REVIEW

METHODS FOR THE SEPARATION OF SOUTH AMERICAN STRYCHNOS AND INDIAN CURARE ALKALOIDS

G. B. MARINI-BETTÒLO AND G. C. CASINOVI
Istituto Superiore di Sanità, Rome (Italy)

In the chemical study of the active constituents of curares the research workers[13, 19, 21, 24, 26, 39, 51, 56, 58, 86, 87] are faced with a number of problems, which in spite of century-long investigation are not yet close to a definite solution.

These substances are alkaloids and are found in curares employed by numerous Amazonian tribes as arrow poisons, as well as in the bark of various species of *Strychnos*.

The first great difficulty lies in the fact that for many years the conventional methods did not allow an adequate separation, for analytical or preparatory purposes, of the many alkaloids to be found in the bark and roots of South American *Strychnos* species and in the curares derived therefrom.

The claim is well justified that also in this field the introduction of chromatographic methods has marked a turning point in the research.

In fact, it is due to HEINRICH WIELAND, who employed chromatography on an alumina column, that in 1941 the first pure alkaloids were obtained from calabash curares as well as from *S. toxifera*[91].

In 1952, SCHMID, KARRER and coworkers[85] introduced partition chromatography on a cellulose column for preparatory purposes. For analytical purposes they developed two-dimensional chromatography on paper using known alkaloids; by determining the rates of movement and colour reactions they supplied the basis for the identification of these substances. These methods have been adopted, with slight variations, by THEODOR WIELAND[94] and by MARINI-BETTÒLO and coworkers[1].

The difficulties inherent in the resolution of alkaloid mixtures by chromatographic methods only, whether on a column or on paper using one and two-dimensional systems, have subsequently led us to introduce the use of aqueous solvents in these methods[27], as well as electrophoretic techniques[59, 60], electrophoresis on cellulose columns and counter-current distribution[26].

The object of this review is to set forth and comment on the techniques and practical aspects of the various methods that make possible the solution of problems for which chromatographic methods alone appear to be insufficient.

An outline will, therefore, be given of:

1. Data concerning the starting material.
2. Preliminary assays for orientation purposes.
3. Methods for extracting all the alkaloids from curares and *Strychnos* species.
4. Analytical separation of alkaloids by:

References p. 432/433.

(a) paper strip chromatography;
(b) circular paper chromatography;
(c) paper electrophoresis;
(d) two-dimensional chromatography.

5. Preparative separation of alkaloids by:
(a) absorption chromatography on alumina columns;
(b) partition chromatography on cellulose columns;
(c) separation on paper sheets;
(d) band electrophoresis;
(e) continuous electrophoresis;
(f) column electrophoresis;
(g) counter-current distribution.

1. STARTING MATERIAL

The following two types of material are usually employed for the extraction of alkaloids:

1. Curares prepared by Amazonian Indians.
2. Plants of the genus *Strychnos* from South America.

In both cases there may be considerable differences between the various laboratory materials available.

The curares which contain quaternary curarizing alkaloids of the indole group, are differentiated into: calabash curares and "pot curares"*. As commonly known, they are prepared by Amazonian and Orinoco Indians by boiling extracts from various plants, including *Strychnos* bark; a sticky black mass is thus obtained containing concentrated alkaloids together with other substances.

From data reported in the literature it may be assumed that calabash curares contain about 8–10% of quaternary curarizing alkaloids.

Work on curares, which undoubtedly are a valuable material owing to their high concentration of active principles, is somewhat hampered by the presence of other alkaloids from other plants added during the preparation.

A more interesting material may be obtained from the bark and roots of *Strychnos* species. This has not undergone other transformations and is substantially made up of most of the alkaloids to be found in Indian curares. The alkaloid percentage, however, is very low; on an average it lies between 0.1 and 0.2%, and exceptionally 1%.

On the other hand two main difficulties are likely to be met with when using botanical material: first the exact botanical determination of the plant; second the finding of a sufficient quantity of material of the same species, since one rarely finds plants of the same species in one and the same region. This accounts for the fact that more than 1–2 kg of bark of a single species is not often available.

* One cannot at the present moment consider calabash curares as the only source of indole curarizing alkaloids. These alkaloids may be found in curares stored in various containers and even in glass bottles (see Symposium on Curares and Curarizing Substances, Rio de Janeiro, August 1957).

References p. 432/433.

Furthermore, it must be borne in mind that for alkaloid extraction the part of the *Strychnos* bark close to the ground and the roots are useful, whereas neither the branches nor the fruit can be utilized, since according to our observations they do not contain alkaloids even when these are plentiful in the bark and roots.

Another difficulty is the possible variation, as was found in some cases, in the alkaloid compositon in one and the same species according to its geographical distribution and the season when it was gathered.

2. PRELIMINARY ASSAYS

Since the available material is so heterogeneous, it may be necessary—particularly for the plant collector—to have a method of detecting the presence of curarizing alkaloids.

This can be carried out, in a preliminary way, by treating a few hundred milligrams of material on a spot plate with 4–5 drops of 5% acetic acid (or 10% tartaric acid) and then absorbing the coloured liquid with a bit of filter paper within a few minutes[23]. As soon as the paper has dried it should be treated with a 1% ceric sulphate solution[46]. If indole curarizing alkaloids are present the paper will turn red or violet or blue or green.

This test is so simple and rapid that it can be carried out in the field much to the advantage of the material collector. He is thus spared the trouble of gathering plants and carrying them hundreds of km before finding that they are of no use from the chemical point of view.

This method is applied today in Brazil when gathering botanical material.

3. METHODS OF EXTRACTING ALKALOIDS

The process for the extraction of alkaloids is more or less the same whether the curares are prepared by Indians or obtained from plant bark, since in both cases the curarizing alkaloids have to be separated from inert substances and from tertiary alkaloids.

WIELAND utilized extraction with methanol followed by boiling water. The alkaloids were then precipitated with mercuric chloride and converted to the reinecke salts which were subjected to chromatography on alumina columns[89–91].

SCHLITTLER AND HOHL[80] in their work on *S. melinoniana* percolated the powdered bark first with water and afterwards with methanol containing 3% acetic acid. KING, on the other hand, usually employed a 3% aqueous solution of tartaric acid[48], while SCHMID AND KARRER[85] used methanol and acetic acid.

One or two percent acetic acid is more convenient than tartaric acid, because the latter has to be removed as alkaline tartrates, which is not necessary in the case of the former.

The extracts obtained by the above methods are concentrated to small volumes, brought to pH 8 with ammonia or alkali and extracted with chloroform to separate

References p. 432/433.

tertiary bases. The solution is then treated with dilute hydrochloric acid and the alkaloids are precipitated either as reineckates or picrates.

The reinecke salts of the quaternary and some of the tertiary bases can be fractionated owing to their different solubilities in acetone–water[85], though this method is not always reliable.

The reinecke salts can also be purified directly on alumina columns (WIELAND and SCHLITTLER), but today it is considered better to transform them into chlorides by the Kapfhammer method, *i.e.*, by treating them with silver sulphate and then with barium chloride.

BOEKELHEIDE[20] extracts the calabash curares with water and then with methanol. The concentrated liquids are brought to pH 8 and then extracted with methylene chloride, thus eliminating the tertiary bases. The quaternary bases are afterwards precipitated with picric acid.

The mixture of alkaloid picrates is passed over an ion exchange resin (Dowex-II-X4 Cl) to recover the quaternary alkaloid chlorides.

4. ANALYTICAL SEPARATION OF ALKALOIDS

(a) *Paper strip chromatography*

It must be pointed out here that the peculiarities of quaternary ammonium derivatives make it rather difficult to decide which solvents are best suited for these separations.

TABLE I

COMPOSITION OF SOLVENTS USED IN CHROMATOGRAPHIC SEPARATIONS OF *Strychnos* AND CURARE ALKALOIDS*

Solvent composition	*Designation*	*Author*	*Reference*
Acetic ester–water–pyridine 200:200:90	A	KARRER	82
Methyl ethyl ketone–water–cellosolve 300:70:15	B	KARRER	82
Methyl ethyl ketone saturated with water and 1–3% methanol	C	KARRER	85
	2	MARINI-BETTÒLO	27
Acetic ester–pyridine–water 7.5:2.3:1.65	D	KARRER	85
	1	MARINI-BETTÒLO	27
Acetic ester–acetic acid–water 7.5:0.9:0.9	E	KARRER	85
n-Butanol–chloroform–water 10:10:0.6	F	KARRER	85
Ethyl acetate–methyl ethyl ketone–glycol monomethyl ether–water 200:300:154:200	A	TH. WIELAND	92
Acetic ester–pyridine–water 200:90:200	B	TH. WIELAND	92
Ethyl acetate–pyridine–water 1000:600:300	C	TH. WIELAND	92
Ethyl formate–acetone–water 1000:1450:400	D	TH. WIELAND	92
Methyl ethyl ketone–pyridine–water 1400:300:300	E	TH. WIELAND	92
Methyl ethyl ketone saturated with water containing 10% methanol	3	MARINI-BETTÒLO	27
5 g of $CH_3COONa \cdot 3H_2O$ in 100 ml 10% acetic acid; the solution is saturated with amyl alcohol	4	MARINI-BETTÒLO	27
10 g $CH_3COONa \cdot 3H_2O$ in 100 ml 10% acetic acid; the solution is treated as above	5	MARINI-BETTÒLO	27
n-Butanol saturated with water		BOEKELHEIDE	20

* It is important to avoid traces of peroxides in solvents. For this reason methyl ethyl ketone must be distilled before use and then passed over an ion-exchange column (Dowex-50-X4) with ferrous ion as the cation.

Butanol must also be purified by washing it with sodium bisulphite.

References p. 432/433.

