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

PART IV. COMPARISON OF ULTRA-VIOLET SPECTROPHOTOMETRY, COLORIMETRY AND DENSITOMETRY AS METHODS FOR THE QUANTITATIVE DETERMINATION OF OXINDOLE ALKALOIDS IN PLANT MATERIAL

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SUMMARY

Oxindole alkaloids occurring in various species of Mitragyna were determined quantitatively by means of ultra-violet spectrophotometry, colorimetry and densitometry after extracting the total alkaloid from the plant material and separating the individual alkaloids by thin-layer chromatography. The results showed that if there were few alkaloids in the plant there was little to choose between the three methods and that in each case the coefficient of variation was small but if a large number of alkaloids were present the results from all three methods showed greater coefficients of variation, those being obtained by densitometry being less reliable than the others.

INTRODUCTION

Methods for the quantitative determination of some Mitragyna oxindole alkaloids by means of ultra-violet spectrophotometry[1], colorimetry[2] and densitometry[3] after separation by TLC have been described. These methods, however, involved the use of artificial mixtures of isolated alkaloids so that it is necessary to consider the application of the methods to plant material which may contain other alkaloids and also non-alkaloidal constituents. This report gives some account of the application of the three methods to different species of Mitragyna and assesses the reliability of each method.

EXPERIMENTAL

Plant materials

Leaves of *Mitragyna inermis* (Willd.) O. Kuntze, obtained from Ghana.
Leaves of *Mitragyna rotundifolia* (Roxb.), obtained from the Phillipines.

Leaves of *Mitragyna rotundifolia* (Roxb.), obtained from Burma.
Leaves of *Mitragyna parvifolia* (Roxb.) Korth., obtained from India.
Bark of *Mitragyna parvifolia* (Roxb.) Korth., obtained from India.

Extraction of alkaloids

Samples of 5.0 or 10.0 g coarsely powdered leaf or bark were moistened with ammonia and shaken continuously for 1 h in the cold with 3 × 50 ml ethyl acetate successively. The combined macerates were concentrated to about 10 ml under reduced pressure and extracted with 3 × 10 ml 10% sulphuric acid. The acidic solution was then made alkaline with ammonia and extracted with 3 × 10 ml chloroform. After washing the chloroform extract with water and drying with sodium sulphate it was concentrated under reduced pressure, the final volume being adjusted to 10 ml.

Column chromatography (when necessary). Alumina; column 5.0 × 1.5 cm. Eluate: chloroform–methanol (98:2).

Thin-layer chromatography. As given in Parts I[1], II[2] and III[3]. Gradient TLC as described by SHELLARD, ALAM AND ARMAH[4].

Spectrophotometry. As given in Part I[1].

Colorimetry. As given in Part II[2].

Densitometry. As given in Part III[3].

RESULTS AND DISCUSSION

Although it is usual for isomeric pairs of alkaloids to be present in Mitragyna plant material, care must be taken during extraction and isolation processes to prevent further isomerisation of the alkaloids. This precaution is even more necessary when the alkaloids are extracted for quantitative analysis. Experiments indicated that when oxindole alkaloids are refluxed in alkaline solution (*e.g.* pyridine or ethyl acetate containing ammonia) isomerisation quickly occurs but at room temperature isomerisation is very slow and none is detected after 12 h. Consequently the method used for extracting the alkaloids was as described under "Experimental".

For qualitative work the chloroform solution after concentration was examined with thin-layer chromatography including gradient TLC using several systems and various differential spray reagents to ascertain whether any of the six oxindole alkaloids under discussion were present. It was sometimes necessary to separate the mixture by means of column chromatography, small scale preparative chromatography or by a pH buffer solution extraction in order to confirm the identity of the alkaloids.

Even for quantitative work it was sometimes necessary to clean up the alkaloidal mixture by passing it through an alumina column in order to obtain thin-layer chromatograms in which the alkaloidal spots were not contaminated with an alkaloidal material. Table I shows the results of the qualitative analysis. Among the total number of alkaloids, indole alkaloids and oxindole alkaloids other than those under consideration may have been present. Fig. 1 shows the appearance of the scanogram of the alkaloids from *Mitragyna rotundifolia* from the Phillipines before and after passing the mixture of alkaloids through a column of alumina. Figs. 2–5 show some of the chromatograms obtained. Tables II–V give the results of the quantitative determinations (each figure is an average of six applications on one plate). For the densitometric determination, method A was used[3].

