THE QUANTITATIVE DETERMINATION OF SOME MITRAGYNA OXINDOLE ALKALOIDS AFTER SEPARATION BY THIN LAYER CHROMATOGRAPHY*

PART III. DENSITOMETRY

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SUMMARY

A method for the densitometric determination of some oxindole alkaloids after separation by TLC is described. Problems associated with the choice of suitable instruments, parameters and the more important factors relating to the reproducibility of results depending on the separation of the alkaloids on the layer are thoroughly investigated and discussed. A statistical analysis of the results obtained are given and a recommendation is given regarding the preferred method of making the analysis and of calculating the results.

INTRODUCTION

Densitometry is a method whereby the intensity of colour of a substance is measured directly on the chromatogram. It has been utilised in paper chromatography for the evaluation of amino acids, sugars and steroids[1-6]. The application of densitometry to thin layer chromatography has been utilised indirectly by treating the chromatograms in one way or another prior to scanning[7-12]. DALLAS, BARRETT AND PADLEY[13] scanned the coloured substances directly by cutting the normal size thin layer plate (20 × 20 cm) into strips which could be accommodated in the holder of a Joyce Loebl Chromoscan normally used for holding the paper strips.

More recently, GENEST[14], using a photo volt densitometer with a TLC plate holder attached, reported the analysis of individual lysergic acid type alkaloids in Morning Glory seeds but he gave no indication of the problems involved.

This report gives some account of the problems involved in the quantitative determination of the oxindole alkaloids in admixture by densitometry using the Joyce Loebl Chromoscan with Thin Layer Attachment.

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EXPERIMENTAL

(A) Thin layer chromatography

(1)–(6) as given in Part 1[15].

(7) Detection of alkaloids: by means of 0.2 M ferric chloride in 35% perchloric acid followed by heating at 120° for 1 h.
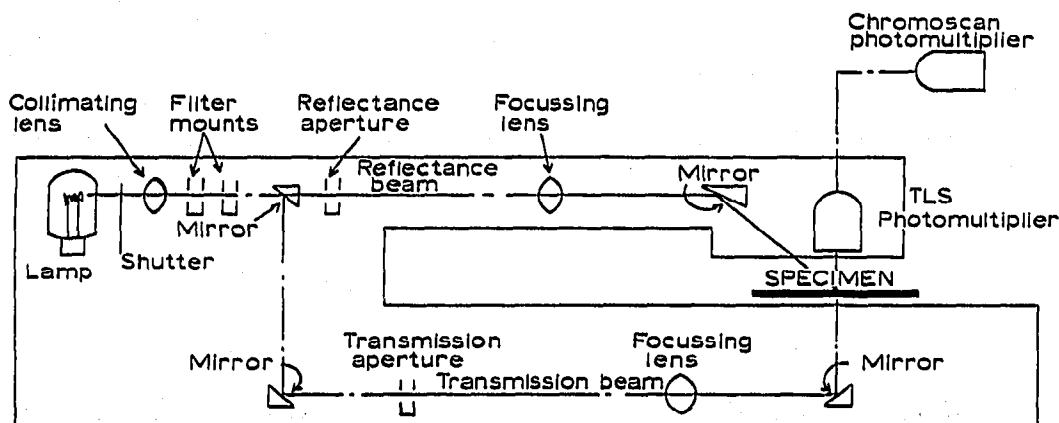

Fig. 1. Optical system of Joyce Loebl densitometer with thin layer attachment.

(B) Densitometry

The instrument used was the Joyce Loebl Double Beam and Integrating Densitometer with Thin Layer Attachment (for optical system see Fig. 1). The filters, apertures and wedge used with the densitometer are as follows:

(i) *Filters*

Code No.	Peak transmission wavelength (in A)
430	4300
465	4650
490	4900
520	5200
590	5900
620	6200

(ii) *Apertures*

Code No.	Dimension
0503	5 mm × 0.3 mm
0505	5 mm × 0.5 mm
1003	10 mm × 0.3 mm
1005	10 mm × 0.5 mm
1010	10 mm × 1.0 mm

(iii) *Grey Balancing Wedge*   2.0 optical density

RESULTS AND DISCUSSION

The reagent must be such that it reacts with the alkaloid to give a coloured compound which contrasts sharply with the absorbent background. Preferably the adsorbent should remain white or should assume a different colour from that given by the alkaloid so that by means of suitable filters it can behave as a white background. There should be no diffusion of the coloured compound on the surrounding adsorbent

and the edges of the coloured area should be sharp and definite. It must be possible to obtain a maximum intensity of colour with the reagent and the colour should be stable to light for a long period of time. The colour intensity should be reproducible and the absorbance of light by the colour must show a definite relationship to the quantity of alkaloid present.

Of the many spray reagents examined only solution of ferric chloride in 35% perchloric acid gave colours which were of value (Table I).

TABLE I

COLOURS GIVEN WITH THE OXINDOLE ALKALOIDS AND 0.2 M FERRIC CHLORIDE IN 35% PERCHLORIC ACID (2:50)

	60–65°/1 h	120°/1½ h
Rotundifoline and isorotundifoline	violet blue	dark brown
Rhynchophylline and isorhynchophylline	violet blue	reddish violet
Mitraphylline and isomitraphylline	violet blue	red
Background adsorbent	white	white

GORDON AND WEBER[16] had used this ferric chloride reagent successfully for the estimation of indoleacetic acid and related compounds but it does not give a colour with Mitragyna oxindole alkaloids in a glass vessel either at room temperature or at elevated temperatures. However, when sprayed on to silica gel layers, though not on to alumina layers, containing the alkaloids, colours are given at 65° and 120°, the final colours being quite different at the higher temperatures, although on their development they pass through violet blue then fade to pale violet before reappearing as brown, reddish violet and red according to the alkaloids. There is a good contrast and the alkaloidal spots have sharp well defined edges. The colours obtained at 65° are not stable but those obtained at 120° after 1.5 h are stable for many days. The colour intensities are reproducible and there is a relationship between the absorbance and the quantity of the alkaloids.

Selection of instrument parameters

The Joyce Loebl Densitometer is designed to enable a choice of filters, apertures, speeds, and method of scanning according to the particular problem.

(i) Filters

In order to achieve optimum results it is necessary for the substance to absorb as much light as possible and for the background adsorbent to absorb as little light as possible and since the substance is coloured it will chiefly be light of the complementary colours which will be absorbed. Since the light source is a tungsten filament lamp giving light of all wavelengths in the visible region it is necessary to use suitable filters to control the wavelengths of the incident light so that light passing through the filter will be within a selected narrow range of wavelengths. Consideration must therefore be given to the colour of the spot when selecting the most appropriate filter. Fig. 2 shows the integrated area readings for the same alkaloidal spots (rotundifoline: dark brown and isorhynchophylline: reddish violet) using different filters with a fixed
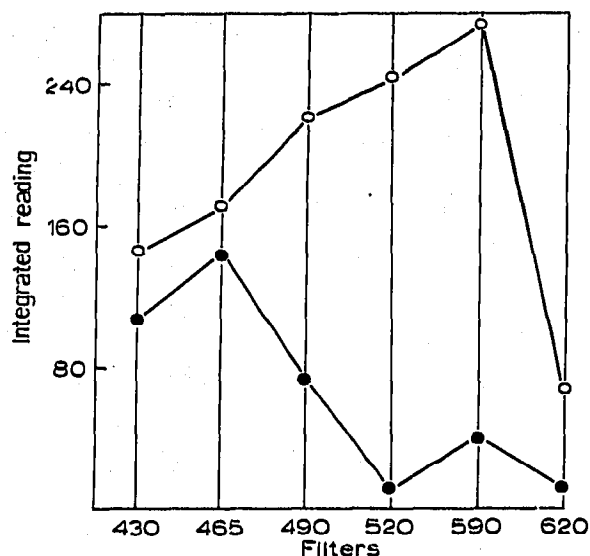
Fig. 2. Integrated readings using different filters. Aperture: 0505; method of scanning: reflectance. (O—O) Rotundifoline; (●—●) isorotundifoline.

