A CONVENIENT SEPARATION OF ALKALOID MIXTURES BY PARTITION CHROMATOGRAPHY, USING AN INDICATOR IN THE STATIONARY PHASE*

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(Received February 1st, 1962)

Partition chromatography is a versatile and precise tool, which has been of great use in the separation of mixtures of closely related organic substances whose resolution by classical chemical methods would be impractical or impossible. When the two immiscible phases employed are neutral (*i.e.*, when there are no acidic or basic solvents, and no buffer in the polar phase), the advantage is offered of permitting visual detection of bands of acidic and basic materials on the column through the use of an indicator in the stationary phase. Sulfonphthalein indicators, which are highly polar in both forms, cover a wide range of pK_a values, and are readily available commercially, are quite ideal for this purpose.

Such a method has been extensively used for the separation of organic acids, both on silica gel and on Kieselguhr, using Bromcresol Green as the indicator on the column; some early papers describing this are listed at the end of this paper[1–5]. However, application of the method to resolution of alkaloid mixtures is apparently without precedent. Two related experiments have been reported: CLAYTON AND STRONG in 1954[6] separated volatile aliphatic amines on Celite, with phenolphthalein as an indicator in the polar phase; and TRAUTNER AND ROBERTS in 1948[7] made use of an indicator to follow the separation of hyoscine and hyoscyamine on silica gel, but the indicator was not on the column during the separation; it was added at various stages in a non-polar solvent, the band positions were marked, and the indicator solution was removed.

EXPERIMENTAL

A. Selection of a partition system

A mathematical treatment of partition chromatography indicates that the best resolution of bands on a column (*i.e.* the greatest ratio between the difference of R_F's and the difference of partition coefficients) occurs in the area where the partition coefficient in the column is equal to 1 (at about $R_F = 0.5$). As the average pressure-packed Kieselguhr column such as those described below has a retention volume of

* This investigation was supported in part by research grants from the National Institutes of Health (H-2952 (C4) and CY-4500 (C2)).

** Woodrow Wilson Foundation Fellow, 1959–60; Co-operative National Science Foundation Fellow (University of Wisconsin), 1960–62.

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parghly three times the volume of the stationary phase, the actual ideal average routition coefficient of a mixture (mobile phase: stationary phase) is about 0.3.

The partition coefficient for the mixture to be separated can be determined in a 10-ml Erlenmeyer flask, using a variety of solvent mixtures, and (for the case of most alkaloids) Mayer's test on the upper and lower phases as a qualitative estimate of the amount of material in each. If the mixture contains a large amount of alkaloidal tar (which would probably have an R_F of near 0 in the system useful for separating the pure bases), this should be removed first by adsorption chromatography, precipitation of solid fractions, or simple extraction of the mixture with the mobile phase of the system to be used.

In choosing a solvent mixture, a number of factors must be considered. Primary among these is the solubility of the alkaloids of the mixture to be separated in various solvents. Most complex organic molecules, such as many alkaloids, contain both polar and non-polar portions and thus are ideally suited to separation by partition chromatography. It should be possible to find, for such compounds, a good polar and non-polar solvent which can be rendered essentially immiscible by addition of one or two indifferent solvents; for example, many alkaloids are relatively soluble in ethylene dichloride and in methanol, and these solvents may be separated by addition of hexane and water, in which many alkaloids are relatively insoluble. These four components are the ones which have proved most useful in this Laboratory, but many other variations are possible, depending upon the nature of the compounds to be separated. If possible, one should avoid highly volatile solvents (such as pentane or chloroform), and non-polar solvents which have a high solvation action on polar solvents (such as ethyl acetate), for a stable equilibrium will be difficult to obtain if these are used. If the non-polar phase draws in a large amount of the polar solvent, not only will the equilibrium of the solvent system be unstable but, furthermore, the indicator will be eluted from the column. Other important considerations are: (1) the polar phase should not be buffered (as the indicator will work poorly); (2) the mixture to be separated should be reasonably soluble (at least 10 %) in the mobile phase; (3) no component of the system should be highly toxic, acidic or basic, highly reactive, or corrosive; (4) no component of the mobile phase should be excessively high-boiling, for this would make necessary tedious post-extractions of the fractions to recover the material; (5) the system need not normally contain over four components; and (6) the polar phase should not be more than 50 % of the system, as much would be wasted.

