An Alkaloid Screening Procedure Utilizing Thin-Layer Chromatography

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Any procedure for screening plant material for alkaloids should yield the maximum information concerning the alkaloid content of the sample, with a minimum of effort. Most of the procedures reported in the literature (1, 2, 4, 7–10) are time consuming and leave much to be desired with regard to the detection of all types of alkaloids. The development of a new procedure must, therefore, take several factors into consideration.

Raffauf (6) has very effectively summarized the difficulties involved in testing for alkaloids and has indicated that, from personal experience, none of the common and generally accepted methods is acceptable. The possibility of false-positive alkaloid reactions with the general alkaloid detecting reagents, the failure of some reagents to detect certain types of alkaloids (false-negative results), difficulties involved in purification of extracts without involving a loss of certain types of alkaloids, and the failure of most procedures to detect quaternary alkaloids have been mentioned as the major difficulties in the development of an acceptable procedure (6).

Through the use of purified plant extracts, in conjunction with the rapid procedure of thin-layer chromatography, we have developed a screening procedure capable of detecting small quantities of alkaloids in 2 g samples of powdered plant material. The procedure also involves a partition purification of secondary and tertiary alkaloid-amines to remove material often found responsible for false-positive reactions. A second fraction is also prepared from the same sample which is intended to contain any water-soluble quaternary bases. This fraction is purified to contain alkaloids free from many interfering plant pigments. Evidence for the effectiveness of the method is presented through a screening of 28 plants known to contain varying concentrations and chemical types of alkaloids. In addition, 8 plants free from alkaloids were included to serve as controls.

MATERIAL AND METHODS

Plant specimens used in the study.—Samples[1] for evaluation were selected to include a variety of different chemical types of alkaloids present in the plant material. Also, samples representative of bark, leaf, seed, root, rhizome, whole plant etc. were included so that if materials present in these plant parts were to present problems to the method proposed, these would become evident during the course of the study. The 28 alkaloid-containing plant samples and 8 alkaloid-free plant samples used in this study are listed in table 1.

Preparation of Fraction I.—All seed drugs were first defatted with petroleum ether (bp 30–60°C). Two g of dried plant material was thoroughly moistened with 28 per cent NH_4OH solution and then dried on a steam bath. The prepared

[1]All samples were obtained from the S. B. Penick Company, New York 8, N. Y. except *Rivea corymbosa* and *Ipomea tricolor* seeds which were supplied by Riker Laboratories, Northridge, Calif.; *Coffea arabica* seeds (unroasted), purchased locally; and *Catharanthus roseus* (whole plants), which were grown from seed in Pittsburgh, Pa., and represented 8 month old plants at harvest. All samples were finely powdered prior to use in the screening procedure.

sample was then placed in a 50 ml Erlenmeyer flask fitted with an air condenser, 15 ml of chloroform was added, and the mixture was refluxed on a steam bath for 30 minutes. The refluxed mixture was cooled to room temperature (*ca* 25°C) and filtered through Whatman no. 1 filter paper. The marc was returned to the flask, an additional 15 ml of chloroform was added, and the 30 minute reflux period was repeated. After cooling, filtering, and combining the filtrates, the mixture was evaporated on a steam bath to *ca* 2 ml. Thin-layer chromatography