TABLE I

OXINDOLE ALKALOIDS IDENTIFIED IN MITRAGYNA PLANT MATERIAL

Plant material	*Total No. of alkaloids present*	*Oxindole alkaloids*
M. parvifolia, bark	2	mitraphylline, isomitraphylline
M. parvifolia, leaves (both from India)	3	mitraphylline, isomitraphylline
M. rotundifolia, leaves (from Burma)	5	rhynchophylline, isorhynchophylline
M. rotundifolia, leaves (from the Phillipines)	7	rhynchophylline, isorhynchophylline mitraphylline, isomitraphylline
M. inermis, leaves (from Ghana)	11	rhynchophylline, isorhynchophylline rotundifoline, isorotundifoline

It was often necessary to use several TLC systems in order to obtain good separation of the alkaloids although with *Mitragyna rotundifolia* leaves it was not found possible to separate isorhynchophylline and isomitraphylline sufficiently to permit them to be determined by the colorimetric method. They were determined differentially by the U.V. spectrophotometric method[1] and by scanning in a direction perpendicular to the line of development by the densitometric method[3]. Silica gel/ether was employed for these determinations but the estimation of mitraphylline and rhynchophylline was made from a silica gel/chloroform–acetone (5:4) system. With *Mitragyna inermis* leaves it was not possible to estimate rotundifoline and isorhynchophylline by the densitometric method since they could not be separated from

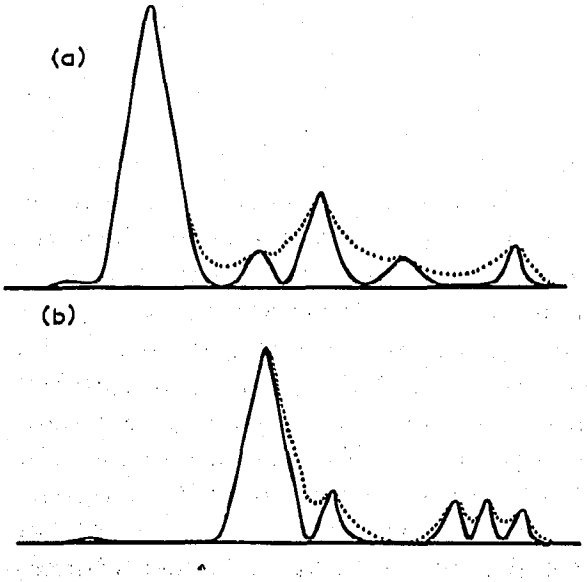

Fig. 1. Scanograms. Total alkaloidal extract of *Mitragyna rotundifolia* from the Phillipines. (a) Silica gel/chloroform–acetone (5:4); (b) silica gel/ether. (· · · · ·) before passing through column; (———) after passing through column.

