Journal of Chromatography, 98 (1974) 1–54
Chromatographic Reviews

CHREV. 67

ION-EXCHANGE CHROMATOGRAPHY OF NITROGEN COMPOUNDS

PAVEL JANDERA and JAROSLAV CHURÁČEK
Department of Analytical Chemistry, Institute of Chemical Technology, Pardubice (Czechoslovakia)
(Received May 9th, 1973)

CONTENTS

1. INTRODUCTION

This review is divided into sections dealing with amines and other nitrogen bases, urea and cyanamide derivatives, amides of sulphonic acids and nitro compounds. The survey does not include nucleic acid constituents nor amino sugars, which are best dealt with from a biochemical and not from an analytical viewpoint.

This is the third review in a series devoted to the ion-exchange chromatography of organic compounds. Previous reviews dealt with carboxylic acids, and with sulphur compounds, phenols, phosphorus compounds and esters of carboxylic acids.

2. AMINES, NITROGEN-CONTAINING BASES AND RELATED COMPOUNDS

A. Sorption behaviour on cation exchangers

The sorption of amines and other basic organic compounds on cation-exchange

resins is related to the protonation of the base B. Only the protonated bases, BH^+ should be taken up by the resin if only electrostatic interactions account for the sorption.

Considering such an idealized sorption model, an equation can be derived for the distribution coefficients that characterize the sorption of trace amounts of the organic bases in a similar manner to that for the anion-exchange sorption of carboxylic acids:

$$D_{BH} = \frac{(BH^+)}{[B] + [BH^+]} = \frac{(BH^+)}{[BH^+]} \cdot \frac{1}{1 + \frac{K}{[H^+]}} = K_C^{BH} \cdot \frac{1}{\alpha_{BH(H)}} \cdot \frac{Q}{[C^+]} \qquad (1)$$

where D_{BH} denotes the volume distribution coefficient of the base (amine), K represents the equilibrium constant for the dissociation of the conjugated monoprotic acid BH^+:

$$K = \frac{[B]\,[H^+]}{[BH^+]} \qquad (2)$$

(protonation with only a single proton is assumed in this simple model) and K_C^{BH} is the selectivity constant describing the equilibrium for the ion exchange of the competing counter-ion C^+ for the base BH^+:

$$K_C^{BH} = \frac{(BH^+)\cdot[C^+]}{[BH^+]\cdot(C^+)} \qquad (3)$$

The terms in parentheses denote the concentrations of the cations in the resin phase and those in square brackets denote concentrations in the outer solution: Q is the cation exchange capacity of the resin[1].

If the hydrogen form of the cation-exchange resin is brought into contact with a solution containing an organic base, acid–base interaction would be expected between the acidic cation exchanger and the base. For a strongly acidic sulphonated cation exchanger, the following equation applies:

$$R\text{–}SO_3^- \cdot H^+ + B \rightleftarrows R\text{–}SO_3^- \cdot H^+ B \qquad (4)$$

In this case, the equation for the distribution coefficient of the base D_{BH} has to be modified:

$$D_{BH} = K_H^{BH} \cdot Q \cdot \frac{1}{K + [H^+]} \qquad (5)$$

where

$$K_H^{BH} = \frac{(BH^+)\cdot[H^+]}{[BH^+]\cdot(H^+)} \qquad (6)$$

and the meaning of the other terms is the same as in eqn. 1.

In practice, however, the sorption behaviour of organic bases is complicated by other interactions of a non-electrostatic nature, which influence the values of the distribution coefficients in an analogous manner to other organic compounds.

A number of workers studied the selectivities of the sorption of aliphatic[2-11] and aromatic[9,12-21] amines, amides[18,22] and other, mainly pharmaceutically significant, nitrogen-containing bases[10,24-27] on phenolic[2,4] and conventional polystyrene-based sulphonated[3-27] and acrylic acid polymer-based[10,12,24] cation-exchange resins and macroreticular resins[14,15,22] in aqueous[2,3,5,9-12,16-19,21,24-27], mixed aqueous–organic[6-8,13,15,24] and anhydrous organic[14,22,23] media.

Kressman and Kitchener[2] pointed out the increase in the affinity for sulphonated resins due to the alkyl groups in substituted ammonium ions. The increase in selectivity with increasing ionic size in the aliphatic series, *i.e.*, with the length and number of aliphatic chains, was attributed to Van der Waals' forces. This relationship applies to sulphonated cation exchangers based on polystyrene–divinylbenzene copolymers with a low degree of cross-linking (2–6%)[11], while the reverse order of selectivity has been found on the highly cross-linked resins[3,5].

Schwarz and Boyd[28] studied the ion exchange of tetramethylammonium ions with sodium ions on Dowex 50W polystyrene sulphonated cation-exchange resin cross-linked with 0.5, 2, 4 and 8% of divinylbenzene. At low degrees of cross-linking, the larger tetraalkylammonium ion is taken up by the resin more readily than the small sodium ion. The preference for the tetramethylammonium ion, when present in trace amounts, decreased with the degree of cross-linking in the resin.

An explanation of this selectivity behaviour of alkylammonium ions was proposed in which it was suggested that so-called "contact ion pairs" due to the electrostatic interactions can predominate in highly cross-linked resins with a comparatively low water content and considerably high molarity of the internal solution, in contrast to resins with a low degree of cross-linking, where the "water structure-enforced ion pairing" of a hydrophobic nature should be decisive[7].

A constant increase in the distribution coefficients with increasing degree of cross-linking of Dowex 50W (8–16%) has been found, on the other hand, in the monoalkylammonium ions series. The sorption increases with the length of the hydrocarbon chain (ion diameter) from the methylammonium to the *n*-propylammonium ion. Based on the correlation of the selectivity coefficients with the steric and polarity factors of Taft's equation, it was suggested that the exchange of the cations of organic bases on cation-exchange resins is controlled by steric effects[9].

The selectivity of lower alkylammonium ions (methyl- and ethylammonium) for cation-exchange resins in both the hydrogen and lithium forms has been found to be greater in methanol than in water, although the opposite effect was found with the higher members (butyl- and octylammonium). Selectivity maxima have been observed for methyl- and ethylammonium ions in binary alcohol–water solutions in contact with the hydrogen form of the resin at about 80 mole-% of methanol, while a steady increase or decrease in selectivity takes place with the lithium form and higher alkylammonium ions[8]. The presence of alcohol promotes the formation of water structure, decreasing the hydration of the ions present in solution. This effect, together with the increased ion pair formation between the alkylammonium ions and the functional groups in the resin due to the lower dielectric constant of the alcoholic media, is more distinct with the lower alkylammonium ions, and accounts for the

increase in their sorption in alcohol over that in water. The dispersion forces between the hydrocarbon chains of alkylammonium ions and the resin network become predominant with higher alkylammonium ions and are responsible for their higher selectivity in water.

The occurrence of sorption maxima in binary alcohol–water solutions has been tentatively explained by assuming a decrease in the rate of diffusion of hydrogen ions from the resin into the outer solution with an increase in the amount of alcohol which should oppose the effects of water structure and ion pair formation.

The neutral amine molecules become protonated in the inner phase of the cation-exchange resin in the hydrogen form, where the concentration of hydrogen ions is considerable. This effect cannot take place with the metal ionic form of the cation-exchange resin and, consequently, the uptake of amines by the resin is decreased considerably in the latter case[21].

The sorption of the non-protonated aromatic amines on sulphonated cation-exchange resins exceeds the ion-exchange capacity of the resin in the hydrogen form. The sorption increases with increasing basicity of the amine[13] and with decreasing degree of cross-linking. Greater uptake of amines has been found in aqueous solutions than in non-aqueous methanol, acetic acid or dioxane. The larger the number and lengths of the aliphatic side-chains in molecules of aromatic amines are, the greater is the selectivity for the resin that the amine shows in aqueous solutions as compared with organic solvents[8].

Sulphonated polystyrene-based cation-exchange resins in different metal ionic forms take up amines to a considerable extent, while no sorption of aniline was observed using polyacrylic acid resin in the sodium form, and limited sorption took place with more basic amines[12].

Selectivity minima have been observed for various aromatic amines on the hydrogen form of cation-exchange resins in a number of mixed aqueous–organic solvents (including alcohols, acetone, acetronitrile, dioxan and tetrahydrofuran) at an organic solvent concentration of about 80%[15]. The occurrence of selectivity minima has been explained by the simple concurrence of the two opposite effects: the increase in dielectric constant of the solution brought about by the addition of water to the organic solvent accounts for the decrease in the electrostatic forces between the functional groups of the resin and the protonated amine cations. This effect results in a decrease in the distribution coefficients. A similar decrease in sorption will be caused, on the other hand, by better solvation and higher solubility of the organic base in the outer solution after the addition of an organic solvent to water.

Several workers have studied the sorption of amines and other weak bases in completely non-aqueous organic solvents[14,22,23]. Great care should be taken to ensure perfectly anhydrous conditions in order to obtain reproducible results. The resins used must be dried to a constant weight under vacuum at elevated temperature, completely dry solvents and glassware have to be used and even brief exposure to the atmosphere in the experimental steps is to be avoided. Otherwise, the resin will take up a certain amount of water and the swelling and sorption properties will change as the resinous phase would then contain a binary organic–aqueous solvent mixture.

The swelling of conventional gel-type ion-exchange resins is seriously limited in organic solvents. No swelling or sorption of a weak base (*p*-nitroaniline) on Dowex 50-X8 (H^+) was observed in a non-polar solvent (benzene)[14]. High *p*-nitroaniline

sorption was found, however, when using a highly porous, rigid, macroreticular sulphonated cation-exchange resin (Amberlyst 15) in benzene solutions[14].

Sorption in non-aqueous media is highly dependent on the type of solvent used and the porosity and particle size of the resin. The solvation of the sulphonic acid groups of the resin seems to be the most important parameter in the sorption process. Other factors, such as hydrogen bonding, matrix attraction and steric hindrance, must also be considered[22,23].

B. Chromatography of nitrogen-containing bases, drugs, barbiturates and related compounds on anion exchangers

Owing to non-ionic interactions with the resin matrix, numerous organic bases are retained on anion-exchange resins to some extent. Katz and Burtis[29] studied the elution positions of a variety of basic and neutral nitrogen-containing organic compounds in a high-resolution anion-exchange system using Dowex 1-X8 resin with small particles of a narrow size distribution and sodium acetate–acetic acid buffer as the eluting medium[30,31].

A survey of the elution data for some pyrimidine, imidazole, purine, pyridine and indole derivatives, alkaloids and other nitrogen-containing compounds is given in Table 1. Many of the basic compounds are eluted with no retention at the column void volume. Some functional groups, such as hydroxyl, amide, imide and methoxy, apparently have no effect on the retention of bases. Other functional groups, such as carboxyl and carbonyl, however, contribute to the retention of the solute on the resin.

The imidazole structure appears to have less effect on the retention than other ring structures tested, and imidazole compounds are eluted much earlier than comparable benzene or other heterocyclic compounds. It also appears that the pyridine ring contributes less than the benzene ring to later elution. The elution volumes of the indole derivatives are, in most instances, approximately the same as those for analogous benzene derivatives. The members of the purine and pyrimidine group are eluted early, with the exception of those which contain carboxylic groups. The presence of the carbonyl group strongly favours later elution. The contribution to the retention caused by an amino group in the 2- or 6-position is stronger than that of the carbonyl group and the methylamino group appears to be even more effective than the amino group. The inclusion of a carbohydrate structural element may cause shifts in the elution position in either direction and the nucleosides are usually separated from their base compounds by a few millilitres. The unsaturation in the side-chain and other functional groups (hydroxyl, carboxamide) may increase the retention. The introduction of a methyl group on the nitrogen atom of the primary structure seems to account for earlier elution.

Anion-exchange chromatography in acetate media has been utilized for the automated high-resolution analyses of UV-absorbing constituents in urine, blood serum and other physiological fluids. Nitrogen-containing bases, amphoteric compounds and related derivatives represent a large proportion of the compounds that have been separated.

The utility of such an automated, high-resolution analytical system, which enables large numbers of molecular constituents to be quantified in a single analysis, has been emphasized by recent medical research, which has indicated that hundreds of the molecular constituents of human body fluids may be related to bodily functions

TABLE I

ELUTION VOLUMES OF SOME NITROGEN-CONTAINING BASES AND RELATED COMPOUNDS

Ion-exchange resin: Dowex 1-X8, 10 μm. Column: 0.45 × 316 cm. Eluting agent: sodium acetate–acetic acid buffer, pH 4.4, concentration varying from 0.015 to 6 *M*. Flow-rate: 28 ml/h. Temperature increased from 25 to 60° after 15 h. Pressure: 1500–2300 p.s.i.

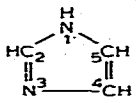
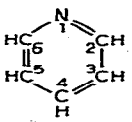
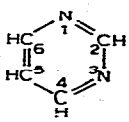
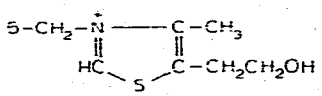
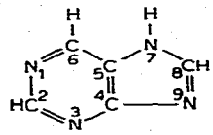
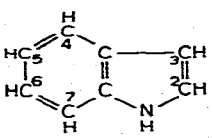
Compound	*Primary structure*	*Additions to primary structure*	*Elution volume (ml)*
Imidazole derivatives			
Histidine		4-$CH_2CH(NH_2)COOH$	14
4-Amino-5-imidazole-carboxamide		4-NH_2, 5-$CONH_2$	72
Urocanic acid		4-CH=CHCOOH	381
Pyridine derivatives			
Trigonelline		1-CH_3^+, 3-COO^-	15
Pyridoxamine		2-CH_3, 3-OH, 4-CH_2NH_2, 5-CH_2OH	25
Pyridoxal		2-CH_3, 3-OH, 4-CHO, 5-CH_2OH	25
Pyridoxine		2-CH_3, 3-OH, 4-CH_2OH, 5-CH_2OH	25
Nicotinamide		3-$CONH_2$	42
N-Methylnicotinamide		1-CH_3, 3-$CONH_2$	49
Nicotinuric acid		3-$CONHCH_2COOH$	569
Nicotinic acid		3-COOH	581
Pyrimidine derivatives			
Thiamine		2-CH_3, 4-NH_2, 	14
Cytosine		2=O, 4-NH_2	20
Cytidine		1-β-D-Ribofuranosyl, 2=O, 4-NH_2	24
Deoxycytidine		1-(2-Deoxy-β-D-ribofuranosyl), 2=O, 4-NH_2	25
Pseudouridine		1-H, 2=O, 3-H, 4=O, 5-β-D-ribofuranosyl	29
Uridine		1-β-D-Ribofuranosyl, 2=O, 3-H, 4=O	38
Deoxyuridine		1-(2-Deoxy-β-D-ribofuranosyl), 2=O, 3-H, 4-O	39
Uracil		1-H, 2=O, 3-H, 4=O	42
Thymidine		1-(2-Deoxy-β-D-ribofuranosyl), 2=O, 3-H, 4=O, 5-CH_3	45

TABLE I *(continued)*

Compound	*Primary structure*	*Additions to primary structure*	*Elution volume (ml)*
Purine derivatives and related compounds			
Theobromine		1 –H, 2 =O, 3 –CH_3, 6 =O, 7 –CH_3	39
Caffeine		1 –CH_3, 2 =O, 3 –CH_3, 6 =O, 7 –CH_3	50
Deoxyinosine		1 –H, 6 =O, 9 –(2-deoxy-*β*-D-ribofurano-syl)	62
Inosine		6 =O, 9 –*β*-D-ribofuranosyl	66
Hypoxanthine		3 –H, 6 =O	75
Deoxyadenosine		6 –NH_2, 9 –(2-deoxy-*β*-D-ribofuranosyl)	94
Adenosine		6 –NH_2, 9 –*β*-D-ribofuranosyl	101
1-Methylguanine		1 –CH_3, 2 –NH_2, 3 –H, 6 =O	118
7-Methylguanine		2 –NH_2, 3 –H, 6 =O, 7 –CH_3	120
Theophylline		1 –CH_3, 2 =O, 3 –CH_3, 6 =O	137
Adenine		6 –NH_2	152
Xanthosine		1 –H, 2=O, 3 –H, 6 =O, 9 –*β*-D-ribo-furanosyl	163
Xanthine		1 –H, 2 =O, 3 –H, 6 =O	172
Guanosine		1 –H, 2 –NH_2, 6 =O, 9 –*β*-D-ribofura-nosyl	214
Deoxyguanosine		1 –H, 2 –NH_2, 6 =O, 9 –(2-deoxy-*β*-D-ribofuranosyl)	217
6-Methylaminopurine		6 –$NHCH_3$	240
Guanine		2 –NH_2, 3 –H, 6 =O	241
Uric acid		1 –H, 2 =O, 3 –H, 6 =O, 8 =O, 9 –H	550
Indole derivatives			
Serotonin		3 –$CH_2CH_2NH_2$, 5 –OH	14
N-Acetyltryptophan		3 –$CH_2CH(NHCOCH_3)COOH$	25
Tryptamine		3 –$CH_2CH_2NH_2$	25
Tryptophan		3 –$CH_2CH(NH_2)COOH$	110
3-Indoleacetamide		3 –CH_2CONH_2	598
3-Indoleacetic acid		3 –CH_2COOH	1106
5-Hydroxyindoleacetic acid		3 –CH_2COOH, 5 –OH	1114
3-Indoleacrylic acid		3 –CH=CHCOOH	1440
Other compounds			
Adrenaline			14
Cyanocobalamin			14
3,4-Dimethylphenyl-ethylamine			14
Dopamine			14
Insulin			14
3-Methoxytyramine			14
Metanephrine			14
Noradrenaline			14
Normetanephrine			14
p-Tyramine			14
Urea			21

and to various pathological states. Drugs and their metabolites can be analyzed in the same way. Dowex 1-X8 resin columns connected with an ultraviolet flow-photometer as detector have been used, and over 100 peaks could be resolved in about 48 h at 2000–4000 p.s.i. A concentration gradient of sodium acetate or ammonium acetate–acetic acid buffer (pH 4.4) has been used with a concentration increasing from 0.015 to 6.0 *M* during the course of the analysis[30,31].

Anion-exchange resins containing 8% of divinylbenzene, giving a final degree of cross-linking of 10% (due to methyl bridging in the chloromethylation step), gave the best operation, while resins with a higher degree of cross-linking gave poorer separations. Resins with a final degree of cross-linking of only 4% showed a poor mechanical stability of the resin bed. Small particles of ion-exchange resin (*ca.* 10 μm) were decisive in achieving a good separation with a column pressure drop of 3000–4000 p.s.i. Bio-Rad Aminex A-27 has been found to be satisfactory for use in columns 150 cm long and 0.62 cm I.D.[32]. A high-pressure sampling valve has been used for introducing samples[33], and this system was able to separate up to 150 chromatographic peaks from urine in 40 h, which represents a considerable improvement over the previous technique. The use of a pH gradient and coupled ion-exchange columns with different properties shows promise for achieving a further increase in resolution and decrease in analysis time.

The identification of the constituents separated from physiological liquids is a difficult task owing to the small amounts of sample involved and their dilution by the eluting solvent. A certain success has been achieved, however, and several compounds identified[29,34,35]. As an example, two metabolites of phenacetin (4-hydroxyacetanilide and 3-methoxy-4-hydroxyacetanilide) were isolated from human urine and identified by their infrared, mass and ultraviolet spectra[35].

A different approach is that of using the chromatogram obtained as a profile or fingerprint in evaluating body functions. Reference profiles of "normal" subjects are compared with profiles from patients with metabolic or pathological abnormalities. When using this approach, accurate and precise reference profiles must be employed. A satisfactory reproducibility with a relative standard deviation of 1–4% was observed between the elution times of similar peaks from three chromatographic runs of 200-μl urine samples on a 0.24 × 100-cm column packed with 12–15-μm Aminex BRX anion-exchange resin using a linear acetate gradient at a pressure of 1000–1600 p.s.i., the column temperature being maintained at 21° for the first 4 h and then increased to 60° for the final 16 h of the run[36].

The acquisition of chromatographic information by digital conversion and recording on paper tape and subsequent evaluation by a digital computer is the best procedure for quantifying large numbers of chromatographic peaks. Manual procedures are of little value for clinicians because of the inordinate amount of time required. The development was reported of a small on-line digital computer capable of serving several analytical systems of the type described above[37].