TABLE II

R_C VALUES AND PHYSICO-CHEMICAL PROPERTIES OF ALKALOIDS FROM SOUTH AMERICAN *Strychnos* SPECIES AND CALABASH CURARES*

*Alkaloid***	R_C		*Colour*	*Fluorescence*	*Colour with $Ce(SO_4)_2$ (Spot plate)*	*Reference*
	C	*D*				
Erythrocurarine	0.12	0.31	Red	Red		64
Alkaloid A	0.23	0.55			Blue-violet	85
Solimoesine 3	0.27	—			Purple	63
Guiacurarine I	0.31	0.42	Brown-yellow		Blue-violet	64
Alkaloid B	0.34	0.51			Red-violet	85
Alkaloid C	0.34	0.51			Red-violet	85
Alkaloid D	0.35	0.68			Red-violet	85
Alkaloid E	0.36	0.58			Blue	85
Toxiferine I	0.42	0.67			Red-violet	85
Alkaloid F	0.49	0.73			Blue-violet	85
Guiacurarine 10	0.50	1.05			Violet	76
Alkaloid S chloromethylate	0.51	1.35			Purple-red	73
Macrophylline A	0.52	1.6			Violet	38
Rubrocurarine 4	0.52	0.50	Red	Red		64
Guiacurarine 8	0.58	0.56	Yellow	Yellow	Blue-violet	63
Rubrocurarine 3	0.63	0.54	Red	Red	Pale blue	64
Rubrocurarine 2	0.65	0.51	Red	Red		64
Alkaloid G	0.65	0.73			Blue	85
Fluorosolimoesine 2	0.67	1.2	Yellow	Yellow		63
Alkaloid R	0.68	0.92			Purple	73
Caracurine I	0.70				Purple-red	4
Alkaloid H	0.71	0.99			Red-violet	85
Erythrocurarine 3	0.72	1.00	Red	Red	Blue	76
Guiacurarine 9	0.73	0.94	Orange-red	Orange	Violet-blue	76
Guiacurarine 2	0.76	0.99			Violet-blue	64
Rubrocurarine 1	0.78	0.92	Red	Red		64
Fluorosolimoesine 3	0.79	3.1		Green-yellow		63
Fluorosolimoesine 4	0.79	2.0		Green-yellow		63
Caracurine II	0.8				Purple	4
Caracurine III	0.8	1.32			Purple-red	4
Calebassine (C-Toxiferine II = C-Strychnotoxine)	0.8	1.03			Blue-violet	85
Solimoesine 2	0.85	—			Red	63
Alkaloid I	0.89	1.06			Blue-violet	85
Solimocurarine	0.93	0.82			Blue-violet	63
Curarine	1.00	1.00			Blue	85
"Rotes Alkaloid"			Red			85
Caracurine IV	1.00				Violet	4
Guiacurine I	1.05	1.34			Blue-violet	64
Guiacurarine 2	1.07	1.33			Blue	64
Caracurine Va	1.1				Purple-blue	4
Alkaloid J	1.04	1.12			Red-violet	85

(Contd. on p. 416)

* At present there is some confusion in the nomenclature of alkaloids from *Strychnos* bark and calabash curares.

To avoid these difficulties a Commission to unify the nomenclature of these alkaloids was appointed at the International Symposium on Curare and Curarizing Substances in Rio de Janeiro, August 1957.

In this review we have used the names given in the original papers, with the exception that WIELAND'S *C-Alkaloid* term has been simplified to *alkaloid*. The former term is now inaccurate because many C-alkaloids, *i.e.* calabash alkaloids, have been found in *Strychnos* species. Moreover the calabash alkaloids have also been found in other curares. The term C-alkaloid followed by letters was necessary in the past to avoid confusion with alkaloids from other plants. In this review no confusion is possible since only alkaloids of *Strychnos* and curare are referred to.

** The alkaloids are in the form of the chlorides unless otherwise stated.

References p. 432/433.

TABLE II (*Continued*)

Alkaloid	R_C		*Colour*	*Fluorescence*	*Colour with $Ce(SO_4)_2$ (Spot plate)*	*Reference*
	C	*D*				
Guaianine	1.12				Pale blue-violet	36
Caracurine IX chloromethylate	1.12				Violet	6
Alkaloid 2 fluorescent	1.18	1.05		Blue	Blue-violet	76
Alkaloid K (Dihydrotoxiferine)	1.22	1.14			Violet	85
Solimoesine I	1.3	1.54			Purple-violet	63
Caracurine V	1.4	—			Purple-red	4
Caracurine VII chloromethylate	1.40	—			Purple-orange	4
Erythrocurarine 2	1.43	1.15	Red	Red	No colour	64
Alkaloid M	1.45					3
Melinonine H	1.47					8
Melinonine K	1.52					8
Alkaloid Y	1.59	2.22			Red-violet	3
Caracurine VI	1.6				Purple	3
Macrophylline B	1.64	1.86			Violet	38
Calebassinine	1.68	1.38			No colour	85
Guiacurarine 3	1.70	1.64	Orange	Green-yellow	Green-blue	64
Diaboline	1.76	2.10			No colour	38
Fedamazine	1.9			Orange	Blue	7
Melinonine J	2.01					8
Melinonine F	2.01					8
Pseudofluorocurine	2.10		Yellow	Green-yellow	Orange-red	73
Caracurine VII	2.1				Orange	3
Fluorocurine	2.10	1.70	Yellow	Green-yellow	Orange	84
Fluorocordatine	2.13	1.20		Blue	Blue	76
Melinonine E	2.18					8
Fluorocurinine	2.23	1.65	Yellow	Yellow-green	Pale red	85
Fluorocurarine (C-Curarine III)	2.25	1.71	Pale yellow	Blue	Pale blue	85
Alkaloid UFC						85
Melinonine F	2.01					8
Melinonine M	2.42				Pale blue	8
Alkaloid L	2.50	1.99			Red	84
Desacetyldiaboline	2.64	2.42			Pink-red	76
Mavacurine	2.70	2.23			Red-violet	8,63
Precurarine	2.78	3.8			Blue	63
Fluorescent Alkaloid 1	2.90	1.69		Green-yellow	Pale red	76
Kryptocurine	2.95	3.10				72
Melinonine G	3.00					8
Fluorosolimoesine 1	3.00	1.5	Yellow	Yellow-green		63
Alkaloid X	3.5	4.9			Red	85
Alkaloid O	3.95	4.30			No colour	36
Premavacurine	4.0	3.6			Red-violet	63
Alkaloid P	—	—			Blue	36
Alkaloid 1						92
Alkaloid 2					Red-violet	92
Xanthocurine	—	1.58	Orange		Green-blue	36
Alkaloid UB		1.13			Brown	46
Alkaloid Q		4.92				73
Croceocurine			Orange-red			72
C-Strychnotoxine Ia	*					92
Nor-C-dihydrotoxiferine					Purple	6

* R_F 0.25 in TH. WIELAND'S solvent A.

References p. 432/433.

Various solvents have been tried out by different authors (see Table I), but except special cases the most suitable are KARRER AND SCHMID'S C and D, as they give fair resolutions and most of the displacement values recorded in the literature have been obtained with these solvents.

If a two-dimensional system is used, these solvents give in many cases a fairly satisfactory separation of many alkaloids.

In Table II the R_C values (displacements calculated with respect to curarine) of these substances are given in both solvents C and D, as well as other characteristics by which they can be recognized. In some cases when displacement rates refer to other alkaloids such as mavacurine or melinonine B, it is convenient to convert them into R_C values, by calculation, in order to attain a uniform expression.

In view of the low velocity of these alkaloids, chromatography must always be carried out continuously for 12 to 18 hours if good resolutions are to be attained.

The determination of R_C in the case of these alkaloids very often gives rise to considerable errors: it has been noticed, for instance, that although the same chromatographic conditions are maintained an increase in the quantity of the substances employed is sufficient to produce a strong variation in the R_C (Fig. 1).

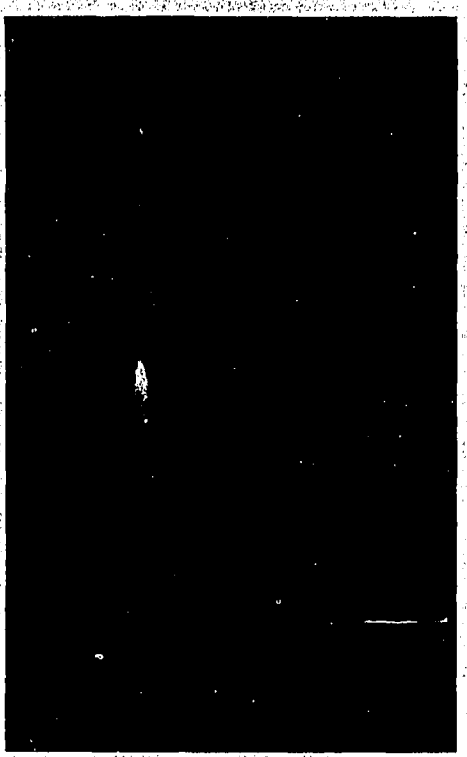

Fig. 1. Chromatograms of *S. parvifolia* alkaloids. Influence of the different weights of the sample on the R_F values.

SCHMID AND KARRER[85] had already pointed out that the R_C values are reproducible only when a 50 γ sample of alkaloid chloride is taken. Moreover, it is necessary that the standard curarine chloride should have a medium displacement of 11 cm for all alkaloids in solvent "C", and in solvent "D", 24 cm for alkaloids with $R_C < 1$, and 18 cm for those with $R_C > 1$.

References p. 432/433.

In the second place, it should be noted that when using different alkaloid mixtures an alteration and often an inversion occurs in the R_C values of the single alkaloids. Furthermore, the presence of large quantities of some alkaloids influences the movement of the others.

In fact, it is not uncommon that an alkaloid mixture which appears to be simple in two-dimensional chromatography turns out to be much more complex when a large quantity of the same mixture is used, the same experimental conditions being maintained.