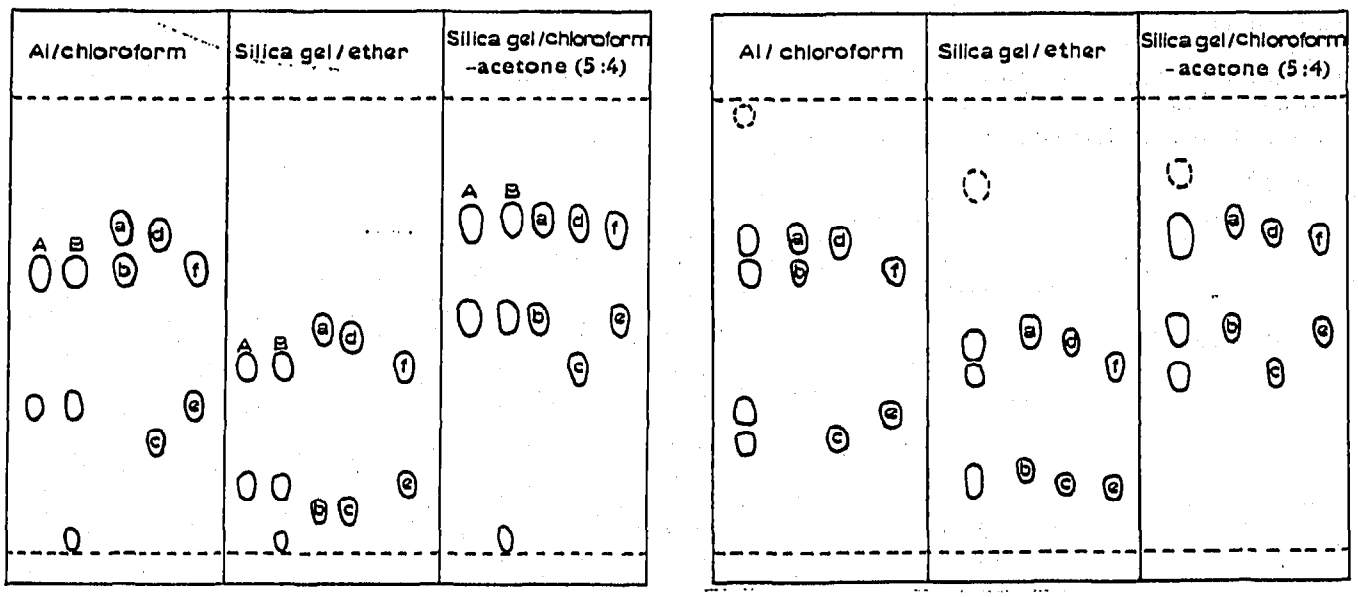

Fig. 2. Chromatograms of alkaloidal extracts of (A) leaves and (B) bark of *Mitragyna parvifolia* from Dehradun, India. a = Rotundifoline; b = isorotundifoline; c = rhynchophylline; d = isorhynchophylline; e = mitraphylline; f = isomitraphylline.

Fig. 3. Chromatograms of alkaloidal extracts of the leaves of *Mitragyna rotundifolia* from Burma. a–f, see the legend to Fig. 2.

further alkaloidal material with any solvent systems along with silica gel. They were determined as binary mixtures by both the U.V. spectrophotometric and colorimetric methods[1,2].