The anion-exchange separation of brominated salicylanilides is of practical interest because of the germicidal activity of these compounds. When added to soaps, brominated salicylanilides reduce the growth and metabolism of skin flora and thus decrease body odour. Chromatography on a Dowex 2 (200–400 mesh) anion-exchange column (13 × 150–400 mm) using gradient elution with acetic acid in methanol has been used to analyse brominated salicylanilides in commercial prepara-

tions[38]. The components are eluted in order of increasing acidity, which depends on the number and position of the bromine atoms as substituents. Substitution on the salicylic ring contributes much more to the ionization of the hydrogen atom of the hydroxyl group than does substitution on the anilide ring. For example, although 4',5-dibromosalicylanilide contains two bromine atoms, compared with one in 5-bromosalicylanilide, the dibrominated compound is only slightly more acidic than the monobrominated compound and shows only a moderately increased affinity for the anion-exchange resin. Using gradient elution with 0.2% methanolic acetic acid being continually introduced into a mixing flask containing methanol, salicylanilide and 4'-bromosalicylanilide are eluted as separated bands. Gradient elution is then continued with 2% acetic acid instead of the 0.2% acid in order to separate 5-bromosalicylanilide and 4',5-dibromosalicylanilide (Fig. 1). 3,5-Dibromo- and/or 3,4',5-tribromosalicylanilide remain sorbed on the resin, if present in the mixture, and glacial acetic acid is required in order to elute them. The compounds eluted are determined by ultraviolet spectrophotometry.

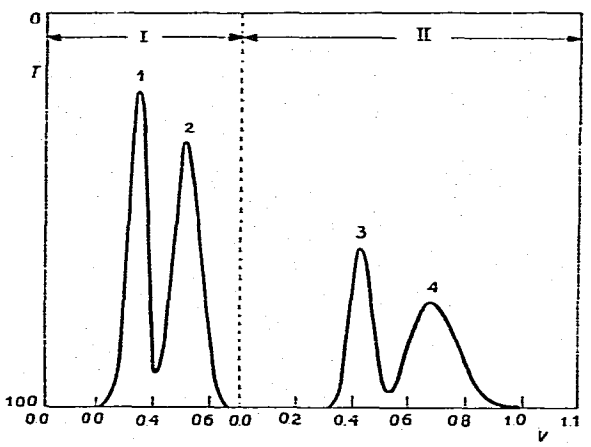


Fig. 1. Separation of brominated salicylanilides by anion-exchange chromatography. (1) Salicylanilide; (2) 4'-bromosalicylanilide; (3) 5-bromosalicylanilide; (4) 4',5-dibromosalicylanilide. Ion-exchange resin: Dowex 2-X8 (CH_3COO^-), 200–400 mesh. Column dimensions: 13 × 150 mm. Mobile phase: gradient elution; I, 0.2% methanolic acetic acid in reservoir, methanol in the mixing flask; II, 2% methanolic acetic acid substituted for the 0.2% solution in the reservoir. T = transmittance (%); V = volume of eluate (l).

Mixtures that contain only salicylanilide, 5-bromosalicylanilide, 3,5-dibromosalicylanilide and/or 3,4',5-tribromosalicylanilide can be separated by stepwise elution with fixed concentrations of acetic acid in methanol. Salicylanilide is eluted with 3% acetic acid, 5-bromosalicylanilide with 20% acetic acid and highly brominated salicylanilides with glacial acetic acid.

Most compounds encountered in pharmaceutical analysis are salts of nitrogenous bases and amphoteric compounds. Ion-exchange chromatography surpasses other liquid chromatographic methods for the separation of individual components because of the greater advantage it can gain from the differences between the acid–base properties of individual compounds. Further, most pharmaceutical liquid

preparations are compatible with the aqueous mobile phase and therefore no conversion of salts into free bases is required and samples can be applied directly, in contrast to some liquid–liquid separations. In addition to cation-exchange chromatography, work with anion exchangers is suitable for the analysis of various drugs.

A few practical examples of anion-exchange separations of pharmaceuticals are as follows. Morphine was separated from codeine on a column of a strongly basic, quaternary ammonium-type anion exchanger in the hydroxyl form. In aqueous solutions, codeine was washed through the column while morphine was sorbed because of its phenolic structure, and then recovered with a dilute acid solution[39]. Tropane alkaloids were separated from morphine in an analogous manner by applying a methanolic solution of sample to a column of Dowex-1 (OH^-) resin[40].

A mixture containing morphine and narceine was separated on a column containing Amberlite IRA-400 (OH^-) anion-exchange resin by elution of the morphine with 0.1 *M* acetic acid–ammonium acetate buffer at pH 4.6 with subsequent recovery of narceine using 0.1 *M* acetic acid–ammonium acetate buffer at pH 4.0 (ref. 41).

Strychnine could be separated from quinine on a column packed with an anion-exchange resin (Dowex 1-X2, 100–200 mesh) in the oxalate form. Strychnine was eluted with 1 % potassium oxalate solution, and quinine was then eluted with 1 *N* alcoholic ammonia solution[42].

Anion-exchange chromatography was used to separate diphenylhydramine hydrochloride, doxylamine succinate, triplenamine hydrochloride, phenindamine

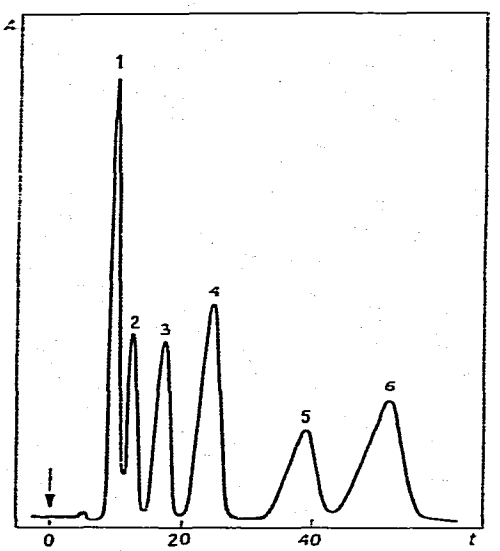


Fig. 2. Highly efficient, high-speed anion-exchange separation of a phenethylamine mixture. (1) Ephedrine; (2) dexedrine; (3) MDA; (4) STP; (5) benzphetamine; (6) α-benzyl-3,4-dimethylphenethylamine (less than 1 μg of each component). Ion-exchange resin: Durrum DA-X4, spherical strongly basic quaternary ammonium anion-exchange resin, 20 ± 5 μm. Column dimensions: 500 × 3 mm. Mobile phase: 0.2 *M* sodium nitrate solution, pH 3.15. Flow-rate: 0.26 ml/min. Input pressure: 500 p.s.i. Temperature: 55°. Detector: UV, 254 nm. Instrument: Chromatronix liquid chromatograph, Series 500. Sample size: 30 μl. *A* = absorbance; *t* = elution time (min).

tartrate, chloroprophenpyridamine maleate and pyrrobutamine diphosphate salts in tablets, elixirs and ointments, using 60% ethanol as the eluent. The amount of the free base released from the column was determined by titration with 0.1 *N* hydrochloric acid. With preparations that contain ephedrine, this component is removed first by using a cation-exchange column of Amberlite IRC-50 (ref. 43).

The development of new, highly efficient chromatographic methods made possible the anion-exchange separations of much more complicated mixtures of drugs. Fig. 2 shows an example of a highly efficient, high-pressure separation of a mixture containing six different phenethylamines on a 3 × 500 mm column packed with the strongly basic, spherical, fine-mesh anion-exchange resin Durrum DA-X4, using 0.2 *M* sodium nitrate solution of pH 3.15 for elution in less than 60 min[44].

Interesting examples of the practical use of specific interactions between nitrogen-containing bases and counter-ions in the resin are represented by separations of alkaloids on columns containing Dowex-1 anion-exchange resin in the chromate and hexacyanoferrate(II) forms. Quinine was separated from other alkaloids by chromatography on the chromate form of the anion-exchange resin. A neutral alcoholic mixture of alkaloids was introduced on to the column and all alkaloids except quinine were eluted with water. The latter was subsequently recovered with 1 *N* ammonia or 1 *N* formic acid in 96% ethanol[45].

For the separation of brucine from strychnine, the anion exchanger in the hexacyanoferrate(II) form was used. A mixture of alkaloids in hydrochloric acid solution was poured on to the column. Brucine was eluted with 1 *N* hydrochloric acid and the elution of strychnine followed with ammonia in 96% ethanol[46].

C. Chromatographic separations on cation exchangers

(a) Separations on cation-exchange resins by elution with solutions of acids

Mixtures containing various aliphatic and aromatic amines, quaternary ammonium compounds, biogenic amines, nitrogen-containing drugs and other weak bases were successfully separated on cation exchangers with solutions of acids, buffers or bases as the eluting agents.

In strongly acidic solutions, the effects of differences in the charges on ionic groups are decreased considerably and, on the other hand, non-ionic sorption on the resin becomes a very important factor influencing the distribution of protonated organic bases between the cation-exchange resin and the mobile phase.

As early as 1947, Tsuda and Matsumoto[47] reported the partial separation of a mixture of methylamine, dimethylamine and trimethylamine. In their experiments, they used a series of 10 columns packed with a cation-exchange resin in the sodium form. The sample was applied as an aqueous solution and the separation was effected using 3–4 l of water as the mobile phase. The columns were subsequently disconnected and each was extracted with 1 *N* hydrochloric acid. The sorption was found to decrease in the order methylamine, trimethylamine and dimethylamine. The first to third columns contained methylamine, the fourth a mixture, the fifth and sixth trimethylamine, the seventh a mixture and the eighth dimethylamine. Similar results were achieved with ethylamine, diethylamine and triethylamine mixtures[47].

This method was also applied to the separation of 2-picoline from pyridine. The first to fifth columns contained 2-picoline, the sixth a mixture and the seventh to

tenth pyridine. Complete separation of 2- and 3-picoline and 2,6-lutidine in pyridine oil was impossible by this technique[48].

A number of workers used hydrochloric acid solutions for the elution chromatography of amines on sulphonated or carboxylic acid cation-exchange resins. Rosenthal and Tabor[49] devised a method for the separation of putrescine, spermidine and spermine in bacterial and animal tissue extracts. The sample was deproteinized with trichloroacetic acid, neutralized and separated on a 0.5 × 4.5 cm column packed with Dowex 50-X2 (H^+), 100–200 mesh, sulphonated cation-exchange resin. Putrescine was eluted with 0.5 *N* hydrochloric acid and spermidine with 2.5 *N* hydrochloric acid. For the separation of all three compounds, elution with a concentration gradient of hydrochloric acid was used. Using this method, the complete separation of putrescine from several contaminating amino acids and the separation of spermidine or spermine from S-adenosylmethionine could not be achieved. The successful separation of the latter compounds was accomplished on a 0.5 × 6 cm column packed with Amberlite XE-64 carboxylic cation-exchange resin. Spermidine was eluted first with 0.13 *N* acetic acid and then the elution of adenosylmethionine followed with 2.5 *N* hydrochloric acid as the eluent. An acetic acid gradient was used for the separation of more complex mixtures[49,50].

This method was later modified by Holder and Bremer[51], using a 0.9 × 50 cm column of Dowex 50-X8 (200–400 mesh). The elution was performed with a concentration gradient of hydrochloric acid (the concentration was continuously changed from 1 to 2 *N*). The successful separation of 1,3-diaminopropane, putrescine and cadaverine was achieved, these substances being eluted in the opposite order compared with elution from an Amberlite XE-64 column.

Parrish[52] attempted the separation of mixtures containing polyethylene polyamines using a 1.2 × 30 cm column packed with Bio-Rad AG 50-X8 (H^+) sulphonated cation-exchange resin. With 2 *M* hydrochloric acid as the eluent, only ethylenediamine could be eluted as a satisfactorily separated elution band. Protonated cations of polyamines containing a higher number of amino groups were held so strongly on this resin that their elution with hydrochloric acid solutions was impossible. The use of a resin with a lower degree of cross-linking (and hence a lower affinity of the cations for the resin) was also unsuccessful. A combination of elution with 600 ml of 2 *M* hydrochloric acid, which removed ethylenediamine, and elution of the higher polyamines with 6% ammonia solution resulted in the partial separation of diethylenetriamine, triethylenetetramine and tetraethylenepentamine from each other. When elution with ammonia only was used, ethylenediamine appeared first as a sharp peak followed by another band containing a mixture of higher polyamines[52].

Hydrochloric acid solutions have been used successfully as eluents in chromatographic separations of naturally occurring quaternary nitrogen compounds on columns of cation-exchange resins. The separation of carnitine, betaine, trimethylamine oxide and creatinine was achieved by chromatography on a heated column (64.6°) packed with Dowex 50-X12, 200–400 mesh, eluting with 1 *N* hydrochloric acid. The two last peaks, however, overlapped seriously. Choline was completely sorbed on the resin under these conditions and could not be eluted as a defined peak even with 12 *N* hydrochloric acid. Choline could be removed from a mixture of quaternary compounds prior to chromatography on a Dowex 50 column, by elution with a sodium acetate–acetic acid mixture at pH 7.0 from a column of Amberlite IRC-50 carboxylic

cation-exchange resin[53]. A similar procedure has been used to resolve choline, serine and ethanolamine in phospholipid hydrolyzates[54].

Christianson *et al.*[55] developed a more general method for the quantitative separation of complex mixtures of naturally occurring quaternary nitrogen compounds, including betaine, choline, stachydrine, trigonelline and thiamine, using elution with solutions of hydrochloric acid. The concentration was increased in steps during the elution (1.0, 2.5, 4.0 and 6.0 *N*). Dowex 50W-X8, 200–400 mesh, was used in a column of 3.2 cm I.D. and bed height 60 cm. The column effluent fractions were analyzed for quaternary nitrogen compounds by measuring the ultraviolet absorption of their periodide derivatives. Carnitine, betaine, choline, γ-butyrobetaine, tetramethylammonium chloride, stachydrine, N-methylnicotinamide chloride, trigonelline, ergothioneine, thiamine chloride and trimethylamine oxide hydrochloride were tested and most were eluted as sharp, separated bands. Amino acids (serine, glycine, γ-aminobutyric acid, histidine, proline, etc.) and certain other nitrogen-containing compounds (ethanolamine, methylamine, ammonia, adenine, guanine and disulphides of ergothioneine and thiohistidine) were also separated in this system. A number of compounds that contain the same ionic groups but differ in their carbon skeletons could be separated on the basis of differences in their non-ionic sorption to the resin. The introduction of a non-basic polar group, such as hydroxyl, into the molecule reduces the non-ionic sorption: thus carnitine is eluted ahead of its parent compound, γ-butyrobetaine. Under the strongly acidic conditions used, ionization of carboxyl groups is completely suppressed, so that they are only slightly more effective than hydroxyl groups in decreasing the binding to the resin. This effect is illustrated by the proximity of the elution positions of betaine and choline. Most of the quaternary nitrogen compounds were eluted after their related amino acids or amines, but in a similar sequence. This could be explained by the contribution of additional methyl groups in the quaternary nitrogen compounds to non-ionic sorption.

The resulting satisfactory resolution of serine, glycine, ethanolamine, betaine and choline in close proximity in early fractions is advantageous for the analyses of mixtures that contain these biologically related substances. The method has been applied successfully to the chromatography of corn extracts. Twenty compounds were separated, identified and their amounts determined quantitatively[55].

Elution with 6 *N* hydrochloric acid has been used for the separation of histamine from contaminating substances in human urine. Sulphonated cation-exchange resins (*e.g.* Dowex 50W-X4, 100–200 mesh) are more suitable for this purpose than carboxylic cation exchangers (*e.g.*, Amberlite IRC-50) (ref. 56).

Chromatography on columns packed with cation-exchange resins has been used for the separation and determination of thiamine, cocarboxylase and other thiamine phosphate esters, as well as pyridoxine and cyanocobalamin in pharmaceutical preparations containing the above three vitamins. Stepwise elution was carried out with a series of eluents (including distilled water, boric acid–acetic acid mixtures and hydrochloric acid in aqueous and dioxan solutions)[57–59].

Mixed aqueous–organic solutions of hydrochloric acid have been widely used to improve the elution of compounds with greater affinity to ion-exchange resins, mainly in the aromatic series. Jones[60] separated N-*n*-amylpiperidine from contaminating bases on a sulphonated polystyrene cation-exchange resin, using 1 *N* hydro-

chloric acid in 50% ethanol to elute the bases. N-*n*-Amylpiperidine was the first compound eluted. This method has been used in studies of the hydrogenation of coal tar bases[60].

Watkins and Walton[12] compared the separations of some aromatic amines on Dowex 50W-X8 (50–100 mesh) and Dowex 50W-X4 (100–200 mesh) sulphonated cation-exchange resins using hydrochloric acid as the eluent in both aqueous and mixed aqueous–organic solutions. It was possible to achieve a clear-cut separation of aniline from pyridine on Dowex 50W-X8 by elution with 1.0 and 2.0 *N* hydrochloric acid in ethanol, while the elution curves in aqueous medium overlapped. The separation of benzylamine from the other amines studied (aniline, pyridine and *n*-butylamine) in aqueous hydrochloric acid was incomplete even at a very slow flow-rate.

Seki and Morimoto[61] separated N-2,4-dinitrophenyl derivatives of various lower aliphatic amines (methyl to amyl) by chromatography on the carboxylic cation-exchange resin Amberlite IRC-50 or Duolite CS-101 (H^+), packed in 8–10 × 600 mm columns, using tetrahydrofuran–methyl ethyl ketone–1% aqueous hydrochloric acid (4:3:13) or tetrahydrofuran–methyl ethyl ketone–water (4:3:13) as the eluting agent.

Churáček and Jandera[62] used elution with methanolic or ethanolic hydrochloric acid for the separation of derivatives of aliphatic amines and alcohols (coloured amides and esters of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid, which are more basic than dinitrophenylamines). Cation-exchange resins with a low degree of cross-linking (*e.g.*, Dowex 50W-X2) have been found to give better separations owing to the improved accessibility of the ion-exchange sites and accelerated diffusion in contrast to resins with higher degrees of cross-linking (*e.g.*, Dowex 50W-X8). An increased concentration of hydrochloric acid in the mobile phase decreases the sorption and speeds up the elution. The differences in the distribution coefficients of homologous derivatives reach a maximum in solutions that contain about 80–90% of alcohol, the distribution coefficients having minimum values, which offers optimum separation conditions. Both homologous esters and amides are eluted in order of increasing polarity, amides having higher distribution coefficients than those of the corresponding esters. Secondary amides are sorbed more strongly than primary amides. Compounds with longer aliphatic chains are eluted before the lower homologues. The separation of four aliphatic esters is shown in Fig. 3.

The elution of quaternary salts of higher molecular weight alkaloids from a strong cation exchanger is often incomplete with aqueous hydrochloric acid, even if a resin with a very low degree of cross-linking is used. The presence of alcohol will usually improve the elution rate considerably and is essential for some compounds[63]. The quantitative elution of hyoscine, codeine, papaverine, berberine, strychnine and other alkaloids from a Dowex 50-X1 column is easy to achieve with alcoholic solutions of mineral acids, while the recovery with water as eluent is incomplete[64].

A number of mixtures containing alkaloids, drugs and other pharmaceuticals could be separated by cation-exchange chromatography in aqueous–organic solutions of acids. Phenylephrine could be separated from codeine or dextromethorphan, and antihistamines on a cation-exchange column. Phenylephrine was eluted with 8.0 *N* phosphoric acid and codeine or dextromethorphan with 1.0 *N* hydrochloric acid in 60% methanol. Finally, the antihistamine (chloropheniramine, promethazine, pheniramine or methapyrilene hydrochloride) was eluted with 3.5 *N* hydrochloric acid in 40% or 50% methanol[65,66].

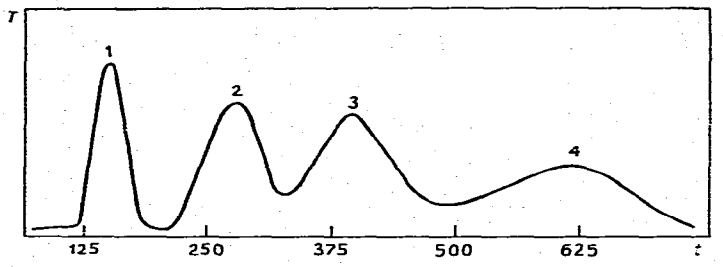


Fig. 3. Separation of some primary aliphatic esters of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid. (1) *n*-Nonyl ester; (2) *n*-amyl ester; (3) *n*-propyl ester; (4) methyl ester (20 μg of each component). Ion exchanger: Dowex 50W-X2 (H^+), ~ 50 μm. Column dimensions: 315 × 3.0 mm. Mobile phase: 0.199 *M* hydrochloric acid in 76.5% ethanol. Flow-rate: 0.09 ml/min. Inlet pressure: 11 atm. Temperature: ambient. Detector: photometric at 510 nm. Sample size: 40 μl. *T* = transmittance; *t* = elution time (min).

