THE ESTIMATION OF TROPANE ALKALOIDS IN VEGETABLE DRUGS BY PAPER PARTITION CHROMATOGRAPHY, WITH SPECIAL REFERENCE TO THE ALKALOIDS OF *DATURA SANGUINEA*

BY R. E. A. DREY and G. E. FOSTER

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ALTHOUGH the estimation of tropane alkaloids in plant products has been investigated extensively, no generally accepted method of determination of the individual alkaloids is yet available, and the problem continues to be the subject of research. Assay processes for the official solanaceous drugs estimate the total alkaloidal contents, expressed as hyoscyamine, while, following the early chromatographic work of Roberts and James[1] and of Trautner and Roberts[2], Evans and Partridge[3,4] devised a technique for separation and estimation of hyoscine and hyoscyamine using kieselguhr columns, which have been used similarly by Schill and Ågren[5].

These methods suffer from three main disadvantages. Firstly, there is no provision for establishing with certainty that complete separation of the alkaloids has been achieved since they are estimated titrimetrically. Secondly, if either of the alkaloids is present in small amount only, its estimation by adsorption on a column, followed by elution with an appropriate solvent and titration, is subject to a relatively large error. For example, the titration equivalent to 10 g. of drug containing 0·01 per cent. of hyoscine is only 0·165 ml. of 0·02N acid. Thirdly, when a mixture of tropane alkaloids is separated on a column in a routine analytical laboratory account cannot be taken of the possible presence of alkaloids other than hyoscine and hyoscyamine, as such work presents considerable experimental difficulties and is time consuming. In this connection Hills and Rodwell[6], who investigated the alkaloids of *Duboisia myoporoides* using the column technique, have stated that alkaloids other than hyoscine can account for 30 to 90 per cent. of the total alkaloidal content and that, even in samples yielding maximal amounts of hyoscine, it may not be possible to account for more than half of the total alkaloid.

In view of the above considerations, it was felt that a more specific assay process might be developed by paper partition chromatography using buffered papers such as employed by Brindle, Carless and Woodhead[7] for the separation of hyoscine and hyoscyamine. By this method it is possible to identify all non-volatile alkaloids which differ in R_F value from hyoscyamine and hyoscine, providing that an adequate range of reference alkaloids is available.

Accordingly, we have applied ourselves to this problem and have devised an assay process based upon paper partition chromatography. Our results are described in the present paper, which gives details of the method and its application to the assay of certain solanaceous drugs.

Preliminary Experiments

In our preliminary investigations we followed closely the work of Brindle *et al*[7]. Suitable volumes of a solution of alkaloids in chloroform were applied to filter paper buffered to *p*H 7·4 and the bases separated chromatographically by running with water-saturated *n*-butanol. After development of the chromatograms the alkaloidal spots were located by treatment with Dragendorff's reagent. Some estimate of the relative amounts of alkaloids could be obtained by comparison of the intensities of the spots, but it was considered that removal of the alkaloids from the paper and subsequent assay would afford greater precision. For this purpose, reference chromatograms carrying standard spots of hyoscine, hyoscyamine and other appropriate alkaloids were developed simultaneously with the chromatograms carrying the test material. Only the reference chromatograms were treated with Dragendorff's reagent and the spots, so obtained, employed to mark the alkaloid carrying areas on the test chromatograms. The untreated papers were then cut into strips carrying the individual alkaloids, which were then removed by elution with ethanol. Suitable volumes of eluate were freed from interfering buffer salts and the alkaloidal contents determined by the Vitali-Morin reaction, which unfortunately is not given by all tropane alkaloids.

In experiments, using a standard solution containing hyoscine and hyoscyamine, we were unable to recover completely the alkaloids by the above chromatographic process, in spite of the fact that the paper strips after elution of the alkaloids gave no reaction with Dragendorff's reagent. Similar losses have been reported in separations of amino-acids[8], thiohydantoins[9], and ergot alkaloids[7]. We observed, however, that in the case of these alkaloids any such losses occurred substantially in proportion to the amount of alkaloid present. Typical results are summarised in Table I.