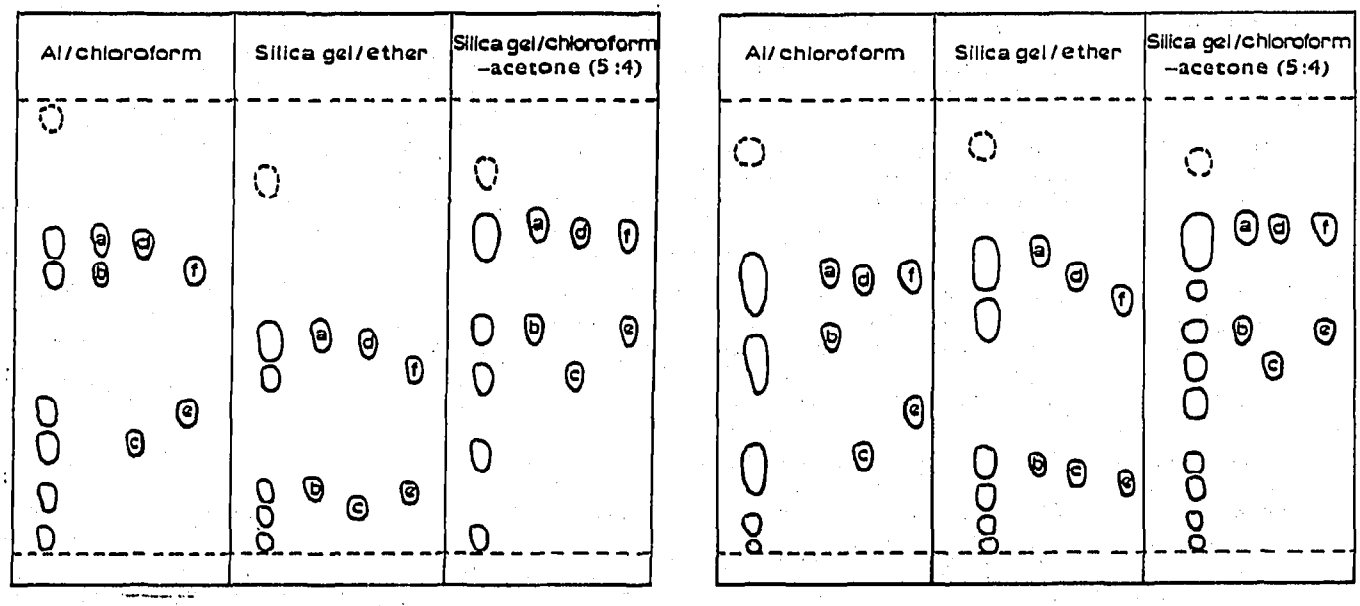

Fig. 4. Chromatograms of alkaloidal extracts of the leaves of *Mitragyna rotundifolia* from the Phillipines. a–f, see the legend to Fig. 2.

Fig. 5. Chromatograms of alkaloidal extracts of the leaves of *Mitragyna inermis* from Ghana. a–f, see the legend to Fig. 2.

TABLE II

DETERMININATION OF THE IDENTIFIED OXINDOLE ALKALOIDS IN THE BARK AND LEAVES OF *Mitragyna parvifolia* FROM INDIA

Two and three alkaloids present respectively.

(A) BARK

Alkaloid	*Amount (mg) in 10 g bark*		
	U.V. spectrophotometry	*Colorimetry*	*Densitometry*
Mitraphylline	6.25 8.75 6.28 6.28 6.21 6.41 6.32 6.31 6.21 6.18 Average 6.32	5.91 6.81 6.28 6.41 6.29 6.49 6.12 6.21 6.23 6.87 Average 6.36	6.27 6.52 6.01 6.28 6.28 6.21 6.81 6.48 6.49 6.61 Average 6.40
Isomitraphylline	5.28 5.12 5.28 5.18 5.01 5.01 5.01 4.92 4.28 4.98 Average 5.01	5.12 4.89 4.92 5.12 4.89 4.92 5.81 5.21 5.12 5.12 Average 5.17	5.21 5.32 5.41 5.41 4.92 4.81 4.82 5.21 5.41 5.89 Average 5.24

% alkaloid found and % coefficient of variation

Alkaloid	*U.V. spectrophotometry*		*Colorimetry*		*Densitometry*	
	% alkaloid	*% coefficient of variation*	*% alkaloid*	*% coefficient of variation*	*% alkaloid*	*% coefficient of variation*
Mitraphylline	0.003	2.02	0.064	5.32	0.064	3.66
Isomitraphylline	0.050	4.36	0.052	1.98	0.052	6.67

(B) LEAVES

Alkaloid	*Amount (mg) in 10 g leaves*		
	U.V. spectrophotometry	*Colorimetry*	*Densitometry*
Mitraphylline	9.25 9.05 9.02 9.38 9.18 9.01 9.01 9.29 9.41 9.32 Average 9.19	8.92 9.05 9.28 8.92 9.12 9.40 8.95 8.92 9.42 9.41 Average 9.14	9.58 9.41 9.82 8.92 8.81 9.28 9.21 9.21 9.41 9.29 Average 9.29
Isomitraphylline	7.29 7.12 7.42 7.81 7.29 7.39 7.49 7.49 7.21 7.33 Average 7.38	7.01 6.92 7.41 7.41 7.29 7.48 7.44 7.52 7.28 7.88 Average 7.36	6.92 7.48 7.48 6.99 7.18 7.81 7.58 7.28 7.92 7.28 Average 7.39

% alkaloid found and % coefficient of variation

Alkaloid	*U.V. Spectrophotometry*		*Colorimetry*		*Densitometry*	
	% alkaloid	*% coefficient of variation*	*% alkaloid*	*% coefficient of variation*	*% alkaloid*	*% coefficient of variation*
Mitraphylline	0.092	1.80	0.091	1.72	0.093	4.42
Isomitraphylline	0.074	4.32	0.074	3.46	0.074	4.75

TABLE III

DETERMINATION OF THE IDENTIFIED OXINDOLE ALKALOIDS IN LEAVES OF *Mitragyna rotundifolia* FROM BURMA

Five alkaloids present.