The systems which have been found most applicable in this Laboratory are those containing ethylene dichloride and Skellysolve B in various ratios less than 2:3 (over which point the mobile phase takes up too much of the polar solvents), methanol, and enough water to effect separation (see below for specific systems). A system containing benzene, methanol, and water was also used successfully in one case, but was not very stable. In another Laboratory, hexane, heptane, and cyclohexane were found to be interchangeable with Skellysolve B[8]. Systems containing carbon tetrachloride and ethanol were also satisfactory, though less so than the ones eventually used most. A system employing Methyl Cellosolve and butanol gave a satisfactory coefficient in one case, but the alkaloid mixture to be separated was very sparingly soluble in the mobile phase.

It must be emphasized that this procedure for selection of a solvent system is

only applicable to mixtures of individual compounds which are fairly close in chemical properties. Although this method is of exceptional utility in separation of mixtures which would be unaffected by ordinary chemical resolutions, it is by no means a substitute for chemical separations of crude and complex mixtures. Thus, a mixture of crude *Buxus* alkaloids will give a large number of overlapping bands from R_F 0.0 to R_F 1.0 when added to a column such as described in the section below. However, after the mixture has been chemically separated into various fractions by salt precipitation, pH fractionation, and extraction with various solvents, each of the resulting fractions is then readily separable by this method—with somewhat different systems being used for different fractions. Similarly, a mixture of the bisbenzylisoquinoline bases isochondrodendrine (which is insoluble in chloroform) and tetrandrine (which is soluble in chloroform) is easily separated by a simple extraction, but behaves in a confusing fashion under the above treatment (because the two components differ markedly in an important chemical property).

B. Selection of an indicator

Sulfonphthalein indicators are highly polar in both forms, and normally will be retained in the stationary phase on a column, unless the mobile phase contains moderate amounts of a polar solvent. Fortunately, they also cover a very wide range of pK_a values. Those most likely to be applicable to alkaloid work are listed in Table I; most of the latter are readily available commercially.

TABLE I

COMMON SULFONPHTHALEIN INDICATORS

No.	*Name*	*pH range*	*Color*	
			acid	*base*
1.	Bromcresol Green	3.8–5.4	yellow	blue
2.	Chlorphenol Red	5.0–6.6	yellow	red
3.	Bromcresol Purple	5.2–6.8	yellow	purple
4.	Bromphenol Red	5.4–7.0	yellow	red
5.	Dibromophenol-tetrabromophenol sulfonphthalein	5.6–7.2	yellow	purple
6.	Bromthymol Blue	6.0–7.6	yellow	blue
7.	Phenol Red	6.8–8.4	yellow	red
8.	Cresol Red	7.2–8.8	yellow	red
9.	Metacresol Purple	7.4–9.0	yellow	purple
10.	Thymol Blue (basic range)	8.0–9.6	yellow	blue

That indicator should be chosen which is farthest down the table, and will clearly turn to the basic color when a solution of about 0.1 mg of its acid form in one ml of the stationary phase of the system to be used is treated with one mg of alkaloid mixture. The use of indicators farther down the list (with a higher pH range) will result in minor bands remaining invisible on the column; if those higher up the list (with a lower pH range) are used, the areas between bands will not return to a yellow color, separation will be observed poorly if at all, and the whole column will soon become discolored.

C. Testing on a column

After a partition system and an indicator have been selected for a given mixture, they should be incorporated into a small column to check the separation; the procedure may then be extrapolated to preparative scale (see below).