TABLE 1. *Plants used in the screening procedures.*

Official title or common name	Botanical origin	Family	Plant part used
A. Plants containing alkaloids			
ARECA N.F. XI	*Areca catechu*	Palmae	seed
BELLADONNA LEAF U.S.P. XVI	*Atropa belladonna* or its var. *acuminata*	Solanaceae	leaf and flowering or fruiting top
BELLADONNA ROOT N.F. XI	*Atropa belladonna*	Solanaceae	root
CINCHONA N.F. IX	*Cinchona succirubra*	Rubiaceae	bark
COCCULUS N.F. VII	*Anamirta cocculus*	Menispermaceae	fruit
COFFEE	*Coffee arabica*, or *Coffea liberica*	Rubiaceae	seed
COLCHICUM CORM N.F. IX	*Colchicum autumnale*	Liliaceae	corm
COLCHICUM SEED N.F. X	*Colchicum autumnale*	Liliaceae	seed
EPHEDRA	*Ephedra equisetina*	Gnetaceae	above ground plant
ERGOT N.F. XI	*Claviceps purpurea*	Hypocreaceae	sclerotium
HYDRASTIS N.F. X	*Hydrastis canadensis*	Ranunculaceae	rhizome and roots
HYOSCYAMUS N.F. XI	*Hyoscyamus niger*	Solanaceae	leaf
IPECAC U.S.P. XVI	*Cephaelis ipecacuanha*, or *Cephaelis acuminata*	Rubiaceae	rhizome and roots
IPOMEA TRICOLOR	*Ipomea tricolor*	Convolvulaceae	seed
KOLA N.F. VIII	*Cola nitida*	Sterculiaceae	cotyledon
LOBELIA N.F. X	*Lobelia inflata*	Lobeliaceae	leaf and top
MUSTARD, BLACK N.F. XI	*Brassica nigra*, or *Brassica juncea*	Cruciferae	seed
OLOLIUQUI	*Rivea corymbosa*	Convolvulaceae	seed
PEPPER, BLACK	*Piper nigrum*	Piperaceae	fruit
PHYSOSTIGMA	*Physostigma venenosum*	Leguminosae	seed
PILOCARPUS	*Pilocarpus jaborandi*, *Pilocarpus microphyllus*, or *Pilocarpus pinnatifolius*	Rutaceae	leaflet
RAUWOLFIA SERPENTINA N.F. XI	*Rauvolfia serpentina*	Apocynaceae	root
SANGUINARIA N.F. XI	*Sanguinaria canadensis*	Papaveraceae	rhizome
STRAMONIUM N.F. XI	*Datura stramonium* or its var. *Tatula*	Solanaceae	leaf and flowering or fruiting tops
VERATRUM ALBUM	*Veratrum album*	Liliaceae	rhizome
VERATRUM VIRIDE N.F. X	*Veratrum viride*	Liliaceae	rhizome and root
VINCA ROSEA	*Catharanthus roseus*	Apocynaceae	whole plant
YELLOW ROOT	*Zanthorrhiza apiifolia*	Ranunculaceae	rhizome and roots
B. Plants devoid of alkaloids			
APOCYNUM N.F. VIII	*Apocynum cannabinum*, or *Apocynum androsaemifolium*	Apocynaceae	rhizome and roots
CHERRY, WILD U.S.P. XVI	*Prunus serotina*	Rosaceae	bark
DIGITALIS U.S.P. XVI	*Digitalis purpurea*	Scrophulariaceae	leaf
FRANGULA N.F. IX	*Rhamnus frangula*	Rhamnaceae	bark
HAMAMELIS N.F. IX	*Hamamelis virginiana*	Hamamelidaceae	leaf
KAVA	*Piper methysticum*	Piperaceae	root
MULLEIN	*Verbascum thapsus*	Scrophulariaceae	leaf
QUILLAJA N.F. IX	*Quillaja Saponaria*	Rosaceae	bark

of this material indicated that a high pigment content of most samples interfered with the interpretation of chromatograms after spraying with the Munier and Macheboeuf modification of Dragendorff's alkaloid reagent (5). Therefore, this chloroform extract was purified in the following manner.