Alkaloid	*Amount (mg) in 10 g*		
	U.V. spectrophotometry	*Colorimetry*	*Densitometry*
Rhynchophylline	8.21 8.29 8.41 7.81 7.92 8.23 7.99 8.21 8.42 8.38	8.21 7.81 8.21 7.91 7.92 8.18 7.81 8.41 8.28 8.81	8.10 7.90 7.92 8.41 8.51 8.61 8.21 8.01 8.29 8.92
	Average 8.19	Average 8.16	Average 8.29
Isorhynchophylline	25.61 24.21 23.81 23.61 23.81 24.14 22.91 25.02 25.12 25.21		25.91 22.21 22.91 25.21 23.81 22.91 26.12 24.17 24.18 22.01
	Average 24.35		Average 24.00
Mitraphylline	4.28 4.71 4.21 4.18 4.42 4.53 4.62 4.38 4.28 4.39	4.65 4.09 4.19 4.45 4.45 4.39 4.52 4.38 4.32 4.32	4.09 4.08 4.81 4.28 4.28 4.42 4.39 4.32 4.52 4.62
	Average 4.39	Average 4.38	Average 4.38
Isomitraphylline	4.52 4.28 4.62 4.19 4.12 4.70 4.48 4.42 4.28 4.28		4.21 4.08 4.68 4.71 4.21 4.72 4.41 4.91 4.19 4.06
	Average 4.39		Average 4.42

% alkaloid found and % coefficient of variation

Alkaloid	*U.V. spectrophotometry*		*Colorimetry*		*Densitometry*	
	% alkaloid	*% coefficient of variation*	*% alkaloid*	*% coefficient of variation*	*% alkaloid*	*% coefficient of variation*
Rhynchophylline	0.082	2.56	0.082	3.80	0.083	3.96
Isorhyncho-phylline	0.24	2.80			0.24	2.02
Mitraphylline	0.044	8.10	0.044	2.98	0.044	5.12
Isomitraphylline	0.044	4.32			0.044	6.03

ASSESSMENT OF THE THREE METHODS

The accuracy and reproducibility of the results associated with each method depends to a considerable extent upon the behaviour of the substances on the thin layer, though the factors differ with each of the methods.

As with all elution techniques, the accuracy and reproducibility of the results obtained with ultraviolet spectrophotometric and colorimetric methods depend to

TABLE IV

DETERMINATION OF THE IDENTIFIED OXINDOLE ALKALOIDS IN THE LEAVES OF *Mitragyna rotundifolia* FROM THE PHILLIPINES

Seven alkaloids present.

Alkaloid	*Amount (mg) in 10 g*		
	U.V. spectrophotometry	*Colorimetry*	*Densitometry*
Rynchophylline	3.25 3.09 3.31 3.00 3.02 3.09 3.15 3.42 3.59 3.09 Average 3.20	2.91 3.14 3.21 3.41 3.09 3.08 3.09 2.99 3.48 3.21 Average 3.16	3.01 3.35 3.48 3.48 3.49 3.87 3.01 3.81 2.92 2.90 Average 3.33
Isorhynchophylline	12.81 11.91 11.28 11.01 11.07 12.82 10.99 12.52 12.51 11.81 Average 11.92		10.91 11.21 12.91 11.81 11.91 11.81 11.09 12.09 12.07 12.21 Average 11.80
Mitraphylline	1.88 1.28 1.21 1.51 1.28 1.30 1.52 1.25 1.26 1.51 Average 1.36	1.41 1.28 1.21 1.12 1.51 1.52 1.21 1.29 1.19 1.19 Average 1.29	1.52 1.52 1.88 1.21 1.88 1.49 1.81 1.68 1.32 1.23 Average 1.50
Isomitraphylline	3.25 3.22 3.18 3.51 3.52 3.28 3.21 3.20 3.09 3.08 Average 3.23		3.51 3.51 3.21 3.09 3.03 3.88 3.61 3.21 3.31 3.89 Average 3.43