TABLE II

% COEFFICIENT OF VARIATION DUE TO FILTERS 465 AND 520 WITH ISORHYNCHOPHYLLINE
(Based on 15 replicates from 5 plates).
Chromatographic system: silica gel/chloroform–acetone (5:4). Method of scanning: reflectance.
Aperture: 0505.

Filter	Amount (µg)						
	8	20	32	40	48	60	80
465	10.13	8.35	6.73	5.58	5.08	6.65	4.88
520	6.12	5.72	7.60	7.42	9.55	8.39	9.95
No filter	10.76	7.01	9.09	7.69	8.30	8.79	11.44

aperture and the reflectance method of scanning. As expected from the fact that reflectance depends on the absorption coefficient and shows a maximum near the position of maximum absorptivity, the reddish violet colour spot of isorhynchophylline

TABLE III

% COEFFICIENT OF VARIATION USING FILTER 465 WITH ISORHYNCHOPHYLLINE AND ROTUNDIFOLINE
(Based on 15 replicates from 5 plates)
Chromatographic system: silica gel/chloroform–acetone (5:4). Method of scanning: reflectance.
Aperture: 0505.

Alkaloid	Amount (µg)						
	8	20	32	40	48	60	80
Isorhynchophylline	10.13	8.35	6.73	5.58	5.08	6.65	4.88
Rotundifoline	8.25	6.25	5.95	6.09	7.09	7.12	6.28

gives the highest reflectance integrated reading with Filter No. 590, while the brown spot of rotundifoline is given by Filter No. 465 and although with isorhynchophylline the reading is low compared with that obtained using Filter No. 520 or 590, it is reasonably high. Thus it would appear that Filter No. 465 is the most suitable filter to use with all the Mitragyna alkaloids. It is compared statistically with Filter No. 520 which gives high readings with isorhynchophylline but very low readings with rotundifoline (Tables II and III).

(ii) Aperture

The choice of the aperture must depend to a marked extent on the shape and size of the alkaloidal spot to be scanned but naturally the smaller the aperture the higher the integrated reading will be (Fig. 3). It was shown experimentally that different
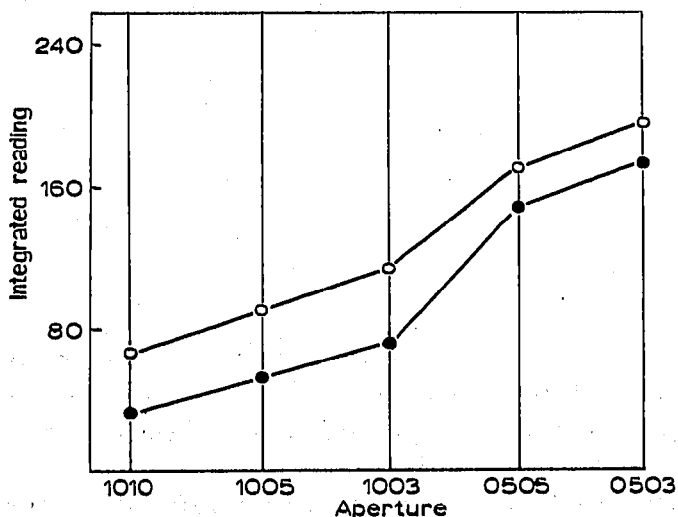

Fig. 3. Integrated readings using different apertures. Filter: 465; method of scanning: reflectance. (O—O) Rotundifoline; (●—●) isorotundifoline.

sized apertures do not have any appreciable effect upon the relationship between the integrated area readings and the amount of the alkaloid. Statistical comparison of the various apertures shows that when the slit length is smaller than the width of the spot being examined there is a lower percentage of coefficient of variation of the results than when the slit length is greater than the width of the spot. Slightly better results are obtained when the slit width is 0.5 mm wide than when it is 0.3 mm wide (Table IV).

(iii) Method of scanning

The behaviour of a beam of light when it strikes an absorbing material, irregularly distributed both upon and within the surface of a semi-opaque solid, is very complex and far from being completely understood. SHIBATA[17] has given some consideration to the problem in his work on the spectrophotometric determination of substances in biological tissue and although this is not strictly comparable to the absorbance of light by coloured substances adsorbed on thin layers, his remarks and observations can be applied to some extent.

TABLE IV

% COEFFICIENT OF VARIATION DUE TO DIFFERENT APERTURES WITH ISORHYNCHOPHYLLINE
(Based on 15 replicates from 5 plates.)
Chromatographic system: silica gel/chloroform–acetone (5:4). Method of scanning: reflectance.
Filter: 465.

Aperture	Amount (µg)						
	8	20	32	40	48	60	80
1005	11.05	6.77	9.53	6.80	7.97	8.17	6.67
1003	14.90	9.56	8.66	8.41	11.43	7.07	10.42
0505	10.13	8.35	6.73	5.58	5.08	6.05	4.88
0503	8.63	9.81	6.78	8.15	6.67	5.78	4.88

The optical phenomenon in any transluscent material can be described as:

$$I_0 = I_a + I_t + I_r$$

where

I_0 = incident light
I_a = absorbed light
I_t = transmitted light
I_r = reflected light

SHIBATA[17] not only distinguished between these major components of incident light but also recognised at least six other types of resultant light. Much of the light incident upon the substance is scattered and this depends on the shape and size and uniformity of the coloured substance absorbed on the supporting medium.

Thus the integrated area readings which are a measurement of the difference between the incident and the non-adsorbed light which is collected by the photomultiplier(s) will depend also upon the method used for scanning the spots. Two methods are possible:

(a) Reflectance. When the instrument is working with the thin layer scanning attachment, the specimen beam is provided by a second source of light, housed in the TLC attachment itself as shown in Fig. 1 and the reference and specimen beams are separately balanced by two photomultipliers, one in the main instrument and the other in the TLC attachment. The specimen beam after scanning the coloured substance is reflected back to the second photomultiplier and the final difference in the intensities of the two beams is measured by the photomultiplier in the main instrument.

Thus any light which is not reflected directly back to the photomultiplier is considered as absorbed light. Some of this may be transmitted light, some will be scattered light and some will be diffused light, and it is clear that variation will occur in the result due to variation in the amount of transmitted or scattering or diffused light from plate to plate.

(b) Transmittance. When the thin layer attachment is used the set up is the same as with the reflectance method except that by suitable arrangement the incident light is made to pass through the material from opposite directions (Fig. 1) and is then directed to the photomultiplier of the thin layer scanning attachment.

Hence in the transmittance method the light passes through the glass plate before it reaches the material. Depending upon the depth of the alkaloidal spot embedded in the layer so the light will pass through a certain thickness of the adsorbent layer. Both the glass and the adsorbent will cause considerable scattering of light to take place before it reaches the alkaloidal material. In order to reduce the scattering of the incident light by the particles of the absorbent, the adsorbent layer can be rendered transparent by treatment with light petroleum and liquid paraffin. As with the reflectance, there will be some variation in the results depending upon the extent of scatter by the glass and the adsorbent from plate to plate.

Table V shows the percentage of standard deviation due to the different methods of scanning.

TABLE V

% COEFFICIENT OF VARIATION DUE TO DIFFERENT METHODS OF SCANNING. ALKALOID: ISORHYN-CHOPHYLLINE

(Based on 15 replicates from 5 plates.)
Chromatographic system: silica gel/chloroform–acetone (5:4). Filter: 465. Aperture: 0505 with reflectance; 1005 with transmittance.