(*b*) *Circular paper chromatography*

In the case of very complex alkaloid mixtures it has been found that one-dimensional separation on paper is rendered still more difficult by the partial overlapping of a long series of alkaloids, which have R_C values that are very close to each other. In such cases the components come out much more clearly if the chromatogram is run on a small band only a few centimetres wide[27].

Better results are obtained by means of Rutter's circular paper chromatography in that clear-cut bands appear which are well differentiated from neighbouring bands.

This system, though very convenient for a preliminary investigation of alkaloid mixtures, has, however, the drawback that comparison data are very scanty in the literature. Nevertheless, it is extremely useful for selecting the most suitable separation solvents and for making a rapid examination of certain mixtures.

(*c*) *Electrophoresis on paper*

By means of electrophoresis on paper it is possible to separate numerous curarizing alkaloids from *Strychnos* species, as was shown for the first time by MARINI-BETTÒLO AND LEDERER[59].

A good separation can be obtained by adjusting the conditions such as the pH of the buffer. Since electrophoresis is based on principles that are quite different to those of paper chromatography, substances that have identical R_C values may often be well separated.

For some alkaloids the mobility of the ions has been measured at various values of the pH; these mobilities are given in Table III.

TABLE III

ELECTROPHORETIC MIGRATION OF ALKALOIDS FROM *Strychnos* SPECIES AND CALABASH CURARES*

Alkaloids	*Displacement towards the cathode in 3 hours (in mm)*					
	pH 2.3	*pH 4.3*	*pH 6.4*	*pH 8.5*	*pH 10.5*	*pH 11.4*
Mavacurine	31	—	24	30	24	19
Fluorocurine	77	—	80	90	80	42
Alkaloid D	65	—	90	90	100	52
Calebassine	65	—	80	90	78	50
Curarine	106	98	110	108	—	64

* G. B. MARINI-BETTÒLO AND J. A. COCH FRUGONI, *Gazz. chim. ital.*, 86 (1956) 1326.

References p. 432/433.

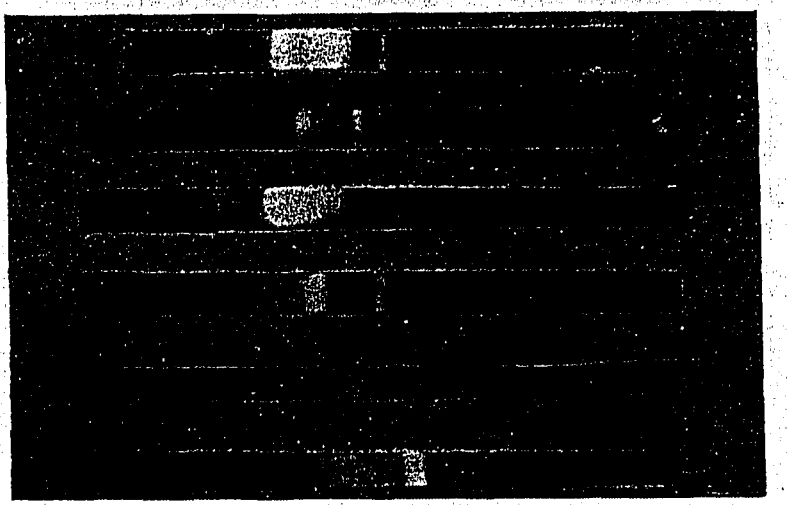

Fig. 2. Electrophoretic separation of *S. trinervis* alkaloids (fluorescent zones under U.V. light) (G. B. MARINI-BETTÒLO AND M. LEDERER, *Nature*, 174 (1954) 133).

Electrophoresis on paper strips chiefly permits the rapid identification of alkaloid groups rather than the single components that are present (Fig. 2).

To this end the portable apparatus designed by MARINI-BETTÒLO AND COCH FRUGONI may be usefully employed in the field[67].

(*d*) *Two-dimensional chromatography*

Two-dimensional chromatography is usually performed in the first direction with KARRER'S solvent "D" (Table I) and in the second direction with KARRER'S solvent "C" (Table I).

TH. WIELAND suggests other solvents as reported in Table I[94]. BOEKELHEIDE[20] found it more convenient to use a water–*n*-butanol mixture in one direction and KARRER'S solvent C in the other, thus avoiding the use of pyridine which is always troublesome.

For the determination of the relative displacement, curarine has generally been

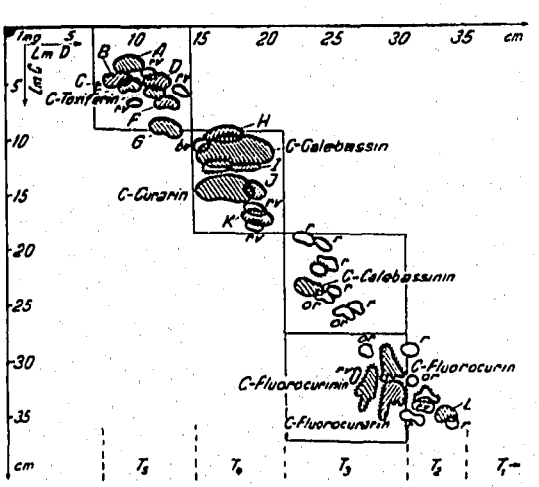

Fig. 3. Two-dimensional chromatogram of calabash curare alkaloid chlorides (H. SCHMID, J. KEBRLE AND P. KARRER, *Helv. Chim. Acta*, 35 (1952) 1864).

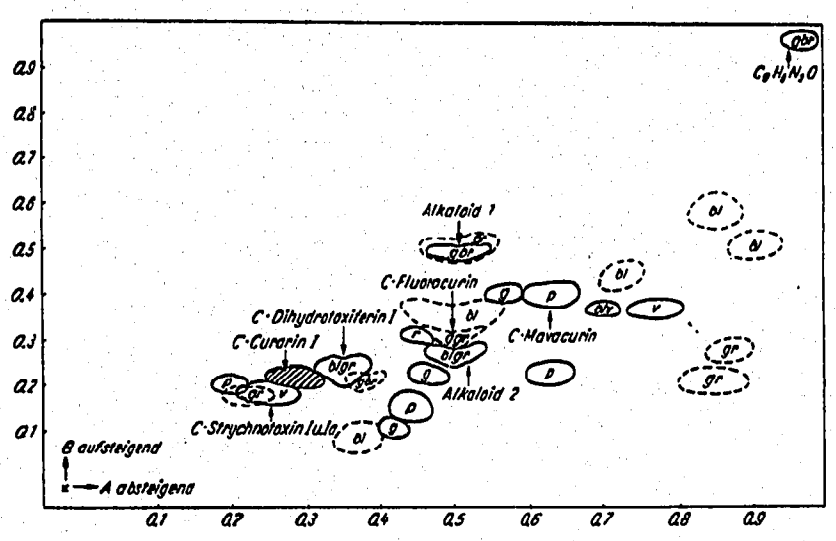

Fig. 4. Chromatogram of calabash curare alkaloid chlorides (TH. WIELAND AND H. MERZ, *Ber.* 85 (1952) 731).

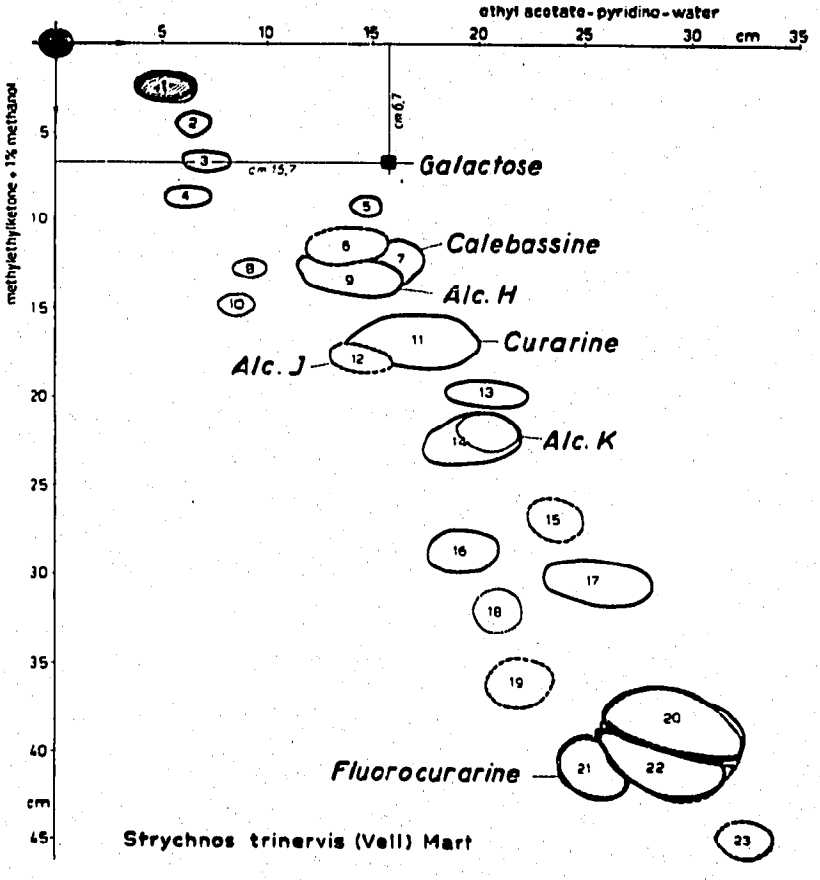

Fig. 5. Chromatogram of *S. trinervis* alkaloid chlorides (K. ADANK, D. BOVET, A. DUCKE AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 83 (1953) 966).

References p. 432/433.

used as standard; other substances have, however also been taken, as previously stated, such as galactose and mavacurine[1,8].