The total chloroform extract (*ca* 2 ml) was introduced into a micro-separatory funnel[2] and 1.0 ml of 1 per cent HCl (w/v) was added. The ends of the separatory funnel were suitably closed and the mixture shaken and allowed to separate into two zones. The chloroform layer was removed and sufficient 28 per cent NH_4OH

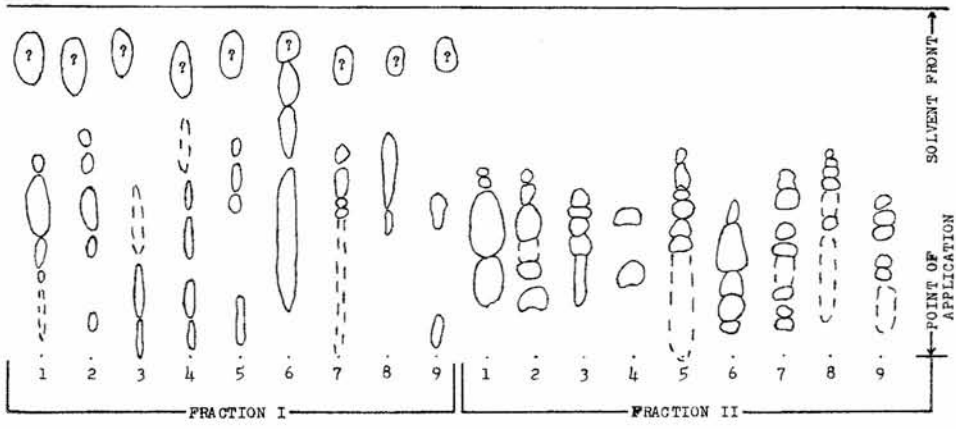

FIG. 1. *Thin-layer chromatograms of fractions I and II from samples 1–9. 1, Cinchona; 2, Hydrastis; 3, Ipecac; 4, Pilocarpus; 5, Rauwolfia; 6, Sanguinaria; 7, Vinca rosea; 8, Veratrum viride; 9, Yellow root. Solid lines indicate a strong positive reaction for alkaloids after treatment with Dragendorff's reagent; broken lines indicate a weak positive reaction; (?) indicates a questionable alkaloid reaction. Chromatograms were developed with BAW (4:1:1) on a matrix of Silica Gel G.*

solution was added to the aqueous acid layer in the micro-separatory funnel to render it distinctly alkaline to litmus. The liberated free bases were removed by two successive 0.5 ml chloroform extractions of the alkaline mixture. The chloroform extracts were combined and evaporated to dryness. Exactly 0.1 ml of chloroform was added to each residue, which contained the alkaloids equivalent to 2 g of powdered air-dried sample. This extract was labeled Fraction I and was considered to contain the majority of any secondary and/or tertiary nitrogenous bases present in the sample.

Preparation of Fraction II.—The air-dried chloroform-exhausted marc remaining after the preparation of Fraction I was introduced into a 50 ml Erlenmeyer flask

[2]A micro-separatory funnel was prepared by attaching a small piece of suitable chloroform-resistant tubing to the narrow (exit) end of a medicine dropper of *ca* 4 ml capacity. A small clamp was used to prevent a loss of chloroform-acid mixture from the funnel.

and refluxed for 30 minutes with 15 ml of ethanol containing 0.5 per cent HCl (w/v). Following the reflux period, the mixture was cooled to room temperature, filtered through Whatman no. 1 filter paper and concentrated on a steam bath to dryness. Initially this solution was concentrated to 4 ml and directly chromatographed. Interpretation of the chromatograms after the Dragendorff spray was almost impossible due to the presence of pigments and therefore a purification of this extract was attempted.

After concentration of the alcohol extract to dryness, 1.0 ml of distilled water was added and mixed well with a micro-stirring rod. This suspension was centrifuged at 1500 rpm for 10–15 minutes. The supernatant was removed with a

