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A GRADIENT METHOD FOR THE COUNTER-CURRENT SEPARATION OF ALKALOIDS USING A HEAVY ORGANIC PHASE

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SUMMARY

A method is proposed for the separation of all the components of a complex mixture of acids or bases by counter-current distribution with progressive variation of the pH. The function that controls the double distribution and dissociation equilibrium in the case of a weak base was investigated. Since the separation depends on the difference in the product of the dissociation constant K_b and the distribution coefficient K_r, two possibilities are considered: the use of a lower buffer phase and an upper organic phase whose composition is progressively changed in such a way as to vary K_r, the bases being eluted in order of increasing $K_r \cdot K_b$, and the use of a lower organic phase with an upper buffer phase whose pH is varied from neutrality to increasingly acidic values in such a way as to extract the alkaloids in order of decreasing $K_r \cdot K_b$. With the aid of this second process, it was possible to isolate the known nine alkaloids of *Strychnos nux-vomica*, *i.e.* strychnine, α- and β-colubrines, brucine, pseudostrychnine, pseudobrucine, icajine, vomicine, and novacine, as well as four others that had not been discovered previously. The separation of strychnine and brucine on the basis of the difference in the product $K_r \cdot K_b$ was also examined. Where this product was equal (*e.g.* for colubrines and brucine between chloroform and water), partial modification of one phase (addition of 35 % of ethyl acetate) leads to non-proportional changes in the K_r values, and so permits separation.

INTRODUCTION

The separation of basic and acidic substances by distribution at a fixed pH has had, and still has, important applications both in chromatography and in counter-current processes. Both methods have been improved by techniques (some of them patented) involving the use of different pH values. Thus columns have been prepared with zones of absorbent saturated with buffers to give a variation of the pH from the top to the bottom of the column[1], and counter-current distributions (CCD) have been carried out with buffers whose pH values varied from one test tube to the next as the stationary phase[2].

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These methods are very laborious and are not readily reproducible with the various materials to be separated. Moreover, the results are unsatisfactory in the separation of complex mixtures. They are therefore rarely used. CCD with movement of both phases in opposite directions is also rarely used, since it does not allow the the separation of mixtures of more than two or three substances simultaneously[3].

In the present article, we describe a simple method by which complex mixtures of alkaloids (more than thirteen in the case of *Strychnos nux-vomica*) can be separated with a CCD apparatus consisting of 200 tubes. We believe that this method is useful not only from the point of view of large-scale preparations but also for the isolation of the "minor alkaloids", which are present in quantities of up to 1 % with respect to the predominant components. Due to the fact that this method allows the isolation of substances present in very small quantities it may be used in structural investigations by means of mass spectrometric and X-ray methods.

Preparative chromatography does not offer acceptable solutions, both because of the difficulty of recovery and because of the frequency with which a single spot corresponds to more than one substance (the mixture of thirteen alkaloids from *Strychnos nux-vomica* chromatographed in equal quantities gives at most five or six spots depending on the mobile phase used).

In using CCD for the separation of alkaloids, it is necessary to take into account, and even to use, the difference in their pK_a values (*e.g.*, strychnine 7.27, pseudobrucine 5.60 (ref. 4)), to which there should correspond a pH that differs from one case to another for a suitable distribution between immiscible solvents. Thus by variation of the two phases in the CCD apparatus, various authors[5] have separated one or two substances at a time from a mixture. The use of aqueous stationary phases having various pH values with a lighter organic phase has not given positive results[2].

In this work we used a heavier organic stationary phase, which remained the same throughout the process, with a buffered aqueous phase, the pH of which was varied discontinuously from neutrality to increasingly acidic values; this system gave selective extraction of substances from the stationary organic phase. The substances could thus be collected on emerging from the last test tube of the CCD apparatus as the pH in the reservoir tube was changed. To obtain a better understanding of the interdependence of the factors on which this method depends, the general validity of which is shown by the results obtained, it is useful to consider the following theoretical treatment. The double dissociation and distribution equilibrium, which was first discussed by IRVING AND WILLIAMS[6], is outlined below from the analytical point of view.