Ephedrine or amphetamine, barbiturates and antihistamines can be separated and determined in pharmaceutical preparations using a 25 cm × 12 mm cation-exchange column of AG 50W-X4 (H^+), 100–200 mesh. The sample is passed through the column and the excipients and colouring agents are eluted together with barbiturates with aqueous isopropanol (1:1). Then 0.055 and 0.27 *N* alcoholic hydrochloric acid is used to remove the remaining excipients and colourants. Amphetamine or ephedrine is eluted with 0.60 *N* alcoholic hydrochloric acid and elution of the antihistamine follows with 2.50 *N* alcoholic hydrochloric acid. Barbiturates can be separated from excipients and colourants on an anion-exchange column of AG1-X2 (Cl^-)[67].

Mixtures containing *p*-aminosalicylic acid and *m*-aminophenol have been separated on a column of the strong cation-exchange resin Dowex 50W-X8 (200–400 mesh). A solution of the mixture in dimethylformamide is passed through the column and the column is washed with the solvent; the eluate contains *p*-aminosalicylic acid. *m*-Aminophenol is then eluted from the column with ethanolic hydrochloric acid[68,69].

(b) Separations on cation-exchange resins by elution with neutral solutions of organic solvents

Some very weakly basic nitrogen-containing compounds show the behaviour of neutral compounds in mixed aqueous–organic or non-aqueous solvents. It is possible to utilize the differences in selectivities between individual components for the separation of mixtures containing such compounds by chromatography on cation exchangers with non-aqueous or mixed solvents as the eluting agents.

Caffeine, ethoxybenzamide and propylphenazone could be separated by elution with 96% ethanol or isopropanol using a column of Dowex 50-X2 cation-exchange resin[70]. Mixtures containing benzoic acid and acetanilide were separated on a Bio-Rad AG 50W-X8 (H^+) cation-exchange column by elution of the first compound with anhydrous acetonitrile and the other with a mixture of acetonitrile and dimethylformamide (5:1)[23]. Stepwise elution with acetonitrile and mixed acetonitrile–dimethylformamide (15:1) can be used for the separation of 2,4-dinitroaniline and *o*-nitroaniline, using the same cation-exchange column[23]. Other workers, however, encoun-

tered serious difficulties when attempting separations under similar conditions. The separation of acetanilide and N-*n*-propylacetanilide deteriorated seriously owing to the tailing of the first compound eluted (acetanilide) when the conventional resin with 8% cross-linkage (Dowex 50W-X8) was used with anhydrous methanol–acetonitrile mixtures as eluents. The separation was improved to a considerable extent by using macroreticular resins instead of the conventional resins, accelerating the diffusion rate in the resin[22]. On the other hand, the differences in sorption on cation-exchange resins when using anhydrous and aqueous–organic solvents can be used for separating even high-molecular compounds. A column packed with a low cross-linked cation exchange resin, Dowex 50W-X4 (H^+), was used for the conversion of chlorophylls *a* and *b* into the corresponding pheophytins and the subsequent rapid separation and determination of the pheophytins by elution with anhydrous acetone (pheophytin *a*) followed by elution with 85% acetone (pheophytin *b*)[71].

(c) Separations on carboxylic cation-exchange resins by elution with buffer and salt solutions

The most efficient separations of amines and other nitrogen-containing compounds have been achieved by elution with buffer solutions that offer the possibility of precise pH control. It was shown that an amine salt could be eluted from a cation exchanger by using a buffer solution (0.05–0.15 *M*) at a pH approximately 1.5 units above the p*K* value of the amine. This pH corresponds to the value at which about 90% of the compound exists as the neutral molecule. By raising or lowering the pH of the buffer solution, the retention volume can be decreased or increased. The retention volume can be further adjusted by modifying the column temperature or the ionic strength of the buffer.

Sulphonated polystyrene resins retain certain amines and bases too strongly for convenient separation when buffer solutions are used for the elution and a number of workers preferred weakly acidic carboxylic resins in order to avoid the use of powerful eluents.

Ion-exchange chromatography on the carboxylic cation exchanger Amberlite XE-64 was used for separations of various amines, including spermine, spermidine, cadaverine, putrescine, propanediamine, agmatine, pyrrolidine, choline, acetylspermine, thiomethylpropylamine and amino acids. Initially, the elution was performed with a concentration gradient of sodium sulphate in a solution buffered with sodium phosphate at pH 7.2 (ref. 50). Later, this method was modified and a potassium chloride concentration gradient in 0.1 *M* potassium phosphate buffer of pH 7.1 was used for elution[51]. An example of the separation of amines in a urine sample achieved under these conditions is shown in Fig. 4. The recoveries of the amines added to the sample were within the range 85–95%.

Elution with phosphate buffer concentration gradient (from 0.001 to 0.005 *M*, pH 6.7) has been used successfully for the separation of the metabolites of 1-methyl-2-aldoximinopyridinium iodide, which is one of the most effective oximes for antagonizing alkyl phosphate intoxication. The carboxylic cation exchanger Amberlite CG-50, 200–400 mesh, was packed in an 11 cm × 0.63 cm^2 column and gradient elution yielded well-separated elution bands of 1-methyl-2-aldoximinopyridinium, 1-methyl-2-methoxypyridinium and 1-methyl-2-cyanopyridinium ions[72].

Blau[73] used McIlvaine buffer at pH 5.0 as the eluent for the chromatography

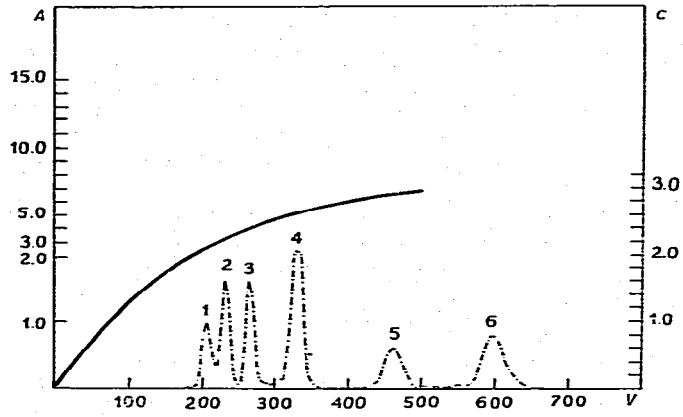


Fig. 4. Separation of amines in a urine sample by elution with a potassium chloride concentration gradient in solution buffered with potassium phosphate. (1) 2,2'-Dithiobis(ethylamine); (2) cadaverine; (3) putrescine; (4) 1,3-diaminopropane; (5) spermidine; (6) spermine. Ion exchanger: Amberlite IRP-64 (K^+). Column dimensions: 300 × 9 mm. Mobile phase: potassium chloride concentration gradient (0.1 *M* potassium chloride, buffered with potassium phosphate buffer at pH 7.1, was continually mixed in a 200-ml flask originally containing water). After 500 ml had been eluted, the elution was completed with saturated potassium chloride solution. Flow-rate: 0.5 ml/min. Temperature: 37°. Sample size: 10 ml. *A* = absorbance; *V* = volume of eluate (ml); *c* = concentration of potassium chloride (equiv./l).

of primary and secondary amines in urine on columns packed with the carboxylic cation exchanger Zeo-Karb 226, *ca.* 100 mesh, buffered at pH 7.3 before use. Amino acids, urinary pigments, purines, pyrimidines, proteins and all anions were removed in a preliminary step by sorption on a column containing a strongly basic anion-exchange resin. The separation achieved was reasonably efficient and a number of peaks could be resolved in satisfactory yield. Elution volumes of some amines chromatographed under the conditions described are summarized in Table 2.

Perry and Schroeder[74] studied the chromatographic behaviour of a number of aromatic, heterocyclic and aliphatic amines on a 30 × 1.0 cm column packed with Amberlite CG-50 (200–400 mesh) carboxylic cation exchanger, using 0.1 and 0.2 *M* pyridinium acetate buffers of pH 6.32 and 6.12, respectively, as the eluting agents. Their results are summarized in Table 3. Some general trends can be deduced from this table about the influence of chemical structure on the elution volumes of amines. An increase in the number of amino groups in polyamines delays the elution, and when as many as four are present, as in spermine, the elution cannot be effected with the buffer used. The longer is the carbon chain of aliphatic amines or the side-chain of aromatic amines, the greater are the elution volumes. On the other hand, methylation or acetylation of the amino group and hydroxylation of the β-carbon atom in the side-chain of phenylethylamine derivatives or hydroxylation of the benzene ring of aromatic amines accelerates the elution from the resin. Chromatography in pyridinium acetate medium on Amberlite CG-50 has been used successfully for the analytical separation and determination of amines in human urine of normal and pathological subjects. When only a fraction of the number of amines listed in Table 3 is present, it is often possible to achieve the complete separation of most of the amines present[74].

Using more concentrated pyridinium acetate buffers (0.438 *M*, pH 5.7; 0.5 *M*,

TABLE 2

ELUTION VOLUMES OF SOME AMINES IN CHROMATOGRAPHY ON A CARBOXYLIC CATION-EXCHANGE RESIN

The elution volume is the total volume eluted at the amine peak with, in each instance, a 112.5 × 0.8 cm I.D. column filled with Zeo-Karb 226 buffered at pH 7.3, and eluted with McIlvaine buffer at pH 5.0. Counting was started after rejection of the first 25 ml after application of the sample.

Amine	*Elution volume (ml)*	*Shape of peak*	*Recovery*
Trimethylamine N-oxide	12	Very sharp	Quantitative
Creatinine	20	Very sharp	Quantitative
Tetramethylammonium	79	Sharp	Quantitative
Diethylamine	81	Sharp	Quantitative
n-Amylamine	109	Sharp	Quantitative
Isoamylamine	111	Sharp	Quantitative
Trimethylamine	118	Sharp	Quantitative
Piperidine	128	Sharp	Quantitative
n-Butylamine	130	Sharp	Quantitative
Pyrrolidine	148	Sharp	Quantitative
n-Propylamine	150	Sharp	Quantitative
Dimethylamine	162	Sharp	Quantitative
Ethylamine	184	Sharp	Quantitative
Tyramine	260	Broad	85%
Glucosamine	270	Broad	85%
Methylamine	280	Broad	Quantitative
Canavanine	290	Very broad	80%
Ethanolamine	300	Broad	80%
Adrenaline	310	Very broad	Not determined
3-Hydroxytyramine	430	Very broad	90%
Noradrenaline	460	Very broad	70%
5-Hydroxytryptamin	465	Very broad	80%
Ammonia	490	Broad	Quantitative
pH 5.0 breakthrough	600	—	—

pH 4.4) and a 0.9 × 7 cm column packed with the smaller particle size carboxylic cation exchanger Bio-Rex 70 (—400 mesh), good separations were achieved of several polyamines which were not examined in the study of Perry and Schroeder. These amines included all of the commonly occurring oligoamines and their acetyl derivatives. Table 4 gives the elution times under these experimental conditions. The separations between 1,3-diaminopropane and putrescine, and cadaverine and acetylspermidine B, could be improved by slightly lowering the ionic strength of the elution buffer, which resulted in an increase in the time of analysis. Iminobispropylamine could not be separated from spermidine in this system[75].

Wall[76] found excessive swelling of carboxylic cation exchangers (polymethacrylic acid exchanger Amberlite CG-50 and polyacrylic acid exchanger Zeo-Karb 226) with low degrees of cross-linking in pyridinium acetate solutions. Consequently, the exchangers were softened in such a way that the flow-rate was unacceptably slow with resins of small particle size. For this reason, he preferred to use potassium citrate buffer as the eluent. Amberlite CG-50 gave more efficient separation of the amines under study, but its insufficient mechanical stability under the experimental conditions

TABLE 3

ELUTION VOLUMES OF AMINES CHROMATOGRAPHED ON AMBERLITE CG-50

Authentic compounds were chromatographed in mixtures on Amberlite CG-50 columns 45 cm in length and 0.9–1.0 cm I.D. at a flow-rate of 10 ml/h and a temperature of 40°. Chromatograms were developed with pH 6.32–0.1 *N* pyridinium acetate buffer for the first 250 ml, and thereafter with pH 6.12–0.2 *N* pyridinium acetate buffer.

Compound	*Range of elution (ml)*	*Elution peak* (ml)*
Ethanolamine	32– 40	36
Ammonia	33– 42	38
Ethylamine	38– 46	42
Pyrrolidine	49– 58	54
N-Acetylhistamine	49– 65	
Pyridoxamine	68– 79	74
N-Methylmetanephrine	78– 89	
Metanephrine	102–115	109
Epinephrine	101–124	
Normetanephrine	112–130	121
1-Methylhistamine	112–130	121
Norepinephrine	116–132	
Synephrine	118–135	
Isoamylamine	119–136	128
Mescaline	124–142	
3,4-Dimethoxybenzylamine	127–142	
Octopamine	132–147	
3-Methoxy-4-hydroxybenzylamine	134–150	
Epinine	135–151	
3,4-D...ethoxyphenylethylamine	140–154	
3-Methoxytyramine	141–158	
3-Hydroxy-4-methoxyphenylethylamine	145–163	
Putrescine	140–171	159
p-Hydroxybenzylamine	151–171	162
Cadaverine	163–183	
Dopamine	163–190	
Benzylamine	169–189	178
p-Tyramine	170–196	186
3-Ethoxy-4-hydroxybenzylamine	177–193	
m-Tyramine	183–202	
p-Methoxybenzylamine	192–215	203
Histamine	202–225	214
Bufotenin	205–235	
Phenylethylamine	228–254	240
o-Tyramine	218–262	241
p-Methoxyphenylethylamine	228–261	244
Kynuramine	229–261	244
2,2'-Dithiobis(ethylamine)	277–297	289
Serotonin	283–303	
Agmatine	305–321	312
N,N-Dimethyltryptamine	310–335	
5-Methoxytryptamine	328–352	339
Tryptamine	380–415	397
Spermidine	408–466	438
5-Methyltryptamine	465–505	483

* Elution peaks were not obtained for a number of amines that gave no colour or weak colours with ninhydrin.

TABLE 4

ELUTION TIMES OF POLYAMINES AND RELATED COMPOUNDS

Column (0.9 × 7 cm) packed with the weakly acidic carboxylic cation exchange resin Bio-Rex 70 (− 400 mesh). Elution with 0.438 *M* pyridinium acetate, pH 5.7. After 100 ml of this buffer had passed through the column, the eluting agent was changed to 0.5 *M* pyridinium acetate, pH 4.4. Flow-rate: 2 ml/min. Temperature: 80°.

Compound	*Time (min)*
Arginine	4
Putrescine	25
1,3-Diaminopropane	27
Cadaverine	32
Acetylspermidine B	34
Acetylspermidine A	42
Agmatine	59
Spermidine	64
Iminobispropylamine	64
Acetylspermine	68
Spermine	78

prevented its use. Satisfactory separations of amines were obtained with Zeo-Karb 226-X4.5 resin with a bead diameter of 20 ± 8 μm, packed in a 22 × 0.5 cm column. Elution with 1.1 *M* potassium citrate buffer of pH 6.15 was performed at 52° and about 80 p.s.i. With the flow-rate maintained at 28.5 ml/h, the elution times were ammonia 31, *p*-tyramine 81, cadaverine 95, putrescine 104, histamine 140 and tryptamine 165 min[76].

Ion-exchange chromatography on Amberlite IRC-50 carboxylic cation-exchange resin has been used for the separation and determination of non-volatile amines (choline, tyramine, histamine and agmatine) in various kinds of saké[77].

Amberlite IRC-50, 200–400 mesh, in a 40 × 0.9 cm column was also used for the chromatographic separation of phenolic amines (DOPA, adrenaline, noradrenaline and dopamine) in biological extracts, with recoveries of 83–97%. Catecholamines were eluted in the above order with 0.4 *M* ammonium acetate buffer at pH 5.0 and determined in fractions by absorption at 279 nm. The quality of the separation is strongly dependent on the pH and ionic strength of the eluting buffer used[78].

A carboxylic cation exchanger (Duolite CS-101 (NH_4^+), 100–200 mesh) could be used for the separation and determination of various alkaloids. Mixtures of strychnine or brucine with berberine were sorbed on a Duolite column; strychnine or brucine was eluted with 1 *N* ammonium chloride solution and berberine was subsequently eluted with 1 *N* sodium carbonate solution[79]. Hyoscine, atropine and berberine could be separated from each other on a column of the same resin by stepwise elution with water (hyoscine), 0.2 *N* ammonium chloride solution (atropine) and 1 *N* sodium carbonate solution (berberine)[80]. Similarly, berberine is separated from other *Phellodendron* alkaloids on a Duolite CS-101 column by passing 1 *N* ammonia, 1 *N* ammonium chloride and 1 *N* sodium carbonate solutions consecutively through the resin. Berberine is again contained in the carbonate fraction[81].

The separation of several other pharmaceutically important mixtures can be also effected by chromatography on carboxylic cation exchangers. Salicylic acid,

chinophen and aminopyrine have been separated from each other on a 9 × 170–250 mm column packed with Amberlite CG-50, 200–400 mesh. Using a buffer solution of pH 4.6 containing 10 vol. % of ethanol, salicylic acid and, successively, chinophen were eluted and then 0.1–0.2 *N* hydrochloric acid was used for the elution of aminopyrine[82]. Similarly, the separation of sulpyrine, antipyrine and aminopyrine by the use of an Amberlite CG-50 column (9 × 300 mm) was reported. Water or buffer solution of pH 5.5 was used for eluting sulpyrine followed by antipyrine, and 1 *N* hydrochloric acid was then used to elute aminopyrine[83].

(d) Separations on strongly acidic sulphonated cation exchangers by elution with buffer and salt solutions

As has been mentioned already, both polymethacrylic acid and polyacrylic acid carboxylic cation exchangers, namely those with lower degrees of cross-linking or irregular particle shapes, suffer from mechanical instability. Very fine particles are often formed during the chromatographic run, which leads to a decrease in the permeability of the column, so that adequate flow-rates cannot be used. Unacceptably long analysis times must be avoided and the use of more mechanically stable sulphonated polystyrene-based cation exchangers is to be preferred in many instances. On the other hand, strongly acidic polystyrene cation exchangers have the disadvantage that many amines are sorbed too strongly, especially those with a large number of amino groups and some aromatic amines, thus making their elution from the column difficult. In order to effect the elution, buffers with high pH values are often required. The elution can sometimes be facilitated by the addition of an alcohol to the eluting agent.

Perry and Schroeder[74] compared the chromatographic behaviour of various aliphatic, aromatic and heterocyclic amines on a carboxylic cation exchanger (Amberlite CG-50) and a strongly acidic polystyrene-based sulphonated cation exchanger (Amberlite CG-120) in pyridinium acetate buffers. Their results with Amberlite CG-50 are summarized in Table 3 and Table 5 gives elution volumes obtained with the Amberlite CG-120 column. The elution behaviour of the amines on this resin was similar to that on Amberlite CG-50 columns in that the increase in the number of carbon atoms in the aliphatic chain delayed the emergence from the resin, while hydroxylation of the aliphatic chain or substitution of a methyl or acetyl group on the amino group accelerated the elution. An increase in the number of amino groups increased the uptake of the amine on the resin to such an extent that diamines failed completely to be eluted from Amberlite CG-120 under the experimental conditions used, in contrast to the behaviour on Amberlite CG-50 (ref. 74).

Chromatography of many aromatic amines on strongly acidic sulphonated cation exchangers has been considered to be impractical owing to the strong bonds formed between most aromatic amines and the resin[84]. The elution of these compounds, however, can be improved by an appropriate choice of eluting agents.