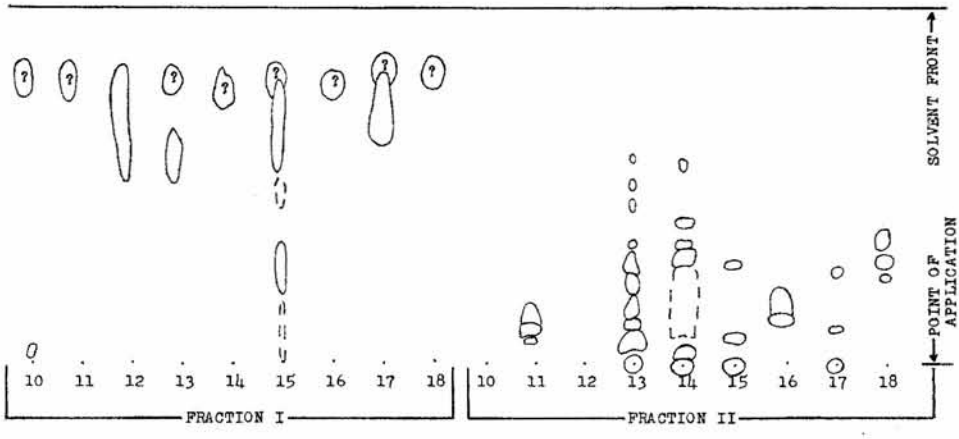

FIG. 2. *Thin-layer chromatograms of fractions I and II from samples 10–18. 10, Areca; 11, Coffee; 12, Ephedra; 13, Ergot; 14, Hyoscyamus; 15, Ipomea tricolor; 16, Kola; 17, Black Pepper; 18, Physostigma. Solid lines indicate a strong positive reaction for alkaloids after treatment with Dragendorff's reagent; broken lines indicate a weak positive reaction, (?) indicates a questionable alkaloid reaction. Chromatograms were developed with BAW (4:1:1) on a matrix of Silica Gel G.*

pipet and was labeled Fraction II. Fraction II was considered to contain any quaternary alkaloids present in the sample but residual secondary and/or tertiary bases could be present due to incomplete extraction of the original ammoniacal sample with chloroform. This procedure served to eliminate practically all of the pigments that interfered with the interpretation of chromatograms prepared from the original acid-ethanol extract.

Thin-layer chromatography of Fractions I and II.—The DeSaga[3] apparatus

[3]DeSaga Thin-Layer Chromatography Apparatus. Brinkmann Instruments, Inc., Great Neck, Long Island, New York. The spreading applicator designed to produce a matrix 250 μ in thickness was used.

was used and the matrix was prepared by thoroughly mixing 30 g of Silica Gel G with 40 ml of distilled water. After stirring for 60 seconds an additional 20 ml of water was added with continued stirring for 30 seconds. The mixture was then transferred to the spreading applicator which was then drawn across the surface of five 200×200 mm glass plates. The plates were allowed to set for 5 minutes and were then activated in a circulating-air oven at 105–110°C for 30 minutes. After cooling to room temperature the plates were stored in a desiccator over soda lime until ready for use.