THEORY

An alkaloid, as a weak base, satisfies the dissociation equilibrium

$$K_b = \frac{[OH^-]\,[B^+]}{[B\,OH]} \qquad (1)$$

According to the distribution law, the concentrations of the undissociated form in the two phases are related as follows:

$$K_r = \frac{[B\,OH]}{c_o} \qquad (2)$$

where c_o = concentration in the organic phase.

The total quantity (T) of alkaloids is the sum of the following three terms:

$$T = c_o V_o + [\mathrm{B\ OH}] \cdot V_w + V_w \cdot [\mathrm{B^+}]$$

where V_o = volume of the organic phase and V_w = volume of water.

The product $[\mathrm{B\ OH}] \cdot V_w$ may be neglected in the sum, since $[\mathrm{B\ OH}] \ll [\mathrm{B^+}]$ and $[\mathrm{B\ OH}] \ll c_o$. This is because the alkaloid in the aqueous phase is mainly in the form of salt in the pH range in question. We can thus write:

$$T = c_o V_o + V_w \cdot [\mathrm{B^+}] \tag{3}$$

From eqns. (1) and (2) we find

$$[\mathrm{B\ OH}] = c_o \cdot K_r = \frac{[\mathrm{OH^-}]\,[\mathrm{B^+}]}{K_b} \tag{4}$$

From eqn. (3), if $V_w = V_o$ (volumes of the aqueous and organic phases equal), division by V_o gives:

$$\frac{T}{V_o} = c_o + [\mathrm{B^+}]$$

where T/V_o is the analytical concentration, which is constant, and which we shall denote by c_t = total moles/l (volume of one phase). Substitution of $c_t = c_o + [\mathrm{B^+}]$ in eqn. (4) gives:

$$\frac{[\mathrm{B^+}]}{c_t - [\mathrm{B^+}]} = \frac{K_r \cdot K_b}{[\mathrm{OH^-}]} = \frac{K_r \cdot K_b [\mathrm{H^+}]}{K_w}$$

Taking logarithms and changing the sign we obtain:

$$\log \frac{c_t - [\mathrm{B^+}]}{[\mathrm{B^+}]} = \log \frac{K_w}{K_r \cdot K_b} + \mathrm{pH}$$

The expression $\log \{(c_t - [\mathrm{B^+}]) / [\mathrm{B^+}]\}$ (logarithm of the reciprocal of the extraction coefficient) is thus a linear function of the pH. Fig. 1 shows the lines for the unitary values of the term $\log \{K_w / (K_r \cdot K_b)\}$ and those of strychine and of brucine. The slope is 1.

It can be seen that the concentration in the aqueous phase is equal to that in the organic phase, $c_t = 2[\mathrm{B^+}]$ and hence $\log \{(c_t - [\mathrm{B^+}]) / [\mathrm{B^+}]\} = 0$, when $\mathrm{pH} = \log \{(K_r \cdot K_b) / K_w\}$.

Fig. 2 shows the concentration in the aqueous phase as a function of the pH corresponding to unitary values of the expression $\log \{K_w / (K_r \cdot K_b)\}$.

For a base having a given K_b, K_r can be varied by variation of the organic solvent (one phase is always water). Two bases having the same value of the product $K_r \cdot K_b$ can be separated by modification of the organic phase, since the changes in the distribution coefficients K_r of the two bases are generally not proportional, as can be seen in the separation of colubrines and of brucine (see later). Two alkaloids