Method	Amount (μg)				
	8	20	40	60	80
Reflectance	15.02	6.66	3.84	4.97	4.09
Transmittance	11.09	5.88	6.38	6.45	8.75
Treated transmittance	12.92	14.62	8.13	8.32	9.65

(iv) Direction of scanning

There are two directions in which the alkaloidal spots can be scanned (i) along the line of development of the spot and (ii) perpendicular to the line of development of the plate.

Normally the first method would be selected but there may be occasions when the separation of two adjacent alkaloidal spots is not sufficient to enable the base line between the two curves to be re-established. Provided the spot is not an elongated spot comparable results are obtained by either direction of scan. There is a slight difference in the appearance of the two series of curves, those by method (i) show a symmetry with a blunt point at the apex, those by method (ii) a steep initial slope with a flattened apex and then an equally steep return slope. This is explained by the fact that in the two directions of scanning, the distribution of the alkaloid in the spot (and therefore the colour intensity in the spot) is not the same. In the direction of development the maximum amount of the alkaloid is distributed at the upper portion of the spot while in the perpendicular scanning the light path comes across with similar distribution of the alkaloid throughout the spot.

Perpendicular scanning is sometimes necessary because with some solvent systems a narrow trail is left between the spots and this results in the non return to the base line of the recording pen due to some absorbance taking place along this path.

To ensure that the aperture is correctly positioned for scanning each spot, since slight differences in R_F values may mean that they are not accurately aligned, it is

TABLE VI

% COEFFICIENT OF VARIATION USING DIFFERENT REFLECTANCE METHODS OF SCANNING. ALKALOID: ISORHYNCHOPHYLLINE
(Based on 15 replicates from 5 plates.)
Chromatographic system: silica gel/chloroform–acetone (5:4). Filter: 465. Aperture: 1005 with transmittance.

Method	Amount (µg)						
	8	20	32	40	48	60	80
Normal	11.05	6.77	9.53	6.80	7.97	8.17	6.67
Perpendicular	10.91	7.21	8.29	6.72	6.92	7.92	7.29

necessary to adjust the position of the aperture before scanning each individual spot.

Table VI shows no marked difference in the percentage coefficient of variation when the spots are scanned by the two methods.

(v) Speed of scanning

The instrument provides for two rates of movement of the thin layer plate, the gear ratio being 1:2 and 1:4. For any given spots the slower the rate of scan the larger will be the integrated area reading. It is essential therefore to maintain the same gear ratio for all observations, although there is little difference between the percentage coefficient of variations of the results obtained with the two speeds (Table VII).

TABLE VII

%COEFFICIENT OF VARIATION USING DIFFERENT GEAR RATIOS. ALKALOID: ISORHYNCHOPHYLLINE
Chromatographic system: silica gel/chloroform–acetone (5:4). Filter: 465. Aperture: 1005.

Gear ratio	Amount (µg)						
	8	20	32	40	48	60	80
1:2	11.05	6.77	9.53	6.80	7.97	8.17	6.67
1:4	10.12	7.59	8.29	6.73	8.01	9.31	5.30

Measurement of curve area

The optical system of the densitometer is so designed that when there is a balance between the reference beam and the light reflected from the white background adsorbent, the pen records a straight continuous line, the base line. When the light is absorbed by the coloured spot on the plate the pen describes a curve, the peak of which corresponds to maximum absorbance and returning to the base line when the beam of light returns to the white background adsorbent. The area of the curve can be measured by an integrator, the rate at which it counts increasing as it moves away from the base line. If the margin of the coloured spot is really sharp and if the adsorbent in between the spots is white so that the pen returns to the base line every time, the area of the curve would be related linearly to the count of the integrator.

Unfortunately this does not always happen, usually because the adsorbent between the spots is not free from absorbing materials so that a different base line

results. The area of the curve arising from this new base line will give a higher integrator count than the same area of the curve will give from the original base line. For this reason it was found necessary to measure the area of the curve as from its preceding base line. Several methods are available for measuring the area of a curve but the most accurate method is to use a planimeter and all the curve areas given in this report are measured with a planimeter. The results are given in Table VIII A and B and show the importance of measuring the area of the curve by planimeter and not to rely on the integrator counts provided on the instrument.

TABLE VIII A

VARIATION IN THE AREAS OF THE CHROMOSCAN CURVES (sq.cm) (BY PLANIMETER) OF THE SAME CONCENTRATION PER SPOT DUE TO DIFFERENT BASE LINES. ALKALOID: ISOMITRAPHYLLINE
Chromatographic system: silica gel/chloroform–acetone (5:4). Filter: 465. Aperture: 0505. Gear ratio: 1:2. Method of scanning: reflectance.

| Amount of alkaloid (μg) | Distance of base line from left hand side of chromoscan chart (mm) | | | | |
	30.00	35.00	40.00	45.00	50.00
80.00	15.25	17.65	17.45	17.50	17.40
60.00	14.25	16.52	16.59	16.70	16.74
40.00	11.15	12.52	12.69	12.50	12.72
20.00	7.01	8.07	8.12	8.05	8.05
12.00	4.02	4.79	4.82	4.85	4.75
8.00	2.07	2.87	2.89	2.92	2.85

TABLE VIII B

VARIATION IN THE CHROMOSCAN INTEGRATION READINGS OF THE SAME CONCENTRATION DUE TO DIFFERENT BASE LINES. ALKALOID: ISOMITRAPHYLLINE
Chromatographic system: silica gel/chloroform–acetone (5:4). Filter: 465. Aperture: 0505. Gear ratio: 1:2. Method of scanning: reflectance.

| Amount of alkaloid (μg) | Distance of base line from left hand side of chromoscan chart (mm) | | | | |
	30.00	35.00	40.00	45.00	50.00
80.00	178	210	215	235	250
60.00	159	198	205	210	230
40.00	129	156	167	176	221
20.00	81	96	107	126	137
12.00	45	59	68	76	98
8.00	29	37	36	39	54

Nature of the alkaloidal spot

Reference has been made to the problems associated with the behaviour of the beam of light on striking the adsorbed colour complex. The extent to which the light is scattered or reflected will depend on the size and shape of the spot as well as the actual distribution of the colour complex on the adsorbent.

These properties are dependent on a number of factors:

(1) The size of the initial application of the alkaloid,

(2) the nature of the solvent system and adsorbent used,

(3) the distance travelled by solvent front,

(4) the thickness of the adsorbent layer,

(5) the presence of other alkaloids (or other substances) in the mixture applied to the plate.

Some of these factors are more important than others and some are more easy to control than others. In this work the only suitable adsorbent is silica gel since no colour is obtained with the alkaloid on alumina. The thickness of the layer, while not absolutely controllable can be obtained within reasonable limits by using a commercial spreader and adhering closely to the instructions. Nevertheless reproducibility of R_F values cannot be guaranteed and, as will be seen later, the R_F value is an important factor in controlling the size and shape of the spot. The presence of other alkaloids (or other compounds) is unpredictable in the analysis of plant extracts but every effort should be made to eliminate or reduce errors due to these factors. The most important variables and those most easy to control are:

(1) the size of the initial application and,

(2) the nature of the solvent system and,

(3) the distance travelled by the solvent front.

(1) Size of the initial application

FAIRBAIRN AND SUWAL[18] have already drawn attention to the importance of the area of the initial application in quantitative paper chromatography while PURDY AND TRUTER[19] emphasised the importance of the area of the initial application in quantitative TLC where the area of the final spot is involved in the calculation.

Generally the solution of the material is carefully applied to the starting line on the plate as a circular area so that planimeter readings can be related to the initial spot diameter.