The sulphonated polystyrene cation-exchange resin Zeo-Karb 225 with 8% cross-linking, under 200 mesh, in a 26.2 cm × 0.825 cm^2 column was used for the separation of 2-hydroxymethyl-3-hydroxypyridine and 2,6-di(hydroxymethyl)-3-hydroxypyridine. The compounds were eluted in this order with 0.6 *M* pyridinium acetate buffer at pH 5.0 and a good separation was achieved, in contrast to the elution with sodium acetate–acetic acid buffers, when skewed shapes of the peaks and severe

TABLE 5

ELUTION VOLUMES OF AMINES CHROMATOGRAPHED ON AMBERLITE CG-120

Authentic compounds were chromatographed in mixtures on Amberlite CG-120 columns 30 cm in length and 0.9–1.0 cm I.D., at a flow-rate of 30 ml/h and a temperature of 50°. Chromatograms were developed with 0.2 *N* pyridinium acetate buffer of pH 3.50 for the first 600 ml, and thereafter with 0.8 *N* pyridinium acetate buffer of pH 5.50. The breakthrough of the second developer occurred at 636 ml.

Compound	*Range of elution (ml)*	*Elution peak (ml)*
Glucosamine	115–132	121
Galactosamine	115–132	121
N-Acetylethylenediamine	145–160	152
β-Methoxyethylamine	150–165	157
N-Methylethanolamine	156–171	163
3-Amino-1-propanol	175–191	183
Serinol	175–191	183
Dimethylamine	187–200	193
β-Hydroxypropylamine	195–209	201
N-Methylethylamine	198–215	206
Diethylamine	212–224	218
Ethanolamine	210–230	219
2-Aminobutanol	220–236	228
Pyrrolidine	250–270	260
Piperidine	250–272	261
Methylamine	262–280	273
Ethylamine	272–294	282
γ-Methylmercaptopropylamine sulphoxide	290–300	295
Ammonia	296–345	308
n-Propylamine	312–332	322
Isobutylamine	316–345	328
Hydroxylamine	334–371	347
Cyclopropylamine	350–382	364
n-Butylamine	390–420	405
Isoamylamine	430–465	445
γ-Methylmercaptopropylamine	447–490	467
n-Amylamine	504–542	520

tailing were observed. This behaviour has been attributed to the improved competition for exchange sites with molecules of solutes effected by pyridinium ions compared with that of the sodium ions[85].

Successful separations of a variety of amines have been achieved on sulphonated cation-exchange resins by elution with buffers of higher pH. In strongly basic media, however, amines are present as non-electrolytes and ion-exchange equilibria cannot account for the chromatographic separation, which is to be attributed to the non-ionic interactions with the resin and, in most instances, to the effects of salting-out due to the presence of the strong electrolyte in the eluent.

By using 0.05 *M* borax solution at pH 9.2 as the eluent, monoethanolamine, diethanolamine and triethanolamine were completely separated on a 13.4 × 0.8 cm column packed with the sulphonated strongly acidic cation-exchange resin Dowex 50W-X8 (Na^+), 100–200 mesh. The compounds were eluted in order of decreasing

molecular weights. This method has been applied to the analytical separation and determination of diethanolamine and monoethanolamine in commercial triethanolamine samples[86].

Stepwise elution with citrate, borate and salicylate buffers in combination with very fine cation-exchange resins with a narrow particle size range gives an efficient separation of multicomponent mixtures that contain various amines and offers an attractive method for the analysis of mixtures that contain biogenic amines of physiological importance. The weakly acidic cation-exchange resin Amberlite CG-50, which would not retain amines as strongly as strongly acidic sulphonated resins, suffers, however, from mechanical instability, which causes clogging of the column by fine particles. This effect may seriously disturb the chromatographic elution because of the intolerably high pressure which may result. Therefore, more stable sulphonated cation-exchange resins are to be preferred. The analysis of amines was reported using a 15 × 0.9 cm column of the sulphonated cation exchange resin Amberlite CG-120 and the accelerated chromatography of amines was carried out using a 10 × 0.9 cm column[87]. More efficient, accurate and reproducible results have been achieved with Aminex resins designed for high-resolution operations. Aminex A-5, 11.5–15.5 μm, was used in 0.6 × 10 cm columns for the chromatography of primary monoamines and diamines[88]. Chromatography was performed using a commercial amino acid analyzer (Hitachi KLA-2) and the colour produced by the reaction of amines with ninhydrin was continually recorded at 440, 570 and 640 nm. The temperature was maintained at 50° during the run. Three buffers were used for the elution: 0.100 *M* sodium citrate buffer at pH 5.28 (total sodium concentration 0.30 *M*); 0.025 *M* sodium borate buffer at pH 8.02 (total sodium concentration 0.60 *M*) and 0.200 *M* sodium salicylate at pH 11.08 (total sodium concentration 0.23 *M*). In the analysis of primary monoamines, methylamine, ethylamine, allylamine, *n*-propylamine and isobutylamine were separated by elution with the citrate buffer, then elution with the borate buffer separated *n*-butylamine, dopamine, isoamylamine, histamine and *n*-amylamine, and finally tyramine, β-phenethylamine, serotonin and *n*-hexylamine were separated by elution with the salicylate buffer. The complete analysis of this 14-component mixture took about 7 h. A five-component mixture of primary diamines was analyzed in about 8 h under the same conditions as those used for the monoamine mixture and a clear-cut separation was also achieved. Ethylenediamine was eluted with borate buffer and the separation of higher diamines was effected by elution with the borate and salicylate buffers in the following order: 1,3-propanediamine, putrescine, cadaverine and hexamethylenediamine[88]. The tailing of some compounds during the elution with the salicylate buffer can be suppressed by the addition of benzyl alcohol.

The use of a concentration gradient of the borate and salicylate buffers with the pH changing from 10.00 to 12.50 in the third step instead of elution with a constant concentration of the salicylate buffer brought about a considerable improvement in the separation efficiency, in spite of using a coarser particle resin, Aminex A-4 (17 ± 2 μm in diameter) in a 0.6 × 12 cm column[89]. The gradient was formed by continuous mixing in a three-chamber system with individual chambers containing 120 ml of: (1) 0.05 *M* sodium borate buffer at pH 10.0 (total sodium concentration 0.60 *M*; 0.4% of benzyl alcohol added); (2) 0.20 *M* sodium salicylate at pH 11.50 (total sodium concentration 0.65 *M*); and (3) 0.20 *M* sodium salicylate at pH 12.50 (total sodium concentration 0.70 *M*). This system permitted the separation of most of the primary

amines and diamines from the above experiments in a single run. The separation of an authentic mixture of 16 amines is shown in Fig. 5. The total analysis time was 8 h under a 1 kg/cm² back-pressure. The accuracy of this analysis was found to be within ±3.56% and the method has been applied successfully to the analysis of amines produced by putrefaction of marine animals[89].

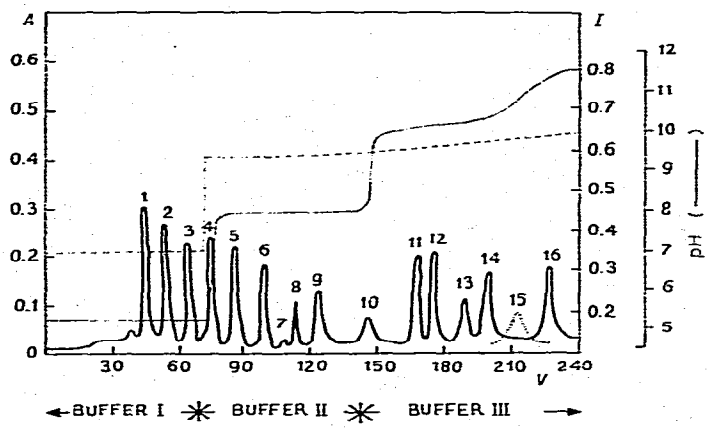


Fig. 5. Separation of a mixture of 16 amines using a combined stepwise-gradient system. (1) Methylamine; (2) ethylamine; (3) allylamine; (4) *n*-propylamine; (5) isobutylamine; (6) *n*-butylamine; (7) 1,2-propanediamine; (8) histamine; (9) isoamylamine; (10) *n*-amylamine; (11) tyramine; (12) putrescine; (13) phenylethylamine; (14) cadaverine; (15) serotonin; (16) hexamethylenediamine; *ca.* 0.4 μmole of each except for serotonin (1 μmole). Ion exchanger: Aminex A-4 (Na^+), 17 ± 2 μm. Column dimensions: 12 × 0.6 cm. Mobile phase: buffer I, 0.116 *M* sodium citrate, pH 5.28; buffer II, 0.025 *M* sodium borate, pH 8.02 (0.5% of benzyl alcohol added); buffer III, gradient system — chamber 1, 0.05 *M* sodium borate, pH 10.00 (0.4% of benzyl alcohol added), chamber 2, 0.20 *M* sodium salicylate, pH 11.50 and chamber 3, 0.20 *M* sodium salicylate, pH 12.50. Flow rate: 30 ml/h. Inlet pressure: *ca.* 1 atm. Temperature: 50°. *A* = absorbance; *V* = volume of eluate (ml); *I* = ionic strength of sodium ion.

In spite of the good results achieved, the use of this method is limited to some extent. Agmatine and higher aliphatic amines (having more than seven carbon atoms) are held so strongly on the resin that no elution is possible using either a constant salicylate buffer concentration or gradient elution. Furthermore, as secondary and tertiary amines do not react with ninhydrin, these compounds cannot be analyzed by this method. Isopropylamine and aromatic amines also give a negative ninhydrin reaction.

In the aromatic series, the difference in protonation constants of pyridine and benzylamine also makes possible the separation of these two compounds. Using a 70 × 6.5 mm column of the strongly acidic cation exchanger Lewatite SP-100 (Na^+), 0.2–0.4 mm, pyridine was completely eluted with $2 \cdot 10^{-2}$ *M* sodium acetate (pH 6) in 50% ethanol and, subsequently, sodium borate (pH 10.5) in 50% ethanol was used for the elution of benzylamine[90].

Pyridine could be separated from 4-methylpyridine on a 34 cm × 0.078 cm² column packed with the strongly acidic cation exchanger KU 2-X8 (NH_4^+) by elution with 3 *N* ammonium chloride solution[91].

Wheaton and Stewart[92] separated and determined a number of phenolic amines on a 135 × 0.64 cm column of the strongly acidic sulphonated polystyrene cation-exchange resin Technicon Chromobeads A, by elution with an ammonia concentration gradient (0.15–3.7 *M*). The column was operated at 60° and a 1.5 ml/min flow-rate with a column back-pressure of 100–400 p.s.i. The concentration of phenolic amines in the eluate was monitored by continually measuring the absorbance in the UV region. It was possible to separate completely a mixture containing octopamine, synephrine, hordenine, tyramine and N-methyltyramine in about 2 h. In general, the monophenolic amines and 3-methoxy-4-hydroxyphenethylamines could be chromatographed satisfactorily. The catechol compounds are unstable under alkaline conditions and attempts to chromatograph them failed. Phenethylamines with no hydroxyl group and fully methoxylated compounds such as 4-methoxyphenethylamine or mescaline were apparently not eluted with ammonia solution.

The separation of phenolic amines was achieved with several different gradients using borate, carbonate or phosphate buffers in the pH range 10–12. The use of the resin in the sodium form instead of the ammonium form offers a more precise control of pH and ionic strength so as to allow separations of very closely related compounds. No interference occurs if an automatic colorimetric procedure is used for monitoring column effluents that contain substances that do not have suitable absorption maxima in the UV region.

Work at 60° is recommended because lower temperatures resulted in poorer resolution and temperatures above 60° did not yield further improvement. The method has been used for analyses of citrus juices with recoveries in the range 98–104%. Other biological fluids, pharmaceuticals and food products are likely to give satisfactory results when analyzed by this procedure with slight modifications[92].

Sargent and Rieman[93] studied the salting-out chromatography of various amines. As anion-exchange resins decompose slowly in the alkaline solutions that are used to suppress the ionization of amines, cation exchangers were preferred. The best results were achieved with a strongly acidic sulphonated cation-exchange resin cross-linked with 4% of divinylbenzene. Dowex 50-X4 (K^+), 200–400 mesh. Solutions of potassium phosphate used as the eluent were much more efficient salting-out agents than were solutions containing sodium chloride and sodium hydroxide. The capacity ratios of 22 amines in solutions with various concentrations of potassium phosphate as the eluent are given in Table 6.

Because of the similarity of their capacity ratios over the whole range of eluent concentrations, *n*-propylamine, *tert.*-butylamine and tetramethylenediamine cannot be separated from each other by salting-out chromatography. A similar situation occurs for mono- and diethanolamine. It is possible, by the choice of appropriate conditions, to effect the separation of all of the other amines listed in Table 6. When a mixture of two amines is to be separated using a column less than 30 cm in length, the ratio of their capacity ratios must be $\geqslant 1.3$ for small monofunctional compounds such as methyl- or ethylamine and $\geqslant 1.5$ for large polyfunctional compounds such as ethanolamines and polyethylenediamines. The elution curves for these large polyfunctional compounds exhibit a large spread which makes their separation difficult. The separation of ethylenediamine and its polymers on a 14.8 cm × 3.90 cm^2 column of Dowex 50-X4, 200–400 mesh, shown in Fig. 6 is not quantitative; a quantitative

TABLE 6

CAPACITY RATIOS (K') OF 22 AMINES ON DOWEX 50-X4 (K^+), 200–400 MESH, IN POTASSIUM PHOSPHATE SOLUTIONS

Amine	*Concentration of potassium phosphate solution (M)*				
	0.44	*0.88*	*1.32*	*1.76*	*2.20*
Monoethanolamine	1.21	1.69	2.26	3.37	4.84
Diethanolamine	1.10	1.56	2.17	3.62	–
Triethanolamine	1.58	2.36	3.78	7.01	–
Ethylenediamine	1.36	2.08	3.39	7.96	–
Diethylenetriamine	1.36	2.31	2.26	13.7	–
Triethylenetetramine	1.18	2.29	–	19.2	–
Tetraethylenepentamine	1.09	2.34	–	31.7	–
Methylamine	1.65	2.23	3.57	6.35	10.34
Morpholine	1.69	2.90	5.57	12.4	–
Ethylamine	2.02	3.31	6.21	13.0	28.9
Isopropylamine	2.51	4.47	9.80	23.1	59.1
Tetramethylenediamine	2.84	5.21	12.0	33.0	–
tert.-Butylamine	2.82	5.55	12.8	–	–
n-Propylamine	3.02	5.51	12.4	–	–
Diethylamine	3.02	6.09	15.2	–	–
sec.-Butylamine	3.85	8.04	20.5	–	–
n-Butylamine	5.29	11.2	28.9	–	–
Triethylamine	5.07	12.7	37.5	–	–
Pyridine	10.0	14.3	29.9	–	–
n-Amylamine	10.3	23.3	–	–	–
Aniline	20.1	29.9	–	–	–
Benzylamine	17.5	–	–	–	–

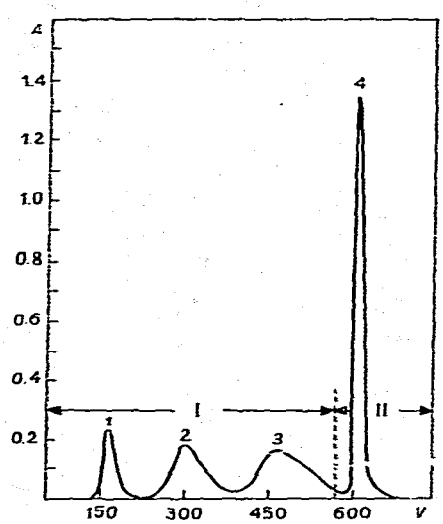


Fig. 6. Separation of ethylenediamine and its polymers by salting-out chromatography on a cation-exchange column. (1) Ethylenediamine; (2) diethylenetriamine; (3) triethylenetetramine; (4) tetramethylenepentamine. Ion exchanger: Dowex 50-X4 (K^+), 200–400 mesh. Column dimensions: 14.8 cm × 3.90 cm². Mobile phase: 1.80 *M* tripotassium phosphate (I), changed at 576 ml to 0.45 *M* (II). Flow rate: 0.5 cm/min. *A* = absorbance; *V* = volume of eluate (ml).

separation would be obtained by elution from a 20-cm column with approximately 1 l of 1.80 *M* potassium phosphate solution.

The separation of another 11-component mixture by salting-out chromatography is shown in Fig. 7 (ref. 93).

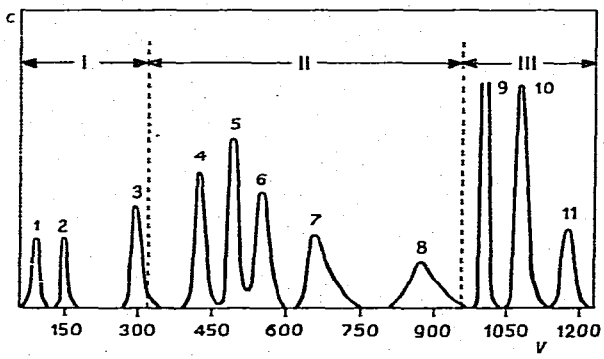


Fig. 7. Separation of an 11-component mixture of amines by salting-out chromatography on a cation-exchange column. (1) Monoethanolamine; (2) methylamine; (3) ethylamine; (4) isopropylamine; (5) *n*-propylamine; (6) diethylamine; (7) *sec.*-butylamine; (8) *n*-butylamine; (9) triethylamine; (10) *n*-amylamine; (11) benzylamine. Ion exchanger: Dowex 50-X4 (K^+), 200–400 mesh. Column dimensions: 27.5 cm × 2.28 cm^2. Mobile phase: tripotassium phosphate: I, 1.75 *M*; II, 1.32 *M*; III, 0.22 *M*; eluent changed at 324 and 960 ml. Flow-rate: 0.4 cm/min. *c* = concentration (arbitrary units); *V* = volume of eluate (ml).

Strongly acidic cation exchangers have been used for the separation of drugs in mixtures. Six principal alkaloids in opium were separated by combination of anion-exchange and cation-exchange chromatography[41]. The sample was passed through a column of cation exchanger in the hydrogen form; narceine and morphine were eluted with 1 *N* aqueous ammonia and then separated by anion-exchange chromatography. Further elution with 1 *N* aqueous ammonia eluted codeine from the cation-exchange column and the remainder of the alkaloids were eluted with 1 *N* alcoholic ammonia. This fraction was re-chromatographed on the same column. Papaverine and narcotine were separated by elution with 0.1 *M* ammonium acetate buffer at pH 8 in 80% ethanol. Thebaine was eluted with 1 *N* alcoholic ammonia.

Caffeine was separated from quinine and strychnine on a column of Dowex 50-X2, 100–200 mesh, by elution of the first compound with acetate buffer (1 *N* in acetic acid and 0.1 *N* in ammonium acetate). Quinine and strychnine were then eluted with 1 *N* alcoholic ammonia[42]. This method can also be used for the separation of caffeine from *p*-phenetidine[94]. It is possible to separate codeine from terpine hydrate by sorption on a column of Dowex 50-X8 followed by elution with 10% ammonia in ethanolic solution[95].

Effective separations of various pharmaceutically important amines, phenolic amines and barbiturates can be achieved by salting-out chromatography on Dowex 50-X8 using solutions with various concentrations of tripotassium phosphate or ammonium sulphate in water or aqueous methanol as the eluents. Ammonium sulphate is a more effective salting-out agent for non-phenolic amines than for phenolic amines[96,97].

(e) Separation of amines and nitrogen-containing basic compounds on cation exchangers other than conventional ion-exchange resins

So-called oleophilic ion-exchange polymers have been prepared with the aim of increasing the swelling of cation-exchange resins in non-aqueous solvents, thus increasing the rate of ion exchange in these media[98]. Lauroylated and partially sulphonated polystyrene cation exchangers showed considerable swelling in a number of non-polar solvents, including saturated hydrocarbons[99].

The affinities of nitrogen-containing organic bases for these exchangers increase with their basicities. For bases of nearly equal strength, secondary interactions between the resinous skeleton and the base have a strong influence on the sorption selectivity. On the basis of differences in selectivity, some chromatographic separations of organic bases could be performed in non-polar media. Pyridine was partially separated from aniline by elution with 0.04 *N* *n*-butylamine in *n*-heptane[100]. The separation of aniline from nicotine was hindered by the relatively strong non-ionic sorption of aniline. These compounds, however, could be partially separated by elution with 0.02 *N* *n*-butylamine in *n*-heptane, if sample overloading was avoided and a fine-mesh resin used. Attempts were also made to separate some indole alkaloids. By elution with pyridine or *n*-butylamine in 1:1 dichloroethane–methanol, it was possible to separate renoxidine from other alkaloids of the reserpine class and to achieve a partial separation of leurosine from vincaleukoblastine[100].

