1.756	+0.13
	200 pg	*100 pg*	*50 pg*								
POPOP	±4.9	±6.6	±11.0	18	0.9998	2.435	+0.01	6	0.997	2.482	+0.05
	10 ng	*5 ng*	*2 ng*								
Rhodamine B in direction of solvent flow	±3.9	±2.6	±10.2	14	0.9999	4.383	+0.07	6	0.997	4.386	+0.07
Rhodamine B at right-angles to solvent flow	±2.9	±2.7	±10.1	14	0.9998	4.502	+0.29	6	0.998	4.507	+0.27
	100 pg	*50 pg*	*20 pg*								
Rhodamine B in direction of solvent flow	±3.2	±5.2	±15.3	16	0.9983	4.058	−0.87	6	0.988	3.987	−0.75
Rhodamine B at right-angles to solvent flow	±3.3	±5.0	±15.8	16	0.9988	4.183	−0.91	6	0.985	4.027	−0.18

amplifier. In a comparative trial, we were able to ascertain that equipment fitted with modern electronics, such as field effect transistors, yields even lower limits of detection and even better reproducibility. The optimization of other equipment components also, such as the optics and the lamp in order to increase the energy of the light, would be expected to lead to an improvement in the accuracy of the measuring procedure by a factor of 2–4.

POPOP [2,2'-*p*-phenylene-bis(5-phenyloxazole)] for scintillation measurement was applied in alternating concentrations of 50, 100 and 200 pg in spots 3 mm apart and developed to a height of 30 mm (Fig. 11). Measurements both in the direction of the solvent flow and at right-angles to the solvent flow gave concurring results. The standard deviations (S_{rel}) for all of the individual values were ±4.9% for 200 pg, 6.6% for 100 pg and 11.0% for 50 pg (see Table II). The regression lines for three concentrations (six bands each) all passed through the origin. The correlation coefficients were between 0.993 and 0.999 and, for all values, 0.9998. We estimate the limit of detection to be 1 pg.

Rhodamine B, a substance that can be excited to produce intense fluorescence, was applied in concentrations between 10 ng and 20 pg in order to ascertain whether the correction curve is also linear at higher concentrations. The results are shown in

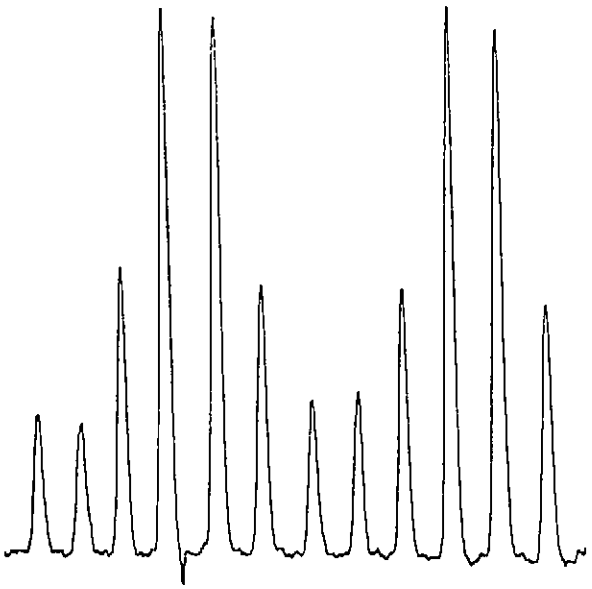
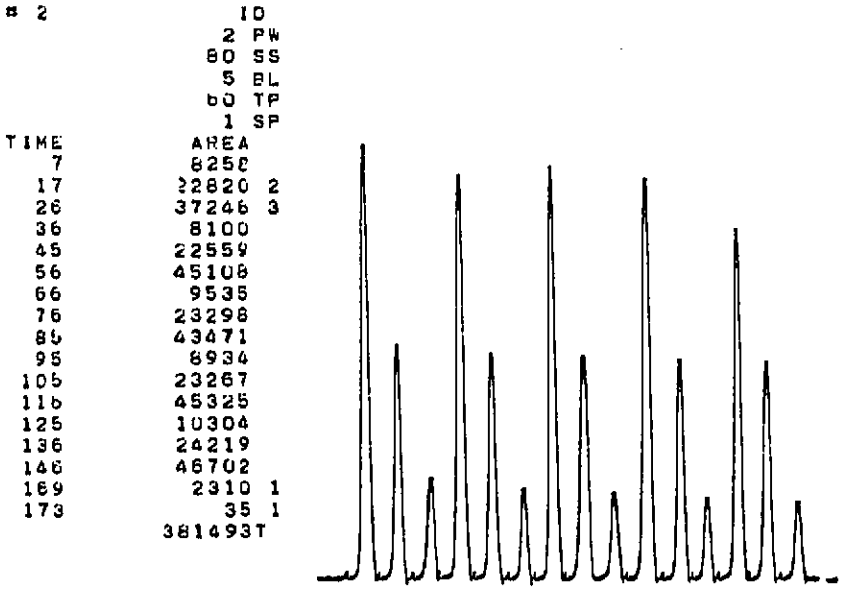