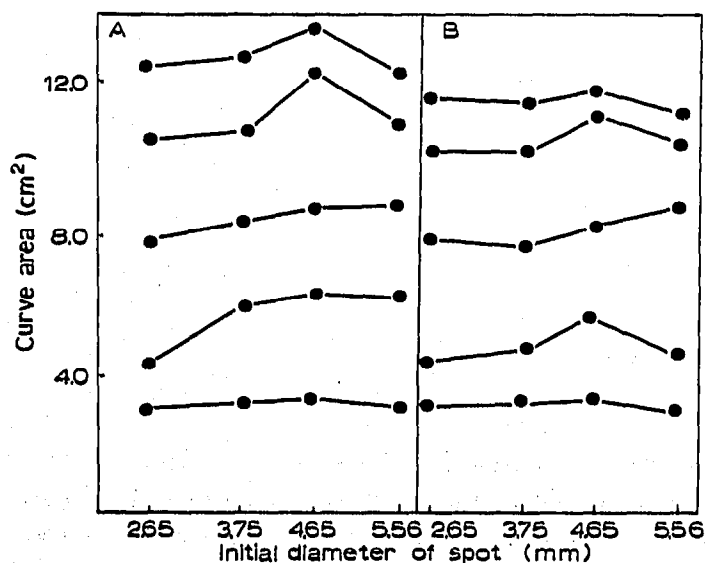Table IXA and B shows the variation in planimeter reading of the curves ob-

Fig. 4. Variation of curve areas with initial diameter of spot. Alkaloid: rotundifoline; chromatographic system: silica gel/chloroform–acetone (5:4); filter: 465; gear ratio: 1:2; aperture: (A) 0505 for reflectance, (B) 1005 for transmittance.

TABLE IX

CHROMOSCAN CURVE AREA (BY PLANIMETER) OF THE SAME AMOUNT OF ALKALOID BUT HAVING STARTING SPOTS OF DIFFERENT DIAMETERS. ALKALOID: ROTUNDIFOLINE

Chromatographic system: silica gel/chloroform–acetone (5:4). Filter: 465. Aperture: 0505. Gear ratio: 1:2. Initial diameter of spot: (a) 2.65 ± 0.35; (b) 3.75 ± 0.39; (c) 4.65 ± 0.57. (d) 5.56 ± 0.49 mm.

Amount of alkaloid (µg)	Planimeter readings a	b	c	d
A. Method of scanning: reflectance				
10.00	3.21, 2.89	2.89, 2.92	3.25, 3.15	2.79, 3.17
	2.79, 2.72	2.89, 3.21	3.16, 3.30	3.00, 2.80
	2.78, 3.12	3.12, 3.09	3.35, 3.45	3.09, 3.01
	3.14, 3.04	3.18, 3.07	3.51, 3.02	3.12, 2.89
	2.89, 2.89	3.00, 3.01	2.98, 2.99	2.79, 2.79
	= 2.953	= 3.037	= 3.216	= 2.945
20.00	4.05, 4.20	5.85, 6.05	6.05, 6.34	6.00, 6.05
	4.07, 4.45	5.85, 6.15	6.45, 6.85	5.69, 5.65
	3.85, 4.12	5.65, 5.65	6.07, 5.95	6.25, 6.17
	4.09, 4.23	5.45, 6.48	5.90, 5.97	5.85, 5.87
	4.75, 4.65	6.05, 6.05	6.47, 6.02	6.07, 6.47
	= 4.246	= 5.923	= 6.207	= 6.007
40.00	8.12, 7.20	8.65, 8.55	9.42, 8.45	8.90, 9.05
	7.45, 8.25	8.34, 8.75	8.12, 8.25	8.95, 7.95
	8.17, 8.24	8.72, 8.25	8.19, 8.10	7.92, 8.15
	7.47, 7.12	8.42, 7.62	9.05, 9.15	9.15, 9.07
	7.49, 7.24	7.62, 7.74	9.42, 8.15	9.12, 9.45
	= 7.675	= 8.266	= 8.630	= 8.77
60.00	10.70, 9.62	10.45, 9.96	12.01, 11.92	10.46, 10.96
	11.00, 10.69	10.23, 11.03	12.12, 11.76	11.09, 10.72
	10.07, 9.69	10.86, 10.96	13.00, 12.92	10.92, 10.62
	10.89, 10.69	10.92, 10.92	12.61, 12.62	11.21, 11.21
	10.71, 10.61	10.72, 10.62	12.42, 12.72	10.70, 10.62
	= 10.467	= 10.671	= 12.410	= 10.851
80.00	12.01, 13.12	12.89, 13.21	14.09, 13.25	12.25, 12.40
	11.89, 12.81	12.85, 12.75	14.12, 14.02	12.21, 12.91
	12.91, 11.91	11.92, 12.82	13.75, 14.12	12.91, 11.82
	13.02, 13.20	12.72, 13.28	13.28, 13.29	12.45, 11.92
	12.81, 12.75	13.19, 12.82	13.40, 13.09	13.09, 11.98
	= 12.664	= 12.846	= 13.640	= 12.390
B. Method of scanning: transmittance				
10.00	2.91, 2.75	2.95, 3.09	3.20, 3.31	2.98, 2.75
	2.65, 2.89	3.12, 3.12	3.09, 2.97	2.65, 2.72
	2.89, 3.01	2.98, 2.98	2.98, 3.10	2.65, 2.59
	2.91, 3.05	2.71, 3.09	3.39, 3.17	2.92, 2.91
	3.01, 3.07	3.10, 3.12	3.31, 3.20	2.91, 2.98
	= 2.914	= 3.017	= 3.172	= 2.806
20.00	3.95, 4.30	4.35, 4.42	5.65, 5.45	3.85, 4.20
	4.25, 4.40	4.12, 4.60	5.70, 5.65	4.70, 4.05
	4.50, 4.49	4.50, 4.47	5.20, 6.12	4.35, 4.20
	4.55, 4.72	4.30, 4.80	5.45, 5.23	4.39, 4.80
	3.72, 3.90	4.95, 4.85	5.45, 5.23	4.95, 4.99
	= 4.278	= 4.536	= 5.513	= 4.448

TABLE IX *(continued)*

| Amount of alkaloid (μg) | Planimeter readings | | | |
	a	b	c	d
Method of scanning: transmittance				
40.00	8.12, 7.20	7.92, 6.85	8.75, 8.62	8.90, 9.05
	7.42, 8.40	7.12, 7.45	8.72, 8.20	10.00, 8.03
	8.50, 8.65	7.65, 7.92	8.07, 7.95	8.92, 8.75
	7.21, 7.19	7.67, 7.21	7.69, 8.01	8.25, 8.15
	7.00, 7.19	7.85, 7.65	8.12, 8.13	8.01, 8.07
	= 7.688	= 7.529	= 8.126	= 8.613
60.00	10.62, 10.52	10.71, 10.69	10.69, 10.91	9.97, 9.81
	10.21, 9.96	10.71, 9.96	10.71, 11.09	9.71, 10.21
	9.96, 9.71	9.78, 9.89	11.12, 11.92	10.21, 10.81
	10.21, 10.91	9.89, 10.02	11.12, 11.91	10.92, 10.98
	10.12, 10.71	10.21, 10.81	11.21, 11.90	10.79, 10.92
	= 10.293	= 10.267	= 11.258	= 10.433
80.00	11.28, 11.81	11.21, 10.92	10.89, 12.08	10.91, 10.98
	10.81, 11.99	11.91, 12.21	12.12, 12.08	11.81, 10.92
	12.01, 11.98	12.01, 12.17	11.98, 11.81	11.81, 11.61
	11.56, 11.62	12.09, 10.89	11.91, 11.91	11.71, 10.81
	11.52, 11.91	11.29, 10.91	11.81, 11.98	10.91, 10.89
	= 11.649	= 11.561	= 11.857	= 11.236

tained from spots of the same amount of alkaloid but initially applied to the plate as different sized areas.

The graphical representation of the results (Fig. 4) shows the general trend of the variation, an increase in diameter of the initial spot leads to increased chromoscan reading but this is not true for every concentration of alkaloid per spot or for different methods of scanning.