Examination of the various chromatograms shows that the spots are ranged diagonally (Figs. 3, 4 and 5); this happens because the R_C values often have the same sequences in the two solvents. Here the same remarks apply which we made in the preceding pages with regard to reciprocal influences hampering the recognition of substances on the basis of R_C values only. This does not happen, for instance, with amino acids etc.

(e) Chromatography with aqueous solvents

To prevent a diagonal distribution of the alkaloids and thus facilitate the resolution of these mixtures, it is advisable to use other systems. To this end aqueous solvents

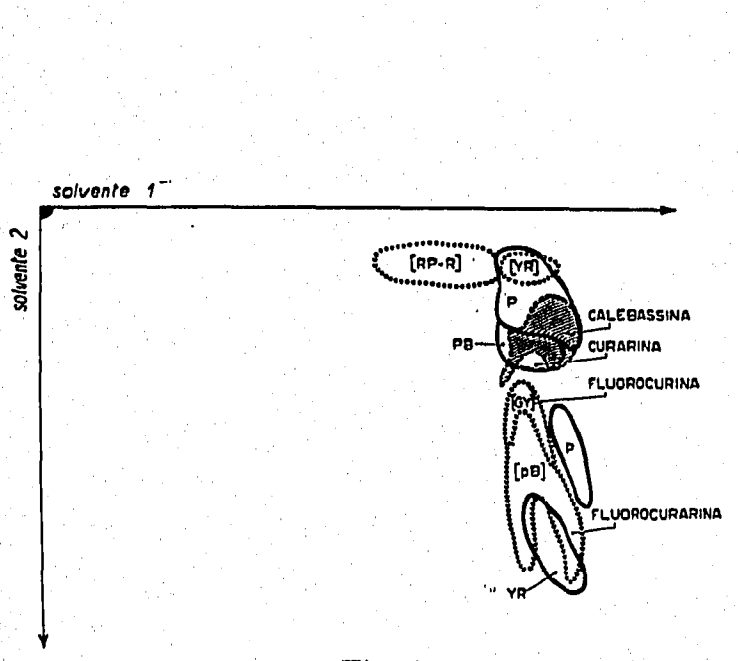

Fig. 6

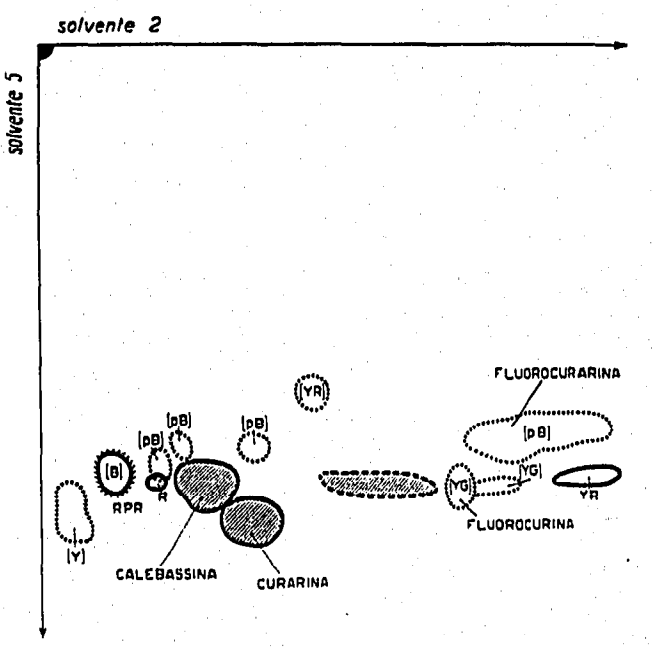

Fig. 7

Fig. 6. Two-dimensional chromatogram of *S. solimoesana* alkaloids (G. C. CASINOVI, M. LEDERER AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 86 (1956) 342).
Fig. 7. Two-dimensional chromatogram of *S. solimoesana* alkaloids. First direction: organic solvent; second direction: aqueous solvent (G. C. CASINOVI, M. LEDERER AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 86 (1956) 342).

have been suggested by CASINOVI, LEDERER AND MARINI-BETTÒLO[27].

Their study has been chiefly directed towards mixtures based on sodium acetate (see Table I), which, in the case of *S. solimoesana* Kruk, produced a clear inversion of the spot sequence (see Figs. 6 and 7) obtainable with KARRER's solvents C and D[27].

(f) Combination of chromatographic and electrophoretic methods

By combining paper electrophoresis with chromatography, a very good resolution may be realized. This system has been suggested by CASINOVI, LEDERER AND MARINI-BETTÒLO[27] (see Fig. 8); electrophoresis should be used first and chromatography

afterwards, and in the former a volatile electrolyte buffer (acetic acid or/and amine) should be employed in order to avoid interferences with the chromatographic stage.

In this case also the various spots are no longer arranged diagonally as in the case, for instance, with *S. tomentosa* alkaloids (Fig. 9).

These methods, however, have the drawback that diffusion occurs which makes the definition of the spots less precise. The same applies to two-dimensional paper electrophoresis when several buffers or a single one with different ionic forces are used[59,60].

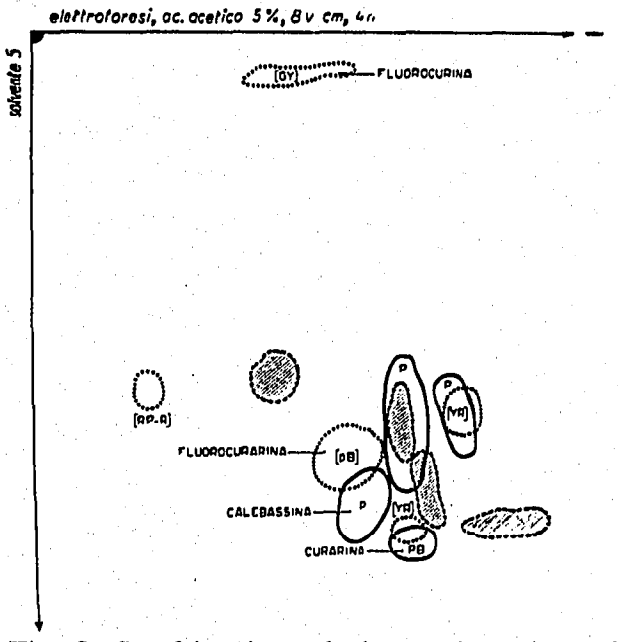

Fig. 8. Combination of electrophoresis and paper chromatography for the separation of *S. solimoesana* alkaloids (G. C. CASINOVI, M. LEDERER AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 86 (1956) 342).

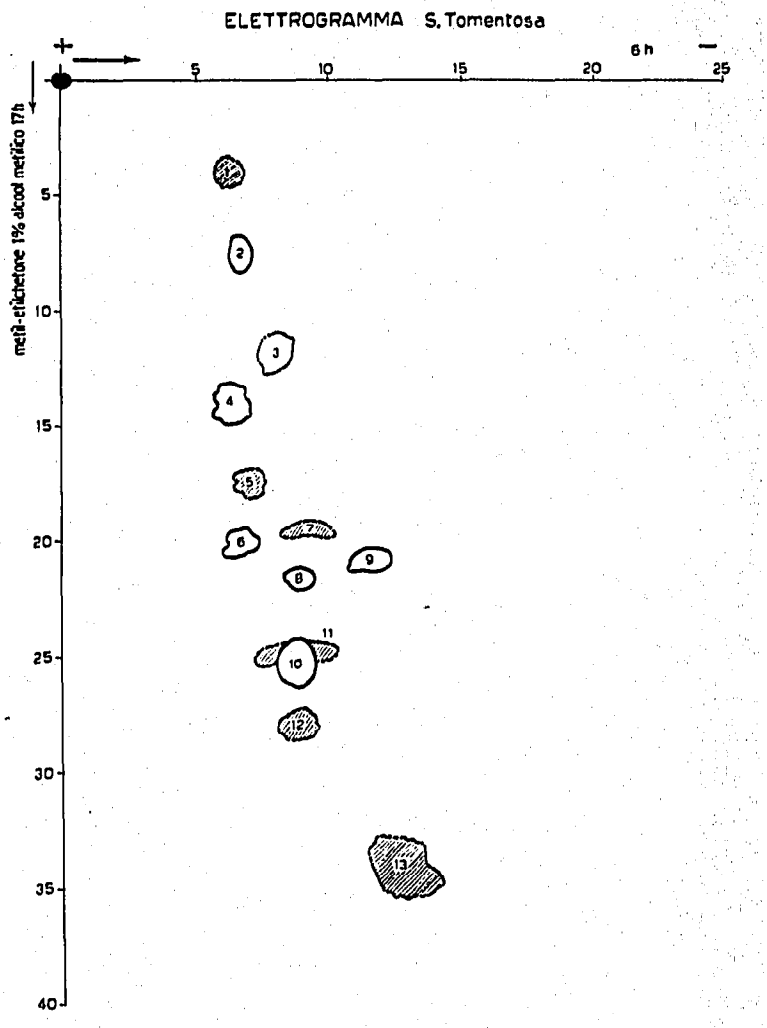

Fig. 9. Combination of electrophoresis and paper chromatography for the separation of *S. tomentosa* alkaloid chlorides (G. B. MARINI-BETTÒLO, M. LEDERER, M. A. IORIO AND A. PIMENTA, *Gazz. chim. ital.*, 84 (1954) 1155).

5. PREPARATIVE SEPARATION METHODS

(a) *Chromatography on absorption columns*

Chromatography on alumina columns has provided the first method for obtaining pure alkaloids either from curares or South American *Strychnos* species.

H. WIELAND, who worked with columns of alumina or Fuller's earth and utilized

alkaloid reineckates or alkaloid perchlorates, was able to separate these alkaloids, whereby the first indication of the great complexity of these mixtures was obtained. SCHLITTLER AND HOHL[80] used alumina columns and eluted the reineckates of *S. diaboli* with acetone and methanol.