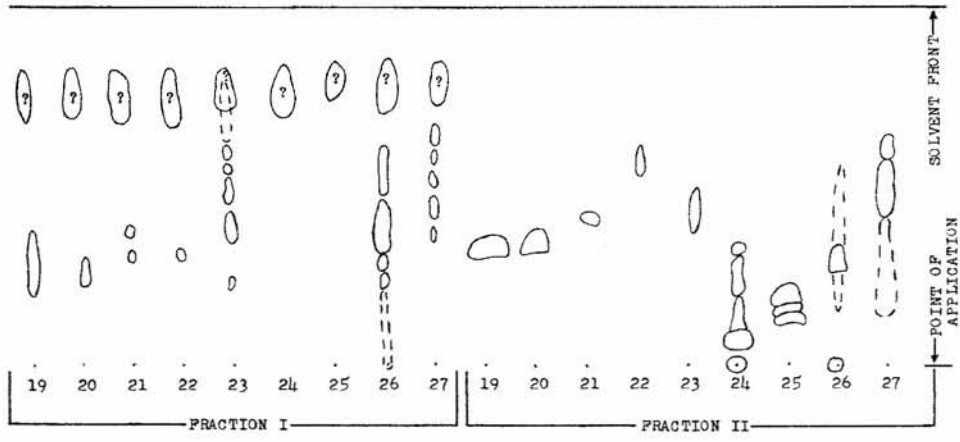

FIG. 3. *Thin-layer chromatograms of fractions I and II from samples 19–27. 19, Belladonna leaf; 20, Belladonna root; 21, Colchicum corm; 22, Colchicum seed; 23, Lobelia; 24, Black Mustard; 25, Ololiuqui; 26, Stramonium; 27, Veratrum album. Solid lines indicate a strong positive reaction for alkaloids after treatment with Dragendorff's reagent; broken lines indicate a weak positive reaction; (?) indicates a questionable alkaloid reaction. Chromatograms were developed with BAW (4:1:1) on a matrix of Silica Gel G.*

In our preliminary work with non-purified Fractions I and II, chromatographic solvent systems of benzene, benzene-methanol (95:5) and methanol were utilized. Little, if any, migration of alkaloid components was noted using benzene and benzene-methanol (95:5) as eluents with most of the alkaloid-containing samples. The high polarity of methanol effectively induced migration of alkaloid components from the point of application of the sample; however, the diffusion and tailing of the components resulted in poor resolution and decreased sensitivity through an increase in the surface area of the alkaloid components. With these results as a guide, additional solvents were utilized hoping to find a system that would yield definite resolution of the alkaloids and also to prevent the tailing observed with the three solvent systems previously utilized. Optimal results were obtained using *n*-butanol-acetic acid-water (4:1:1 v/v) (BAW). The only disadvantage that

we could find with this system was that it was rather slow in comparison to the benzene, benzene-methanol or methanol-types. The amount of time that was required for the BAW to migrate 115 mm from the point of application of the sample could be reduced by about one-third by equilibration of the system through lining the developing chamber with Whatman no. 1 filter paper. Plates were introduced quickly only after the paper had become completely saturated with the BAW mixture. Running time under these conditions was approximately 90 minutes.

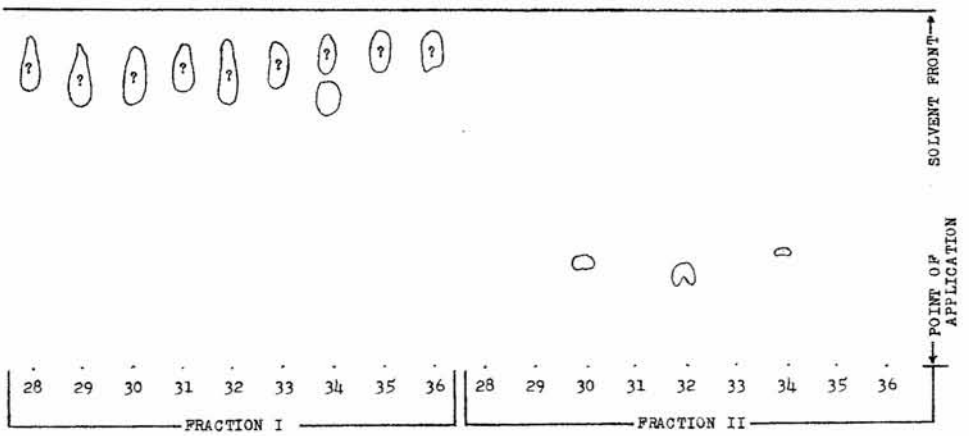

FIG. 4. *Thin-layer chromatograms of fractions I and II from samples 28–36. 28, Apocynum; 29, Wild Cherry; 30, Cocculus; 31, Digitalis; 32, Frangula; 33, Hamamelis; 34, Kava; 35, Mullein; 36, Quillaja. Solid lines indicate a strong positive reaction for alkaloids after treatment with Dragendorff's reagent; broken lines indicate a weak positive reaction; (?) indicates a questionable alkaloid reaction. Chromatograms were developed with BAW (4:1:1) on a matrix of Silica Gel G.*