The results show that with the smaller loads (up to 40 μg) there is a significant difference between the curve area readings even from initial spots having fairly similar diameters but with the higher loads (over 40 μg) the difference in curve area readings from initial spots having fairly similar diameters are not significant.

The results obtained by the reflectance method of scanning are similar to those obtained by the transmittance method, except where the diameter of the initial spot is 3.75 ± 0.39 mm when the transmittance method gives much higher readings with all amounts of alkaloids than does the reflectance method. The fact that spots containing higher amounts have lower readings shows that the larger diameter spots become exceedingly diffuse on development, which in turn give lower readings with both methods of scanning.

It is not very difficult to ensure that the area of the initial spots is kept constant. In paper chromatography FAIRBAIRN AND SUWAL[18] achieved this by making a very light impression on the paper by means of a cork borer of 7 mm in diameter and then a small volume of solution is applied with a micrometer syringe on to those marked areas only. The solvent is evaporated in a constant stream of air so as not to let the solvent spread outside the circumference of this lightly marked circle. Such a technique does not ensure spots of equal area but keeps the area of the spot within the 7 mm diameter. This method cannot be applied to thin layer surfaces as the act of marking would damage the thin layer itself and this would lead to the distortion of the shape of

the spot as it moves on the plate. PURDY AND TRUTER[19] tackled this problem by marking the position of the plate and maintaining the end of the needle 2 mm above the surface of the plate. The drop from the syringe is then taken on the plate by moving the plate upward until it touches the solution of material held at the end of the needle. The solvent is then evaporated before the next drop is taken in the same way. They made no reference, however, to the amount of the solution delivered each time. In this work it was found that the amount of the volume of solution delivered each time is an important factor in getting spots of equal area. It was found that by delivering a constant volume of solution from the syringe using a particular needle and by fixing the position of the needle and the plate with approximately 2 mm gap between, with one side of the plate inclined a little to facilitate taking up the drop from the syringe, a more satisfactory procedure for obtaining areas of constant size was established. A volume of 10 μl could be applied as a circular spot of 3.75 mm diameter by delivering 4 × 2.5 μl.

(2) The nature of the solvent systems

Changes in the solvent system result in different movement of the same substance on the thin layer plate and this results in different shapes and sizes of the final spots. The latter changes are accompanied by a different deposition and distribution on the substance within the spot on the surface of thin layer, depending upon the partition and adsorption isotherm of the substance with respect to the adsorbent layer and the solvent system.

A solvent system which results in a substance having a low R_F value produces a resultant spot which is round, compact and small, while a solvent system which results in the substance having a high R_F value produces a spot which is larger and often diffuse. Thus a definite amount of the substance run on the thin layer, but using different solvents systems, will give different responses with respect to the chromoscan readings.
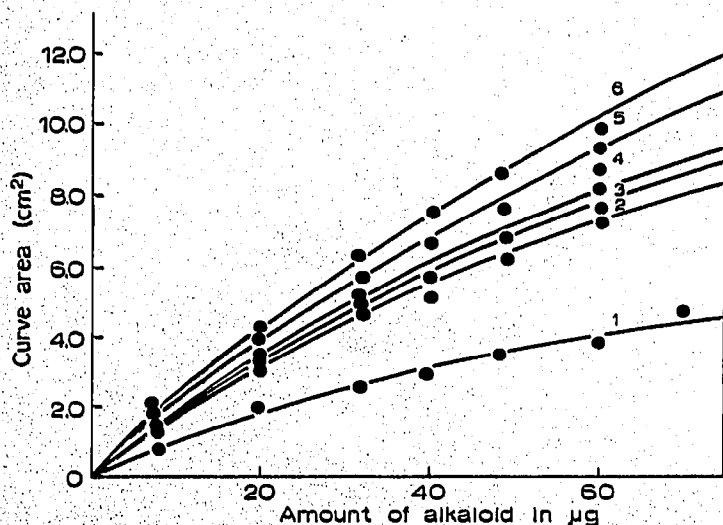

Fig. 5. Variation of calibration curve with different solvent systems. Alkaloid: rotundifoline; filter: 465; aperture: 1005; gear ratio: 1:2; method of scanning: reflectance. Solvent system: (1) chloroform–cyclohexane (7:3); (2) chloroform; (3) benzene–ethyl acetate (7:2); (4) ether; (5) benzene–acetone (1:1); (6) chloroform–methanol (95:5).
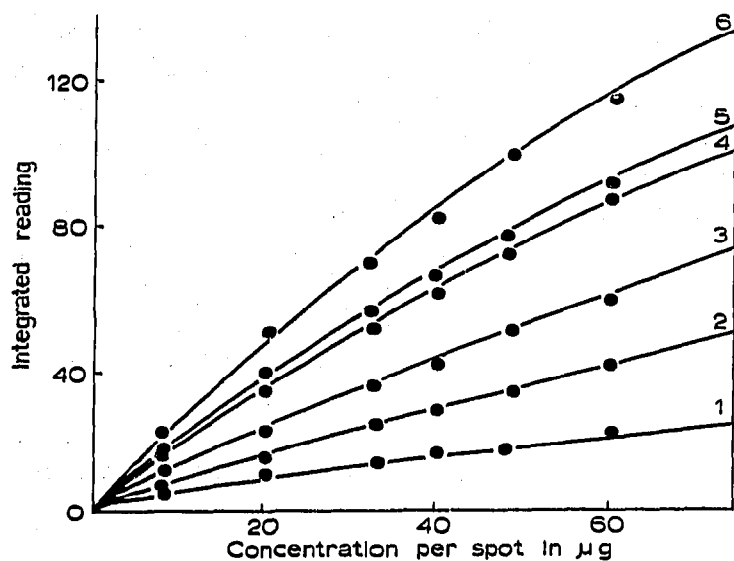
Fig. 6. Variation of calibration curve with different solvent systems. Alkaloid: rotundifoline; filter: 465; aperture: 1005; gear ratio: 1:2; method of scanning: transmittance. Solvent system: (1) chloroform–cyclohexane (7:3); (2) chloroform; (3) benzene–ethyl acetate (7:2); (4) ether; (5) benzene–acetone (1:1); (6) chloroform–methanol (95:5).

Figs. 5 and 7 show the effect on the calibration curve of rotundifoline of different solvent systems and the relation between the peak area and R_F values respectively when the plates are scanned by the reflectance method, while Figs. 6 and 8 show the same effects when the plates are scanned by the transmittance method. It will be seen that for both the reflectance and the transmittance methods of scanning, the chromoscan readings increase with the increase of R_F values of the alkaloid irrespective of the
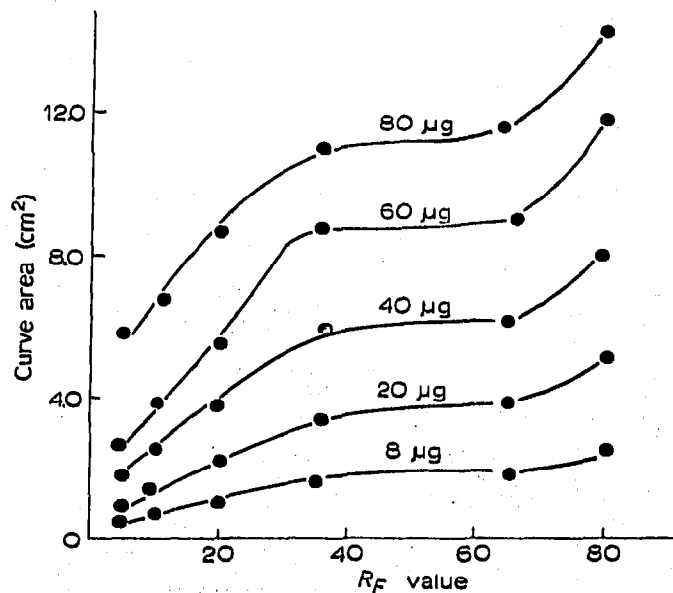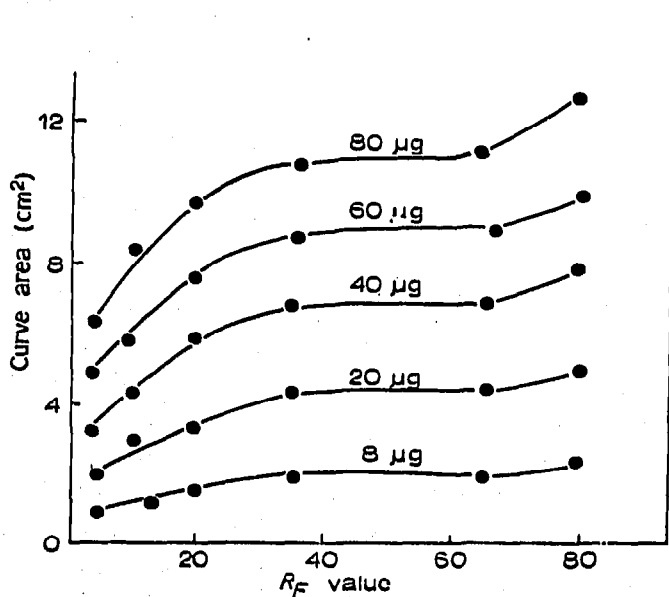