(*b*) *Partition chromatography on cellulose columns*

An improvement on the preceding method, which is, however, still being used for the separation of certain fractions, is the partition chromatography on cellulose columns, subsequently developed by SCHMID AND KARRER[85] and by TH. WIELAND[92]. In this separation KARRER's solvent "C" is mainly used as in paper strip or in two-dimensional chromatography. BOEKELHEIDE[20] suggests the use of a specially prepared cellulose column and butanol–water as solvent. After a first separation by elution, slow-moving fractions were extruded and extracted separately.

Yet, even under these conditions, it is only by working with very large quantities of substances and discarding many fractions that appreciable quantities of pure alkaloids can be obtained; when employing small quantities it is extremely difficult to realize preparative separations that are of any use.

Indeed, various factors affect the resolving power of partition columns; the most important of these are packing, height, homogeneity, elution velocity, and weight ratio between substance and absorbent.

As pointed out by MARTIN AND SYNGE[69], the maximum efficiency for a given column is obtained by using very fine charging material and very low elution speeds. This is the main drawback to the method, as the operations last so long that the zones already separated are apt to become mixed up again through diffusion.

A systematic study of the various factors and their influence should be made in order to understand fully their importance; such a study should be carried out with artificial mixtures, the resolution obtained with the various types of column being observed.

Our experiments in this field, however, have shown that the resolving power of a given column is not the same in the case of different groups of substances; thus although the performance of a column may prove satisfactory with some test substances, *e.g.* dyes, its behaviour may be quite different with alkaloids from *Strychnos* species and curares.

These results may possibly be attributed to the fact that other phenomena, besides mere partition, have an influence on the resolving power of a given column.

These considerations have already been brought forward by MARTIN AND SYNGE[69], by CRAIG[29] and by HECKER[37].

The necessity of examining the other characteristics of a column is a logical consequence of the above remarks. For testing purposes, samples of the substances to be separated should be used; but since pure samples of *Strychnos* alkaloids are difficult to obtain, particularly of the high degree of purity required for all the tests necessary for the systematic study, the problem cannot yet be investigated fully.

Yet, despite the lack of numerical and systematic data for direct comparison of

References p. 432/433.

the various columns employed, such a comparison can still be made by using paper chromatograms of mixtures of alkaloids that can easily be obtained and chromatograms of the same mixtures, or others containing the same alkaloids on various columns.

For instance, the usual procedure when investigating the content of alkaloids in a given plant is to determine the composition of the mixture by common methods. Once the composition is known, the problem of separation on a preparatory scale arises.

As a rule, the available material is both scarce and of a complex composition, due to the great number of alkaloids and the closeness of their R_C values; therefore, an efficient resolution is indispensable, also from a quantitative point of view.

A comparison between the resolving power of a strip of paper and column of

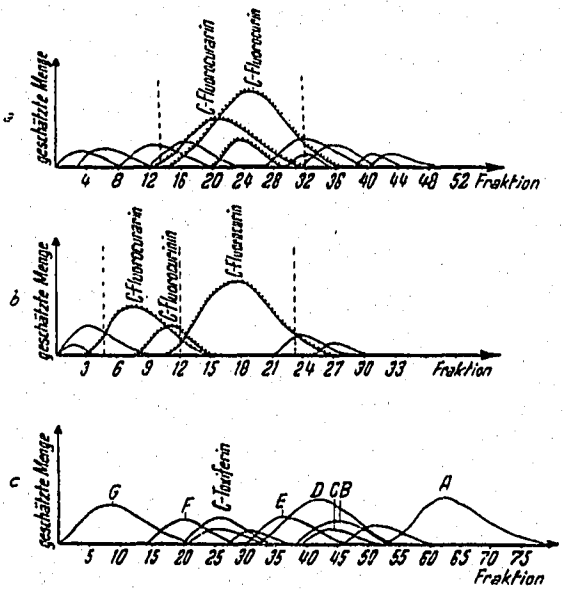

Fig. 10. Separation diagrams of calabash alkaloid chlorides (H. SCHMID, J. KEBRLE AND P. KARRER, *Helv. Chim. Acta*, 35 (1952) 1864).

equal length shows that the efficiency of the latter is very small, even when the charging density of the two is about the same and the weight ratio between mixture and cellulose is the same.

In their work on calabash curarizing alkaloids SCHMID AND KARRER[85] claim that the resolving power of the cellulose column coincides quantitatively with that of the corresponding paper chromatograms, but they only give schematic diagrams on the subject (Fig. 10).

A closer examination of these results shows, for instance, that in the T_5 group of the NIII fraction, alkaloids A (R_C: 0.55) and G (R_C: 0.73) are present together with other alkaloids, and that for their separation, contrary to expectation, many chromatograms using both solvents were necessary in order to secure complete resolution. Similar examples may be found in the work of the above-mentioned authors[85].

In the case of *S. melinoniana* alkaloids, BÄCHLI, VAMVACAS, SCHMID AND KARRER[8] give a diagram (Fig. 11) of the column chromatography of a mixture of at least 14 alkaloids, but the graph (see Fig. 11) shows only two well-separated maxima. Evi-

References p. 432/433.

dently, the resolving power is not high. As a further confirmation, reference may be made to the work of CASINOVI[26], who, when separating a relatively simple mixture of *S. amazonica* alkaloids, proved the inefficiency of a column whose resolving power was considerable for dye mixtures; consequently he had to resort to counter-current distribution in order to obtain satisfactory separations.

In an investigation of the fractionation of *S. macrophylla*[38] alkaloids by means of a cellulose column under normal conditions, it was found, by subjecting the fractions

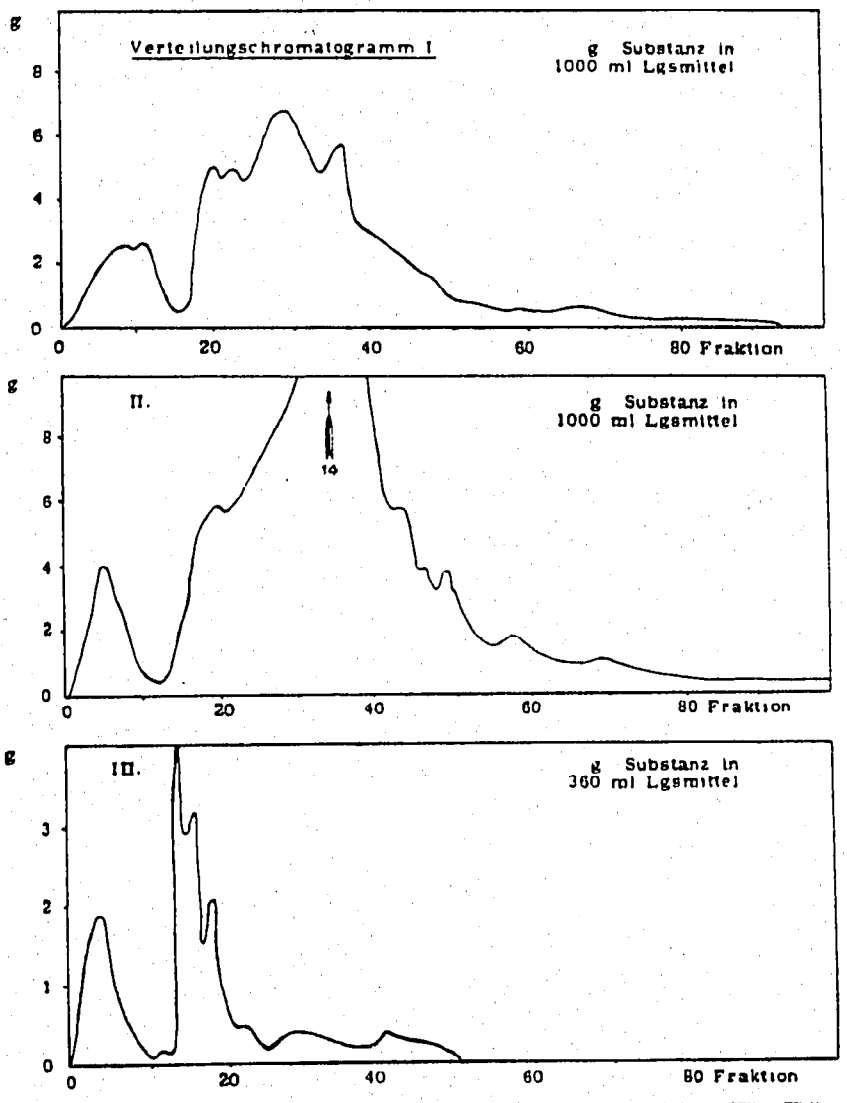

Fig. 11. Separation diagrams of *S. melinoniana* Baill. alkaloid chlorides (E. BÄCHLI, C. VAMVACAS, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 40 (1957) 1181).

to paper chromatography, that the fractionation is fairly satisfactory for those alkaloids (Mavacurine – Macrophylline B or Macrophylline A and B) whose R_C ratio is very high, but that it is practically *nil* for alkaloids whose R_C values are very close together, *e.g.*, Fluorocurarine–Mavacurine, which on a paper strip of equal length would be easily separated (Fig. 12).

When the mixture consists of a great number of alkaloids, whose R_C values are

References p. 432/433.

very close together, as is the case with *S. solimoesana* Kruk., the cellulose column can only perform a preliminary separation into various groups and the isolation of small quantities of the single components is only possible by means of repeated chromatography on paper sheets[63].

The same considerations apply to alkaloids from *S. guianensis*[64] (Fig. 13) and *S. subcordata*[76], and to WIELAND'S calabash curares (Fig. 14).