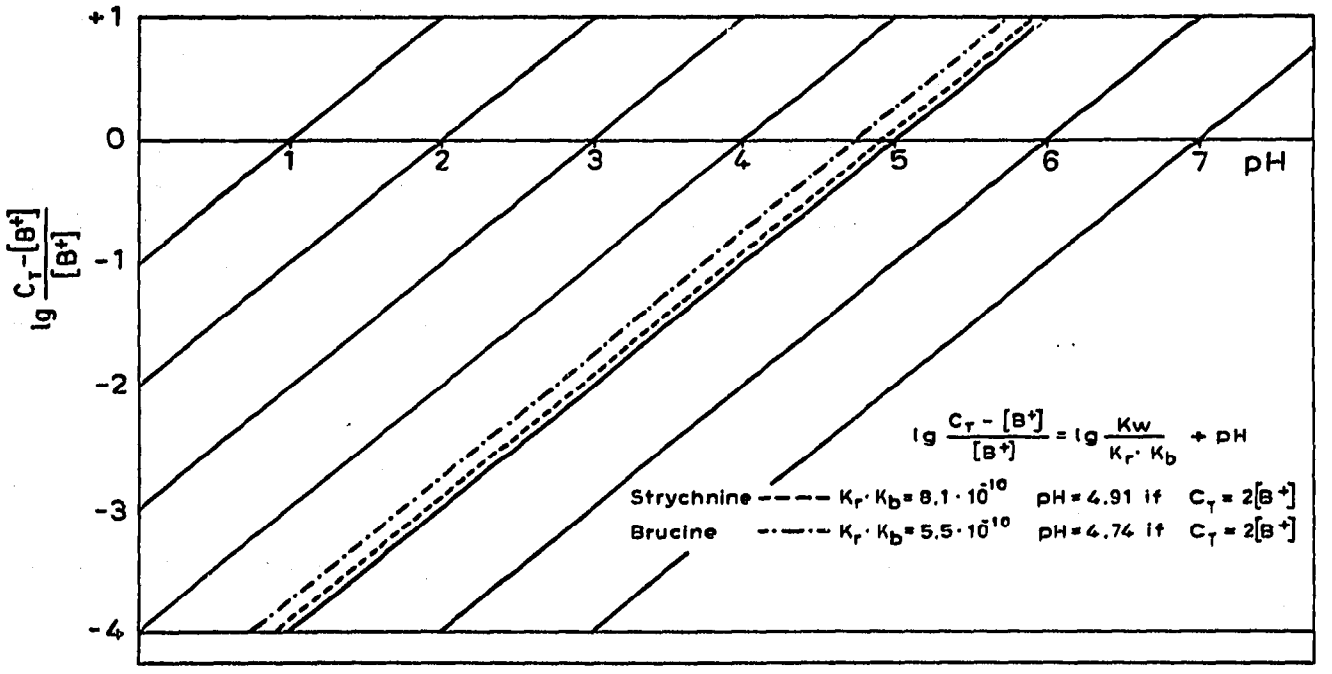

Fig. 1. Logarithm of the reciprocal of the extraction coefficient c_t — $[B^+]/[B^+]$ as a function of the pH corresponding to unitary values of the term log $\{K_w/(K_r \cdot K_b)\}$.