Fig. 7. Variation of curve area with R_F value. Alkaloid: rotundifoline; filter: 465; aperture: 1005; gear ratio: 1:2; solvent system: chloroform–acetone (5:4); method of scanning: reflectance.

Fig. 8. Variation of curve area with R_F value. Alkaloid: rotundifoline; filter: 465; aperture: 1005; gear ratio: 1:2; solvent system: chloroform–acetone(5:4); method of scanning: transmittance.
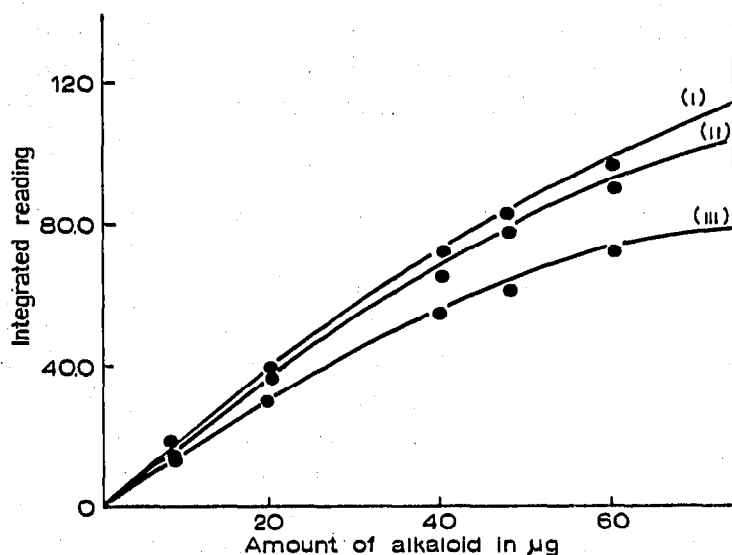
Fig. 9. Variation of calibration curve with distance travelled by solvent. Alkaloid: rotundifoline; filter: 465; aperture: 1005; gear ratio: 1:2; solvent system: chloroform–acetone (5:4); method of scanning: reflectance; distance travelled by solvent: (i) 15 cm, (ii) 10 cm, (iii) 5 cm.

amount of the alkaloid. The almost flat position of the curves of Figs. 7 and 8 show that, in fact, the chromoscan readings remain constant between hR_F value of 35 to 65.

(3) Distance travelled by the solvent front

The distance a substance moves on a thin layer plate will also depend on the
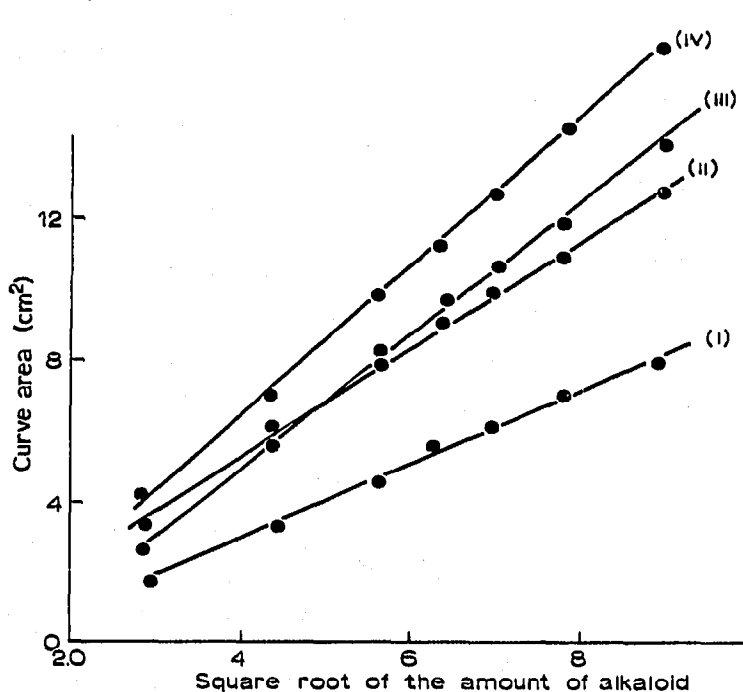
Fig. 10. Relationship between curve area and $\sqrt{}$ amount of alkaloid. Alkaloid: isorotundifoline; filter: 465; solvent system: chloroform–acetone (5:4). (I) Aperture: 1005, gear ratio: 1:2, method of scanning: reflectance; (II) aperture: 0505, gear ratio: 1:2, method of scanning: reflectance; (III) aperture: 0505, gear ratio: 1:2, method of scanning: perpendicular reflectance; (IV) aperture: 0505, gear ratio: 1:4, method of scanning: reflectance.

distance travelled by the solvent front. This is usually 10 or 15 cm on 20 × 20 cm plates or on 20 × 5 cm plates and 5 cm on microscope slides. Fig. 9 shows the effect of the differences on the distance moved by the solvent system on the chromoscan curve area.

Relationship between chromoscan integrated area readings (curve area by planimeter) and the amount of alkaloid applied

A curvo-linear relationship of the asymptotic type exists between the curve area and the amount of the alkaloid applied, irrespective of the mechanical and optical variation of the instrument, *i.e.* using different filters, different apertures, scanning by reflectance or transmittance methods, scanning in the direction of development or perpendicular to the direction of the development or scanning by different speeds of the movement of thin layer plate carrier, except when the plates are sprayed with petroleum–liquid paraffin (1:1) to make them transparent and then scanned by the transmittance method, in which case the relationship between the peak area and the amount of the alkaloid in the spot is linear.

This curvo-linear relationship shown by untreated plates can be converted into a linear relationship by two methods: (i) by plotting the product of the scanogram curve area, the initial diameter and the final area of the spot against the amount of the alkaloid; however, this method is likely to introduce significant errors due to wide variations in the accurate determination of the areas of the initial and final spots; (ii) by plotting the curve area against the square root of the amount of alkaloid (Fig. 10). This linear relationship is confirmed by the regression analysis, Table X. Fig. 11 shows the regression lines of rotundifoline and isorhynchophylline by the reflectance method

TABLE X

DETAILS OF THE REGRESSION ANALYSIS OF THE DATA FOR ROTUNDIFOLINE

Source of variation		Sum of squares	$F°$	Mean squares
Between concentrations		587.452	6	97.908
Within concentrations		9.383	63	0.149
	Total	596.835	69	—

$F_{63}° = 657.10$ $p = 0.001$

i.e. the greater mean square is significantly greater than the smaller mean square.