From what has been said about columns it follows that it is necessary on the one hand to improve their resolving capacity—a problem which is now being studied—and on the other to resort to more efficient methods in which the undesirable effects

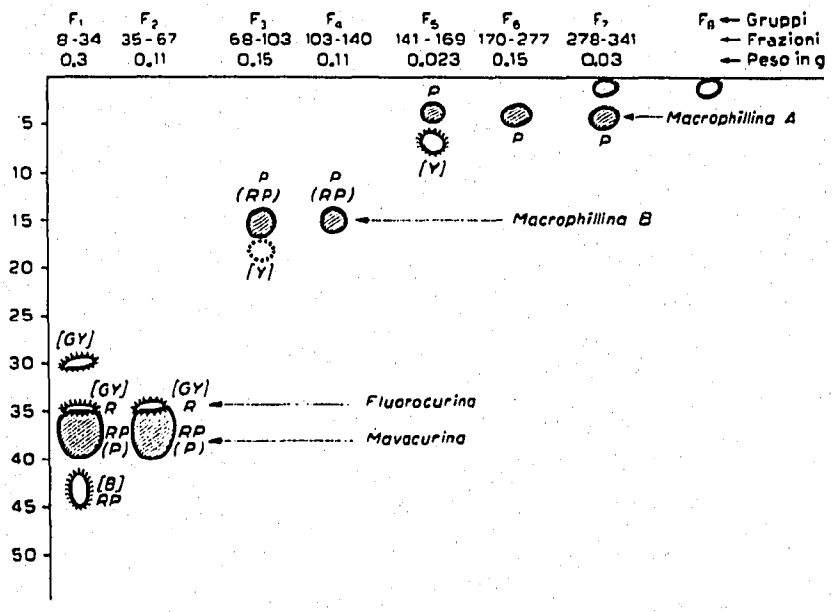

Fig. 12. Schematic representation of the separation of *S. macrophylla* Barb. R. alkaloid chlorides (M. A. IORIO, O. CORVILLON, H. MAGALHÃES ALVES AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 86 (1956) 923).

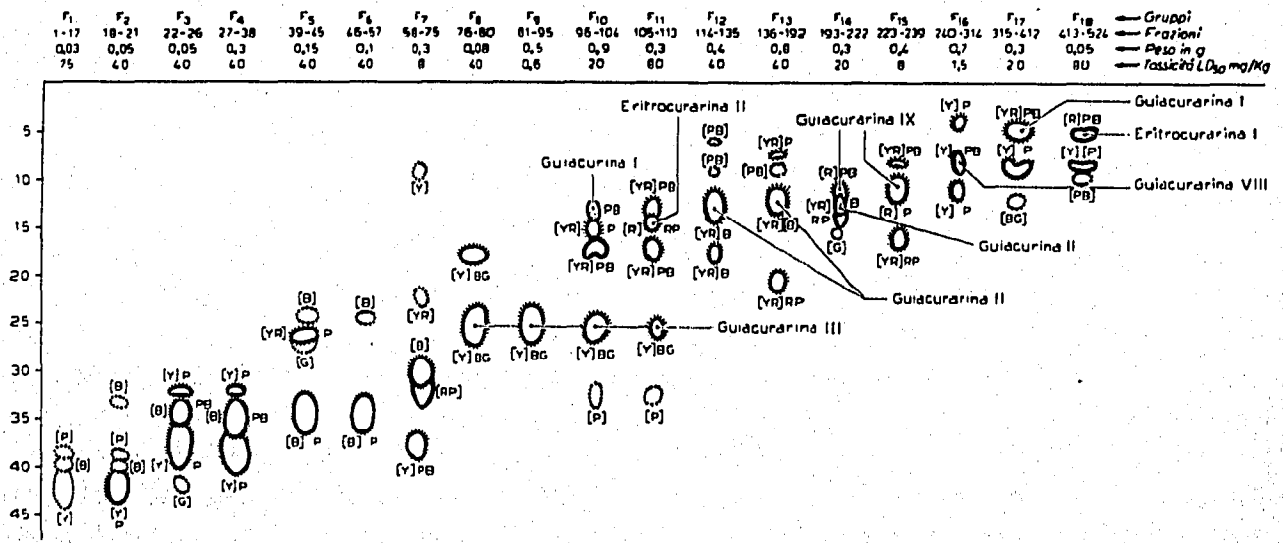

Fig. 13. Schematic representation of the separation of *S. guianensis* Aubl. Mart. alkaloid chlorides (G. B. MARINI-BETTÒLO AND M. A. IORIO, *Gazz. chim. ital.*, 86 (1956) 1305).

References p. 432/433.

of powdered cellulose are avoided, such as column electrophoresis and counter-current distribution, the first results of which seem to be rather promising.

(c) *Preparative separation on paper sheets*

Whenever the resolution of mixtures on cellulose or alumina columns is found to be difficult, one may resort to paper sheet chromatography since it allows the isolation of small quantities of pure alkaloids.

To this end, from 10 to 30 mg of total alkaloids, or fractions containing a number of alkaloids, are placed on sheets of Whatman paper No. 1 or 3 MM along a line and

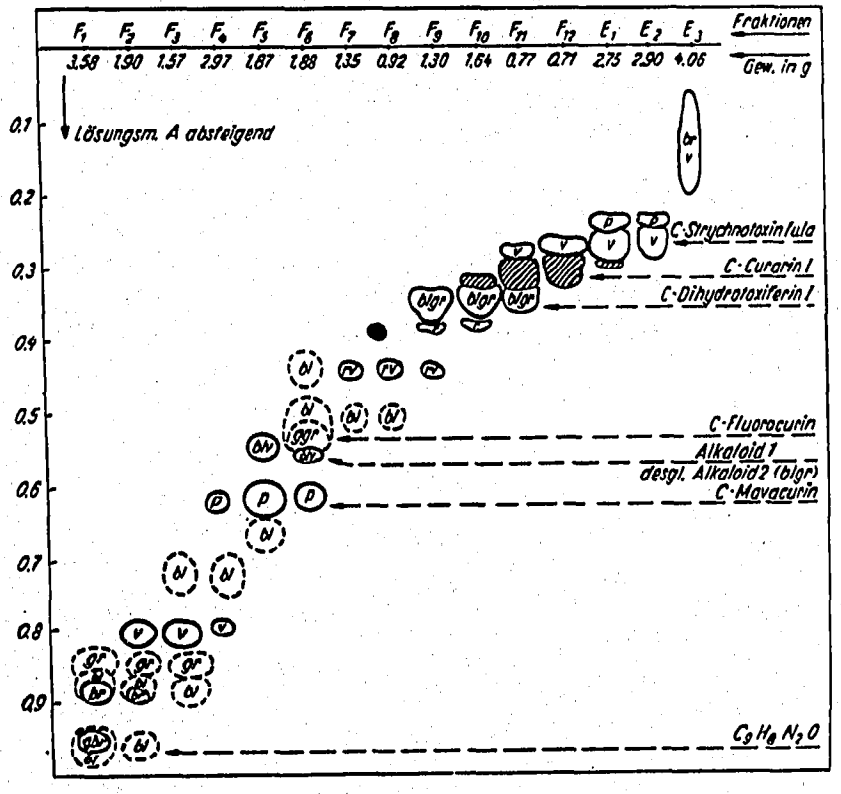

Fig. 14. Schematic representation of the separation of calabash alkaloid chlorides (TH. WIELAND AND H. MERZ, *Ber.*, 85 (1952) 731).

chromatography is effected in the usual way. Bands can be detected under a Wood's lamp or they can be revealed on the strip by applying colour reagents. The bands can then be cut out and eluted. The alkaloid can be recovered from the eluate and again subjected to chromatography, until a fraction is obtained that is chromatographically pure.

In the case of very complex mixtures with strongly differing R_C values, after cutting out the bands whose resolution appears satisfactory, another sheet of paper should be used and chromatography continued until a satisfactory resolution of the other bands is reached.

This method has been successfully employed with mixtures of alkaloids from *S. solimoesana*[63], *S. guianensis*[62] and *S. subcordata*[76]; numerous pure alkaloids can be obtained in this way (Fig. 16).

References p. 432/433.

(d) Band electrophoresis

Besides band chromatography, it may occasionally be advantageous to carry out a band electrophoresis in order to separate certain components. The fractionation of *S. guianensis* alkaloids may serve as an example of this point[64].

Since electrophoresis brings about a considerable diffusion of the bands, it is not advisable to repeat the operation, as is the practice in chromatography; instead electrophoresis should be followed by chromatography of the eluate on a paper sheet.

(e) Continuous electrophoresis

A method that has been tested by LEDERER AND MARINI-BETTÒLO* with *S. guianensis* and *S. Froesii* is that of continuous electrophoresis (see Fig. 17). A good fractionation can be obtained by this method if dilute acetic acid is used as electrolyte; the results are, however, not particularly good since the long duration of the operation causes alteration of the products.

(f) Column electrophoresis

The application of column electrophoresis to the analytical study of mixtures of South American *Strychnos* alkaloids has already given promising results; although by itself it does not yield a complete resolution of mixtures, in conjunction with paper chromatography it has proved a very useful subsidiary means of research.