All aliquots initially applied to the Silica Gel G plates were 5.0 μl for Fraction I (chloroform extract) and 10.0 μl for Fraction II (aqueous extract). Fraction I contained the extracted alkaloids from 100 mg of powdered drug sample whereas Fraction II contained the alkaloids from 20 mg of sample. After application of the samples, the plates were introduced into the equilibrated developing chambers containing 100 ml of BAW mixture and development was continued until the solvent front had reached a point 115 mm from the point of sample application. The plates were removed, air-dried and sprayed lightly with the Munier and Macheboeuf modification of Dragendorff's reagent (5). It should be pointed out that considerable difficulty is observed if the plates are not completely dry prior to application of this reagent. Color produced by reaction of the reagent and the alkaloids is not typical, and the sensitivity appeared to be considerably reduced.

All zones containing alkaloids, as evidenced by a typical orange color after treatment with the spray reagent, were outlined with a stylus five minutes after reagent application. In certain extracts, orange spots appeared several hours after treatment, but these were not considered to be indicative of the presence of alkaloids.

More pigment was present in the Fraction II samples than in the Fraction I samples but the colors of these substances on the chromatograms did not interfere with the interpretation of results. It should be pointed out that in all cases, a material of high R_F value was observed to be present in Fraction I (figs. 1–4) that

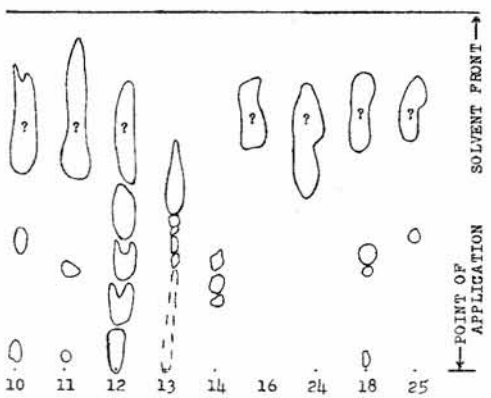

FIG. 5. *Thin-layer chromatograms of fraction I from selected samples. 10, Areca; 11, Coffee; 12, Ephedra; 13, Ergot; 14, Hyoscyamus; 16, Kola; 18, Physostigma; 24, Black Mustard; 25, Ololiuqui. Solid lines indicate a strong positive reaction for alkaloids after treatment with Dragendorff's reagent; broken lines indicate a weak positive reaction, (?) indicates a questionable alkaloid reaction. Chromatograms were developed with BAW (4:1:1) on a matrix of Silica Gel G.*

reacted with the spray reagent after several minutes to produce salmon-colored spots, increasing in intensity to a maximum about 24 hours after initial treatment. The color was characteristic enough to be differentiated from the typical orange color produced by most alkaloids. Only rarely did we observe alkaloid components in the extracts to migrate as far as these components on the chromatograms, and no difficulty was experienced in interpreting the results.

Since these atypical, high R_F components were present at the same general point in each of the Fraction I samples, they were not interpreted to be alkaloids. This reaction was not observed in the Fraction II extracts. The results of screening Fraction I and Fraction II from each of the 36 plants used in this study are shown in figures 1–5.