TABLE I

Assay	Alkaloid	Weight placed on chromatogram	Weight recovered by assay
1	Atropine	0·10 mg.	0·062 mg.
	Hyoscine	0·10 mg.	0·0665 mg.
2	Atropine	0·10 mg.	0·076 mg.
	Hyoscine	0·10 mg.	0·073 mg.

It therefore became possible to obtain satisfactory results by determination of the mixed alkaloids by classical methods, followed by chromatographic separation, elution and colorimetric estimation of the individual alkaloids. The chromatographic results may be used to calculate the relative proportions of the individual alkaloids and these figures, in conjunction with the total alkaloidal content, give the percentages of hyoscine and hyoscyamine present. This method of assay has been successfully applied to solanaceous plants containing hyoscine and hyoscyamine together with very small amounts of other alkaloids.

Determination of Total Alkaloids

Numerous methods have been described for the determination of total tropane alkaloids in plant materials. In our work the following process, based upon Allport's[10] procedure, has been used.

Transfer 10 g. of drug in No. 60 powder to a 150-ml. beaker. Add 20 ml. of ethanol (95 per cent.), mix well, allow to stand for 10 minutes and treat with 3 ml. of dilute ammonia solution. Mix well and, after 10 minutes, add with stirring 50 ml. of chloroform and set aside for a further 15 minutes. Transfer the mixture to a percolator plugged with cotton wool, collecting the percolate in a graduated 350-ml. separating funnel. Gently compress the contents of the percolator with a glass plunger and continue percolation with chloroform until complete extraction of the alkaloid is achieved (about 200 to 250 ml. required). Extract the percolate with 4 quantities (30 ml.) of 6 per cent. acetic acid in 5 per cent. ethanol, and extract the mixed acid layers with three quantities (25, 20 and 15 ml.) of chloroform, washing each chloroform extract with the same 20 ml. of 6 per cent. acetic acid. Reject the chloroform extracts and add the washings to the main acid solution. Render distinctly alkaline with dilute ammonia solution and extract 4 times with chloroform (25 ml.), washing each extract with the same 20 ml. of water. Transfer the mixed extracts to a wide-necked flask and remove the solvent by evaporation. Add 3 ml. of absolute ethanol, again evaporate to dryness and dry the residue at 100° C. for 30 minutes to remove volatile bases. Dissolve the residue in 10 ml. of 0·1N sulphuric acid, warming the flask to assist solution, and titrate the excess of acid with 0·02N sodium hydroxide, using methyl red as indicator.

Each millilitre of 0·02N sulphuric acid is equivalent to 0·005787 g. of hyoscyamine or 0·006067 g. of hyoscine, the actual factor used for calculating the total alkaloidal content being selected according to which alkaloid predominates, as shown by chromatographic examination.

CHROMATOGRAPHY

REAGENTS

Sörensen's Phosphate Buffer Solution, p*H* 7·4

Sodium Phosphate, anhydrous, Na_2HPO_4	— 0·76 g.
Potassium Dihydrogen Phosphate, Anhydrous, KH_2PO_4	— 0·18 g.
Water	to 100 ml.

Buffered Papers (p*H* 7·4)

Cut sheets of Whatman's filter paper No. 1 into strips 22½ in. long and of suitable widths. Immerse the strips in phosphate buffer solution *p*H 7·4 for about 5 minutes. Remove surplus buffer by draining and dry the strips in air for not less than 5 hours.

Dragendorff's Reagent[11]

(a) *Stock Solution.* Mix 0·85 g. of bismuth subnitrate with a mixture of 40 ml. of water and 10 ml. of glacial acetic acid. Add 50 ml. of 50 per cent. potassium iodide solution and shake the liquid until all solid matter has dissolved. Store the solution in an amber glass bottle.

(b) *Reagent.* Add 10 ml. of stock solution to 20 ml. of glacial acetic acid and adjust the volume to 100 ml. with water. The reagent should be freshly-prepared before use.

Standard 1 : 1 *Atropine-Hyoscine Solution in Chloroform*

Dissolve 0·723 g. of hyoscine hydrobromide in 15 ml. of water, render alkaline with 2 ml. of dilute ammonia solution and extract 4 times with chloroform (25, 25, 20 and 20 ml.). Wash each chloroform extract with the same 12 ml. of water and filter through a dry filter into a graduated flask containing 0·500 g. of atropine. Adjust the volume to 100 ml. with chloroform and mix well. To prepare a 0·2 per cent. solution dilute the above 1 per cent. solution in the ratio of 1 to 5 with chloroform.