Inorganic ion-exchange crystals have been used for some separations of nitrogen bases. Aluminium silicates show molecular sieve properties and a complete replacement of the sodium ions initially present in the zeolite structure by organic ions is not possible and the exchange reaction is confined to the large cavities in the crystal. The maximum extent of the exchange decreases with increasing molecular weight and polarizability of the cations. It is less for the di- and trialkyl derivatives than for the monoalkyl ammonium ions of comparable molecular weight, for which the steric hindrance is not pronounced to such an extent[101].

The synthetic inorganic cation exchanger Decalso F (K^+) has been used for the separation of alkaloids into cationic and anionic fractions[102]. Two columns in series, the upper filled with activated 60–80 mesh Decalso and the lower with 60–80 mesh Supersorb, were used for the separation of vitamins B_1 and B_2. Thiamine is a stronger base and is sorbed on the Decalso column and the less basic riboflavin passes through and is sorbed on the column of Supersorb. Thiamine is eluted from the Decalso column with 25% potassium chloride solution and riboflavin is recovered from the Supersorb column with 20% pyridine in 2% acetic acid[103].

Rebertus[104] studied the chromatographic separations of some substituted ammonium ions on columns of zirconium phosphate, 100–200 mesh, in the hydrogen form. Alkane diammonium ions were found to have considerably higher affinities for zirconium phosphate than any of the monoammonium ions examined, which is explained by the fact that two ammonium groups of diammonium ions are bound to the exchanger. The separation of octylammonium, ethylammonium, methylammonium and ethylenediammonium ions was achieved on the 0.55 × 42.5 cm column of zirconium phosphate by elution with 0.1 *N* hydrochloric acid. The elution curves showed severe tailing, which could be decreased by increasing the temperature. Thus, the monoammonium ions were eluted at 50° and the ethylenediammonium ions at 90°. A more difficult separation, that of triethanolammonium, ethanolammonium and

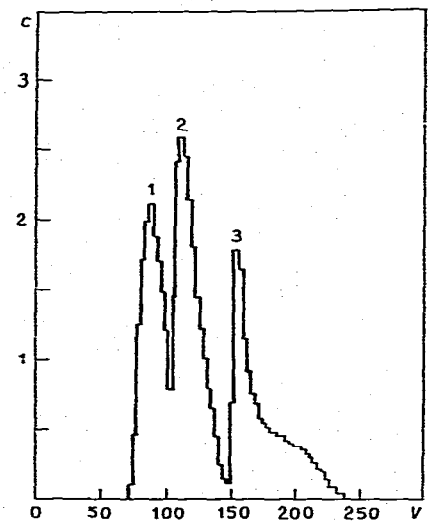


Fig. 8. Separation of triethanolammonium (1), ethanolammonium (2) and ammonium (3) ions on zirconium phosphate. Ion exchanger: Bio-Rad ZP-1 (H^+), 100–200 mesh. Column dimensions: 0.55 × 170 cm. Eluting agent: 0.1 N hydrochloric acid. Flow rate: 0.1 ml/min. Temperature: 80°. c = concentration of salt in effluent (mg/ml); V = volume of eluate (ml).

ammonium ions, was partially effected on a 0.55 × 170 cm column of zirconium phosphate at 80° (Fig. 8).

Cross-linked dextran cation exchangers are useful for some separations of nitrogen-containing bases. *p*-Bromoaniline was separated from *o*-bromoaniline on a 50 cm × 2.75 mm column packed with the weakly acidic carboxymethyl cation exchanger CM-Sephadex C-25. A complete separation was achieved in 36 min using 1 *N* hydrochloric acid for the elution at a flow-rate of 4.0 cm/min (ref. 105).

A strongly acidic sulphoethyl dextran cation exchanger, SE-Sephadex C-25, was used for the chromatographic separation of some biogenic amines (imidazole, indole and catechol compounds) by stepwise elution with buffers of increasing pH. A sample was applied on a 17 × 200 mm column packed with ion exchanger and equilibrated with 0.05 *M* ammonium formate buffer at pH 2.1. DOPA, dopamine, tryptophan, 5-hydroxytryptophan, norepinephrine and melatonin were eluted with 0.05 *M* sodium phosphate at pH 6.1 and the elution of 1,4-methylhistamine and 5-hydroxytryptamine followed with 0.1 *M* sodium phosphate buffer of pH 8.2 as the eluting agent. All compounds were found in the effluent with 95–100% recovery[106].

A 50 × 0.9 cm column packed with SE-Sephadex C-25 has been used in a semi-automated method for the separation and determination of opiates in illicit narcotic preparations. The compounds were determined by continuous ultraviolet monitoring of the column effluent and colorimetric assay was used for lactose and mannitol. A 0.2 *M* sodium dihydrophosphate buffer of pH 4.6 was used for the elution at ambient temperature and was pumped through the column at a 9.0 ml/h rate. Peak elution volumes for compounds of forensic interest are presented in Table 7. Some compounds, such as meperidene and morphine, cannot be separated from each other and are eluted in one elution band with a non-Gaussian shape or a shoulder. Thin-

TABLE 7

PEAK ELUTION VOLUMES FOR THE MOST COMMON COMPONENTS OF NARCOTIC PREPARATIONS

Column (50 × 0.9 cm) of SE-Sephadex C-25, coarse. Elution with 0.2 *M* NaH_2PO_3, pH 4.6. Flow-rate: 9.0 ml/h.

Compound	*Peak elution volume (ml)*
Lactose	27.0
Mannitol	31.5
Heroin	57.6
Codeine	57.6
N-Allylnormorphine	57.6
Ethylmorphine	60.3
6-Monoacetylmorphine	67.5
Narcotine	69.7
Meperidine	71.1
Morphine	72.0
Procaine	76.5
Methadone	77.4
Strychnine	82.8
Succinylcholine	103.5
Methapyrilene	112.6
Papaverine	114.8
Quinine	155.8

layer chromatography can be used for further separations of the unresolved compounds. This peak overlapping, however, does not diminish the usefulness of the procedure described. A hypothetical separation of the compounds found most frequently in narcotic mixtures is illustrated in Fig. 9 (ref. 107).

Alginic acid, a weak cation exchanger introduced as a thin-layer chromatographic stationary phase[108], has been used for the column chromatography of primary aromatic amines[109]. Various dilute acids were tested as the eluting agents: 0.1 *M* hydrochloric acid and 1 *M* monochloroacetic acid are stronger eluents than 1 *M* acetic acid. The affinity of amines for alginic acid is considerably reduced when these acids are used and the efficiency of the separation is decreased. These acids can be used, however, if the separation is required of amines with a higher number of amino-groups, which are more strongly sorbed on the exchanger. Elution with aqueous–organic solvents gave no advantages over the use of aqueous solutions.

The best separations of a variety of amines were achieved with 1 *M* acetic acid as the eluent. Fig. 10 shows elution curves for aromatic amines on a column with a cross-section of 0.94 cm^2 filled with 4 g of alginic acid, 50–150 mesh, using 1 *M* acetic acid for the elution at a flow-rate of 2 ml/min. It can be seen that a good separation of several isomers is obtained. Mixtures of *o*-, *p*- and *m*-nitroanilines; *o*-, *p*- and *m*-aminobenzoic acids; *o*- and *p*-arsanilic acids; and 4-aminosalicylic and 5-aminosalicylic acids can be quantitatively separated. A clean separation of 4-aminosalicylic acid from *m*-aminophenol is also feasible[109].

Carboxymethylcellulose, which is a weakly acidic cellulose cation exchanger, shows, with respect to alginic acid, a lower affinity for aromatic amines and therefore has fewer possibilities for use in column chromatographic separations. The separation

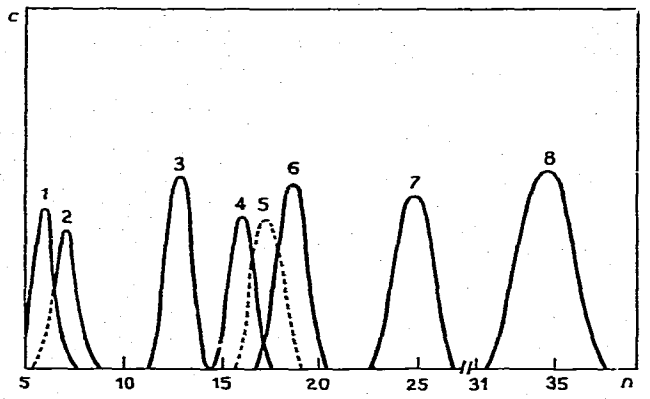


Fig. 9. Separation of compounds occurring in narcotic mixtures on dextran cation exchanger. (1) Lactose; (2) mannitol; (3) heroin (codeine); (4) morphine; (5) procaine; (6) strychnine; (7) methapyrilene; (8) quinine. Ion exchanger: SE Sephadex C-25, coarse. Column dimensions: 50 × 0.9 cm. Mobile phase: 0.2 *M* NaH_2PO_4 buffer, pH 4.6. Flow-rate: 9.0 ml/h. Temperature: ambient. Detector: UV, 240 nm; mannitol and lactose were determined by a conventional colorimetric procedure. Four fractions per hour. *c* = concentration (arbitrary units); *n* = fraction number.

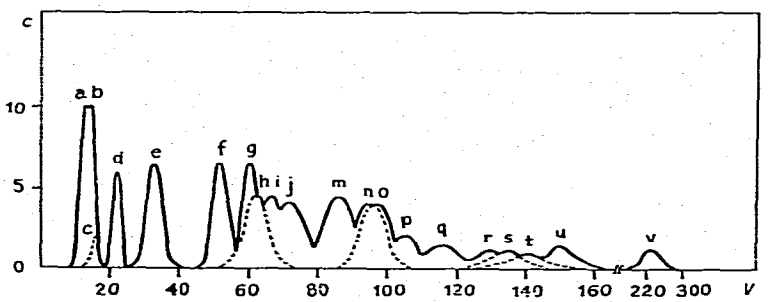


Fig. 10. Elution curves for aromatic amines on an alginic acid column. (a) Sulphanilic, methanilic and orthanilic acids; (b) *o*-arsanilic acid; (c) *o*-nitroaniline; (d) *p*-nitroaniline; (e) *p*-arsanilic acid; (f) 4-aminosalicylic acid; (g) *o*-aminobenzoic acid; (h) sulphanilamide; (i) *p*-aminoacetophenone; (j) 5-aminosalicylic acid; (m) *p*-aminobenzoic acid; (n) *o*-chloroaniline; (o) *p*-aminohippuric acid; (p) *m*-nitroaniline; (q) aniline; (r) *o*- and *p*-toluidine; (s) *m*-aminobenzoic acid; (t) *o*-anisidine and *o*-aminophenol; (u) *m*- and *p*-aminophenol; (v) *α*- and *β*-naphthylamine. Ion exchanger: alginic acid, 50–150 mesh. Column: cross section 0.94 cm^2, filled with 4 g of exchanger. Mobile phase: 1 *M* acetic acid. Flow-rate: 2 ml/min. *c* = concentration (arbitrary units); *V* = volume of eluate (ml).

of several amines can be achieved with water as the eluent, as shown in Fig. 11. As on alginic acid, it is possible to separate the isomers of aminobenzoic acid on carboxymethylcellulose. Amines that are held strongly on the column when eluted with water can be eluted with 1 *M* acetic acid. In this way it is possible to separate benzidine and the *meta*- and *para*-isomers of phenylenediamine[109].

Cellex CM carboxymethylcellulose in a 2.5 × 8 cm column was used for the separation, isolation and determination of water-soluble and water-insoluble long-chain quaternary ammonium compounds from commercial products such as textile softeners, surface-active agents and germicides. After sorption on the ion-exchange column, the compounds were eluted with alcoholic hydrochloric acid, extracted into chloroform and determined by reaction with bromophenol blue[110].

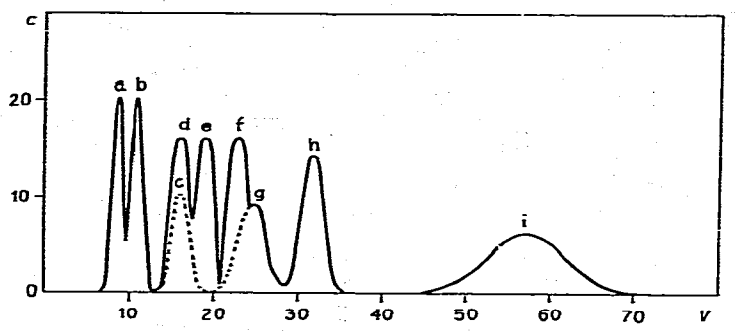


Fig. 11. Elution curves for aromatic amines on a carboxymethylcellulose column. (a) Sulphanilic, methanilic and orthanilic acids; (b) *o*- and *p*-arsanilic acids; (c) *o*-nitroaniline and *o*-aminobenzoic acid; (d) 4-aminosalicylic acid and *p*-nitroaniline; (e) sulphanilamide and *p*-aminohippuric acid; (f) *p*-aminoacetophenone; (g) *p*-aminobenzoic acid; (h) *m*-nitroaniline; (i) *m*-aminobenzoic acid. Ion exchanger: carboxymethylcellulose, H^+ form. Column: cross section 0.94 cm^2, filled with 2 g of exchanger. Mobile phase: water. Flow-rate: 1 ml/min. *c* = concentration (arbitraty units); *V* = volume of eluate (ml).

Chromatography on carboxymethylcellulose columns has been used for the separation and determination of basic drugs in urine[111,112]. Guanethidine and its metabolites (guanethidine N-oxide; 2-(6-carboxyhexylamino)ethylguanidine) could be resolved in 6 h using four ion-exchange columns (1.2 × 6 cm) connected in series by elution with borate buffer of pH 9.7 containing 0.056 *M* sodium tetraborate, 0.044 *M* sodium hydroxide and 0.2 *M* sodium chloride at a flow-rate of 0.16 ml/min[111].

With two carboxymethylcellulose columns of the same dimensions and a suitable choice of the eluting buffer, various drugs can be separated from each other and from the normal basic constituents of human urine. At pH 8.5, cyclizine, pethidine, morphine and amphetamine are separated from interfering substances, while methadone, strychnine and ephedrine are not resolved. By changing the pH of the buffer to 10, these compounds can also be separated from the interfering substances[112].

A combination of ion-exchange chromatography on a Cellex carboxymethylcellulose column and a Dowex 50W-X2 column, using stepwise elution with water, phosphate and acetate buffers (pH 3.5, 4.5 and 5.5) has been applied to the separation of cyanocobalamin, methylcobalamin, cobamide and hydroxycobalamin[113].

Whatman P-11 cellulose phosphate columns together with a continuous polarographic detector have been applied to the separation and determination of catecholamines and related substances[114]. An exponential salt gradient was used during the elution. A solution of 0.5 *M* ammonium acetate in 20% *n*-propanol, pH 6.0, was pumped at a rate of 0.23 ml/min into a mixing vessel containing 25 ml of 0.05 *M* ammonium acetate solution. The mixture was supplied to the column (35 × 0.5 cm) at the same rate. A clear-cut separation was obtained of 3,4-dihydroxyphenylacetic acid, L-tyrosine, DOPA, tyramine, adrenaline, dopamine and noradrenaline in microgram amounts with 95–100% recoveries. This method has been used for investigating the biosynthesis of catecholamines in natural materials[115].

D. High-speed chromatography of nitrogen-containing bases and related compounds on pellicular and controlled surface porosity ion exchangers

The introduction of new, highly efficient ion-exchange packing materials with

a thin, superficially active layer on an impervious solid core made possible numerous separations of nitrogen-containing bases, drugs and related compounds in several minutes.

Strongly acidic cation-exchange columns consisting of the support Zipax SCX coated with a sulphonated fluorocarbon polymer are useful for the separation of aliphatic and aromatic amines. The elution volumes of these compounds can be controlled by an appropriate choice of the eluting medium. Sorption on the Zipax cation exchanger decreases with increasing pH values and/or ionic strength of the mobile phase. Sometimes, the reverse elution order may occur at higher concentration of the salt in the eluting agent, in contrast to the behaviour at low concentrations. For example, quinoline was eluted prior to 8-hydroxyquinoline up to a sodium nitrate concentration 0.10 *M*, while the reverse elution order was observed at higher sodium nitrate concentrations[116].

In addition to changes in pH and ionic strength, small amounts of methanol (up to 10%) may be added so as to modify the mobile phase in order to reduce the retention times of strongly held compounds. The addition of methanol is particularly useful when compounds have a limited solubility in water. Higher concentrations of organic solvents and halide acids and salts as well as the use of aqueous–organic solvents at elevated temperatures, however, may be harmful to the ion-exchange shell and have to be avoided.

Fig. 12 illustrates the separation of four aromatic amines, pyridine, 8-hydroxyquinoline, quinoline and isoquinoline, in less than 10 min on a 1000 × 2.1 mm Zipax strong cation-exchange column with 0.15 *M* sodium nitrate solution as the mobile phase at a column pressure of 1200 p.s.i.[116].

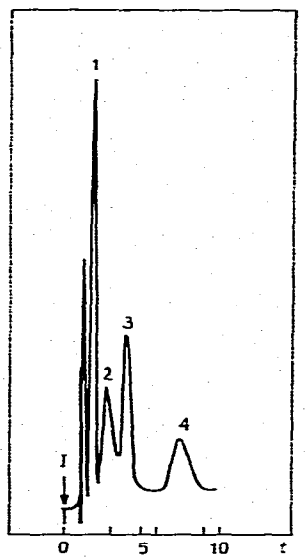


Fig. 12. High-speed, high-efficiency separation of four nitrogen-containing heterocyclic compounds on strong controlled surface porosity cation exchanger. (1) Pyridine; (2) 8-hydroxyquinoline; (3) quinoline; (4) isoquinoline. Ion exchanger: Zipax SCX cation exchanger. Column dimensions: 100 cm × 2.1 mm I.D. Mobile phase: 0.15 *M* sodium nitrate. Flow-rate: 1.5 ml/min. Column input pressure: 1200 p.s.i. Detector: UV, 254 nm. I = inject; *t* = time elapsed (min).

The complete separation of a synthetic mixture of substituted benzimidazoles could be achieved on the same column by elution with 0.025 *N* tetramethylammonium nitrate–nitric acid (pH 1.74) at 60° with a column input pressure of 325 p.s.i. (Fig. 13)[117].

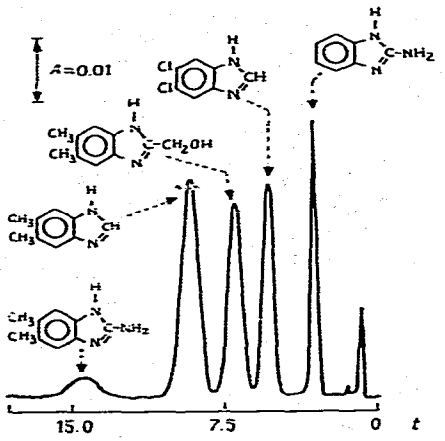


Fig. 13. High-speed, high-efficiency separation of a synthetic mixture of substituted benzimidazoles on a column of strongly acidic controlled surface porosity cation exchanger. Ion exchanger: Zipax SCX cation exchanger, 20–37 μm. Column dimensions: 1000 × 2.1 mm I.D. Mobile phase: 0.025 *N* tetramethylammonium nitrate–nitric acid, pH 1.74. Flow rate: 2.04 ml/min. Inlet pressure: 325 p.s.i. Temperature: 60°. Detector: UV, 254 nm. Sample size: 10 μl of 0.2 mg/ml of each component. *A* = absorbance; *t* = time elapsed (min).

Chromatography on controlled surface porosity exchangers is ideally suited for the analysis of multicomponent drug formulations. Purity and stability tests of drugs and their formulations have been performed, rates of decomposition reactions have been studied and drugs in biological fluids have been determined by this method. The quantitative analysis of a variety of active ingredients in analgesic tablets can generally be completed in about 10 min.

Fig. 14 shows the separation of the components of an APC tablet (aspirin, phenacetin and caffeine) on a 1000 × 2.1 mm Zipax strong cation-exchange column using elution with a buffer at pH 6.86 (ref. 116).