RESULTS

Effect of Alkaloid Concentration on Screening Results.—The samples screened in this study represent materials of varying alkaloid concentration. Although no quantitative alkaloid analysis of each sample was conducted, several general observations can be made with regard to the limits of detection using the described techniques. It has been our experience that no statement can be made relative to the "usual" quantity of alkaloid that can be detected by this method. There are many reasons for this. The area of the spotted sample on the chromatogram will greatly affect the minimum detectable quantity of separated alkaloid on a chromatogram since the surface area represented by a single alkaloid after migration will increase with an increase in R_F value. Thus, the concentration of alkaloid per unit area of the spot will decrease and reduce the lower limit of detection. Therefore, equivalent amounts of two alkaloids, one with a low R_F and the other with a high R_F, will differ in their minimum detectable quantities, all other factors being equal. Ideally then, the prepared fractions for chromatographic application should be as concentrated as possible, should be in a volatile solvent and should be applied to the plates so that the area of application is kept at a minimum. The volumes of solvent used to prepare Fractions I and II in this study are considered to be minimal with respect to rendering the extraction technique workable for all samples. Experience with the method would allow, in certain instances, a much greater increase in the detectable concentration of alkaloids in plant samples. In our studies we have been able to detect as little as 0.5 μg of α-yohimbine by thin-layer chromatography but 200 μg of caffeine or ephedrine could not be detected.

Some of the Fraction I extracts did not appear to contain alkaloids after chromatographing 5 μl aliquots of the extract (figs. 2–3). The chromatographic procedure was repeated using larger volumes of the fraction, and the results obtained were more satisfactory. The results were variable with 10 μl aliquots but alkaloid detection was improved in all samples with the application of 30 μl aliquots. Therefore it would seem necessary to rechromatograph, at 20 or 30 μl, any Fraction I extracts which gave negative results at 5 μl. Chromatograms resulting from the application of 30 μl of samples are shown in figure 5. Application of volumes larger than 10 μl of the aqueous Fraction II is not recommended because of the high degree of sample spreading on application of the sample.

Comparison of Fraction I and Fraction II Results.—One of the original objectives of preparing two fractions from each sample was to allow detection of quaternary ammonium alkaloids, or other water-soluble plant bases not detected by conventional screening procedures. An inspection of the results (figs. 1–4) indicates that in most instances there appears to be some carry-over of Fraction I alkaloid material to Fraction II. This could be prevented by additional chloroform extractions of the basified sample; however, the results that we have obtained do not appear to necessitate this additional step. It is interesting to note that the water-soluble ergot alkaloids are resolved in Fraction II (fig. 2), as well as similar types of alkaloids present in *Rivea corymbosa* (fig. 3) and *Ipomea tricolor* (fig. 2), as can the quaternary alkaloids in Yellow root (fig. 1) and Hydrastis (fig. 1). Sinapine, the only alkaloid reported to be present in Black Mustard (11), is not detected in Fraction I, but appears to be concentrated in Fraction II, in addition to at least 3 other alkaloids (fig. 3). The xanthine-type bases present in Kola (fig. 2) and in Coffee (fig. 2), also are detected more readily in the Fraction II samples than in Fraction I. These observations point out the need for preparing two types of extracts, as described in this study, for an adequate screening procedure.

False-positive Alkaloid Reactions.—Two of eight control plants used in this study gave results indicative of the presence of alkaloids (fig. 4). The results obtained with Kava extracts are explained on the basis of the presence of α-pyrones that have been demonstrated to react with Dragendorff's reagent in a manner similar to alkaloids (3). The anthraquinones present in Frangula also would be expected to react in a similar manner due to the conjugated, ketone functions present in these molecules (3). This feature of Dragendorff's reagent, the ability to react with certain non-alkaloid compounds, is perhaps the most undesirable feature of the screening procedure; however, this reagent is perhaps the most useful and most specific of those available.

SUMMARY

A thin-layer method for the detection of alkaloids in small samples of plant material has been described which will detect water-soluble alkaloids of the quaternary type, in addition to the more commonly encountered secondary and tertiary bases. The method is simple, will detect low concentrations of alkaloid in dry plant material and in addition gives an insight to the complexity of the alkaloids present in the sample. It appears that the chief disadvantage of this screening procedure is in the non-specificity of the detecting reagent. Since no reagent is available that will eliminate this disadvantage, and because there is some information now available on the types of non-alkaloid compounds that will react with this reagent, this does not appear to be a serious disadvantage.

The results of this study have been shown to correlate well with known information about the alkaloids present in the 36 plant samples evaluated.

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