Separation of Alkaloids

Add 3 ml. of dilute ammonia solution to the titrated solution, obtained during the estimation of the total alkaloids, and transfer to a separating funnel, rinsing the flask successively with two quantities of water (10 ml. and 5 ml.) and one quantity of chloroform (25 ml.) and adding the washings to the bulk. Shake, allow the mixture to separate and remove the chloroform layer and wash it with 10 ml. of water. Continue the extraction with 3 quantities, each of 20 ml., of chloroform, washing each extract with the same 10 ml. of water. Remove the solvent from the mixed extracts by evaporation, using a flask with a ground joint to enable it to be well closed with a glass stopper. Add 3 ml. of ethanol to the residue and evaporate to dryness on a water bath.

Dissolve the residue in an accurately measured volume of chloroform (a volume of 6x to 10x ml. is suitable, where x = per cent. of total alkaloids in sample of drug and using at least 1 ml. of chloroform). By means of a micrometer syringe transfer appropriate volumes of the solution to the starting lines of buffered paper strips, according to the scheme shown in Table II. When more than 0·03 ml. of solution is required, apply no more than 0·03 ml. at a time and allow each portion to evaporate before the next application. Develop the chromatograms by irrigating with water saturated *n*-butanol in an atmosphere saturated with water and *n*-butanol, according to the technique of Consden, Gordon and Martin[12].

TABLE II

VOLUME OF STANDARD 1 : 1 ATROPINE-HYOSCINE SOLUTION IN CHLOROFORM APPLIED TO PAPER-STRIP

Buffered Paper-Strip	1 per cent. solution	0·2 per cent. solution	Volume of Alkaloidal Extract applied to Paper-Strip
1.	0·02 ml.	—	0·005, 0·010, 0·015, 0·020 ml.
2.	0·02 ml.	—	0·025, 0·030, 0·040 ml.
3.	—	0·04 ml.	0·020, 0·030, 0·040 ml.
4.	—	0·04 ml.	0.060, 0·080, 0·100 ml.
5.	—	—	0.020, 0.020 ml.
6.	—	—	0·040, 0·040 ml.
7.	—	—	0·060, 0·060 ml.
8.	—	—	0·090, 0·090 ml.

Allow the chromatograms to develop overnight and remove the paper strips from tanks and allow them to dry in the air at room temperature for not less than $2\frac{1}{2}$ hours. Cut strips 5, 6, 7 and 8 length-wise into equal portions, and immerse one half of each, together with strips 1, 2, 3 and 4 in Dragendorff's reagent. Allow to drain and dry in the air.

Approximate Assay

An approximate but useful guide to the hyoscyamine and hyoscine contents of a sample, which will also serve to determine the dilution required for the subsequent colorimetric estimation, may be obtained by matching the "unknown" spots on strips 1 to 4 with the corresponding "standard" spots. If it is found that one alkaloid predominates and the other is present to the amount of no more than 5 per cent. of the total, it will be unnecessary to proceed beyond this stage, owing to the difficulty of estimating very small amounts of alkaloid by the Vitali-Morin reaction. Alkaloids present in minor amounts are estimated semi-quantitatively by visual inspection of the chromatograms.

In addition to the above evaluation of hyoscyamine and hyoscine strips 4 and 8 should be carefully examined for the presence of other alkaloids. In this connection it should be noted that hydrolysis products (tropine and oscine) from the alkaloids, afford relatively weak pink or purple spots, in contrast to the characteristic orange spots given by the parent alkaloids[13]. Similar pink or purple spots are given by norhyoscyamine, pseudo-hyoscyamine, noratropine and pseudo-atropine.

Any spots differing in R_F value from those of hyoscyamine and hyoscine may be identified and estimated semi-quantitatively by running suitable amounts of chloroform extract containing the "unknown" alkaloid or alkaloids alongside small volumes of standard solutions of each suspected alkaloid.