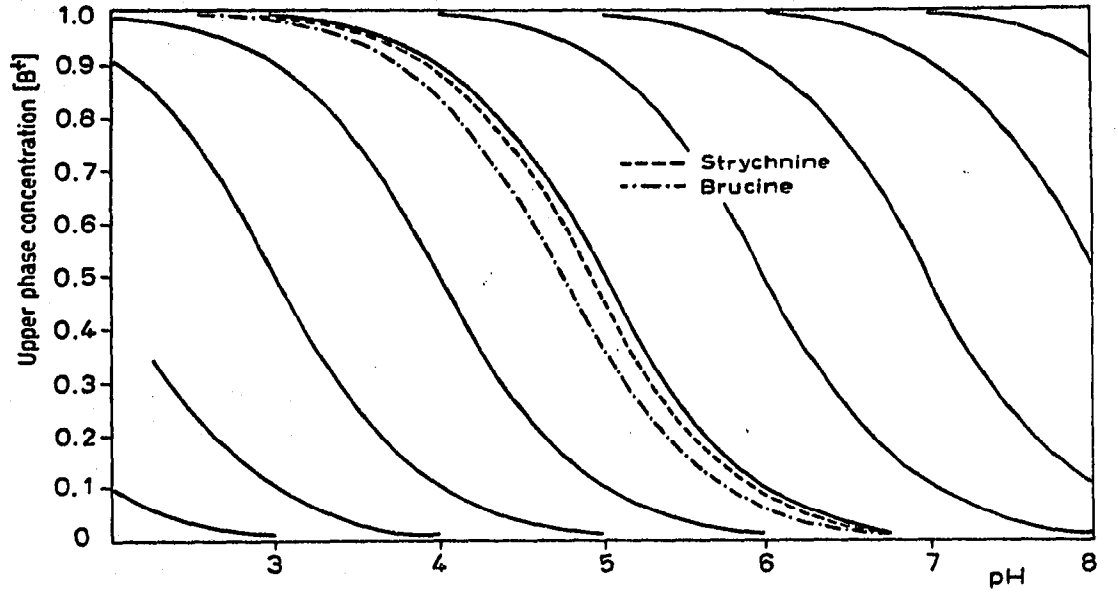

Fig. 2. Concentration of the aqueous phase $[B^+]$ as a function of the pH corresponding to unitary values of the term log $\{K_w/(K_r \cdot K_b)\}$.

having very similar values of K_b can also be separated in a similar manner.The K_b value of strychnine, for example, is 8% higher than that of brucine (K_b of strychnine 10^{-6}, K_b of brucine 9.2×10^{-7}) (ref. 7). The value of K_r between water and chloroform, as found spectrophotometrically by the present authors, is 8.1×10^{-4} for strychnine and 6.0×10^{-4} for brucine; the product $K_r \cdot K_b$ is therefore 8.1×10^{-10}

for strychnine and 5.52×10^{-10} for brucine. The possibility of separation is thus further increased in the distribution, since the product $K_r \cdot K_b$ of strychnine is 32 % higher than that of brucine. Figs. 1 and 2 show the curve for strychnine and brucine.

Since the pH range must be between 7 and 3 to ensure that the alkaloids do not decompose over long periods and to guarantee the absence of emulsions, the product $K_r \cdot K_b$ must be between 10^{-7} and 10^{-11} for the countercurrent separation. Alkaloids having low basicities must therefore have a relatively high K_r* (unfavorable organic solvent) while a very small K_r (very favorable organic solvent) is necessary for more basic alkaloids.

Complex mixtures of alkaloids can be dealt with in two ways. A stationary aqueous phase having a constant pH is used with a lighter organic phase, which is progressively varied in such a way as to decrease K_r and to increase the extractive capacity for the various alkaloids, which are thus carried along in order of increasing $K_r \cdot K_b$. Alternatively, a heavier stationary organic phase may be used with a buffered aqueous phase, the pH of which varies from neutrality to increasingly acidic values in the course of the separation, so that the alkaloids are transported in order of decreasing $K_r \cdot K_b$.

The second of these procedures requires a very small volume of the solvent forming the stationary phase, which may be chloroform, dichloromethane, carbon tetrachloride, or mixtures with other solvents in a wide range of compositions such that the specific gravity is always higher than 1. The first procedure can be carried out by varying the percentages of two or more organic solvents, but requires a large volume of these solvents, and so presents the problem of their recovery and fractionation. Moreover, the lighter organic phase, by the nature of the possible constituents (ether, benzene, ethyl acetate), generally gives higher K_r values than chloroform, and so makes it necessary to work in the alkaline pH range; this presents problems of stability, solubility, and emulsion formation.

In the countercurrent fractionation of the thirteen alkaloids of *Strychnos nux-vomica* in the CCD apparatus, chloroform (2 l) was used as the stationary phase with phosphate buffer of pH between 6.5 and 3.3 as the eluent. The total volume of the eluent was about 80 l for 8000 passes (200-stage Craig apparatus). Eleven clearly separated fractions were obtained, and ten of these contained only one substance each. The only mixed fraction consisted of α- and β-colubrines and brucine, and this was further fractionated with chloroform–ethyl acetate (65:35) as the stationary phase and phosphate buffer of pH 6.2 as the mobile phase.

It is interesting to note that the K_r values of these alkaloids, which had identical $K_r \cdot K_b$ values in chloroform, show distinct and non-proportional changes even when the composition of the organic phase is only partly altered.