Source of variation		Sum of squares	$F°$	Mean squares
Between concentration due to regression		586.277	1	586.277
Deviation from regression		1.175	5	0.235
Within concentration (residual)		9.383	63	0.149
	Total	596.835	69	—

Testing significance of the regression

$F_{63}^1 = 393.47$ $p = 0.001$

hence the regression line is highly significant
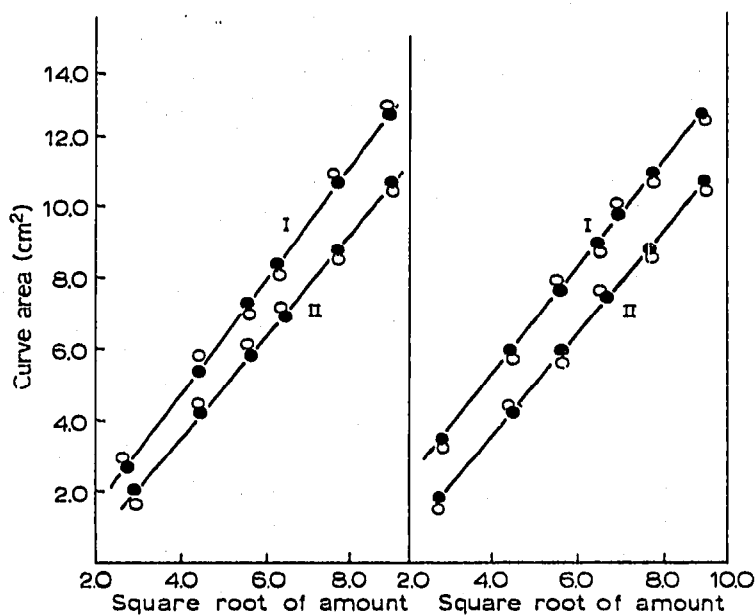
$F_{63}^5 = 1.577$

Fig. 11. Regression lines for (a) rotundifoline, and (b) isorhynchophylline. Filter: 465; gear ratio 1:2. Aperture: (I) 0505, reflectance; (II) 1005, transmittance. (●—●) Regression line points; (○—○) experimental points.

and the transmittance method respectively, which also show the experimental points with respect to the regression lines. From the variance ratio table, $n_1 = 5$, $n_2 = 63$, for $p = 0.05$, $F = 2.35$, and it shows that the regression mean square is not significantly greater than the residual mean square, $i.e.$, the statistical test confirms the linearity of the curve area–amount of alkaloid relationship.

Method of calculating the amount of alkaloid from the curve area readings

The fact that there is considerable variation in the curve area readings for the same amount of alkaloid on the same plate and from plate to plate makes it necessary to utilise the experimental data of the calibration curve of each alkaloid in order to determine the regression line equation for each alkaloid and to use this for calculating the amount of alkaloid present. The regression line equation is

$$y = bx + c \text{ or } x = (y - c) \, b$$

where

x = square root of the amount of alkaloid per spot in μg
y = grand mean or average of a number of readings from one plate
c = intercept
b = slope of the regression line

The constants of the regression lines for each alkaloid separated on silica gel/chloro-form–acetone (5:4) are given in Table XI.

The results of the analysis of a mixture of rotundifoline and isorotundifoline are given in Table XII the analysis of variance (of two-way cross classification type) being carried out in order to analyse the variation associated between readings on different plates and between different readings on the same plate (Table XIII).

TABLE XI

REGRESSION LINE CONSTANTS FOR THE OXINDOLE ALKALOIDS

Chromatographic system: silica gel/chloroform–acetone (5:4). Filter: 465. Aperture: 0505. Gear ratio: 1:2. Method of scanning: reflectance.

Constants	Rotundi-foline	Isoro-tundi-foline	Rhyncho-phylline	Iso-rhyncho-phylline	Mitra-phylline	Iso-mitra-phylline
Slope	1.628	1.896	3.043	1.528	2.002	2.424
Intercept	−1.855	−2.591	−5.928	−0.794	−2.718	−3.059
Variance about the line	0.4885	0.8762	0.7351	0.7923	0.3092	0.6362

It will be seen that for both alkaloids concerned the mean square (variance) between readings on different plates are higher than the mean square (variance) between readings on the same plate. Hence it appears that the average results obtained by a number of readings from one plate would be associated with a lower variation than the average result obtained by a number of readings from different plates. But this

TABLE XII

ANALYSIS OF A MIXTURE OF ROTUNDIFOLINE AND ISOROTUNDIFOLINE

Chromatographic system: silica gel/chloroform–acetone (5:4). Filter: 465. Aperture: 0505. Gear ratio: 1:2. Method of scanning: reflectance.

(a) Curve for rotundifoline

Plate	Replicate observations (sq. cm)						Total	Mean
1	4.45	4.67	4.65	4.42	4.62	4.49	27.30	4.55
2	5.02	5.09	5.20	4.90	4.82	5.20	30.23	5.04
3	5.06	5.01	4.87	4.85	4.85	4.65	29.33	4.88
4	5.12	5.02	4.82	4.82	4.85	4.82	29.45	4.90
5	4.62	4.92	4.82	4.82	4.72	4.82	28.72	4.78
6	4.82	4.82	4.89	4.79	4.79	4.92	29.03	4.83
Total	20.09	29.53	29.25	28.63	28.66	28.90	174.06	
Mean	4.85	4.92	4.87	4.77	4.76	4.81	Grand mean = 4.835	

(b) Curve for isorotundifoline

Plate	Replicate observations (sq. cm)						Total	Mean
1	5.30	5.35	5.22	5.19	5.17	5.18	31.41	5.23
2	5.01	4.92	4.85	4.82	4.72	4.75	29.07	4.84
3	4.75	4.69	4.79	4.82	4.62	4.72	28.39	4.73
4	4.81	4.90	5.01	5.09	5.09	4.92	29.82	4.97
5	5.10	5.20	5.12	5.31	5.21	5.22	31.16	5.19
6	5.21	5.22	5.29	5.30	5.32	5.30	31.64	5.27
Total	30.18	30.28	30.28	30.53	30.13	30.09	181.49	
Mean	5.03	5.04	5.04	5.08	5.02	5.02	Grand mean = 5.04	

TABLE XIII

ANALYSIS OF VARIANCE OF CURVE AREAS

(a) Rotundifoline

Source of variation	Sum of squares	$F°$	Mean squares
Between plates	0.791	5	0.158
Within plates	0.101	5	0.020
Remainder (error)	0.348	25	0.139
Total	1.240	35	—

(b) Isorotundifoline

Source of variation	Sum of squares	$F°$	Mean squares
Between plates	1.5238	5	0.305
Within plates	0.0146	5	0.0029
Remainder (error)	0.1993	25	0.0079
Total	1.7377	35	—

TABLE XIV

VARIATION IN % COEFFICIENT OF VARIATION ACCORDING TO NUMBER OF READINGS PER PLATE. ALKALOID: ROTUNDIFOLINE

	No. of observations				
	1	*2*	*3*	*4*	*5*
Range of % coefficient of variation	4.63 to 7.93	4.98 to 7.76	4.21 to 5.39	3.29 to 5.19	3.31 to 4.16
Average result (μg)	16.965	16.860	16.713	16.863	16.780

TABLE XV

ESTIMATION OF ALKALOIDS (ROTUNDIFOLINE AND ISOROTUNDIFOLINE IN ADMIXTURE). CALCULATED FROM DATA GIVEN IN TABLE XII

Alkaloid (μg)	Method of calculation	Amount of alkaloid found experimentally (μg)			Average	% Coefficient of variation
Rotundifoline (16.00)	A	16.843			16.843	3.95
	B	15.405, 17.800, 16.974, 17.139, 16.810, 16.483			16.769	4.77
Isorotundifoline (16.00)	A	16.080			16.080	4.52
	B	16.966, 15.311, 14.861, 16.793, 15.856, 17.139			16.104	5.63

TABLE XVI

ESTIMATION OF ALKALOIDS IN TWO COMPONENT MIXTURES

Chromatographic system: silica gel/chloroform–acetone (5:4). Filter: 465. Aperture: 0505. Gear ratio: 1:2. Method of scanning: reflectance.

