in the rats. Dichloro substitutions on the 2,4 and 3,4 positions of the phenyl ring of 1-phenyl-2-aminopropane structures, at the doses used, inhibited the behavioral stimulation effects seen with the parent structure. The general lack of responding during the TO contingency after administration of the dichloro-substituted compounds showed that the stimulus control of the changing light sources was maintained. The lowered shock ratios seen in the rats with the *dl* and *d*-1-(3,4-dichlorophenyl)-2-aminopropanes could be termed as an increase in efficiency since the avoidance ratios were only slightly raised, *i.e.*, more protection for the same work output.

Although the *d*-amphetamine and methamphetamine response rates were approximately 30% less at the end than at the start of the study, the mean avoidance response-rate ratios and TO responses with the chloro-substituted compounds were well below those of the amphetamine compounds. For the most part, the chloro compound data fell close to or within the normal standard deviation limits. The difference in the amphetamine data could be due to a development of tolerance to the 1-phenyl-2-aminopropane structure or more probably to a gradual strengthening of the behavior over the period of time covered by the investigation to provide a resistance to drug action.

SUMMARY

Some 1 chlorophenyl-2-aminopropanes have been compared with *d*-amphetamine and methamphetamine for effects on avoidance and discrimination behavior in rats. The doses used were the two lowest that caused suppression of an appetitive-controlled behavior.

After *d*-amphetamine, methamphetamine, and *dl* and *d*-1-(4-chlorophenyl)-2-aminopropane, rats showed increased avoidance response rates as well as responding during time-out periods when it was not necessary. This latter effect was considered as a loss of ability to discriminate. The rats when given dichlorophenyl-2-aminopropanes showed little or no change from normal in avoidance or discrimination behavior.

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Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don) XV

Analysis of Vinca Alkaloids by Thin-Layer Chromatography

By NANCY J. CONE, RUTHANNE MILLER, and NORBERT NEUSS

Thin-layer chromatography of various Vinca alkaloids is described. Adsorbents, solvent systems, and their influence on the R_f values are discussed. R_f values for 26 Vinca alkaloids are given.

THE CLINICAL use of vinblastine[1] (2), a member of a new class of oncolytic alkaloids (3) from the ornamental shrub *Vinca rosea* Linn. (*Catharanthus roseus* G. Don), prompts us to publish the results of the use of thin-layer chromatography (TLC) in the identification of this and other alkaloids obtained from this plant in our laboratories (4). The laborious fractionations and repeated chromatographies required in the preparation of several of these alkaloids were greatly facilitated by continuously monitoring fractions with TLC. The technique was also found to be extremely useful in the course of chemical work leading to the structure elucidation of catharanthine (5) and vindoline (6) as well as the dimeric alkaloids vinblastine (VLB) and leurocristine[2] (LCR) (7). Finally, the behavior of compounds on TLC was often found to be a most efficient criterion of purity. This was especially true in the case of several dimeric alkaloids (8).

The advantages of TLC over paper chromatography, however, should not prevent us from mentioning some of the rules of paper chromatographic technique which also apply here. One cannot, for example, use indiscriminantly the results obtained on pure compounds to identify these components in a crude mixture of alkaloids or complex reaction mixtures without using

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Paper XIV in the series is being published elsewhere (1).

[1] Vinblastine sulfate marketed as Velban by Eli Lilly and Co.

[2] The generic name of leurocristine is vincristine.

TABLE I.—SOLVENT SYSTEMS

System No.	Components
1	Alumina Chloroform-Ethyl Acetate (1:1)
2	Silica Ethyl Acetate-Absolute Ethanol (3:1)
3	Alumina Ethyl Acetate-Absolute Ethanol (3:1)
4	Alumina Benzene (100%)
5	Alumina Chloroform (100%)
6	Silica Chloroform (100%)
7	Alumina Benzene-Chloroform (3:1)
8	Silica Ethyl Acetate-Absolute Ethanol (1:1)
9	Silica 100% Ethyl Acetate
10	Alumina 100% Ethyl Acetate
11	Silica, prepared using 0.5 N KOH instead of H_2O Ethyl Acetate-Absolute Ethanol (1:1)

several solvent systems, different adsorbents, and appropriate developing reagents. Rules similar to those in paper chromatography apply also to the determination of R_f values, preparation of chambers, etc. Finally, additional criteria of identification by various physical methods should always be supplemental whenever this is practical.