The order in which the components appear in the CCD separation between chloroform and buffer is as follows. The alkaloids of the normal series appear first in order of increasing molecular weight (strychnine, α- and β-colubrines, and brucine, the last three appearing together); these are followed by the alkaloids of the pseudo series (pseudostrychnine and pseudobrucine), and finally by those of the N-methyl pseudo series, again in order of increasing molecular weight (icajine, vomicine, and novacine). In the subsequent separation with chloroform–ethyl acetate (65:35) and

* Note that K_r is the ratio of the concentrations of the undissociated form in the aqueous and in the organic phases.

buffer, the above order is reversed, *i.e.* brucine appears first, and is followed by β- and then by α-colubrine.

EXPERIMENTAL

The alkaloid mixture fractionated was the mother liquor from the crystallization of strychnine sulphate, and was supplied by Sandoz of Milan (to whom we wish to express our thanks).

The mixture was made alkaline with dilute sodium carbonate and extracted with chloroform. CCD apparatus used was a 200-stage Post apparatus, volume 10/10 ml. The aqueous phase was a 0.2 *M* phosphate buffer saturated with chloroform. Below pH 4.5, 0.2 *M* monopotassium phosphate was used with hydrochloric acid. The chloroform used as the lower phase contained 0.5 % of ethanol to prevent phosgene formation. Since water extracts ethanol from chloroform, a small quantity (< 0.1 %) of ethanol had to be added to the aqueous solution used.

Five grams of the mixture of free bases were dissolved in 16 ml of chloroform, and the solution was filtered and introduced into the first two tubes of the Craig apparatus. The volume of aqueous phase introduced in each pass was about 10 ml; the agitations for each stage were 10–12. The decantation time gradually decreased from more than 5 min at the beginning to 30–40 sec. The upper phase was collected as it left the 199th test tube. The contents of the tubes (one out of every ten) were periodically chromatographed on Silica Gel $HF_{256+366}$ with benzene–ethyl acetate–diethylamine (7:2:1) as the solvent and examined in UV light. The Dragendorff reagent can be used only after the complete removal of diethylamine at 100°. The pH of the buffer was varied from its initial value of 6.5 as indicated on the abscissa of Fig. 3, the