Determination of Hyoscyamine and Hyoscine

Place the reagent-treated portions of strips 5, 6, 7 and 8, against their respective untreated portions and cut out the alkaloid bearing areas of the latter, along lines across the strips midway between neighbouring spots. Remove the alkaloid from each section by elution overnight with ethanol, collecting the eluate in a 5 cm. diameter glass dish (a large desiccator arranged as a chromatographic chamber forms a suitable apparatus for this work). At the end of each run, dry the paper sections and test them for absence of alkaloid with Dragendorff's reagent.

Adjust each eluate to a suitable volume with ethanol and transfer an amount, equivalent to 0·06 to 0·12 mg. of alkaloid, to a small glass dish and evaporate to dryness. Extract the residue with small volumes of chloroform and filter each extract through a No. 1 Whatman paper into a second glass dish. Evaporate the filtrate to dryness, moisten the residue with 1 ml. of 6 per cent. acetic acid and again evaporate to dryness. Complete the colorimetric determination by the Vitali-Morin reaction, as described by Allport and Wilson[14], but using 0·5 per cent. methanolic potassium hydroxide solution, as recommended by Ashley[15], for development of the colour. Compare the standard test solutions by measuring their light absorption in 1 cm. cells at 550 mμ.

Assay of Solanaceous Drugs

The chromatographic assay has been applied to the examination of *Datura sanguinea*[16], a species of *Datura* which has been little studied. Our

results on specimens of the plant grown from seed at Dartford and also imported from S. America are summarised in Table III. While our chemical work has been in progress, Dr. A. C. C. Newman and Dr. J. M. Rowson have been carrying out a botanical study of the species, of which they hope to publish in due course a full description.

TABLE III

ASSAY RESULTS FOR SAMPLES OF *Datura sanguinea*

Sample		Alkaloidal content, with reference to sample as received			
		Total Alkaloids per cent.	Hyoscine per cent.	Hyoscyamine and Atropine per cent.	Other Alkaloids per cent.
1952 Harvest:					
First year	Dry Leaf	0·511	0·49	0·02	
	" Stalk	0·311	0·28	0·03	
Seedlings	" Root	0·542	0·21	0·29	Unidentified Alkaloid "A" 0·04
1952 Harvest:	Dry Leaf	0·409	0·41	Not more than 0·004	
Plants from cuttings					
Taken in 1951	" Stalk	0·296	0·27	0·02	
	" Root	0·762	0·18	0·42	Unidentified Alkaloid "A" 0·16
1952 Harvest:					
Tops from 1951 Plants	Dry Leaf	0·342	0·34	*nil*	
Wintered under Glass	" Stalk	0·302	0·29	0·014	
Specimens	Dry Leaf	0·267	0·27	nil	Valeroidine not more than 0·004
received from	" Flowers	0·618	0·55	0·04	Valeroidine 0·03; unidentified Alkaloid "A" not more than 0·004
S. America (Ecuador)	" Bark	0·257	0·19	0·02	Valeroidine 0·04; unidentified Alkaloid "B" 0·01; unidentified Alkaloid "A" not more than 0·007
	" Seeds	0·172	0·17	nil	Valeroidine not more than 0·003

NOTE:

REPRESENTATIVE R_F VALUES (Water-saturated *n*-butanol; downward runs)

Hyoscine	0·84
Valeroidine	0·70
Hyoscyamine and Atropine	0·61
*Unidentified Alkaloid "A"	0·49
*Unidentified Alkaloid "B"	0·33

* *Not* noratropine, norhyoscyamine, pseudo-Hyoscyamine, Meteloidine, Nicotine, Oscine, Tropine, or Valeroidine

Samples of leaves of some *Duboisia* species have also been examined for alkaloids and these results are included in Table IV.

TABLE IV

ASSAY RESULTS FOR SAMPLES OF LEAVES OF *Duboisia spp.*

Sample	Total Alkaloids	Hyoscine per cent.	Hyoscyamine and Atropine per cent.	Other Alkaloids per cent.
Duboisia myoporoides	1·60	1·4	0·1	0·1 Valeroidine
Duboisia leichhardtii No. 1	2·10	0·2	1·9	—
Duboisia leichhardtii No. 2	4·07	0·6	3·5	—

DISCUSSION

It will be noted that the proposed method of assay depends upon the proportions of the individual alkaloids, as estimated colorimetrically after chromatographic separation, being the same as in the total alkaloids extracted from the drug. This will be so if the losses, during chromatography, occur in proportion to the amounts of alkaloids applied to the paper. We are satisfied that, in our experiments, this condition was

substantially fulfilled, for close agreement was obtained between the approximate assay figures, estimated by matching of the spots located with Dragendorff's reagent, and the subsequent figures afforded by colorimetric assay using the Vitali-Morin reaction.