Since a bibliography on TLC, which also includes several excellent reviews (9), deals with practically all the phases of this technique and its applications, we shall limit our discussion to a description of the experimental details necessary for the reproducibility of our results.

Preparation of Plates

Alumina.—Our studies were conducted on plates prepared with Fluka[3] special alumina for thin-layer chromatography. Thirty grams of this adsorbent were shaken with 75 ml. of distilled water for 30 seconds, poured into the Desaga[4] applicator, and spread on plates. They were then allowed to dry in the air for about 1 hour and activated in an oven at 110–120° for 15 minutes. Immediately afterward, the plates were allowed to cool in a desiccator and stored for varying lengths of time in a wooden cabinet over drying reagents (Drierite and calcium chloride).

Silica.—The plates were prepared using Merck[4] silica gel in the following manner: Twenty-five grams of the adsorbent was shaken with 50 ml. of distilled water for 30 seconds and applied using the applicator. They were used after having been allowed to air dry for at least 2 hours. These plates are also stored in a wooden cabinet over drying reagents.

Application of Alkaloids

Most of our studies, except where noted, were done using a concentration of 50 mcg. of alkaloid per spot (5 μl. of a 10 mg./ml. solution) in a suitable solvent (in most cases CH_2Cl_2 or $CHCl_3$).

When mixtures of alkaloids were investigated, quantities of 100–200 mcg. were used. Spots were marked 1.5 cm. from the lower edge of the plates. No more than nine samples were applied per plate (10 × 10 cm.).[4]

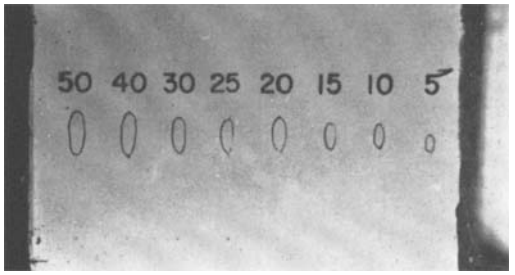
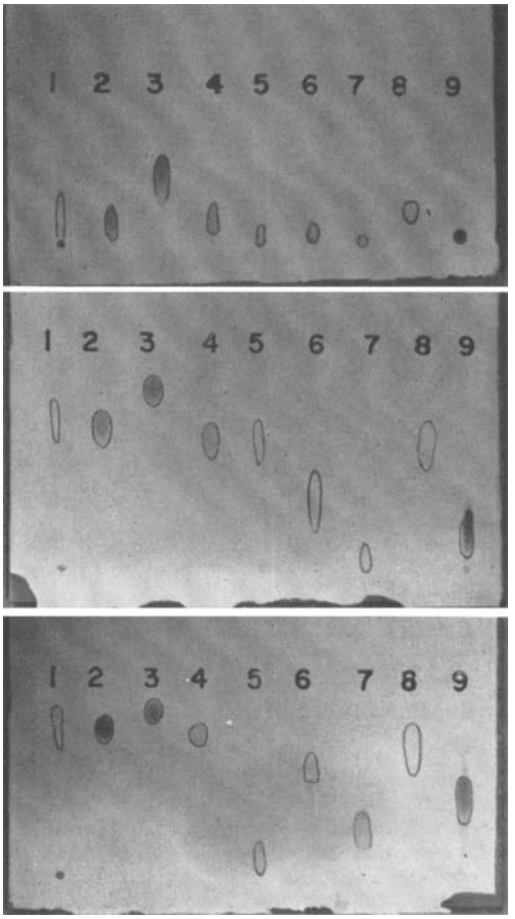


Plate 1—Different concentrations of catharanthine (5–50 mcg): adsorbent silica; solvent system, ethyl acetate.



Plates 2, 3, 4—Dimeric alkaloids on silica adsorbent. Key: carosidine *1*, catharicine *2*, carosine *3*, catharine *4*, pleurosine *5*, neoleurocristine *6*, neoleurosidine *7*, vincarodine *8*, vindolidine *9*. Solvent systems used were ethyl acetate for plate 2 (top); ethyl acetate–ethanol (3:1) for plate 3 (middle); and ethyl acetate–ethanol (1:1) for plate 4 (bottom).