The separation of a similar four-component analgesic with benzoic acid as the internal standard on a Zipax support coated with a strongly basic anion-exchange resin (Zipax SAX) is shown in Fig. 15 (ref. 118). The elution was carried out with a pH 9.2 buffer with the ionic strength adjusted by the addition of 0.005 *M* ammonium nitrate solution. The quantitative separation of components of a tablet of asthma and hay fever medication may be given as a further example[119]. Using 0.01 *M* sodium nitrate solution at pH 5.7 as the eluting agent and a 1500 × 2 mm column packed with Zipax SAX, the components were eluted in order ephedrine, theophylline and phenobarbital in about 15 min with a column pressure drop of 1800 p.s.i.

Little work was devoted to the ion-exchange chromatography of barbiturates and their metabolites prior to the introduction of the high-pressure, high-efficiency technique. Chromatography on pellicular and controlled surface porosity anion

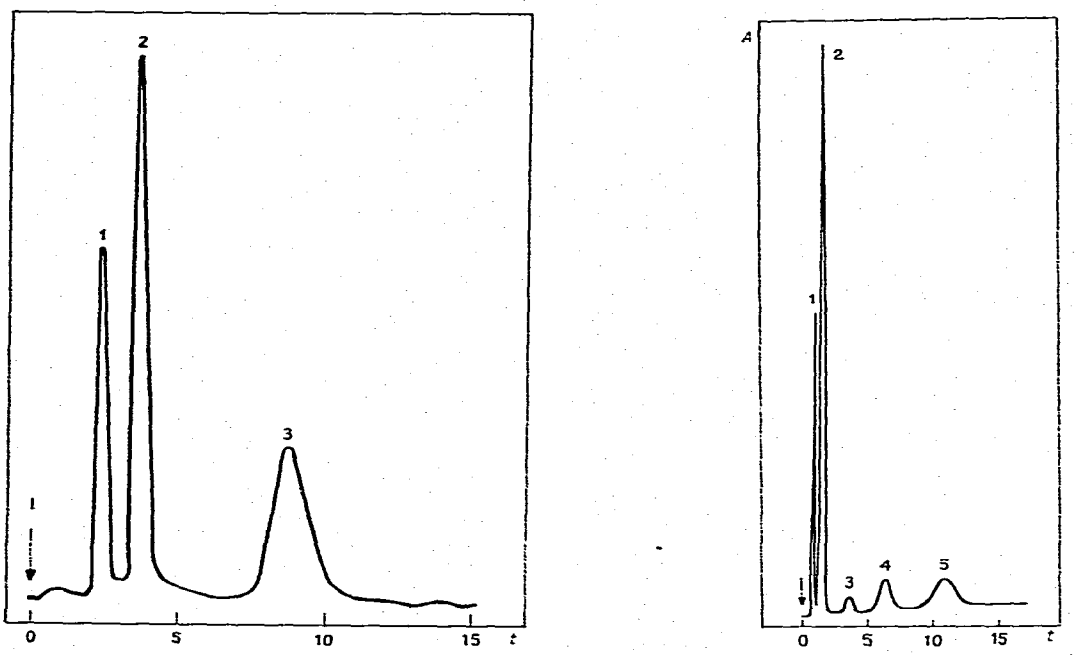


Fig. 14. High-speed, high-efficiency separation of the components of an APC tablet on a column of strongly acidic controlled surface porosity cation exchanger. (1) Acetylsalicylic acid; (2) caffeine; (3) phenacetin. Ion exchanger: Zipax SCX cation exchanger. Column dimensions: 1000 × 2.1 mm I.D. Mobile phase: water buffered at pH 6.86. Flow rate: 1.5 ml/min. Inlet pressure: 1200 p.s.i. Detector: UV, 254 nm. I = inject; t = time elapsed (min).

Fig. 15. Separation of a four-component analgesic with benzoic acid as the internal standard. (1) Caffeine; (2) N-acetyl-*p*-aminophenol; (3) aspirin; (4) benzoic acid (internal standard); (5) salicylamide. Ion exchanger: Zipax SAX controlled surface porosity anion exchanger. Column dimensions: 1000 × 2.1 mm. Mobile phase: buffer, pH 9.2, with ionic strength adjusted by the addition of 0.005 *M* ammonium nitrate. Flow rate: 1.5 ml/min. Inlet pressure: 1200 p.s.i. Detector: UV, 254 nm. Instrument: DuPont Model 820 liquid chromatograph. Sample size: 5 μl. A = absorbance; t = time elapsed (min).

exchangers made possible rapid quantitative barbiturate analyses without the derivatization step that is necessary for gas chromatography. Chromatography on a column containing a pellicular anion exchanger, performed at 80° with 20.0 m*M* phosphate buffer at pH 3.5 as the eluting agent, proved suitable for the separation of phenobarbital and hydroxyphenobarbital. Phosphate buffers proved unsatisfactory eluents for other barbiturate mixtures, as poor peak shapes were obtained. The use of a linear sodium chloride-gradient, however, is well suited for both barbiturates and their metabolites, which are eluted as sharp symmetrical peaks. Good separations of metabolites and the parent compounds can be achieved, as shown in Fig. 16. Alcoholic or ketonic metabolites showed retention volumes less than those of the parent molecules. The phenolic metabolite of phenobarbital is an exception, its retention volume being greater than that of phenobarbital[120].

The chromatography of another barbiturate mixture on the controlled surface porosity anion exchanger Zipax SAX is shown in Fig. 17. Using an input pressure of 1600 p.s.i., it was possible to separate a five-component mixture in less than 15 min[121].

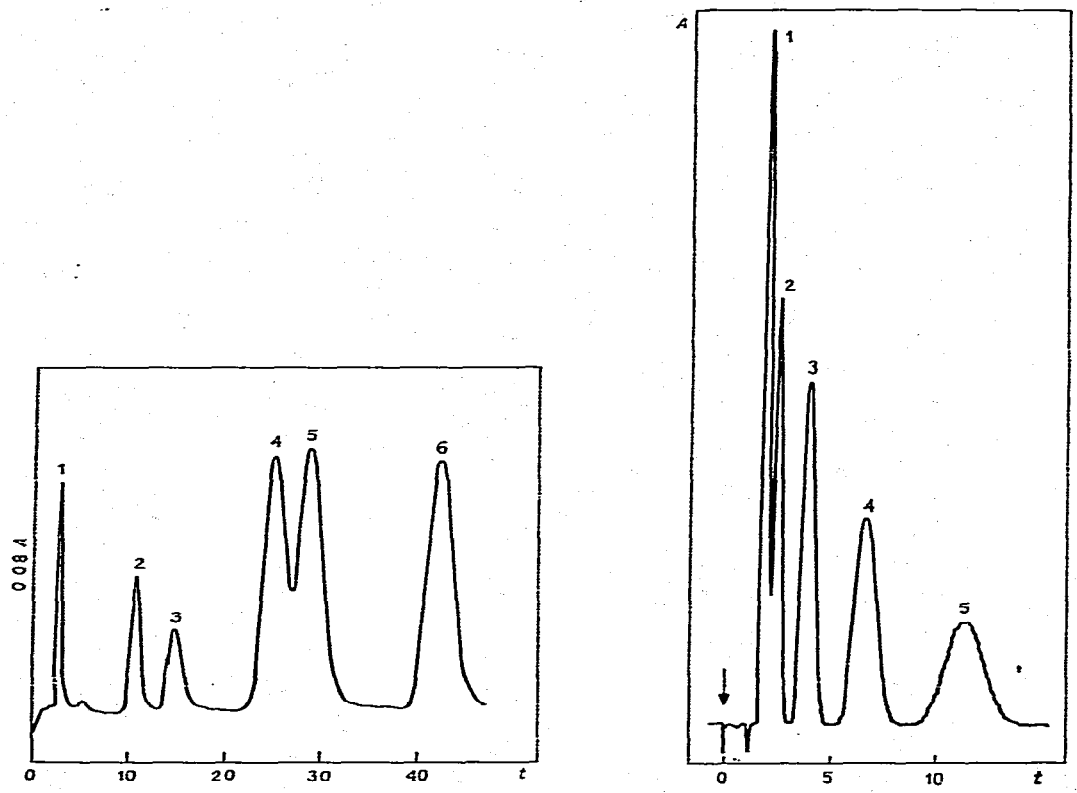


Fig. 16. Chromatography of barbiturates and metabolites on a pellicular anion exchanger. (1) Ketohexobarbital (0.3 μg); (2) hydroxyamobarbital (13 μg); (3) contaminant; (4) amobarbital (5.3 μg); (5) phenobarbital (2.5 μg); (6) hydroxyphenobarbital (3.5 μg). Ion exchanger: pellicular anion exchanger. Column dimensions: 120 × 0.04 in. I.D. Mobile phase: 0.1–1.0 m*M* sodium chloride solution at pH 7. Flow-rate: 26 ml/h. Inlet pressure: 700–900 p.s.i. Temperature: 80°. Detection: UV, 254 nm. Instrument: Varian LCS 1000 liquid chromatograph. *A* = absorbance; *t* = time elapsed (min).

Fig. 17. High-speed, high-efficiency chromatographic separation of a five-component barbiturate mixture using a column packed with a controlled surface porosity anion exchanger. (1) Diethylbarbital; (2) probarbital; (3) butabarbital; (4) phenobarbital; (5) secobarbital. Ion exchanger: Zipax SAX anion exchanger. Column dimensions: 1500 × 2 mm I.D. Mobile phase: 0.01 *M* sodium nitrate, pH 5.7. Flow-rate: 1.2 ml/min. Inlet pressure: 1600 p.s.i. Temperature: 38°. Detector: UV, 254 nm. Instrument: Chromatronix Model 3100 UV. Sample size: 20 μl (approx. 300 ng of each component). *A* = absorbance; *t* = elution time (min).

E. Ligand-exchange chromatography of amines and nitrogen-containing bases on cation exchangers containing a transition metal

In 1954, Stokes and Walton[122] noticed ligand sorption on cation exchangers containing complexing metal ions. Later, this phenomenon was utilized by Helfferich[123] to introduce a new separation technique, ligand-exchange chromatography. In this method, a cation exchanger containing a complexing metal ion (Cu^{2+}, Ni^{2+}, Ag^{+}, Co^{3+}, Fe^{2+}, etc.) is used as a solid sorbent. The potential ligands are sorbed from solutions and form complexes with the metal ions in the resin or displace other

ligands which have previously complexed the metal. The latter remains in the ion exchanger. In contrast to conventional ion exchange, in which counter ions are exchanged, ligand exchange represents the exchange of ligands for other ligands or for solvent molecules in the ionic solvation shells.

Complex formation occurs by a very strong interaction if the proper metal ion is chosen. Thus, ligands are sorbed selectively even when the ligand concentration in the external solution is extremely low and very efficient separations of ligands from non-ligands can be achieved. Moreover, complexes of a metal ion even with rather similar ligands differ greatly in their strengths, and these differences can be utilized for mutual chromatographic separations of ligands on columns of "ligand exchangers" even in instances in which conventional sorbents fail. With an appropriate choice of a complexing metal ion, the selectivities of ligands can be varied widely and even the reversal of the order of selectivity can be achieved[124]. For example, by selecting a suitable metal ion, it is possible to adjust the conditions in such a way that a monodentate ligand is preferentially sorbed from concentrated solutions and a bidentate ligand from dilute solutions. 1,3-Diaminopropan-2-ol was removed from a dilute aqueous solution (0.005 *M*) and from a dilute aqueous mixture containing a large excess of ammonia (0.1 *M* ammonia + 0.001 *M* diamine) by passing the solutions through a small laboratory column containing Amberlite IRC-50 ($Ni(NH_3)_4^{2+}$) carboxylic cation exchanger. Concentrated (15.6 *M*) aqueous ammonia displaced 1,3-diaminopropan-2-ol completely in a highly concentrated and rather small effluent fraction containing the diamine in excess over ammonia[124].

Other amines, however, form nickel complexes with stability constants at least 100 times greater than 1,3-diaminopropan-2-ol, and are held so strongly on the resin that their elution with concentrated ammonia fails. Ethylenediamine was eluted in a wide band from the nickel form of the resin, but the triamine, tetramine and pentamine could not be recovered without stripping the metal from the resin[125].

If the mutual separation of two or more bases is to be achieved, solutions of another ligand are usually used as the eluting agents. The distribution of a base, B, between the resin in the metallic form complexed with a competing ligand, L, and aqueous solution is expressed by the distribution coefficient D_B. When the ligand L is present in a large excess over the base B, and assuming that a single complex ML_n is formed and that neither the ligand L nor the metal M are subject to any other competitive reactions, a simple equation can be written[126]:

$$D_B = \frac{(c_B)_R}{(c_B)_S} = K_L^B \cdot \frac{(c_L)_R}{(c_L)_S} \approx K_L^B \cdot Q \cdot n \cdot \frac{1}{[L]} \tag{7}$$

where $(c_B)_R$ and $(c_L)_R$ denote the total (analytical) concentrations of the base B and the ligand L, respectively, in the resin phase and $(c_B)_S$ and $(c_L)_S$ those in the external solution; K_L^B is the selectivity constant, defined as

$$K_L^B = \frac{(c_B)_R}{(c_B)_S} \cdot \frac{(c_L)_S}{(c_L)_R} \tag{8}$$

$[L] \approx c_L$ is the actual concentration of the free ligand in the solution; Q represents

the cation-exchange capacity of the resin; and n = millimoles of the ligand bound per millimole of the metal ion[126]. The details of the calculation of K_L^B for systems in which the formation of other complexes is to be expected are given elsewhere[124]. The stability constants of the complexes formed and the distribution coefficients of the base B and ligand L must be known, and the number of the free coordination valencies of the metal for the ligands must be taken into account in this approach.

The use of ligand-exchange chromatography, however, has serious practical limitations. One major difficulty is that the metal may be displaced from the resin by the eluent or by one of the ligands to be separated. In this event, not only loss of the ligand-exchange capacity would occur, but complex formation in the external solution would take place, thus counteracting the ligand-exchange selectivity and, consequently, seriously affecting the separations. For this reason, the use of carboxylic cation exchangers or of chelating resins is often recommended. The active exchanging groups of these resins hold the metal ion more tightly by partial complexing.

Walton and co-workers[126–129] compared the elution behaviour of amines on cation exchangers of various types in the nickel and copper forms. Resins with functional phosphoric acid groups (Bio-Rex 63) have a low coordination capacity of the metal ions for amines. Macroreticular sulphonated polystyrene cation-exchange resins (Amberlite XE-219) and cellulose cation exchangers were unsuitable for ligand-exchange chromatography because the nickel ions (and also copper(II) ions) were displaced too readily by the ammonium ions in the aqueous ammonia solutions used as eluents. Chelating resins have been investigated with the aim of reducing the metal leakage during the chromatographic run. These resins contain iminodiacetate functional groups, which block some of the coordination sites of the transition metals, so that a nickel ion can bind only three ammonia molecules instead of six and the attachment of those ligands that are bound is also weakened. The consequence is that the binding of ammonia and other amine ligands is considerably less than the binding by metal-loaded sulphonic cation-exchange resins and the elution volumes from chelating resins in ligand-exchange chromatography are correspondingly smaller. Similar behaviour has been observed with carboxylic cation-exchange resins. The elution sequence from the chelating resins, however, is the same as from the sulphonic exchangers because they have the same cross-linked polystyrene matrix. There is evidence that the affinity of amines for the polystyrene skeleton of the resin can predominate over the affinity for the nickel ions and thus determine the elution order. Steric hindrance effects may also be important for the sorption of amines on cation exchangers loaded with complexing metals[130].

The elution order of amines is different on zirconium phosphate columns in which, in contrast to polystyrene exchangers, non-ionic interactions with the resin matrix are absent. This is very obvious with benzylamine and butylamine, which are held strongly by a polystyrene-based resin but only very weakly by zirconium phosphate. On the other hand, diethanolamine is bound much more strongly by the polar matrix of zirconium phosphate than by cation-exchange resins. Carboxylic resins with a polymethacrylate or polyacrylate matrix give elution sequences different from those on both polystyrene-based resins and zirconium phosphate.

In spite of the difficulties mentioned, a number of interesting separations have been achieved. Ethanolamine was partially separated from diethylamine on a sulpho-

nated cation-exchange resin in the nickel form, while a similar separation on a carboxylic resin was not feasible; 0.365 *M* ammonia solution was used as the eluting agent.

1,3-Propanediamine, 1,4-butanediamine and 1,6-hexanediamine were separated on the nickel form of a sulphonated resin by elution with 1.22 *M* ammonia solution. Ethylenediamine and 1,2-propanediamine were held so strongly on the resin that their elution from the column with aqueous ammonia was virtually impossible. The result of the separation of diamines is shown in Fig. 18 (ref. 126).

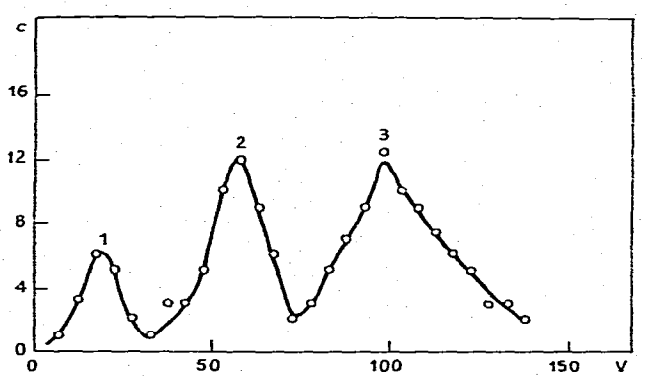


Fig. 18. Separation of 1,6-hexanediamine, 2 mmoles (1), 1,4-butanediamine, 3 mmoles (2), and 1,3-propanediamine, 2 mmoles (3) by ligand-exchange chromatography. Ion exchanger: Dowex 50W-X8 (Ni^{2+}), 50–100 mesh. Column dimensions: 0.7 cm I.D., bulk volume 10 ml. Mobile phase: 1.22 *M* ammonia solution. Detection: flame photometry, 388 nm. *c* = concentration; *V* = volume of eluate (ml).

The separation of a mixture of monoamines was compared on both sulphonated and carboxylic resins in the nickel form (Dowex 50W-X8 and Bio-Rex 70, 50–100 mesh)[128]. The elution order from the sulphonated resin with 0.94 *M* and 1.8 *M* ammonia solutions used subsequently as the eluents was as follows: diethanolamine, ethanolamine, dimethylamine and butylamine. Diethanolamine was clearly separated from ethanolamine, the resolution of butylamine from dimethylamine was not complete and the elution curves of ethanolamine and dimethylamine overlapped very seriously. The elution order from the carboxylic resin (using 0.77 *M* ammonia solution for elution) was different: butylamine, dimethylamine and ethanolamine. The separation of butylamine from the other two amines was superior to that on the sulphonated resin, but no improvement was achieved in the separation of dimethylamine from ethanolamine.

A mixture containing mono-, di- and trimethylamine was resolved on a 1.2 × 30 cm column of the monofunctional chelating resin Chelex 100 (Ni^{2+}), 200–400 mesh, by elution with 0.14 *M* ammonia solution[129]. Mono-, di- and triethanolamine would also be separated from each other. The same eluent was used in the chromatography of a mixture of *n*-propylamine and isopropylamine and a partial separation was achieved on the nickel form of both chelating (Chelex 100) and sulphonated (Dowex 50W) resins of 200–400 mesh size. *tert.*-Butylamine was well resolved from *n*-butylamine on a 1.2 × 28 cm column of the sulphonated resin Dowex 50W-X8 (Ni^{2+}),

200–400 mesh, by elution with 3.0 *M* ammonia solution. The separation of the isomeric bases cannot be attributed to the differences in strength of the metal–amine binding. The stability of the corresponding metal–amine complexes is very small and the differences in the effects on water structure are more likely to account for the separation achieved. The more compact branched isomers enter the water clusters with greater ease than the non-branched isomers and therefore show a descreased affinity for the resin. This behaviour results in shorter elution times for the branched isomers compared with those for the corresponding *n*-isomers.

Hydrazine could be separated from methylhydrazine and 1,1'-dimethylhydrazine on a column of sulphonated resin in the nickel form. Dimethylhydrazine is not retained and methylhydrazine is retained only weakly. These compounds were separated by elution with 0.4 *M* ammonia solution. Hydrazine is sorbed relatively strongly and 5 *M* ammonia solution was used in order to accelerate its elution. The good result of this chromatographic separation is illustrated by Fig. 19 (ref. 129).