Volatile alkaloids in the drug may give rise to error, and care must be taken that these bases are removed from the alkaloids, obtained in the estimation of total alkaloids, before titration. For the plant products used in the present work, heating the alkaloids for 30 minutes at 100° C. was adequate but longer heating may be necessary for other materials. When the sample available is small it may not be possible to titrate the total alkaloids and in such cases the colorimetric assay of Allport[10] may be used. Any fat-containing samples, such as seeds, should be defatted with light petroleum before carrying out the determination of total alkaloids.

Some workers, when using paper chromatography, equilibrate the paper strips with the solvent saturated atmosphere prior to commencement of the runs. In the present work this was found unnecessary. Other factors having little effect upon the development of the chromatograms were (1) minor variations in the composition of the phosphate buffer solution, (2) slight variations in the filter paper used; both Whatman's No. 1 and No. 2 papers gave satisfactory results, and (3) the period of immersion of the papers in the buffer solution. Citrate buffer of *p*H 7·4 was unsuitable for quantitative work owing to large losses of alkaloid. Both downward and upward runs are suitable, but the descending technique was mainly employed in view of the greater distance travelled by the solvent front and alkaloidal spots in a given time. Both A.R. and commercial grades of *n*-butanol have been successfully used and no special precautions were taken to maintain the tanks at constant temperature during the course of runs. Glass tanks have been used throughout the work; in some preliminary experiments using a large ebonite tank the results were unsatisfactory.

The presence of buffer salts as well as traces of moisture is liable to cause unduly rapid fading of the colour in the Vitali-Morin reaction. For this reason it is important that the ethanolic eluates should be freed from buffer salts by extraction with chloroform, as described in the assay. The colour reaction must also be carried out in thoroughly clean and dry apparatus.

Examination of *Datura sanguinea* gave interesting results. The aerial parts contain sufficient hyoscine to make the plant a possible commercial source of the alkaloid. Besides hyoscyamine a number of minor alkaloids, of which valeroidine was identified, are present. It should be noted, however, that under the experimental conditions given above, hyoscine and tigloidine have almost identical R_F values and consequently it was not feasible to take into account the possible occurrence of the latter. Work is in progress with a view to separating tigloidine and hyoscine on two-dimensional paper chromatograms using a suitable pair of solvents. In preliminary experiments a mixture of chloroform, carbon tetrachloride and light petroleum showed promise as a secondary eluent. There is also

no separation of atropine and hyoscyamine and these alkaloids are determined together.

Hyoscyamine and hyoscine predominate respectively in the root and aerial parts of *Datura sanguinea*. Our results with *Duboisia* species confirm known data regarding the alkaloids present.

There has been a revival of interest during recent years in alkaloid biogenesis and much research has been done with solanaceous plants. It is hoped that the present work may find some application in this important field.

Summary

1. A study has been made of the application of paper partition chromatography to the separation and estimation of tropane alkaloids.
2. The separation of the alkaloids on buffered paper and the subsequent elution of the individual alkaloids is described.
3. The estimation of the recovered alkaloids has been carried out by the Vitali-Morin reaction.
4. The chromatographic results taken in conjunction with the total alkaloidal content of a plant material has been used to determine the hyoscine and hyoscyamine contents of the sample.
5. The proposed assay has been applied to samples of *Datura sanguinea* and of *Duboisia* species.

We wish to thank Dr. A. C. C. Newman for supplying the specimens of vegetable drugs used in this investigation. We also wish to thank the Directors of The Wellcome Foundation for permission to publish our results.

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DISCUSSION

The paper was presented by Dr. G. E. Foster.