[3] Available from Gallard-Schlesinger Chemical Manufacturing Corp.
[4] Available from Brinkmann Instruments.

TABLE II.—R_f VALUES OF VINCA ALKALOIDS IN DIFFERENT SOLVENT SYSTEMS

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9	Color: Ceric Ammonium Sulfate
Ajmalicine	0.57	0.68	0.72	0.03	0.51	0.02	0.09	0.68	0.54	Yellow
Carosidine	...	0.58	...	...	...	...	...	0.59	0.10	Yellow
Carosine	...	0.71	...	...	...	...	...	0.65	0.24	Purple-gray
Catharanthine	0.77	0.59	0.74	0.12	0.77	0.03	0.37	0.58	0.38	Green (fades quickly)
Catharine	0.18	0.58	0.76	...	...	...	...	0.56	0.10	Yellow
Catharosine	...	0.56	...	...	...	...	...	0.58	0.08	Purple
Isoleurosine	0.35	0.22	...	0.00	0.23	0.00	0.00	...	...	Gray
Leurosine	0.27	0.35	...	0.00	0.20	...	...	...	...	Gray
Lochnericine	0.15	0.25	0.77	...	...	...	...	0.46	0.03	Blue
Lochneridine	0.00	0.00	0.21	...	...	...	...	0.04	0.00	Blue-green
Lochnerine	0.04	0.35	0.70	...	...	...	...	0.42	0.06	Pale gray
Neoleurocristine	...	0.27	...	...	...	...	...	0.43	0.03	Blue
Neoleurosidine	...	0.06	...	...	...	...	...	0.17	0.00	Yellow-brown
Perivine	0.05	0.30	0.48	...	...	...	...	0.39	0.11	Lt. brown
Pleurosine	...	0.51	0.42	...	...	...	...	0.07	0.03	Yellow
Serpentine	0.03	0.00	0.11	...	...	...	...	0.00	0.00	
Tetrahydroalstonine	0.76	0.60	0.73	0.05	0.66	0.04	0.29	0.76	0.69	Yellow-green
Vinblastine	0.25	0.24	0.66	0.00	0.17	0.00	0.00	0.33	0.04	Purple
Vincamicine	0.03	0.09	0.42	...	...	...	...	0.20	0.00	Bluish orange
Vincarodine	...	0.50	...	...	...	...	...	0.50	0.10	Blue (fades quickly)
Vindolicine	0.24	0.46	0.73	...	...	...	...	...	...	Blue
Vindolidine	...	0.15	...	...	...	...	...	0.29	0.00	Blue
Vindoline	0.44	...	0.68	0.00	0.53	0.03	0.06	0.57	0.20	Crimson
Vindolinine	0.55	0.37	0.70	0.00	0.51	0.00	0.09	0.44	0.13	Orange
Virosine	0.09	0.54	0.63	...	...	...	...	0.48	0.09	Colorless
Sitzirikine	...	0.31	...	...	...	...	...	0.45	0.09	Yellow-green

TABLE III.[a]—SEPARATION OF VARIOUS VINCA ALKALOIDS

	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)	(K)	(L)	(M)	(N)	(O)	(P)	(Q)
Ajmalicine (A)	...	1	1	1	1	1	1	1	1	1	1	1,3	1	1	7	7	1
Catharanthine (B)	1	...	1	1	1	1	1	1	1	1	2	1,3	1	1	1	1	1
Catharine (C)	1	1	...	1	...	3	1	1	1	1	1	4	1	3	1	1	6
Isoleurosine (D)	1	1	1	...	5	...	1	...	1	1	1	3	...	...	7	7	3
Leurosine (E)	1	1	...	5	...	...	1	...	1	1	1	3	...	...	7	7	3
Lochnericine (F)	1	1	3	...	...	...	1	3	6	1	1	3	1	3	1	1	3
Lochneridine (G)	1	1	1,3	1	1	1	...	3	3	6	1	1,3	3	1	1	1	3
Lochnerine (H)	1	1	1,3	...	...	3	3	...	6	3	1	...	3	...	1	1	3
Perivine (I)	1	1	1	1	1	6	6	6	...	3	1	1	3	6	1	1	6
Serpentine (J)	1	1	1	1	1	1	1,6	3	3	...	1	1	6	1	1	1	6
Tetrahydroalstonine (K)	1	2	1	1	1	1	1	1	1	1	...	1	1	1	1	1	1
VLB (L)	1,3	1,3	4	3	3	3	3	...	1	1	1	...	...	4	...	...	3
Vincamicine (M)	1	1	1	...	...	1	3	3	3	6	1	...	...	3	1	1	3
Vindolicine (N)	1	1	3	...	...	3	1	...	6	1	1	4	3	...	1	1	...
Vindoline (O)	7	1	1	7	7	1	1	1	1	1	1	...	1	1	...	3	1
Vindolinine (P)	7	1	1	7	7	1	1	1	1	1	1	...	1	1	3	...	1
Virosine (Q)	1	1	6	3	3	3	3	3	6	6	1	3	3	...	1	1	...