Alkaloid	R_F value	Amount added (μg)	Curve area (sq. cm)				Amount found (μg)	% Coefficient of variation
Rhynchophylline	0.24–0.33	9.60	2.95,	3.40,	2.90,	3.30,	8.90	4.49
			3.32,	3.18				
		19.20	7.32,	6.89,	7.81,	7.28,	19.02	3.71
			7.31,	7.42				
		28.80	10.28,	9.95,	10.81,	9.99,	28.77	11.54
			10.47,	10.92				
Isorhynchophylline	0.63–0.70	10.00	4.12,	4.52,	4.52,	4.02,	10.63	10.23
			3.95,	4.02				
		20.00	6.52,	6.28,	6.72,	6.52,	22.75	4.25
			6.36,	6.63				
		30.00	7.65,	7.28,	7.21,	7.91,	30.30	7.35
			7.95,	7.75				
Mitraphylline	0.38–0.43	7.8	2.85,	2.92,	2.82,	2.95,	7.78	4.97
			2.61,	3.01				
		15.6	4.89,	5.35,	5.45,	5.02,	15.67	6.08
			5.38,	5.20				
		23.4	6.75,	7.25,	6.88,	6.92,	21.74	3.89
			6.82,	6.99				
		31.2	8.25,	8.65,	8.95,	8.95,	30.01	5.29
			9.05,	8.81				
Isomitraphylline	0.60–0.67	7.95	4.00,	3.52,	3.69,	3.59,	7.87	6.09
			4.00,	3.69				
		15.90	6.49,	6.93,	7.08,	6.85,		
			6.75,	6.45			16.39	5.19
		23.85	8.62,	9.05,	9.10,	8.50,	23.77	4.44
			8.62,	8.75				
		31.80	11.01,	10.62,	10.28,	10.17,	31.20	4.21
			10.42,	10.41				

advantage is marred by the fact that the average result obtained from a number of readings from one plate might be much higher or lower than the theoretical readings. This is evident since the average of each plate varies significantly from each other (more marked in the case of isorotundifoline) but average readings taken from different plates do not vary significantly.

It can be concluded that the best results can be obtained by combining readings from different plates (method A) rather than by combining readings from a single plate (method B). The question arises as to the minimum number of readings per plate assuming six plates are used. Table XIV shows the percentage of coefficient of variation according to the number of readings per plate (from rotundifoline readings).

It appears that although the average results obtained do not vary significantly the percentage of coefficient of variation associated with the results do vary significantly and at least five or more readings per plate are necessary to obtain results with low variations.

TABLE XVII

ESTIMATION OF ALKALOIDS IN THREE COMPONENT MIXTURES

Chromatographic system: silica gel/chloroform–acetone (5:4). Filter: 465. Aperture: 0505. Gear ratio: 1:2. Method of scanning: reflectance.

Alkaloid	R_F value	Amount added (μg)	Curve area (sq. cm)				Amount found (μg)	% Coefficient of variation
Rhynchophylline	0.24–0.33	8.00	2.86, 2.65,	2.98, 2.75	2.75,	2.85,	8.19	2.56
		16.00	6.21, 6.21,	6.52, 6.08	6.09,	6.29,	15.93	2.74
		24.0	9.62, 9.09,	8.92, 9.49	8.52,	9.23,	23.61	3.82
Mitraphylline	0.38–0.43	18.0	5.88, 6.18,	6.01, 6.01	6.69,	6.28,	17.46	6.99
		36.0	9.28, 9.57,	9.52, 9.87	9.48,	9.08,	33.91	4.87
		54.0	12.28, 12.31,	12.01, 12.21	11.81,	11.87,	50.83	3.01
Isomitraphylline	0.60–0.67	8.0	3.65, 3.52,	3.65, 3.49	3.95,	3.42,	7.67	6.64
		16.0	6.48, 6.58,	6.28, 6.21	6.12,	6.08,	14.82	4.20
		24.0	9.01, 9.01,	8.48, 8.48	8.58,	9.08,	23.75	4.87

The results obtained from the data given in Table XII (using six readings per plate for method B) are given in Table XV.

The figures for the coefficient of variation of the results of both the alkaloids calculated by the first method are lower than those calculated by the second method. The percentage of coefficient of variation by the first method represents the variation associated mainly with the calibration curve while in the second case the standard deviation is associated with the experimental variations at the time of determining the unknown concentration.

Analysis of the alkaloids in admixture

The analyses were undertaken by method B and calculating the result from the regression line equation.

Two procedures were adapted for determining the curve areas. In the first method where the alkaloidal spots are well separated and the chromoscan curves are well separated, the area of each curve was determined without difficulty using the planimeter.

In the second method, where the alkaloidal spots are not well separated from each other and the chromoscan curves overlap each other where the curves are normally symmetrical it is possible to extrapolate geometrically and complete the curves in order to measure their areas with the planimeter. With isorotundifoline however, the alkaloidal spots are always elongated and the chromoscan curves are asymmetrical (more or less right angled triangular shapes) and such curves cannot be extrapolated satisfactorily. The incomplete curves for this alkaloid were completed by superimposing curves of a known amount of the alkaloid (and thus of known area)

TABLE XVIII

ESTIMATION OF ALKALOIDS IN FOUR COMPONENT MIXTURES

Chromatographic system: silica gel/chloroform–acetone (5:4). Filter. 465. Aperture: 0505. Gear ratio: 1:2. Method of scanning: reflectance.

Alkaloid	R_F value	Amount added (µg)	Curve areas	Amount found (µg)	% Coefficient of variation
Rhynchophylline	0.24–0.33	8.4	2.95, 3.28, 3.09, 2.84, 2.99, 2.61	8.50	5.12
		16.8	6.12, 6.45, 6.29, 6.71, 6.89, 6.81	16.78	4.87
		25.2	9.92, 9.28, 9.09, 9.91, 9.81, 9.72	26.11	3.81
		25.5	10.2, 9.81, 9.28, 9.41, 9.28, 9.09	25.70	5.54
Mitraphylline	0.38–0.43	8.8	3.12, 3.89, 3.81, 3.28, 3.33, 3.44	8.01	10.96
		17.6	5.82, 5.76, 5.81, 5.81, 6.02, 6.42	16.43	9.42
		25.4	7.24, 7.91, 7.58, 7.98, 7.38, 7.99	24.31	6.55
Isomitraphylline	0.60–0.67	7.5	3.56, 3.77, 3.01, 3.42, 3.48, 3.58	7.23	9.44
		15.00	6.52, 6.35, 6.02, 6.72, 6.62, 6.52	15.40	5.15
		22.5	8.82, 8.21, 8.29, 8.81, 8.61, 8.19	22.68	5.17
Rotundifoline	0.66–0.74	7.6	2.61, 2.89, 2.79, 2.59, 2.42, 3.01	7.88	9.38
		15.2	4.85, 5.02, 5.08, 5.01, 4.65, 4.55	16.79	5.45
		22.8	5.52, 5.62, 5.42, 5.91, 5.41, 5.89,	21.28	5.16

on to the incomplete curve and selecting the one which coincided with the curve area. It was then possible to complete the curve and measure its area with the planimeter or to accept the known area of the equivalent curve. The fact that the result of the analysis of isorotundifoline in admixture corresponds with the actual amount present shows that the method is applicable.

The result of the analysis of mixtures of two, three or four alkaloids are given in Tables XVI, XVII and XVIII.

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