From the above it follows that this method can be applied on a preparative scale, particularly for preliminary resolutions of mixtures of alkaloids obtained from partition chromatograms or counter-current distribution. The results of a considerable number of preliminary investigations on the application of column electrophoresis to the purification of *S. amazonica* alkaloids obtained from counter-current distribution, are

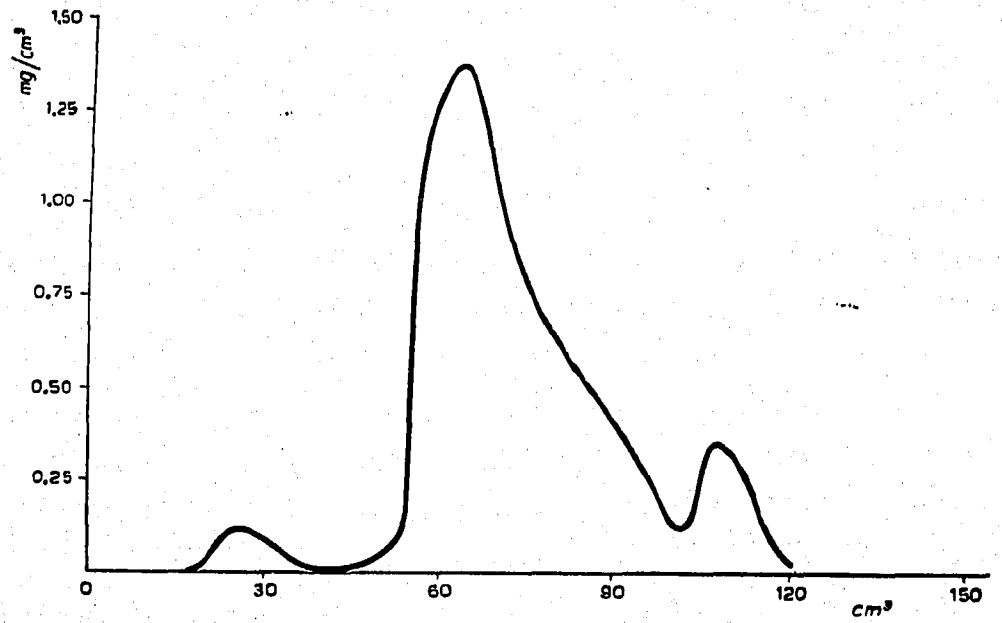

Fig. 15. Elution diagram of column electrophoresis of *S. amazonica* Kruk. alkaloids (G. C. CASINOVI, *Gazz. chim. ital.*, 87 (1957) 1457).

* Unpublished results.

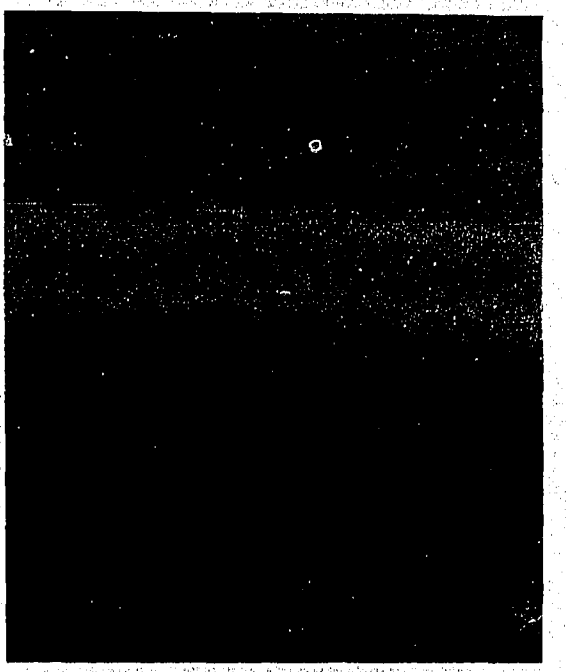

Fig. 16. Separation of *S. subcordata* alkaloid chlorides on a paper sheet.

Fig. 17. Continuous electrophoresis of *S. Froesii* Ducke alkaloid chlorides (M. LEDERER AND G. B. MARINI-BETTÒLO, unpublished results).

References p. 432/433.

given in Fig. 15. This figure shows the elution curves of an electropherogram of crude alkaloids, which was obtained on a column of 2 × 100 cm, a potential of 800 V being applied for 14 hours and 2% acetic acid being used as electrolyte[26].

If the experimental data are plotted in a co-ordinate system in which the number of c.c. of eluate are taken as abscissae and the concentrations as ordinates, it will be seen how the principal alkaloid is separated from small quantities of other alkaloids, which separation could not have been effected so quickly by partition chromatography or counter-current distribution.

The aim of current studies on this subject is to establish whether this method can be applied not only to purification, but also to the preliminary fractionation of groups of substances that are to be subsequently separated by other methods.

(g) *Counter-current distribution*

Counter-current distribution is a highly efficient quantitative mehod, which can also be used on a preparative scale. It has numerous advantages over partition chromatography, such as:

1. The possibility of checking the course of the separation at any time by chromatographing small samples.

2. The absence, thanks to the discontinuity of the process, of diffusion phenomena, which in chromatography might affect the efficiency of the method in case of interruption of the flow in the column or long duration of the process.

3. The absence of irreversible adsorption phenomena, which on columns bring

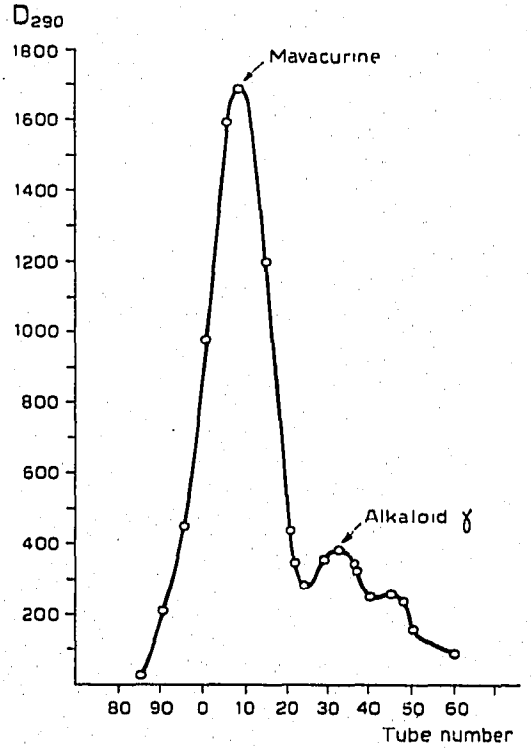

Fig. 18. Distribution diagram of *S. parvifolia* alkaloids in counter-current distribution (solvents: butanol–water).

References p. 432/433.

about a loss of substance and hamper successful performance, as well as the absence of all wall effects, which reduce the column's resolving efficiency.

The disadvantages lie in the complexity of the equipment, which is larger than in chromatography, the long duration of the process and the fact that emulsions are easily formed, which increases the time of operation.

CASINOVI[26] applied this technique to the separation of a fairly simple alkaloid mixture from *S. amazonica*. The apparatus had 15 tubes, the solvents were methyl ethyl ketone–water, and the operation was carried out with 1 g of total alkaloids. After 14, 18, 42 and 56 stages, the process was followed by chromatography. Using the same alkaloid mixture, and employing butanol–water and an apparatus with 25 tubes on the basis of data resulting from previous operations, it is possible to resolve almost entirely the mixture of *S. amazonica* alkaloids.

A third example of the application of this technique is the purification of an alkaloid that shows an intense yellow-green fluorescence under U.V. light. Owing to the low partition coefficient, the distribution was continued for 400 stages with the recycling method.

Once stage 400 had been reached, the distribution curve was plotted from measurements of the fluorescence under U.V. light: the resulting curve clearly proves that the product is homogeneous.

These tests, which up to the present have been carried out with a small apparatus have given interesting results. It may be assumed that by employing multi-tube apparatus the separation of two or more alkaloids whose partition coefficients are close together, will also be possible and that good results will be obtained, as is shown by diagrams obtained with 300 stages in an automatic device (Fig. 18)*.

CONCLUSION

An examination of the present development of methods for separating *Strychnos* quaternary alkaloids, shows that owing to the employment of chromatographic techniques considerable progress has been made in recent years in the separation and identification of these substances.

The peculiar physico-chemical nature of quaternary alkaloids and the complexity of natural mixtures, however, make it sometimes difficult, even nowadays, to obtain pure products and to identify them on the basis of R_C values, a difficulty not met with in the case of sugars, amino acids and nucleotides.

In fact, it should be remembered that, according to what SCHLITTLER AND HOHL[80] wrote some years ago:

"die Bearbeitung der Calebassen-Alkaloide ist ausserordentlich schwierig; sie krankt auch daran, dass beinahe jede einzelne Calebasse bei der Aufarbeitung ein gesondertes Probleme darstellt". The same can be said about the *Strychnos* bark and root alkaloids.

Today, other techniques besides chromatography are available, such as zone-

* Unpublished results.

References p. 432/433.

electrophoresis and counter-current distribution, which are based on different principles and offer new possibilities.

Moreover, it is worth noting that further progress is being made in the field of fractionation by combining various chromatographic techniques; examples are the separation of *S. toxifera* alkaloids where partition chromatography and chromatography on alumina are alternately employed[4], or the separation of alkaloids from *S. guianensis*[64] and *S. amazonica*[26] by combining chromatographic methods with electrophoresis and counter-current distribution.

These methods warrant our belief that it will be possible to resolve many mixtures that could not be fractionated up to the present. We are also of the opinion that here as elsewhere the necessity becomes ever greater of utilizing all available methods of separation.