Fig. 11. Measurement of a plate with 50, 100 and 200 pg of POPOP at right-angles to the solvent flow.



```
# 2                ID
                    2 PW
                   80 SS
                    5 BL
                   60 TP
                    1 SP
TIME              AREA
   7              8250
  17             22820 2
  26             37246 3
  36              8100
  45             22559
  56             45108
  66              9535
  76             23298
  86             43471
  95              6934
 105             23267
 116             45325
 125             10304
 136             24219
 146             46702
 169              2310 1
 173                35 1
                381493T
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Fig. 12. Chromatogram and integrator print-out of 2, 5 and 10 ng of rhodamine B at right-angles to the solvent flow. Time required for measurement, 170 sec.

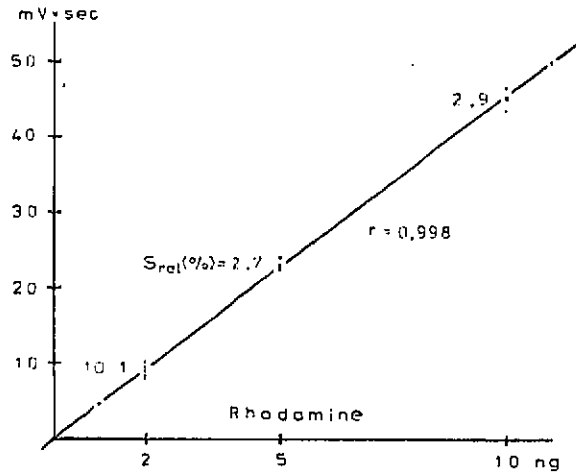

Fig. 13. Regression lines and individual values from the measurement in Fig. 12.

Figs. 12–14. It was found that 6% of the values are significant runaways, which are clearly attributable to errors in sample application, and these values were disregarded in the calculation.

The measurement at right-angles to the solvent flow (Fig. 12), the measuring

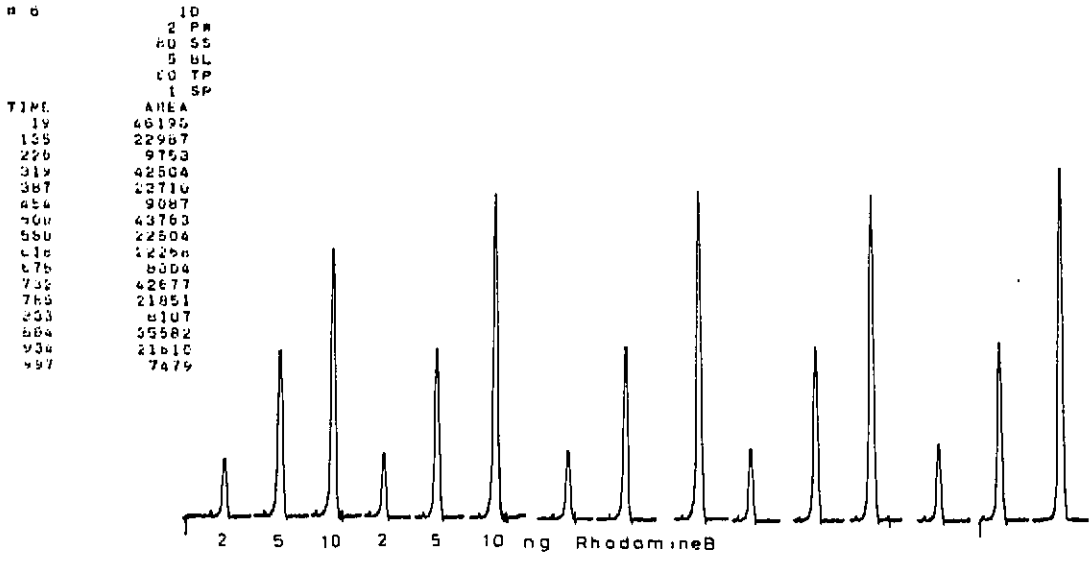

Fig. 14. Chromatogram as in Fig. 12 but measured in the direction of the solvent flow. Minimum time required for measurement, 1000 sec.

time of which, as can be seen from the integrator print-out, was 3 min, was compared with the considerably more tedious measurement in the direction of the solvent flow (Fig. 14). The integrator print-outs show that the time requirement for development in the direction of the solvent flow is six times that for measurement at right-angles to the solvent flow. This discrepancy stems mainly from the adjustments necessary for each individual band. The spot sequence is reversed for the second measurement.

Table II shows no significant differences, either in the standard deviation of the individual values or in the correlation coefficients, and only negligible differences in gradients and coordinate sections.

The linear continuation of the regression lines can be seen for the concentration range from 20 to 100 pg (Table II). The standard deviations clearly show that in the extrapolation of the errors, the error resulting from application dominates, which can readily be explained by the fact that in each concentration series volumes of 100, 50 and 20 nl were applied.

In future work, our interest will be further directed towards increasing the sensitivity of the detection, for example by chemical reaction on the plate, and also by simplifying the analysis procedure by means of clean-up directly on the HPTLC plate.

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