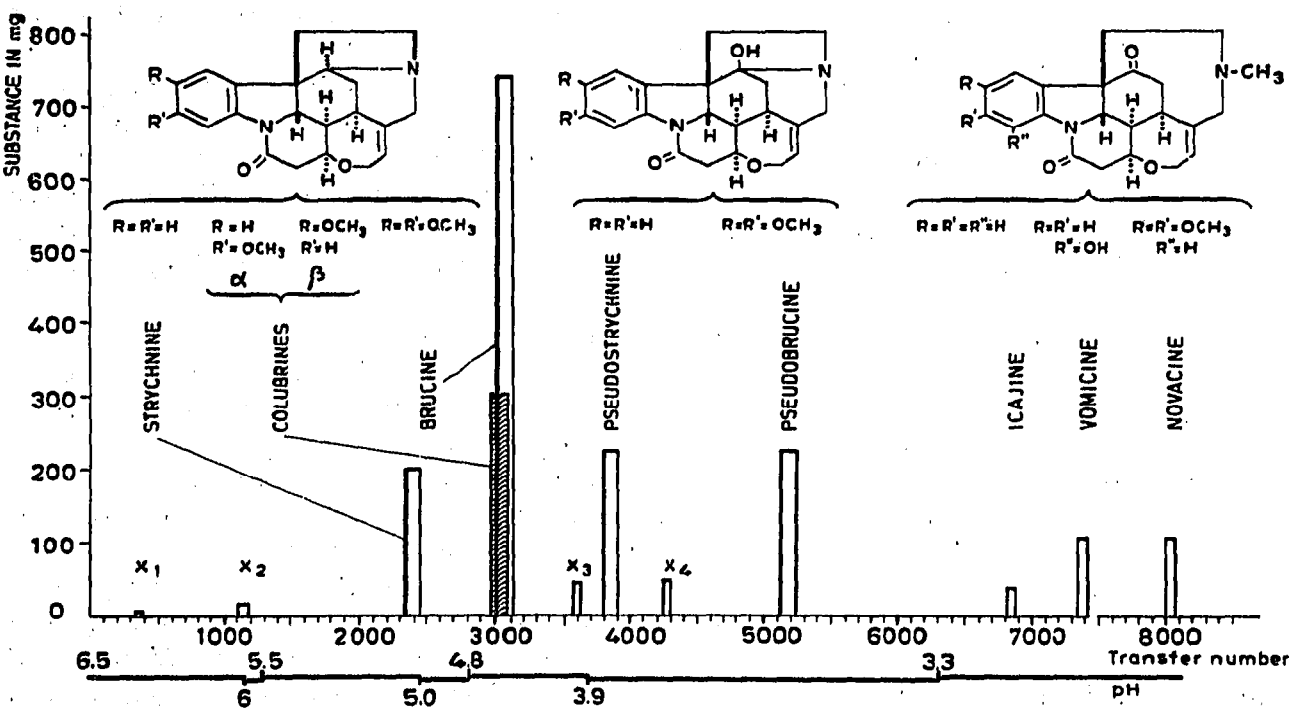

Fig. 3. Separation of *Strychnos nux-vomica* alkaloids by CCD with chloroform and buffer from pH 6.5 to 3.3.

changes being made only when the substance emerging from the 199th test tube was clearly separated from the next.

The magnitude of the pH change in the reservoir container of the apparatus depended on the ease of separation of the substances. This could be estimated from the chromatographic investigations with the aid of the curves in Fig. 2, which give the increase in the concentration in the aqueous phase as a function of the decrease in pH.

The various fractions collected were made alkaline with sodium bicarbonate and extracted with chloroform. Fraction 4, which contained the two colubrines and brucine, was further separated in the Craig apparatus with chloroform–ethyl acetate (65:35) as the stationary phase and phosphate buffer of pH 6.2 as the mobile phase. As can be seen in Table I, the brucine was separated first and collected on leaving the 199th tube, while the two colubrines were separated only after prolonged recycling. All the fractions were crystallized from ethyl acetate, (sometimes mixed with hexane). Alcohol was never used, because of the presence of pseudostrychnine and pseudobrucine, which are readily alkylated to their hydroxyl oxygens. The strychnine, α- and β-colubrines, brucine, pseudostrychnine, pseudobrucine, icajine, vomicine and novacine were identified on the basis of their mass spectra and by comparison of the IR spectra in chloroform and of the chromatographic mobilities in the following four solvent systems: methanol–chloroform (2:8); benzene–ethyl acetate–diethylamine (7:2:1); cyclohexane–chloroform–diethylamine (5:4:1); pyridine–ethyl acetate–water (11.5:75:16.5) (upper phase).

TABLE I

CCD SEPARATION OF FRACTION NO. 4 BETWEEN CHLOROFORM–ETHYL ACETATE (65:35) AND PHOSPHATE BUFFER AT pH 6.2

200-tube apparatus, volume 10/10 ml. Substance 1018 mg.

	Weight (mg)	*Number of passes*	*Procedure*
Brucine	732	520	The upper phase (750 ml) was collected as it left the 199th test tube.
α- and β-colubrines		700	The substances were not separated at the 199th test tube.
Recycling by junction of the 199th to the first test tube.			
α-Colubrine	117	4000	Both phases are collected between test tubes 700 (3 × 200 + 100) and 790 (3 × 200 + 190).
β-Colubrine	106	4000	Both phases are collected between tubes 810 (4 × 200 + 10) and 890 (4 × 200 + 90).

The alkaloids are indicated in Fig. 3 in the order of their appearance from the CCD apparatus with chloroform and buffer (the colubrines are thus superimposed on brucine). The abscissa gives the number of passes and the pH changes, while the ordinate gives the quantity of alkaloids in each fraction. The width of the base indicates the number of passes, and hence the volume in litres (number of passes divided by 100) corresponding to the complete elution of each individual fraction. Finally, the graph also shows four alkaloids denoted by x_1, x_2, x_3, and x_4, which could not be identified with any of the known *Strychnos nux-vomica* alkaloids.

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