REFERENCES

1 K. ADANK, D. BOVET, A. DUCKE AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 83 (1953) 966.
2 W. ARNOLD, W. VON PHILIPSBORN, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 40 (1957) 705.
3 H. ASMIS, E. BÄCHLI, E. GIESBRECHT, J. KEBRLE, J. SCHMID, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 37 (1954) 1968.
4 H. ASMIS, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 37 (1954) 1983.
5 H. ASMIS, E. BÄCHLI, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 37 (1954) 1993.
6 H. ASMIS, P. WASER, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 38 (1955) 1661.
7 H. ASMIS, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 39 (1956) 440.
8 E. BÄCHLI, C. VAMVACAS, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 40 (1957) 1167.
9 F. E. BADER, E. SCHLITTLER AND H. SCHWARZ, *Helv. Chim. Acta*, 36 (1953) 1256.
10 J. BARBOSA RODRIGUES, *L'uiaery ou curare*, Brussels, 1903.
11 K. BERNAUER, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 40 (1957) 731.
12 K. BERNAUER, E. BÄCHLI, H. SCHMID AND P. KARRER, *Angew. Chem.*, 69 (1957) 59.
13 P. DE BERREDO CARNEIRO, *Compt. rend.*, 206 (1938) 1202; 208 (1939) 382, 1429.
14 P. DE BERREDO CARNEIRO, *Bull. soc. chim. biol.*, 21 (1939) 282, 1389.
15 P. DE BERREDO CARNEIRO, *Academica das Ciencias de Lisboa, Biblioteca de Altos Estudos*, (1944).
16 H. BICKEL, E. GIESBRECHT, J. KEBRLE, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 37 (1954) 553.
17 H. BICKEL, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 38 (1955) 649.
18 E. BIOCCA, Pesquisas sobre o metodo de preparaçao do curare pelos indios, *Rev. mus. paulista (São Paulo)*, (N.S.) 8 (1954) 165.
19 R. BOEHM, *Abhandl. kgl. sächs. Ges. Wiss.*, 22 (1895) 201; 24 (1897) 1.
20 V. BOEKELHEIDE, *J. Am. Chem. Soc.*, in the press.
21 J. B. BOUSSINGAULT AND ROULIN, *Ann. chim. et phys.*, [2] 39 (1828) 29.
22 D. BOVET AND F. BOVET NITTI, *Médicaments du système nerveux végétatif*, Karger, Basel, 1948, p. 626.
23 D. BOVET, A. DUCKE, K. ADANK AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 84 (1954) 1141.
24 D. BOVET AND F. BOVET NITTI, *Rend. ist. super. sanità*, 12 (1949) 3.
25 D. BOVET AND F. BOVET NITTI, *Experientia*, 4 (1948) 325.
26 G. C. CASINOVI, *Gazz. chim. ital.*, 87 (1957) 1457.
27 G. C. CASINOVI, M. LEDERER AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 86 (1956) 342.
28 F. DE CASTELNAU, *Expédition de l'Amérique du Sud*, tome 3, Paris, 1851, p. 17.
29 L. C. CRAIG, *Anal. Chem.*, 22 (1950) 1346.
30 A. DUCKE, *Arquiv. jardim. botân. Rio de Janeiro*, 6 (1933) 79.
31 A. DUCKE, O genero Strychnos, *Bol. téc. inst. agron. norte (Belém, Brazil)*, 3 (1945).
32 A. DUCKE, Plantas novas ou pouco conhecidas da Amazonas, *Bol. téc. inst. agron. norte (Belém, Brazil)*, 19 (1950) 20.
33 A. DUCKE, O genero Strychnos no Brasil, *Bol. téc. inst. agron. norte (Belém, Brazil)*, 30 (1955).
34 K. FOLKERS, *J. Am. Pharm. Assoc.*, 27 (1938) 689.
35 K. FOLKERS AND K. UNNA, *Arch. intern. pharmacodynamie*, 59 (1939) 370.
36 E. GIESBRECHT, H. MEYER, E. BÄCHLI, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 37 (1954) 1974.
37 E. HECKER, *Z. Naturforsch.*, 8b (1953) 86.

[38] M. A. IORIO, O. CORVILLON, H. MAGALHÃES, M. H. ALVES AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 86 (1956) 923.
[39] C. JOBERT, *Compt. rend.*, 86 (1878) 121; 89 (1879) 646.
[40] P. KARRER, *Nature*, 176 (1955) 277.
[41] P. KARRER AND H. SCHMID, *Angew. Chem.*, 67 (1955) 361.
[42] P. KARRER AND H. SCHMID, *Helv. Chim. Acta*, 29 (1946) 1853.
[43] P. KARRER AND H. SCHMID, *Helv. Chim. Acta*, 30 (1947) 2081.
[44] P. KARRER AND H. SCHMID, *Helv. Chim. Acta*, 33 (1950) 512.
[45] J. KEBRLE, H. SCHMID, P. WASER AND P. KARRER, *Helv. Chim. Acta*, 36 (1953) 345.
[46] J. KEBRLE, H. SCHMID, P. WASER AND P. KARRER, *Helv. Chim. Acta*, 36 (1953) 102.
[47] J. KEBRLE, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 36 (1953) 1384.
[48] H. KING, *J. Chem. Soc.*, (1949) 955 and 3263.
[49] B. A. KRUKOFF AND H. N. MOLDENKE, *Brittonia*, 3 (1938) 1–74.
[50] B. A. KRUKOFF AND J. MONACHINO, The American Species of Strychnos, *Brittonia*, 4 (1942) 248–322.
[51] B. A. KRUKOFF AND J. MONACHINO, *Bol. téc. inst. agron. norte (Belém, Brazil)*, 11 (1947).
[52] B. A. KRUKOFF AND J. MONACHINO, *Bol. téc. inst. agron. norte (Belém, Brazil)*, 12 (1947).
[53] B. A. KRUKOFF AND J. MONACHINO, *Bol. téc. inst. agron. norte (Belém, Brazil)*, 20 (1950).
[54] B. A. KRUKOFF AND A. C. SMITH, *Bull. Torrey Botan. Club*, 66 (1939) 305.
[55] J. B. DE LACERDA, *Compt. rend.*, 89 (1879) 582, 1034.
[56] LA CONDAMINE, *Hist. Acad. Soc.*, 1745 (Paris), 1749, Mem. 489.
[57] O. DE LAZZARINI PECKOLT, *Rev. soc. brasil. quim.*, 19 (1950) 3.
[58] A. R. MCINTYRE, *Curare, Its History, Nature and Clinical Use*, The University of Chicago Press, Chicago, 1947.
[59] G. B. MARINI-BETTÒLO AND M. LEDERER, *Nature*, 174 (1954) 133.
[60] G. B. MARINI-BETTÒLO, M. LEDERER, M. A. IORIO AND A. PIMENTA, *Gazz. chim. ital.*, 84 (1954) 1155.
[61] G. B. MARINI-BETTÒLO, M. A. IORIO, A. PIMENTA, A. DUCKE AND D. BOVET, *Gazz. chim. ital.*, 84 (1954) 1161.
[62] G. B. MARINI-BETTÒLO AND M. A. IORIO, *Résumés trav. XIVe congr. intern. chim. pure et appl., Zurich, 1955*, p. 152.
[63] G. B. MARINI-BETTÒLO, P. DE BERREDO CARNEIRO AND G. C. CASINOVI, *Gazz. chim. ital.*, 86 (1956) 1148.
[64] G. B. MARINI-BETTÒLO AND M. A. IORIO, *Gazz. chim. ital.*, 86 (1956) 1305.
[65] G. B. MARINI-BETTÒLO AND D. BOVET, *Selected Sci. Papers Ist. Super. Sanità*, 1 (1956) 26.
[66] G. B. MARINI-BETTÒLO, *Festschr. Arthur Stoll*, Birkhäuser, Basel, 1957, p. 2.
[67] G. B. MARINI-BETTÒLO AND J. A. COCH FRUGONI, *J. Chromatog.*, 1 (1958) 182.
[68] D. F. MARSH, *Ann. N.Y. Acad. Sci.*, 54 (1951) 307.
[69] A. J. P. MARTIN AND R. L. M. SYNGE, *Biochem. J.*, 35 (1941) 1363.
[70] C. F. VON MARTIUS, *Flora Brasiliensis*, Wien, 1868.
[71] C. F. VON MARTIUS AND J. B. SPED, *Reise in Brasilien*, München, 1831.
[72] H. MEYER, H. SCHMID, P. WASER AND P. KARRER, *Helv. Chim. Acta*, 39 (1956) 1214.
[73] H. MEYER, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 39 (1956) 1208.
[74] L. PAOLONI AND G. B. MARINI-BETTÒLO, *Nature*, 179 (1957) 41.
[75] J. PELLETIER AND H. PETROZ, *Ann. chim. et phys.*, (2) 40 (1829) 213.
[76] A. PENNA, M. A. IORIO, S. CHIAVARELLI AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 87 (1957) 1165.
[77] W. VON PHILIPSBORN, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 38 (1955) 1067.
[78] W. VON PHILIPSBORN, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 39 (1956) 913.
[79] A. PIMENTA, M. A. IORIO, K. ADANK AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 84 (1954) 1147.
[80] E. SCHLITTLER AND J. HOHL, *Helv. Chim. Acta*, 35 (1952) 29.
[81] H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 30 (1947) 1162.
[82] H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 33 (1950) 512.
[83] H. SCHMID, A. EBNÖTHER AND P. KARRER, *Helv. Chim. Acta*, 33 (1950) 1486.
[84] H. SCHMID, K. SCHMID, P. WASER AND A. EBNÖTHER, *Helv. Chim. Acta*, 34 (1951) 2042.
[85] H. SCHMID, J. KEBRLE AND P. KARRER, *Helv. Chim. Acta*, 35 (1952) 1864.
[86] RICHARD SCHOMBURGK, *Travels in British Guiana, 1840–41*.
[87] ROBERT SCHOMBURGK, *Reisen in Guiana und Orinoco, 1835–39*, Leipzig, 1841.
[88] H. WIELAND, W. KONZ AND R. SONDERHOFF, *Ann.*, 527 (1937) 160.
[89] H. WIELAND AND H. J. PISTOR, *Ann.*, 536 (1938) 68.
[90] H. WIELAND, H. J. PISTOR AND K. BÄHR, *Ann.*, 547 (1941) 140.
[91] H. WIELAND, K. BÄHR AND B. WITKOP, *Ann.*, 547 (1941) 156.
[92] TH. WIELAND AND H. MERZ, *Ber.*, 85 (1952) 731.
[93] TH. WIELAND AND H. MERZ, *Ann.*, 580 (1953) 204.
[94] TH. WIELAND, H. FRITZ AND K. HASSPACHER, *Ann.*, 588 (1954) 1.
[95] B. WITKOP, *Angew. Chem.*, 55 (1942) 85.

Received March 10th, 1958