[a] Numbers refer to solvent systems listed in Table I.

Development of Plates

One-hundred milliliters of the developing solvent system were placed in the bottom of the chamber to insure a 5–7 mm. layer of the solvent. Two 15 × 25-cm. rectangular pieces of Whatman No. 1 filter paper were immersed in the solvent and placed on the two long sides of the chamber. Proper saturation of the system was assured by waiting 30 minutes before using the chamber. After this time the plates were placed in the chamber and rested on a 50-ml. graduate cylinder as a support. Time of development (when the solvent front reached a line marked 10 cm. from the point of application) was about 30 minutes for silica plates and 45 minutes to 1 hour for alumina plates.

TABLE IV.—SEPARATION OF CLOSELY RELATED DIMERIC ALKALOIDS

Compounds	Suitable Systems for Separating
Leurosine VLB Isoleurosine	No. 2 or No. 8
Leurosidine Leurocristine VLB or Leurosine	Develop first in No. 10; air dry 10 min.; then develop in No. 3
Leurosidine Sulfate Leurocristine Sulfate VLB Sulfate or Leurosine Sulfate	No. 11

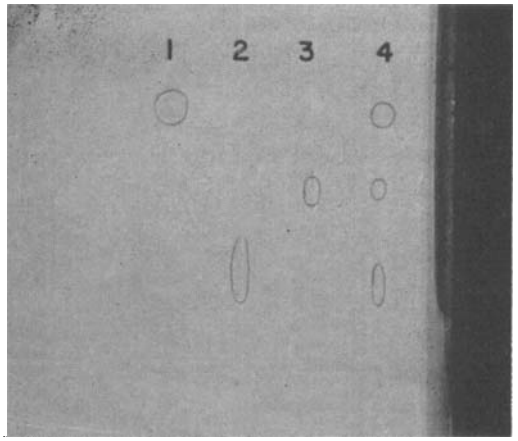


Plate 5.—Oncolytic alkaloids on alumina adsorbent (See Table IV for solvent system.) Key: VLB *1*, leurosine *2*, leurocristine *3*, and mixture *4* of 25 mcg. each of *1*, *2*, and *3*.

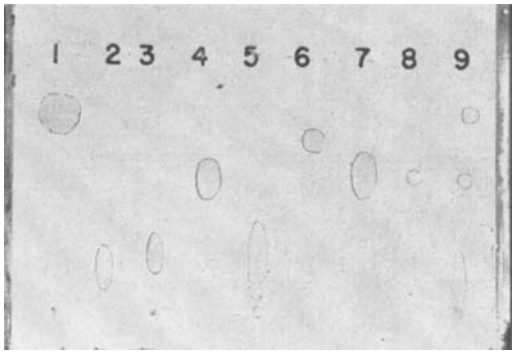


Plate 6.—Monitoring of elution chromatography on alumina. Comparison of an early fraction with several alkaloids known to occur in alkaloidal extract (Fraction A) (4). Key: catharanthine *1*, VLB *2*, isoleurosine *3*, ajmalicine *4*, leurosine *5*, tetrahydroalstonine *6*, vindoline *7*, vindolinine *8*, fraction No. 2 from fraction A *9* (100 mcg.).