% alkaloid found and % coefficient of variation

Alkaloid	*U.V. spectrophotometry*		*Colorimetry*		*Densitometry*	
	% alkaloid	*% coefficient of variation*	*% alkaloid*	*% coefficient of variation*	*% alkaloid*	*% coefficient of variation*
Rhynchophylline	0.032	6.54	0.032	5.55	0.033	10.44
Isorhynchophylline	0.119	6.23			0.118	5.45
Mitraphylline	0.014	9.36	0.013	10.87	0.015	15.75
Isomitraphylline	0.032	5.02			0.034	9.02

a considerable extent upon the recovery of the separated alkaloids from the adsorbent and the nature and amount of the non-alkaloidal material extracted from the adsorbent at the same time. Thus it is essential that alkaloids are well separated so that each can be removed from the adsorbent without contamination with adjacent alkaloids or with non-alkaloidal material which may be present in the plant extract. As far as the six alkaloids are concerned the experimental evidence shows that it is not always possible to effect complete separation, particularly when many alkaloids are present. However, if two adjacent substances which cannot be completely separated have

TABLE V

DETERMINATION OF THE IDENTIFIED OXINDOLE ALKALOIDS IN THE LEAVES OF *Mitragyna inermis* FROM GHANA

Eleven alkaloids present.

Alkaloid	*Amount (mg) in 5 g*		
	U.V. spectrophotometry	*Colorimetry*	*Densitometry*
Rhynchophylline	3.28 3.58 3.62 3.29 3.18 3.69 3.68 3.41 3.12 3.29	3.08 3.68 3.28 3.29 3.29 3.68 3.12 3.19 3.62 3.19	3.81 3.38 3.62 3.21 3.42 4.28 3.82 3.81 4.62 3.68
	Average 3.41	Average 3.34	Average 3.81
Isorhynchophylline	12.52 12.02 11.91 11.72 11.59 11.79 12.41 11.81 12.61 12.01	12.01 12.41 11.72 11.69 12.29 12.91 11.91 11.92 12.94 12.78	
	Average 12.07	Average 12.26	
Rotundifoline	5.29 6.01 5.34 5.48 5.28 5.61 5.61 5.71 5.72 5.49	5.71 5.21 5.29 5.61 5.91 5.21 5.21 5.29 5.41 5.51	
	Average 5.55	Average 5.44	
Isorotundifoline	1.92 2.21 1.92 1.95 2.31 2.01 2.21 2.09 2.19 2.21	1.97 1.92 2.29 2.01 2.21 2.31 1.92 1.91 1.99 2.01	2.87 2.81 2.84 2.41 2.91 1.92 1.99 1.99 2.87 2.81
	Average 2.10	Average 2.05	Average 2.39

% alkaloid found and % coefficient of variation

Alkaloid	*U.V. spectrophotometry*		*Colorimetry*		*Densitometry*	
	% alkaloid	*% coefficient of variation*	*% alkaloid*	*% coefficient of variation*	*% alkaloid*	*% coefficient of variation*
Rhynchophylline	0.068	6.25	0.067	6.98	0.076	10.52
Isorhynchophylline	0.24	0.94	0.25	1.13		
Rotundifoline	0.11	1.82	0.11	1.51		
Isorotundifoline	0.042	6.8	0.041	7.57	0.088	15.92

different spectra it may be possible to determine the amount of each present by measuring the absorbance at two suitable wavelengths and making the necessary calculation. Indeed, differential spectrophotometry has been applied as described in Part I to estimate the amount of alkaloids present when complete separation of several alkaloids cannot be achieved.

With regard to extractable impurities, this is a more significant problem with

the ultraviolet spectrophotometric method than with the colorimetric method since most of the impurities absorb light in the ultraviolet wave band rather than in the visible region.

The extent to which the impurities occur depends upon the nature of the adsorbent, the thickness of the layer and position of the layer from which the impurities are extracted so that care must be taken to extract an equal area of the adsorbent layer from the same plate and at the same R_F value as the substances concerned in order to compensate for the impurities in the solution containing the substances. These precautions are not so essential with the colorimetric method since the evidence shows that variation in the absorption due to impurities extracted from different plates or from different positions on the same plate are insignificant.