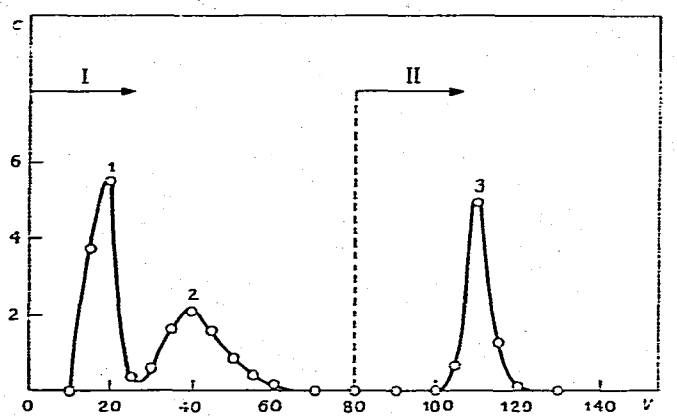


Fig. 19. Separation of hydrazines by ligand-exchange chromatography. (1) 1,1'-Dimethylhydrazine; (2) methylhydrazine; (3) hydrazine (1 mmole of each). Ion exchanger: Dowex 50W-X8 (Ni^{2+}), 200–400 mesh. Column bulk volume: 30 ml. Mobile phase: I, 0.4 *M* ammonia solution; II, 5 *M* ammonia solution. *c* = concentration (arbitrary units); *V* = volume of eluate (ml).

Benzylamine could be separated from pyridine by using a column of nickel-loaded 8% cross-linked sulphonated polystyrene resin by elution with dilute ammonia solution[127].

The sorption of amines on monofunctional iminodiacetic chelating resin in the nickel form increases in the following order: aniline, pyridine and benzylamine. The affinities of the aromatic amines for the resin matrix are more important than the differences in the stabilities of their nickel complexes. These amines can be separated on a column packed with this resin using 2 *M* ammonia solution as the eluent at a flow-rate of 0.3–0.5 ml/min. Lower ammonia concentrations resulted in long retention times[131].

The formation of complexes with iron(III) has been utilized for the separation of phenylenediamine isomers by ligand-exchange chromatography[132]. The mole ratio of the iron(III)–phenylenediamine complexes formed in the resin phase is 1:1 for the *o*-isomer and 1:2 for the *m*- and *p*-isomers. These three isomers were separated suc-

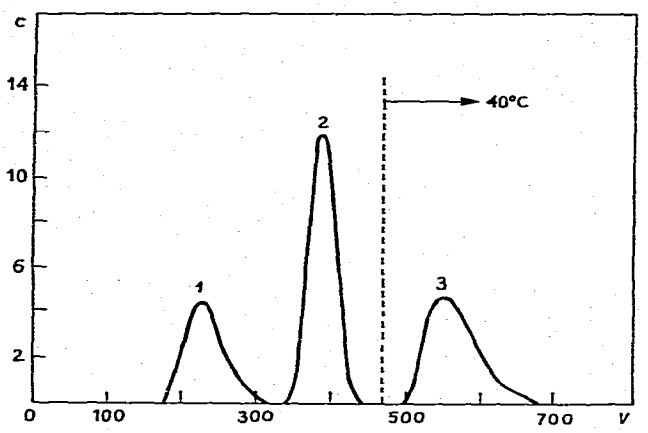


Fig. 20. Separation of phenylenediamine isomers by ligand-exchange chromatography. (1) *p*-Phenylenediamine; (2) *m*-phenylenediamine; (3) *o*-phenylenediamine. Ion exchanger: Amberlite CG-120 (Fe^{3+}). Column dimensions: 753 × 18 mm. Mobile phase: 5×10^{-3} *M* aqueous ammonia, pH 10.4. Flow-rate: 0.5 ml/min. Temperature: ambient (elution of the *o*-isomer was accelerated by working at 40°). *c* = concentration (μg/ml); *V* = volume of eluate (ml).

cessfully using an 18 × 753 mm column of the sulphonated cation exchanger Amberlite CG-120 (Fe^{3+}). The elution was performed with 5×10^{-3} *M* aqueous ammonia at a flow-rate of 0.5 ml/min (Fig. 20).

When ion-exchange celluloses are used for ligand-exchange chromatography, sorption of amines is caused not only by the complexation of the metal ion, but also by the interaction of the amine with the cellulose. Among the metal ions studied (Sb, Co, Hg, Ag), antimony is held the most strongly on ion-exchange celluloses. A sharp separation of aniline from trimethylamine and dimethylamine is possible on a 15 × 1 cm column packed with antimony-pretreated DEAE-cellulose. Aniline is eluted with diethyl ether and trimethylamine and dimethylamine are then eluted in that order with ethanol[133].

Ligand exchange was investigated as a technique for removing, concentrating and determining potential ligands in petroleum products. The results achieved with the macroreticular cation-exchange resin Amberlyst 15 in the nickel, copper and iron forms were promising[134].

Inczédy[90] suggested a method involving the use of a cation-exchange resin in the form of a transition metal together with an eluting solution containing a salt of that metal. In this case, the metal in the resin competes with the metal in the outer solution for the ligand and the distribution coefficient is then controlled by the concentration of the metal ion in the outer solution, [M]. When interferences by acid–base equilibria are prevented, the following equation for the distribution coefficient applies, to a first approximation:

$$D_B = Q \cdot d_B \cdot \frac{k_{MB}}{1 + k_{MB} \cdot [M]} \qquad (9)$$

where Q is the ion-exchange capacity of the resin for the metal M; k_{MB} is the stability constant of the complex MB; and d_B is the distribution coefficient of the free base.

In this derivation, the formation of only one complex species, MB, is considered and the sorption of the base by the resin is assumed to occur only on account of the complex formation with the metal counter ion.

Using this principle, *p*-toluidine was separated from pyridine on a 6.5 × 76 mm column of Lewatit SP-100 (Ni^{2+}), 0.2–0.4 mm, using 10^{-2} *M* nickel(II) chloride (pH 6.6) in 50% ethanol solution as the eluent. Nickel ions form virtually no complexes with *p*-toluidine, which is eluted first, while pyridine is delayed owing to its complex formation with nickel ions[90].

F. Paper and thin-layer ion-exchange chromatography of amines and nitrogen-containing bases

Aliphatic polyamines and other compounds related to the arginine pathway were chromatographed on paper strips impregnated with a strong cation-exchange resin. Good resolution was obtained using acidic buffers or hydrochloric acid as the mobile phase. A quantitative determination was achieved after eluting the spots with strong acid followed by dinitrophenylation[135].

The dinitrophenyl derivatives of amines can be subjected to direct chromatography on cation-exchange papers. Using the commercial cation-exchange resin-containing papers Amberlite WA-2 and Amberlite IRC-50 (H^+), the dinitrophenyl derivative of ethanolamine was separated from the derivative of methylamine and from dinitroaniline using the ascending development technique with methyl ethyl ketone–tetrahydrofuran–water (3:4:13). The spots formed could be recognized by their yellow coloration or by observing them under UV light. The method has been utilized for the analysis of ethanolamine in plant tissues[136].

Possibilities of the chromatographic separation of nitrogen-containing heterocyclic compounds (quinolines, acridines, indoles and carbazoles) on ion-exchange papers have been investigated using various solutions of acids, salts and bases as the mobile phase. There is strong sorption on carboxylic cation-exchange resin paper (Amberlite WA-2) and the separation possibilities do not seem very promising. On anion-exchange papers, on the other hand, most heterocyclic compounds move near the solvent front. There is little difference between cellulose and cellulose derivatives in terms of the separability of various compounds[137].

Some heterocyclic bases (quinoline, isoquinoline, 8-hydroxyquinoline, 6-methylquinoline, 2,6-dimethylquinoline, 5-hydroxyquinoline and acridine) and alkaloids (yohimbine, strychnine, brucine, berberine, dicentrine, corydaline, protopine, narcotine and narceine) were chromatographed on Whatman No. 1 paper strips, impregnated with the liquid anion exchanger di(2-ethylhexyl)orthophosphoric acid. Aqueous solutions of citric acid of varying acidity were tested as the mobile phase. The R_M values were, in most instances, almost linearly dependent on the pH of the mobile aqueous phase. The R_F values increased with increasing acidity of the mobile phase. By an appropriate choice of the pH of the mobile phase, the optimum conditions could be selected for separations. The spots on chromatograms were detected with Dragendorff reagent[138].

A number of alkaloids were chromatographed on Whatman No. 1 paper impregnated with zirconium phosphate. The ascending development technique was applied with 10% acetic acid and 1 *N* hydrochloric acid at 28°. The results achieved using papers impregnated with different amounts of zirconium phosphate are surveyed

TABLE 8

R_F VALUES OF ALKALOIDS ON WHATMAN NO. 1 PAPER IMPREGNATED WITH ZIRCONIUM PHOSPHATE

Conditions: ascending development in containers equilibrated for 24 h at 28 ± 1°. The alkaloids were spotted on the paper as 1% solutions in ethanol (when a salt was employed, the type is indicated in the table). Reagent: the papers were dipped into Dragendorff reagent. Paper: T = Whatman No. 1 paper run as control; 5%, 10%, etc., refer to the degree of impregnation.

Alkaloid	*R_F values*										
	10% acetic acid							*1 N hydrochloric acid*			
	T	*5%*	*10%*	*15%*	*20%*	*25%*	*30%*	*T*	*5%*	*15%*	*30%*
Morphine	0.90	0.36	0.24	0.22	0.20	0.12	0.13	0.81	0.80	0.73	0.58
Apomorphine	0.64	0.12	0.06	0.10	0.10	0.04	0.03	0.36	0.32	0.30	0.26
Heroin	0.95	0.33	0.22	0.20	0.22	0.11	0.15	0.86	0.82	0.73	0.62
Papaverine	0.87	0.27	0.16	0.13	0.09	0.04	0.08	0.71	0.60	0.44	0.30
Hydrastine	0.88	0.15	0.10	0.06	0.07	0.00	0.00	0.81	0.62	0.58	0.43
Quinine	0.88	0.03	0.00	0.00	0.00	0.00	0.00	0.88	0.73	0.56	0.38
Cinchonidine	0.91	0.04	0.00	0.00	0.00	0.00	0.00	0.90	0.73	0.58	0.37
Tropine	0.90	0.55	0.40	0.22	0.19	0.18	0.17	0.92	0.91	0.80	0.64
Atropine	0.92	0.42	0.25	0.22	0.19	0.18	0.16	0.87	0.88	0.79	0.59
Homatropine	0.91	0.42	0.25	0.24	0.19	0.18	0.16	0.90	0.87	0.83	0.64
Cocaine	0.88	0.58	0.34	0.33	0.29	0.26	0.20	0.84	0.83	0.76	0.65
Tropacocaine hydrochloride	0.89	0.36	0.27	0.18	0.16	0.17	0.13	0.83	0.78	0.73	0.68
Hyoscyamine	0.90	0.42	0.30	0.25	0.19	0.25	0.14	0.89	0.88	0.84	0.66
Veratrine	0.87	0.80	0.61	0.68	0.58	0.49	0.48	0.79	0.80	0.76	0.70
Protoveratrine	0.94	0.89	(1) 0.71 (2) 0.90	(1) 0.78 (2) 0.92	(1) 0.64 (2) 0.94	(1) 0.58 (2) 0.78	(1) 0.60 (2) 0.92	0.84	0.91	0.92	0.79
Cinchonine	0.90	0.03	0.00	0.00	0.00	0.00	0.00	0.89	0.73	0.54	0.37
Mescaline hydrochloride	0.92	0.55		0.41			0.22	0.58	0.90	0.81	0.80
Ephedrine	0.77	0.63		0.47			0.30	0.60	0.91	0.80	0.72
Eserine	0.93	0.41		0.27			0.13	0.59	0.93	0.77	0.51
Ergotamine tartrate	0.64	0.03		0.02			0.00	0.31	0.27	0.16	0.17
Yohimbine hydrochloride	0.74	0.40		0.26			0.15	0.60	0.62	0.55	0.53
Colchicine	0.83	0.83		0.72			0.67	0.80	0.71	0.73	0.58
Harmine	0.62	0.04		0.03			0.02	0.28	0.21	0.19	0.11
β-Erythroidine hydrochloride	0.90	0.21		0.16			0.06	0.92	0.81	0.75	0.55
Caffeine	0.84	0.73		0.69			0.51	0.83	0.67	0.64	0.48
Theophylline	0.77	0.71		0.70			0.50	0.71	0.69	0.61	0.55
Emetine	0.90	0.05		0.02			0.00	0.83	0.77	0.80	0.64
Solanine	0.88	0.84		0.69			0.53	0.81	0.82	0.70	
Arecoline	0.92	0.45		0.26			0.16	0.92	0.92	0.86	0.76
Aspidospermine	0.88	0.59		0.28			0.14	0.84	0.80	0.75	0.72
Diaboline	0.86	0.38		0.17			0.15	0.85	0.84	0.74	0.66
Brucine	0.81	0.17		0.07			0.05	0.69	0.68	0.54	0.49
Vomicine	0.76	0.52		0.20			0.19	0.63	0.65	0.57	0.53
Strychnine nitrate	0.80	0.13		0.08			0.04	0.74	0.66	0.57	0.53
Jervine	0.76	0.28		0.18			0.12	0.96	0.95	0.87	

in Table 8. The R_F values suggest that many useful separations can be achieved[139].

Satisfactory separations of some alkaloid salts (strychnine, scopolamine, atropine, pilocarpine and codeine) were accomplished on paper impregnated with the phenol sulphonate cation exchanger K 26–28 (H^+). The measuring error for the range 0.2–0.6 mg of examined alkaloid salts did not exceed $\pm 3\%$ in various analyses of drugs[140].

Mixtures of quinine, strychnine and nicotine in blood extracts were separated by chromatography on a cellulose cation-exchange paper using aqueous buffer at pH 4.5 as the mobile phase. The separated compounds were detected by their fluorescence or absorbance in UV light[141].

Various compounds of toxicological interest were separated by combined chromatography and ionophoresis on ion-exchange paper. A mixture of promazine, quinine, sulphacetamide and acetophenetidine was chromatographed on DEAE-cellulose paper using a 0.1 *M* solution of the disodium salt of ethylenediaminetetraacetic acid as the mobile phase. Promazine and quinine were separated from each other and from a mixture of the two other compounds. These were separated by subjecting the wet sheet to ionophoresis in a direction at right-angles to the chromatographic solvent flow, at a constant current of 10 mA for 30 min. The compounds were detected in UV light[142].

When a mixture containing barbiturate, salicylate, acetophenetidine and *p*-acetylaminophenol is subjected to chromatography on Whatman DE-20 cellulose anion-exchange paper in dilute ammonia solution (0.2 *N*), salicylate is clearly separated from the other compounds, which move together with only partial resolution. However, if the wet paper is subjected to ionophoresis after chromatography, again in 0.2 *N* ammonia solution, the complete resolution of all components of the mixture can be effected[143,144]. This method has been applied to analyses of salicylate, phenobarbitone and other barbiturates in blood[145,146].

Mixtures of barbiturates can be separated by chromatography on Whatman DE-20 anion-exchange paper modified by the introduction of diethylaminoethyl groups. A mixture of phenobarbitone, barbitone, butobarbitone and quinalbarbitone was resolved in 4 h using a mixture of *tert.*-amyl alcohol–0.1 *M* ethylenediaminotetraacetic acid (1:1) as the eluting solvent. Aqueous solvents were unsatisfactory. A mixture of phenobarbitone, amylbarbitone and quinalbarbitone could not be separated by this technique. Partial resolution was obtained with butanol–0.1 *M* EDTA (1:1)[147].

Ion-exchange papers can be used for the rapid, simple and specific preliminary isolation of narcotic drugs, tranquillizers and barbiturates from urine and other biological fluids prior to thin-layer chromatography[148–150].

Amberlite WA-2 paper containing a weakly acidic carboxylic cation-exchange resin, equilibrated with an acetate buffer solution, has been used for the chromatographic separations of water-soluble vitamins and their salts, thiamine, riboflavine, pyridoxine, nicotinamide, ascorbic acid, folic acid and *p*-aminobenzoic acid, using the ascending technique with water as the mobile phase[151]. The anion-exchange papers Amberlite SB-2 and Amberlite WB-2 (CH_3COO^- and Cl^-) were also tested for this purpose[152].

The chromatographic behaviour of primary aromatic amines on thin layers of weak ion exchangers has been investigated. Carboxymethylcellulose and alginic acid were compared as the sorbents[153]. The R_F values on both cation exchangers in various

TABLE 9

R_F VALUES OF AROMATIC AMINES ON THIN LAYERS OF ALGINIC ACID (1–6) AND CARBOXYMETHYLCELLULOSE (7, 8)

Eluents: (1) 1 *N* acetic acid; (2) 1 *N* formic acid; (3) 1 *N* chloroacetic acid; (4) 0.1 *N* hydrochloric acid; (5) 1 *N* chloroacetic acid in 25% isopropanol; (6) 1 *N* chloroacetic acid in 50% isopropanol; (7) water; (8) 1 *N* acetic acid.

Amine	*Eluent*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
m-Aminobenzoic acid	0.10	0.21	0.48	0.62	0.45	0.43	0.14	0.44
o-Aminobenzoic acid	0.23	0.30	0.55	0.67	0.58	0.72	0.44	0.57
p-Aminobenzoic acid	0.16	0.24	0.51	0.61	0.57	0.61	0.28	0.45
Sulphanilic acid	0.95	0.94	0.94	0.96	0.76	0.51	0.96	0.96
o-Arsanilic acid	0.82	0.78	0.86	0.81	0.86	0.96	0.92	0.91
p-Arsanilic acid	0.41	0.42	0.64	0.72	0.55	0.50	0.85	0.81
m-Aminophenol	0.08	0.18	0.49	0.60	0.45	0.31	0.01	0.38
o-Aminophenol	0.09	0.18	0.51	0.60	0.46	0.34	0.01	0.40
p-Aminophenol	0.08	0.18	0.51	0.60	0.45	0.29	0.00	0.40
α-Naphthylamine	0.06	0.12	0.34	0.40	0.44	0.43	0.00	0.28
β-Naphthylamine	0.06	0.12	0.34	0.40	0.48	0.47	0.00	0.26
m-Anisidine	0.08	0.18	0.53	0.62	0.51	0.37	0.02	0.38
o-Anisidine	0.10	0.21	0.56	0.67	0.54	0.37	0.02	0.40
p-Anisidine	0.08	0.18	0.52	0.62	0.48	0.35	0.01	0.38
Benzidine	0.00	0.00	0.04	0.13	0.00	0.00	0.00	0.08
m-Bromoaniline	0.08	0.16	0.44	0.56	0.44	0.48	0.03	0.40
o-Bromoaniline	0.09	0.19	0.51	0.63	0.50	e.s.*	0.03	0.52
p-Bromoaniline	0.06	0.16	0.44	0.58	0.46	0.46	0.02	0.38
m-Chloroaniline	0.08	0.18	0.45	0.58	0.46	0.50	0.04	0.40
o-Chloroaniline	0.13	0.25	0.48	0.64	0.53	0.70	0.04	e.s.*
p-Chloroaniline	0.08	0.18	0.43	0.60	0.46	0.49	0.03	0.38
m-Phenylenediamine	0.01	0.02	0.08	0.28	0.02	0.00	0.01	0.18
o-Phenylenediamine	0.06	0.09	0.20	0.63	0.17	0.11	0.05	0.35
p-Phenylenediamine	0.00	0.02	0.08	0.27	0.02	0.00	0.00	0.16
m-Nitroaniline	0.11	0.21	0.40	0.59	0.44	0.62	0.25	0.49
o-Nitroaniline	0.55	0.52	0.62	0.51	0.90	0.97	0.52	0.52
p-Nitroaniline	0.47	0.47	0.56	0.52	0.83	0.97	0.47	0.55
m-Toluidine	0.08	0.25	0.50	0.66	0.48	0.47	0.02	0.40
o-Toluidine	0.08	0.26	0.50	0.66	0.45	0.44	0.03	0.44
p-Toluidine	0.07	0.25	0.50	0.66	0.49	0.48	0.01	0.41
p-Aminodimethylaniline	0.00	0.04	0.14	0.36	0.09	0.00	0.01	0.24
p-Aminosalicylic acid	0.26	0.27	0.46	0.53	e.s.*	0.28	0.50	0.42
p-Aminoacetophenone	0.20	0.29	0.51	0.67	0.61	0.73	0.35	0.58

* e.s. = elongated spot.

eluents are surveyed in Table 9. The results show that water gives a very good separation of aminobenzoic acids on carboxymethylcellulose plates in the hydrogen form. *p*-Aminosalicylic acid can be well separated from the impurity formed in its preparation, *m*-aminophenol.