A convenient size for the test column is 1.3 cm i.d., with a Teflon stopcock on the bottom and a 100 ml bulb on the top (many variations are possible, of course). About 250 ml of the system is made up and equilibrated, 6 ml of the polar phase and 100 ml of the non-polar phase are withdrawn and combined with 1 mg of the indicator, and the whole mixture is swirled and poured rapidly onto 5 g of Johns-Manville "Celite" 503 (Hyflo "Supercel" is equally satisfactory). The resulting suspension is shaken vigorously; the stationary phase becomes uniformly distributed on the Celite. If the indicator chosen has a pK_a of 6 or less, the Celite will change it partially or wholly to the basic form; in such a case, the flask containing the mobile phase and support is waved briefly over a bottle of conc. hydrochloric acid until the indicator on the support just regains its yellow color. The mixture is then slurried in portions into the open column, the latter having been checked for verticality and protected at the bottom by glass wool, sea sand, and a filter paper disk. When all of the support has been transferred to the column, it is packed tightly with about 10 lbs. air pressure (from a line or rubber bulb); a snug-fitting filter paper disk is dropped onto the top of the packing, and compressed with a tamp until the top of the column is about the same density as the bottom. The column is then ready for use; after the excess solvent has been passed through or withdrawn, 10–50 mg of alkaloid mixture may be added in 0.2 ml of mobile phase, including a non-polar dye to mark the front (American Cyanamid Calco Oil Red or Blue). Elution then may proceed at a rate not exceeding one drop per five seconds. If the column and system are satisfactory, separation should be observed immediately; the bands may be collected as they leave the bottom of the column.

D. Large-scale separations

Large columns (2.0 to 25.0 cm in diam. or even larger) are best constructed by dry-packing; a good dry-packed column about 5 cm in diam. will, in general, give far better comparative resolution than a good slurry-packed one even of small size. The column should be perfectly cylindrical (to prevent extensive wall effects, which are particularly serious in this method of visual band detection) and have no constrictions above the base; a close-fitting solid wood or metal tamp should be constructed for the packing operation.

A 4.5 cm column may be packed with 200 ml of stationary phase and 80 mg indicator on 330 g of Celite 545. The stationary phase and indicator are added to the dry Celite, and the mixture is equilibrated by shaking or rolling it until all lumps are gone. It is then packed into the column with the aid of a powder funnel in at least twenty increments, tamping each one down very thoroughly before adding the next. A layer of fine-grain sea sand and a snug-fitting thick filter paper disk (such as Whatman 3 mm) are placed on top of the completed column (which will be about 5 cm in length), the tamp is placed on the top disk, the lower stopcock is opened, and about 1000 ml of mobile phase is added. The mobile phase running from the bottom is recycled until the entire column is fully wet (*i.e.*, no pressure ridges are visible; this will take about 48 h of running).

The total packing time should not exceed one hour. The final column should be tested with a narrow dye band before use (for channeling, irregularities, or undue diffusion). The alkaloid mixture (1–3 g) may be added in 10–15 ml of mobile phase, and passed through at a rate of 5 ml/min.

The R_F value of a given alkaloid in a given system will be slightly higher in this column than in the smaller slurry-packed one above; although the dry-packing gives a far tighter column, not as much stationary phase can be placed on the carrier.

Such a large column in our Laboratory has been used over thirty times with no obvious decrease in efficiency, and has been used successfully after standing idle under a variety of temperature and humidity conditions for six months.

RESULTS

The following specific applications of this method (summarized in Table II) have been investigated to date. It is highly recommended that an experimenter new to partition chromatography and/or this method try one of these, or another known example, before attempting investigation of an unknown mixture, as a certain degree of technique is required which comes best with experience.

A. Separation of the alkaloids of the Buxaceae[9,10] (see Fig. 1)

This general method was first developed to separate the various complex fractions of the alkaloids of *Buxus sempervirens* L. The alkaloids of this and related plants can be considered as representative of compounds having a highly non-polar saturated nucleus to which are attached a few polar functional groups.

Two solvent systems, (A) ethylene dichloride–Skellysolve B–methanol–water (5:10:2:0.3) and (B) ethylene dichloride–Skellysolve B–methanol–water (1:10:2:0.16) have served to separate all fractions so far investigated into their component individual alkaloids, which in many cases crystallize directly from the column fractions. The compounds are sufficiently strong bases that Phenol Red may be used for an indicator. Bromthymol Blue, Thymol Blue, and Cresol Red have also been employed in another Laboratory[8]; and substitution of hexane, heptane, or cyclohexane in system A does not affect the resolution[8]. Two examples of separations are described here, as typical of the success of this method.