Dr. W. Mitchell (London) said he was particularly interested to learn that valeroidine had been detected in *Datura sanguinea*. That alkaloid along with tigloidine, poroidine and *iso*poroidine, was first isolated from *Duboisia myoporoides* by Martin in about 1935, and he (Dr. Mitchell) subsequently established their structures. The hydrobromides

of tigloidine, poroidine and *iso*poroidine could readily be extracted from reasonably concentrated aqueous solutions of the total hydrobromides by means of chloroform. Valeroidine was not extracted because its hydrobromide was even more soluble in water. By applying that technique could not the authors make a preliminary separation of the total alkaloids and apply the material so extracted to a separate chromatogram? That should certainly eliminate any doubt as to tigloidine being obscured by the hyoscine spot. Was it possible that either of the unknown alkaloids "A" and "B" referred to in the paper was the base "D" that he isolated from *Duboisia leichhardtii* and believed probably to be *iso*valeryltropene, or could they be either poroidine (*iso*valerylnortropene) or *iso*poroidine (α-methylbutyrylnortropene) from *D. myoporoides*?

DR. W. C. EVANS (Nottingham) said he desired to correct the impression given by the authors that the method of assaying the drugs was an absorption process. It was not. It had a purely partition effect. Judging by the inability of the authors to obtain complete recovery of their alkaloids, it would seem that absorption on cellulose was a far greater problem than absorption on kieselguhr. How did the authors identify their valeroidine? Was it by the preparation of crystalline derivatives or by the measurement of the R_F value? That value was liable to be misleading, since a number of alkaloids gave similar R_F values. In that connection he asked whether the authors could give figures for the other alkaloids which were listed at the bottom of Table III as not being identical with the positions of the unidentified alkaloids A and B.

MR. H. B. WOODHEAD (Manchester) said that the recoveries in Table I were said to be typical results, but he wondered whether there was any reason for the recoveries in assay 1 being different from those in assay 2. At what stage of the process was alkaloid being lost?

DR. J. B. STENLAKE (Glasgow) said that Shute in *Nature* had shown that when alkaloids and acids are chromatographed on paper with water as solvent, ion exchange occurred between the ions of the alkaloid or acid and certain ions which were present either in or on the surface of the paper; losses might be explicable on that basis. There was no doubt that under the *p*H conditions which the authors were using any such ion exchange would be minimised, but if such a process occurred, Shute had also shown that there was a definite tailing of spots, and he asked the authors whether they had observed any tailing which might be indicative of ion exchange. It might be possible at least to repress ion exchange of that type, by saturating the paper in a salt solution such as potassium chloride.

MR. J. E. CARLESS (Manchester) said that he would have thought it would have been better to show a wider range of alkaloidal loads than the 0·1 mg. of alkaloid in each case as shown in Table I.

MR. R. E. A. DREY, in reply, said that after the experimental work described had been completed, the effect on their method of the solubility in chloroform of tigloidine hydrobromide was investigated. It was concluded that if tigloidine and also poroidine and *iso*poroidine were to

be determined quantitatively in solanaceous plant materials it would be necessary to adjust the method of extraction, described under "determination of total alkaloids," so that no chloroform liquor or washings would be rejected. It would also be necessary for a second chromatographic separation to be carried out, when tigloidine hydrobromide, and presumably the hydrobromides of poroidine and *iso*poroidine, would be found to have moved with the solvent front, leaving the hydrobromides of the other tropane alkaloids on or close to the starting line. They were unable to identify the alkaloids "A" and "B" with poroidine, *iso*-poroidine or "base D," as no samples of the latter alkaloids were available for comparison. They did not attempt to identify valeroidine beyond running a "marker" spot of pure valeroidine alongside the alkaloidal extract obtained from the vegetable material, in view of the small quantity of alkaloid available. Noratropine, norhyoscyamine and pseudo-hyoscyamine gave relatively weak pink spots of R_F slightly less than that of hyoscyamine. Meteloidine gave very weak ill-defined spots. Tropine and hyoscine gave weak pink spots at a relatively short distance from the starting point, whilst nicotine (only found in *D. myoporoides*) had an R_F value identical with that of hyoscine. No explanation for the losses of alkaloid on the paper (Table I) could be offered apart from the more obvious explanation of losses by retention of alkaloidal matter on the paper and decomposition of alkaloid in the course of runs. It was particularly difficult to attribute such losses to any specific cause, as they might occur both during chromatographic separation and in the course of subsequent elution. Little trouble was experienced due to "tailing."