Problems associated with elution do not affect the densitometric method and this gives certain advantages to this method in as much as it is an extremely simple and quick method. Further, incomplete separation of the substances is not a major disadvantage since the scanning can be made in a direction which is perpendicular to the line of development or it may be possible to complete the curves by geometric methods.

A major factor of importance with densitometry however, is the shape, position and size of the spots, a factor which has no significance for the two elution techniques. The shape, position and size of the spots depend upon the nature of the adsorbent, the thickness of the layer, the solvent system and upon the shape and size of the initial application. Thus with the densitometric method the choice of the chromatographic system is limited and considerable care must be taken to ensure that each initial application is of constant size and shape and this calls for some skill on the part of the operator.

The elution techniques have an advantage over the densitometric method in as much as the latter required larger loads for quantitation, whereas by using larger cells with the ultraviolet spectrophotometric method and colorimetric method, much smaller quantities can be used.

As far as accuracy of results are concerned there is no significant difference between those obtained by the three methods, though it must be said that ultraviolet spectrophotometry is the most accurate and the densitometry method the least accurate. The percentage of coefficient of variation of the ultraviolet spectrophotometric results is much smaller than that of the colorimetric results while that of the densitometric results are the highest. This means that in practice, more determinations must be made by the densitometric method in order to be certain of obtaining satisfactory results.

These facts are not altogether surprising when consideration is given to the actual techniques involved. With the ultraviolet spectrophotometric technique, the major source of variation is the elution technique *i.e.* the method of removal of the adsorbent from the plate and the choice of the solvent. This is a physical operation and as such can be adequately controlled. Provided that the entire procedure is carried out with the reasonable care necessary for analytical work, there should be little variation in the results obtained. With colorimetry there are, in addition to the elution procedure, chemical reactions which depend upon the concentration and the quantity of the reagent and the time and temperature necessary for the reaction to proceed to completion. Although it is possible to standardise the experimental conditions, it is

not possible to control them entirely, so that some variation in the result must be expected. With densitometry no elution is involved and only one chemical reaction takes place. However, the fact that there is no elution of the colour complexes, means that the amount of substance present is not determined in solution but by measuring the amount of light absorbed by the substance adsorbed on a solid phase. This adds considerably to the likely variations. The factors have already been discussed in relation to the behaviour of the incident light when it strikes the adsorbent and adsorbed colour complex. The most important factors are thickness of the layer, method of scanning, size, shape and position of the spots and also the particle characteristics of the adsorbent so that the thin-layer chromatogram plays a major role in the actual determination of the amount of substance present. This is not so with the other two methods. Although it is possible to standardise the experimental conditions, they are less easy to control so that considering all the possible sources of error it is remarkable that this method gives such accurate and reproducible results.

With ultraviolet spectrophotometry only one calibration is necessary for each alkaloid in order to calculate amounts of alkaloids present. The calibration curves are quite independent of TLC methods used for the separation of the alkaloids. This is almost true with the colorimetric method but different calibration curves are necessary with different adsorbents. Some advantage accounts from this in as much as the results are independent of the solvent systems used or the distance travelled by the solvent front. With the densitometric method any change in the chromatographic system requires the preparation of a different calibration curve. Calibration curves can be used with all the three methods but with the colorimetric method and certainly with the densitometric method, due to high % coefficient of variation, it is better to use the regression line analysis data—in fact with the latter method it is essential to use this means of calculating the results.

These methods have been applied to the analysis of the oxindole alkaloids content of five Mitragyna plant materials. The plant material was selected because all six alkaloids were involved, some of them being associated with many other alkaloids, some of them in mixtures which could be easily separated by TLC and some of them which could not be separated satisfactorily either from each other or from other alkaloids which might be present. It will be seen that as the number of alkaloids present in the plant material increases the results obtained by the three methods become less consistent and the % coefficient of variation increases. The increased value of the % coefficient of variation is more marked with the densitometric method and this can only be explained by the fact that since this method depends so much on the nature of the alkaloidal spot the presence of other alkaloids may have caused some distortion in the shape, size and R_F value. The choice of method to be employed for the analysis of plant material must depend on many factors not the least important being the complexity of the mixture. With mixtures of four components which can easily be separated by TLC there is little to choose between the three methods, provided the limitations of each method are known.

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