The "acetone-insoluble" fraction gave, in system A, four major bands of R_F's 0.76, 0.68, 0.59 and 0.48; the first three of these were readily crystallizable from acetone or methanol, the last crystallizable with difficulty. A, minor band at R_F 0.18 was also crystallized from methanol with difficulty.

The "Skellysolve B-soluble" fraction gave, in system B, five major bands at R_F's 0.90, 0.78, 0.68, 0.55 and 0.45, of which the first one and last two only have been crystallized at this time; three minor bands appear at R_F's 0.31, 0.21 and 0.10, of which only the first has been crystallized. To save time and solvent, the last of these bands is best removed by a preliminary pass of the mixture through a large column of system A. The material running near the front is then added to the columns of system B.

Although a column 4.5 cm in diam. will handle only 2 g of mixture per run, no other convenient method has been found in this Laboratory to give any crystalline alkaloids from these mixtures. Preliminary work on other fractions has been prom-

TABLE

APPLICATIONS TO VARIOUS

Mixture		*Buxus sempervirens (acetone-insoluble)*	*Buxus sempervirens (Skellysolve B-soluble)*	*Rauwolfia vomitoria (crude)*	*Zygadenus paniculatus (refined)*
System used		*A*	*B*	*F*	*E*
Indicator used		*Phenol Red*	*Phenol Red*	*Bromcresol Purple*	*Bromthymol Blue*
Band I	R_F	0.76	0.90	0.75	0.36
	%	22 %	20 %	12 %	23 %
	identity by	new alkaloid a b c	new alkaloid a c	"*a*" c	neogermitrine b c
Band II	R_F	0.68	0.78	0.60	0.19
	%	35 %	8 %	25 %	20 %
	identity by	new alkaloid a b c	new alkaloid c	"*b*" + trace "*a*" + one minor base c	neogermidine + germidine etc. b c
Band III	R_F	0.59	0.68	0.36	0.16
	%	14 %	26 %	25 %	50 %
	identity by	new alkaloid a b c	new alkaloid c	ajmaline + trace "*b*" c	zygacine b c
Band IV	R_F	0.48	0.55	0.25	
	%	8 %	16 %	22 %	
	identity by	new alkaloid a c	new alkaloid a b c	Three minor bases + trace ajmaline c	
Band V	R_F	0.18	0.45	0.12	
	%	1 %	8 %	6 %	
	identity by	new alkaloid a c	new alkaloid a b c	Two minor bases c	
Other bands			Three minor bands R_F's 0.31, 0.21, 0.10		

ising, and two more crystalline alkaloids have been obtained from one of these fractions by use of system A.

This method also gave five crystalline alkaloids when used for the mixture of bases of genus *Pachysandra*, employing the system ethylene dichloride–Skellysolve B–methanol–water (2.5:15:2:0.3) (C)[8].

The systems Skellysolve B–methyl cellosolve–*n*-butanol–water (8:1.5:0.5:0.4); Skellysolve B–benzene–ethanol–water (8:2:5:0.9) and Skellysolve B–carbon tetrachloride–methanol–water (5:5:5:0.6), were all satisfactory for the "acetone-insoluble" fraction in terms of partition coefficient, but were inferior to system A above for various reasons (possession of a poor solvent action, a poor equilibrium, and a toxic component, respectively).