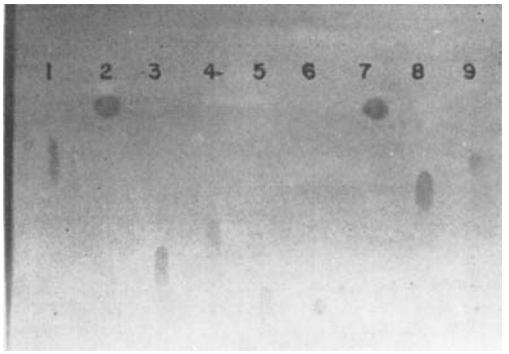


Plate 7.—Examples of results from Table IV on alumina with a chloroform-ethyl acetate (1:1) solvent system. Key: ajmalicine *1*, catharanthine *2*, catharine *3*, leurosine *4*, perivine *5*, serpentine *6*, tetrahydroalstonine *7*, vindoline *8*, vindolinine *9*.

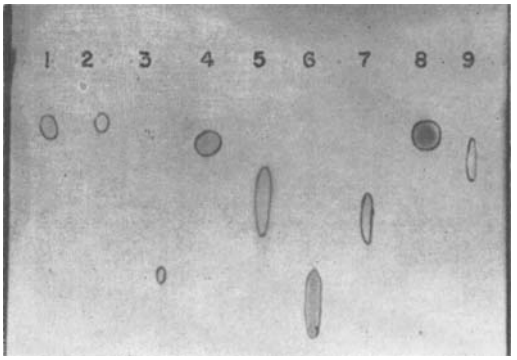


Plate 8.—Examples of results from Table IV on silica with a ethyl acetate-absolute ethanol (3:1) solvent system. Key: catharine *1*, lochnericine *2*, lochneridine *3*, lochnerine *4*, perivine *5*, serpentine *6*, vincamicine *7*, vindolicine *8*, virosine *9*.

Solvent Systems

The solvent systems used in this study are listed in Table I.

Alkaloidal Standards

The alkaloids[5] used in our study had physical characteristics in accordance with those described in the literature (4) and the "Lilly Collection of Physical Data of Indole and Dihydroindole Alkaloids" (10).

Spraying Reagents

Dragendorff's Reagent.—A stock solution is prepared using 2.6 Gm. of bismuth subcarbonate, 7.0 Gm. of dry sodium iodide, and 25 ml. glacial acetic acid. This solution is boiled about 4 minutes and allowed to cool overnight. It is then decanted or filtered from any insoluble material. For every 25-ml. solution obtained, 100 ml. ethyl acetate is added to make the stock solution. The reagent is made fresh daily with 24 ml. ethyl acetate, 10 ml. glacial acetic acid, 4 ml. stock solution, and 2 ml. distilled water, added dropwise.

Ceric Ammonium Sulfate Reagent.[6]—A 1% solution is prepared by dissolving 1 Gm. of ceric ammonium sulfate in 99 Gm. of syrupy phosphoric acid. It is necessary to heat the mixture on a hot plate 5–10 minutes before solution is complete.

DISCUSSION OF RESULTS

The results are tabulated in Tables II, III, and IV. Table II is self-explanatory. From Tables III and IV one can find a system and an adsorbent suitable for the separation of given alkaloids. As far as the reproducibility of R_f values is concerned, we noted that it varies with differences in concentration (Plate 1), the nature of the mixture, the quality of plates used, temperature, and related factors. This has been observed before by Stahl and others (9).

A few examples were chosen which indicate an efficient separation of certain dimeric alkaloids (see Plates 2–5). Plate 6 illustrates the use of thin-layer chromatography to monitor chromatographic fractions. Plates 7 and 8 are representative of the chromatograms which were used to compute the R_f values found in Tables II and III.

[5] We thank Drs. G. H. Svoboda and M. Gorman for authentic samples of several of the alkaloids used in this study and Mr. W. E. Kruse for art and photographic work.

[6] This reagent was introduced for the analysis of Vinca alkaloids by I. Jakovljevic, from the Lilly Control Laboratories, whom we thank for permission to use it in our studies.