Large dry-packed columns containing the above system A were very stable, could be used over wide variations in temperature, gave no loss of efficiency after

II

ALKALOID MIXTURES

Commercial "Veratrine" (refined)	*Commercial "Veratrine" (front-running)*	*Cissampelos pareira (crude)*	*Cyclea peltata (crude)*	*Cyclea peltata (front-running)*
D	*E*	*D*	*D*	*E*
Bromthymol Blue	*Bromthymol Blue*	*Bromcresol Purple*	*Bromcresol Purple*	*Bromcresol Purple*
above 0.50 80 % cevadine + veratridine see next column	0.33 62 % cevadine a b c	0.65 10 % *l*-curine b c	above 0.70 70 % tetrandrine + fangchinoline see next column	0.45 57 % tetrandrine b c
0.31 9 % sabadine + minor bases b c	0.15 38 % veratridine b c	0.32 3 % hayatin + isochondrodendrine b c	below 0.70 10 % isochondrodendrine + minor bases b c	0.25 43 % fangchinoline b c
0.11 2 % various minor constituents b c				

Code: Systems: A, ethylene dichloride 5 : Skellysolve B 10 : methanol 2 : water 0.3.
B, ethylene dichloride 1 : Skellysolve B 10 : methanol 2 : water 0.16.
D, ethylene dichloride 8 : Skellysolve B 12 : methanol 3 : water 0.6.
E, ethylene dichloride 3 : Skellysolve B 12 : methanol 2 : water 0.24.
F, ethylene dichloride 6 : Skellysolve B 10 : methanol 2.5 : water 0.5.

Methods of identification: a = by crystallization; b = by infrared spectrum; c = by paper chromatography, or rarely by a single sharp narrow band on the partition column being used for the separation.

repeated use or prolonged disuse, and gave excellent separations of very closely related compounds. An occasional large slurry-packed column gave equally good results, but most columns packed by that method were loose and gave much diffusion of bands.

B. Separation of the alkaloids of the Menispermaceae[11]

Good results were obtained in the separation of the bisbenzylisoquinoline alkaloids of *Cyclea peltata* Diels and *Cissampelos pareira* L., although the presence of the chloroform-insoluble isochondrodendrine in both mixtures created some difficulty.

The systems used were ethylene dichloride–Skellysolve B–methanol–water (8:12:3:0.6) (D) and ethylene dichloride–Skellysolve B–methanol–water (3:12:2:0.24) (E). Bromcresol Purple was used as an indicator, and the tests were conducted in a slurry-packed column of 1.8 cm i.d., with 15 ml of stationary phase on 15 g of Celite.

The mixed *Cyclea* alkaloids were first chromatographed in the stronger system (D); all running at R_F greater than 0.7 was collected, and run in the weaker system (E). That running behind R_F 0.7 had an infrared spectrum similar to isochondrodendrine; paper chromatography showed it to be a mixture of several bases, possibly including isochondrodendrine. The separation in the weaker system gave two bands of R_F's 0.45 and 0.25, which were shown by infrared spectra and paper chromatography to be pure tetrandrine and fangchinoline, respectively.

The mixed *Cissampelos* alkaloids were separated in the stronger system (D), and gave only two bands; a large amount of the mixture (isochondrodendrine and related bases, and tars) remained insoluble in the mobile phase. The front band (R_F 0.65) had

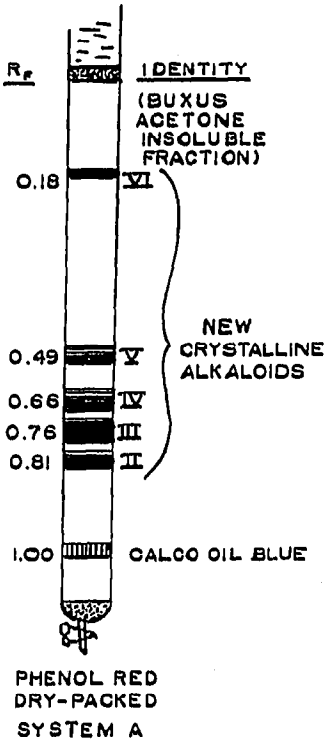

Fig. 1. Diagram of a column separation of the *Buxus* alkaloids.

an infrared spectrum and paper-chromatographic behavior identical with those of *l*-curine. The second band (R_F 0.32) was a mixture, probably containing hayatin and isochondrodendrine.

Thus, although neither of these alkaloid mixtures has been fully separated into individual components by this method, both have yielded some pure alkaloids with a minimal amount of effort and loss of material.