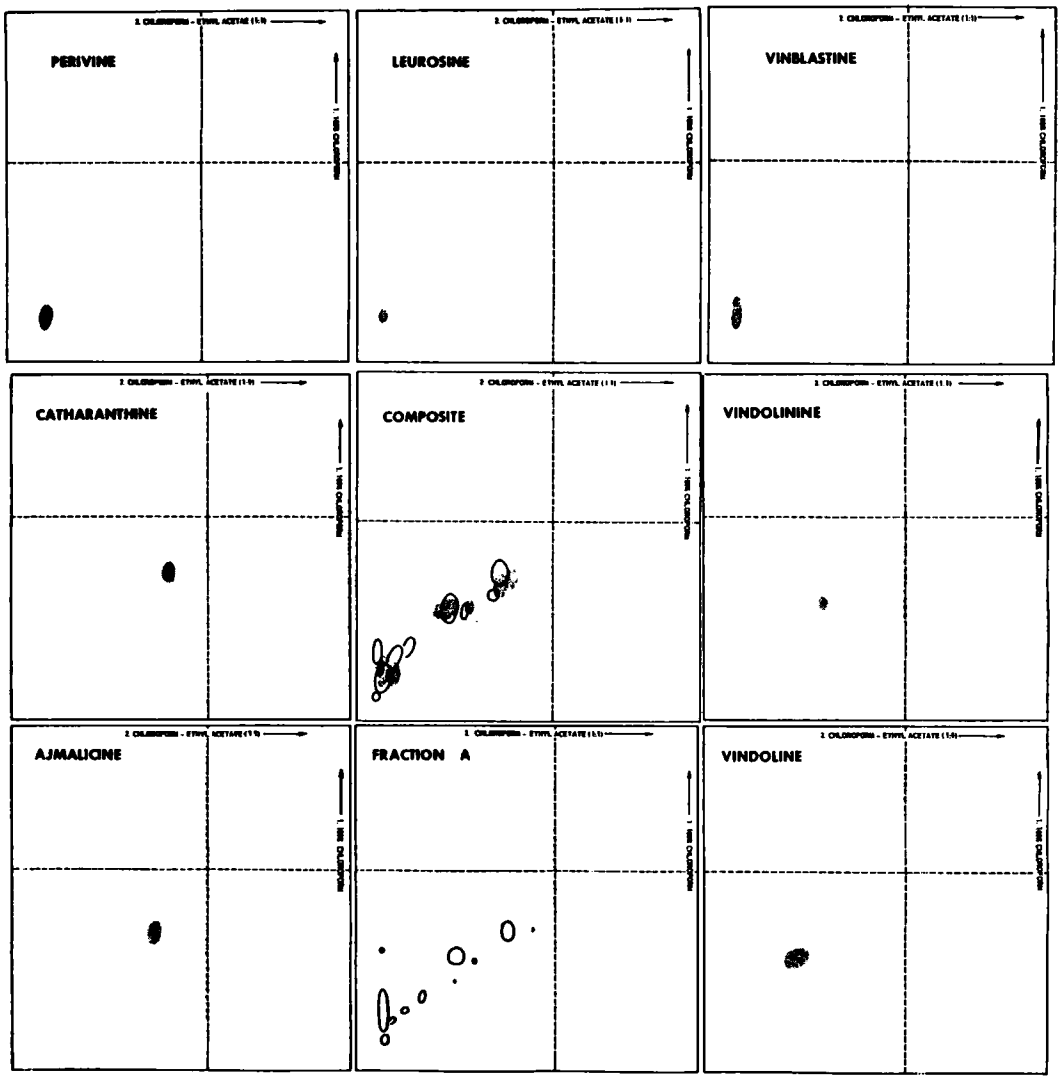


Fig. 1.—Didimensional chromatography on alumina using 1, chloroform (100%) and 2, chloroform-ethyl acetate (1:1) solvent systems. The concentration of Fraction A was 100 mcg. and for the composite 50 mcg. of the following alkaloids applied: ajmalicine, catharanthine, leurosine, perivine, VLB, vindoline, and vindolinine.

When complex mixtures (*e.g.*, Fraction A) (4) were subjected to solvent systems Nos. 1–6 in one dimension, separation was unsatisfactory. A better resolution was obtained by the use of didimensional chromatography on either alumina or silica.

Comparison of the position of spots from individual alkaloids in didimensional chromatograms with the position of spots in the mixture, run under identical conditions, gives a good indication of the nature of the mixture's different components. This procedure is illustrated in Fig. 1. The use of the ceric ammonium sulfate reagent, which gives specific color reactions, was also found to be very useful in this particular technique.

The figure, however, also illustrates that it is not possible to identify spots in the composite unequivocally. Even under similar conditions the location of spots is not always reproducible. Many materials, such as the dimeric alkaloids used in this composite, occur at nearly the same location; it is not possible to distinguish between them in this system.

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