C. Separation of indole-type alkaloids

A complex mixture of alkaloids from *Rauwolfia vomitoria* Afzel., containing at least eight alkaloids (but no reserpine), was separated visually into fractions of R_F's 0.75 (1), 0.60 (2), 0.36 (3), and 0.25 (4) by the system ethylene dichloride–Skellysolve B–methanol–water (6:10:2.5:0.5) (F), using Bromcresol Purple as an indicator. System A with the same indicator could also be used. Fraction 1 contained only one alkaloid (*a*); fraction 2 contained a small amount of *a* and of another base, but was

mostly a third alkaloid (*b*); fraction 3 contained a small amount of *b*, but was mostly ajmaline (identified by paper chromatography); and fraction 4 contained a small amount of ajmaline and three minor bases. Some of the mixture (about 5 %) remained insoluble in the mobile phase. Bases *a*, *b*, and ajmaline were the major components of the total mixture; further work on these, including cleaner separations on better columns and crystallization of the major bases, is in progress.

In a co-operating Laboratory, a variety of other mixtures of indole alkaloids (including ajmaline and ibogaine) have been separated into crystalline components by the use of systems A and C. It was noted that some of these mixtures could be better resolved by alumina chromatography[8].

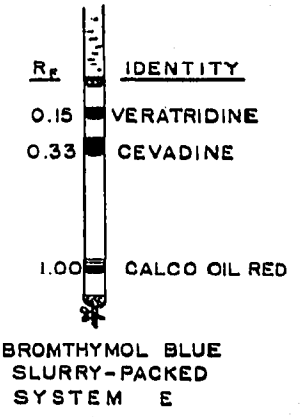

Fig. 2. Separation of a natural mixture of cevadine and veratridine.

D. Separation of the alkaloids of the tribe Veratreae (see Fig. 2)

This general method, which has been shown to be applicable to non-polar highly saturated alkaloids and fairly polar aromatic bases, required surprisingly little modification to be adapted to the highly polar Veratrum-type polyhydroxy ester alkaloids.

A sample of total alkaloids from *Zygadenus paniculatus* Nutt., chromatographed in system E with Bromthymol Blue as indicator, gave three bands only. The germine tri-esters—almost totally neogermitrine—ran at R_F 0.36; the germine diesters (mostly neogermidine and germidine) ran at R_F 0.19; and almost pure zygacine appeared at R_F 0.16. Only small amounts of the aromatic zygadenine-3-esters isolated from another sample[12] were present.

Striking success was encountered in the separation of a natural mixture of veratridine (veracevine-3-veratroate) and cevadine (veracevine-3-angelate) from commercial "Veratrine" (an extract of *Schoenocaulon officinale* Gray). Using system E, cevadine ran at R_F 0.33 and veratridine at R_F 0.15; there was a wide area between the bands which contained no solid, and each band was completely homogeneous. The cevadine band crystallized readily from acetone–water (m.p., becomes anhydrous at 140°, then melts at 204–207°). Veratridine has never been crystallized; efforts on this sample were equally without success. When compared with previous methods[13], this represents a highly efficient resolution of these two compounds.

Slower-running bands in Veratrine could be collected by the use of system D. Two bands, at R_F's 0.31 and 0.11, were visible; the first of these contained three bases, but was probably mostly sabadine; it represented about 10 % of the total Veratrine mixture.

A system, benzene–methanol–water (10:4:0.6), was also used for these bases; although it gave fair results, its equilibrium was highly unstable, probably because the benzene took much methanol into the non-polar phase.

Further work on all of these separations, and more applications of this general method, will be reported in future papers.

SUMMARY

A convenient method has been developed for the separation of the alkaloids of *Buxus sempervirens* L. This method involves the use of partition chromatography on Kieselguhr, with an indicator in the stationary phase; the separate bands are detected visually, collected as a whole and in many cases crystallized directly. General considerations are presented concerned with the application of this method to other alkaloidal mixtures. The method is shown to give rapid and convenient separations of mixtures of bisbenzylisoquinoline alkaloids; of complex mixtures of indole alkaloids; and of mixtures of veratrum ester alkaloids, including an efficient and complete separation of a natural mixture of cevadine and veratridine.

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