The retention of amines is greater on alginic acid plates than on carboxymethylcellulose. The R_F values increase with increasing strength of the acids used for elution. A change in the concentration of the eluent did not produce such appreciable differences in elution behaviour as did the change in the nature of the

eluting acid. In most instances, the isomers have the same R_F values under the same conditions. Isomeric nitroanilines, however, can be separated with acetic acid as the mobile phase. Amines varying in substitution can be separated because of their different acidities and basicities. The affinity for the stationary phase increases with increasing basicity of the amine. Certain amines, however, do not show any decisive effect of their basicity on the chromatographic behaviour (aminophenols and naphthylamines).

The addition of alcohol to the acidic eluent brought about different changes in the R_F values of the isomers, but the results were generally less satisfactory. High-voltage electrophoresis sometimes enables amines that differ only very little in their acidity or basicity to be separated[153].

3. UREA, CYANAMIDE DERIVATIVES AND RELATED COMPOUNDS

Urea and related compounds contain basic amino groups in the close neighbourhood of a carbonyl group. Other basic and acidic groups may also be present and become important in determining the ion-exchange selectivities of these compounds.

Cation-exchange resins in the hydrogen form have been most frequently used for separations of urea-type compounds. Guanidine, biguanide and guanylurea were chromatographed on columns packed with strongly acidic Amberlite IR-120 using hydrochloric acid for elution[154]. Ammelide, ammeline and melamine were separated on an Amberlite IR-120 column by stepwise elution with 0.05, 0.5 and 2 *N* hydrochloric acid[155]. Melam and melem, deammoniated condensation products of melamine, could be separated by ion-exchange chromatography on Amberlite IR-112 columns by elution with 0.5 and 2 *N* hydrochloric acid[156]. Ion-exchange chromatography on Amberlite IR-112 columns has been used for the separation and determination of intermediate products obtained during the production of melamine by heating dicyanodiamide. Dicyanodiamide, cyanomelamine (ammeline), guanidine, melem, melamine, biguanide, guanylmelamine and melam were successively eluted with 0.01–1 *N* hydrochloric acid[157]. Small amounts of melamine in acetoguanamine were separated on a 15 × 0.8 cm column of Dowex 50W-X8 (H^+). Acetoguanamine was eluted with 300 ml of 1.0 *M* hydrochloric acid and the elution of melamine with 250 ml of 2.1 *M* hydrochloric acid followed[158].

Cation-exchange chromatography on a Dowex 50W column has been used for the purification of the antineoplastic agent hexamethylmelamine in biological fluids prior to quantitative determination. After preliminary purification on a Dowex 1 (Cl^-) anion-exchange column, samples were fractionated by cation-exchange chromatography using stepwise elution with 0.1, 0.5, 1, 2.4 and 6 *N* hydrochloric acid[159]. The elution behaviour of some compounds related to urea has been studied on strongly acidic cation-exchange resins Dowex 50-X8, Amberlite CG-120 and Aminex III X-12 (H^+ and Na^+) as well as on the anion-exchange resin Amberlite CG-400 (Cl^-) with water and hydrochloric acid as the eluents. The results for biuret, *tert.*-butylurea, dicyanodiamide, ethylurea, guanidine, methylurea, nitroguanidine, phenylurea, thiourea and urea are presented in Fig. 21 (ref. 160).

Urea and methylurea showed large retention volumes on the hydrogen form of the sulphonic acid resin, while biuret, thiourea and dicyanodiamide elute early, in

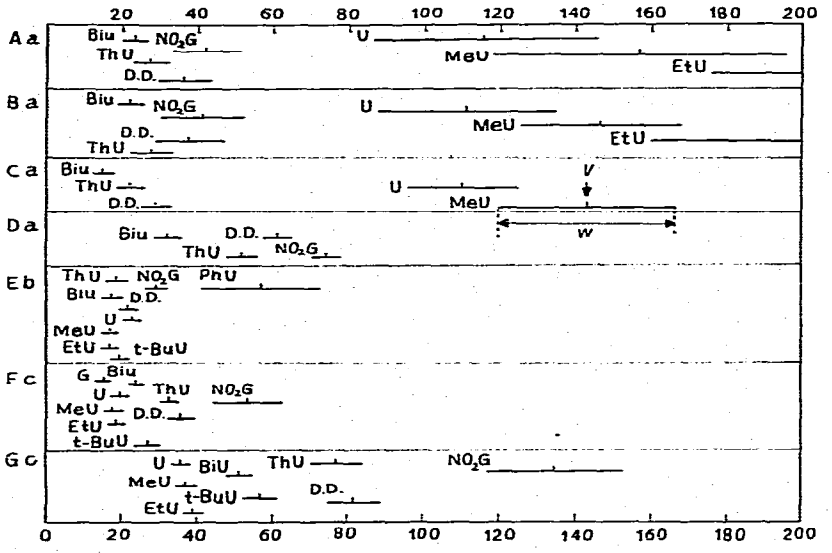


Fig. 21. Peak width and retention volumes of compounds related to urea in chromatography on ion exchangers. Biu = biuret; t-BuU = *tert.*-butylurea; D.D. = dicyanodiamide; EtU = ethylurea; G = guanidine carbonate; MeU = methylurea; NO_2G = nitroguanidine; PhU = phenylurea; ThU = thiourea; U = urea. Separation columns: (A) Dowex 50-X8 (H^+), 200–400 mesh, regraded, 0.8 × 15 cm, 0.01 *N* HCl; (B) Dowex 50-X8 (H^+), 200–400 mesh, regraded, 0.8 × 15 cm, 0.1 *N* HCl; (C) Amberlite CG-120 (H^+), 325–400 mesh, regraded, 0.8 × 15 cm, H_2O; (D) Aminex III X-12 (H^+), 400–600 mesh, 0.8 × 30 cm, H_2O; (E) Amberlite CG-120 (Na^+), 325–400 mesh, 0.8 × 15 cm, H_2O; (F) Amberlite CG-400 (Cl^-), 325–400 mesh, 0.8 × 15 cm, H_2O; (G) Amberlite CG-400 (Cl^-), 325–400 mesh, 0.8 × 50 cm, H_2O. Flow-rate: 0.46 ml/min. Temperature: separation column, 35°; detection column, 30°. Detection: thermal. Detection columns: (a) Dowex 50-X8 (H^+), 200–400 mesh, 0.8 × 7 cm; (b) Dowex 50-X8 (Na^+), 200–400 mesh, 0.8 × 7 cm; (c) Dowex 1-X8 (Cl^-), 200–400 mesh, 0.8 × 7 cm. *V* = retention volume (ml); *w* = apparent peak width (ml).

spite of the fact that the acidity constants differ only slightly from that of urea. This indicates the importance of the non-ionic (molecular) sorption mechanism of these compounds. On the other hand, guanidine, urea and alkylureas have small retention volumes on the anion-exchange resins. The acidic nitro group causes relatively stronger sorption of nitroguanidine. Fig. 21 may be helpful for making an appropriate choice of conditions for the separation of urea-type compounds.

Elution with buffer solutions has been also applied to the cation-exchange chromatography of urea-type compounds. Biuret in physiological fluids was sufficiently separated from other peaks using the procedure for neutral and acidic amino acids on the Beckman amino acid analyzer, using a buffer of pH 4.25 at 33° for the elution[161]. Over 25 monosubstituted guanidines of known structure could be separated on cation-exchange columns of an amino acid analyzer and the occurrence of several as yet unidentified guanidines in the physiological fluids of plants and insects has been revealed[162].

Anion-exchange chromatography on Amberlite IRA-410 columns has been used for the separation of cyanamide from cyanourea[154]. Ammeline, ammelide, cyanuric acid and cyameluric acid were separated on Amberlite IRA-411 with 0.05–1 *N* sodium hydroxide solutions as the eluting agents[157]. Anion-exchange columns of

AV-17 (OH^-) (100–180 × 8 mm) have been applied to the separation of melam from melamine using water and 2 *N* potassium hydroxide solution for successive elutions[163]. Ammeline and ammelide could be determined in melamine on these columns. Melamine was eluted with water and the other two compounds with 0.1 *N* sodium chloride solution. The stepwise elution with water and 0.5 *N* potassium hydroxide solution from AV-17 columns has been used for the separation of dicyanodiamide from cyanamide[163].

The separation of urea and dicyanodiamide could be effected on a 0.8 × 7 cm column packed with Dowex 1-X8 using 0.01 *N* hydrochloric acid for the elution at 30° (ref. 160).

UV spectrophotometry or colorimetry after reaction with ninhydrin or Sakaguchi reagent have been used with advantage for the analyses of column effluent fractions and for the continuous monitoring of urea-type compounds in eluates. The use of a continuous thermal detector has also been reported[160].

4. AMIDES OF SULPHONIC ACIDS

Anion-exchange chromatography has been used for the separation of some mixtures containing amides of sulphuric acid and sulphonic acids. Sulphamide and its deammonation condensation products, imidodisulphuric diamide (imidodisulphamide) and trisulphimide, were separated quantitatively on a 10 × 1.0 cm column packed with Dowex 1-X8 (Cl^-), 100–200 mesh. The elution of sulphamide was carried out with pure water; 0.2 *M* potassium chloride solution was then used to remove imidodisulphamide and, finally, trisulphimide was eluted with 0.50 *M* potassium chloride solution[163]. This procedure may be expected to be useful also for the separation of other, more condensed, sulphamides.

Mixtures of sulphanilamide and N-acetylsulphanilamide could be separated on a column of Dowex 1 (Cl^-), 100–200 mesh. Distilled water with the pH adjusted to 5.6 was used for the elution. The recovery was about 98.7%. This method has been applied to analyses of biological fluids after the preliminary removal of proteins[165].

Certain sulphonamide mixtures were separated by means of paper impregnated with cation exchangers of the phenolsulphonic type. Sulphanilamide and prontosil could be separated using a mixture of acetone and water (3:2) in the presence of ammonia as the mobile phase. Separations of other sulphonamides (sulphanilurea, sulphanilacetamide, sulphanilguanidine, sulphanilacetamine and sulphanilamidothiazole) on cation-exchange paper are also feasible[166].

5. NITRO COMPOUNDS

Aromatic and aliphatic nitro compounds (except those which contain other highly polar groups, such as carboxylic, phenolic or amino groups) do not show any tendency to ionize in aqueous solutions. For this reason, the ion-exchange mechanism cannot be utilized for chromatographic separation of these compounds. However, successful separations of various nitro compounds could be achieved by using salting-out chromatography on ion-exchange columns.

Kemula and Brzozowski[167] reported the separation of some mixtures of nitroalkanes, nitro alcohols and isomeric mononitrobenzoic acids on the strongly acidic

cation exchangers Dowex 50 and Wofatit KPS-200 using ammonium salts for elution. The concentration of nitro compounds in the eluate was continuously measured with a polarographic flow-through detector using a dropping mercury electrode (chromatopolarographic method). A constant potential (−1.0 V) corresponding to the diffusion current of nitro compounds was applied on the dropping electrode and the corresponding current was continuously recorded[167].

The detection of polarographically inert compounds is also possible, if these compounds are surface-active, by measuring continuously the suppression of polarographic maxima or by using the formation of polarographically active complexes with copper[168]. The use of a.c. polarography for this purpose has also been suggested[169].

It was established that the nature of the anion of the ammonium salt used for the elution influences to a large extent the retention volume of the compound eluted. The salting-out coefficient may even change to negative values (*e.g.*, 2-nitropropane, if the thiocyanate anion is used). Ammonium sulphate yielded the highest salting-out coefficients of all the salts tested, and was therefore used as the eluent.

An elevated temperature was applied in order to desorb those compounds strongly retained on the column. It has been found that the distribution coefficient decreases with increasing temperature and its logarithm is a linear function of temperature.

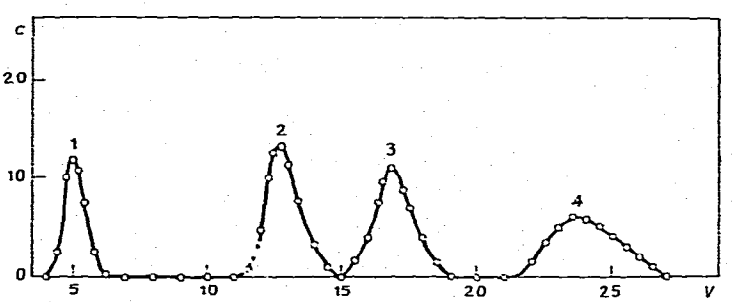


Fig. 22. Separation of a mixture of four nitro alcohols by salting-out chromatography. (1) 2-Hydroxymethyl-2-nitropropane-1,3-diol; (2) 2-methyl-2-nitropropanol; (3) 2-nitrobutanol; (4) 2-hydroxymethyl-2-nitropentanol (10 μmoles of each). Ion exchanger: Wofatite KPS-200 (NH_4^+), < 0.06 mm. Column dimensions: 370 × 7 mm. Mobile phase: 1.0 *M* ammonium sulphate solution. Flow-rate: 6 ml h. Temperature: ambient. Detector: polarographic. *c* = diffusion current (μA); *V* = volume of eluate (ml).

Fig. 22 gives an illustration of the separation of four aliphatic nitro alcohols on a 370 × 7 mm column packed with Wofatit KPS-200 (NH_4^+), <0.06 mm. The elution was carried out with 1.0 *M* ammonium sulphate solution at ambient temperature. The complete separation of five nitroalkanes could be effected on a 115 × 7 mm column of Dowex 50 by elution of the first three compounds with 1.0 *M* ammonium sulphate solution at 25°, followed by, in order to accelerate the operation, elution of the last two compounds with 0.5 *M* ammonium sulphate solution at 71°. This separation is shown in Fig. 23. The complete separation of the three isomeric nitrobenzoic acids was possible using a 210 × 7 mm column of Dowex 50. The *o*-nitrobenzoic acid

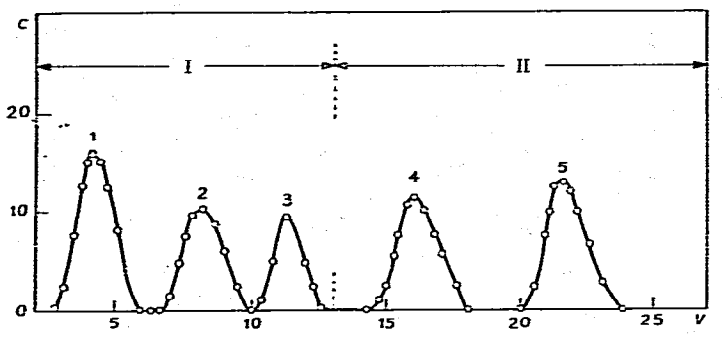


Fig. 23. Separation of five nitroalkanes by salting-out chromatography. (1) Nitromethane; (2) 2-nitropropane; (3) 1,3-dinitropropane; (4) 1-nitrobutane; (5) 1-nitropentane. Ion exchanger: Dowex 50 (NH_4^+), < 0.06 mm. Column dimensions: 115 × 7 mm. Mobile phase: I, 1.0 *M* ammonium sulphate solution; II, 0.5 *M* ammonium sulphate solution. Flow-rate: 7.5 ml/h. Temperature: I, 25°; II, 71°. Detector: polarographic. *c* = diffusion current (μA); *V* = volume of eluate (ml).

was eluted with 0.1 *M* ammonium sulphate in 0.019 *M* hydrochloric acid at ambient temperature. At this temperature, however, the remaining two acids were strongly sorbed and their elution curves were very broad and overlapped. A clear-cut separation could be achieved by eluting *p*-nitrobenzoic acid at 56° and, finally, *m*-nitrobenzoic acid at 82° (ref. 167).

Nitrobenzene could be separated from β-naphthol on a column of Dowex 50-X8 by elution with aqueous ethanol (*ca.* 30%)[170].

An interesting possibility for the complete chromatographic separation of the isomeric *o*-, *m*- and *p*-nitroethylbenzenes in mixtures has been reported[171]. Columns packed with a Ni(γ-picoline)$_4$(CNS)$_2$ type clathrate as the stationary phase were used and the mobile phase contained 2 *M* ammonium rhodanide, 0.3 *M* γ-picoline and 40–60 vol.-% of organic solvent. The clathrates of the type used are highly selective in caging aromatic compounds. This selectivity is based on the shape rather than on the volume of the caged molecules and particularly efficient resolution of isomers is possible by chromatography on these sorbents. Clathrates, of course, are not ion exchangers and the sorption mechanism can be considered as a special case of ligand exchange. However, the potential practical attraction of these unusual sorbents for difficult separations of isomeric compounds rather than material or sorption mechanism considerations made us feel that it was appropriate to mention this method here.

6. SUMMARY

The review presents a systematic survey of ion-exchange methods for the analysis of nitrogen-containing compounds. The main interest is in cation-exchange separations of amines, nitrogen-containing bases and related compounds by elution with solutions of acids, buffers and salts and aqueous organic solvents and in high-speed chromatography of these compounds on pellicular and controlled surface porosity ion exchangers. Anion-exchange, ligand-exchange, and ion-exchange paper

and thin-layer chromatography are also dealt with. Cation- and anion-exchange chromatography of urea type compounds and salting-out chromatography of nitro compounds and amides of sulphonic acids are included. The review pays particularly attention to papers published in 1962–1970.

REFERENCES

1 J. Inczédy, *Kunstharz-Ionenaustauscher Symposium, Leipzig, 4–7 June, 1968*, p. 558.
2 T. R. E. Kressman and J. A. Kitchener, *J. Chem. Soc.*, (1949) 1208.
3 H. P. Gregor and J. I. Bregman, *J. Colloid Sci.*, 6 (1951) 323.
4 T. R. E. Kressman, *J. Phys. Chem.*, 56 (1952) 118.
5 D. K. Hale, D. I. Packham and K. W. Pepper, *J. Chem. Soc.*, (1953) 844.
6 A. B. Chizevskaya, G. L. Starobinets and P. V. Martsinkevich, *Vestsi Akad. Navuk Belarusk. SSR. Ser. Khim. Navuk*, (1965) 90.
7 V. T. Athavale, C. V. Krishnan and C. Venkateswarlu, *Indian J. Chem.*, (1967) 496.
8 G. L. Starobinets, A. B. Chizhevskaya P. V. Martsinkevich and L. M. Ovsyanko, *Teoria Ionnogo Obmena i Khromatografii*, Nauka, Moscow, 1968, p. 78.
9 V. Taláček, *Collect. Czech. Chem. Commun.*, 33 (1968) 731.
10 A. V. Mikhalev, N. L. Isaeva, L. F. Yakhontova and B. P. Bruns, *Kolloid. Zh.*, 31 (1969) 102.
11 N. G. Donskaya and G. S. Libinson, *Zh. Fiz. Khim.*, 44 (1970) 1561.
12 S. R. Watkins and H. F. Walton, *Anal. Chim. Acta*, 24 (1961) 334.
13 J. E. Gordon, *J. Phys. Chem.*, 67 (1963) 16.
14 D. J. Pietrzyk, *Talanta*, 13 (1966) 209.
15 D. J. Pietrzyk, *Talanta*, 13 (1966) 225.
16 I. M. Vagina and G. S. Libinson, *Zh. Fiz. Khim.*, 41 (1967) 2060.
17 G. S. Libinson and I. M. Vagina, *Zh. Fiz. Khim.*, 41 (1967) 2933.
18 I. M. Vagina and G. S. Libinson, *Dokl. Akad. Nauk SSSR*, 175 (1967) 1312.
19 I. M. Vagina and G. S. Libinson, *Termodin. Ionnogo Obmena*, (1968) 200.
20 T. G. Matsaberidze and N. F. Voitsekhovskaya, *Tr. Tbilis. Gos. Univ.*, 126 (1968) 139.
21 S. V. Nikitina and A. M. Koganovskii, *Zh. Fiz. Khim.*, 44 (1970) 784.
22 J. E. Cassidy and C. A. Streuli, *Anal. Chim. Acta*, 31 (1964) 86.
23 J. E. Gordon, *J. Chromatogr.*, 18 (1965) 542.
24 H. Watanabe, *Nippon Kagaku Zasshi*, 82 (1961) 461.
25 G. L. Starobinets and I. P. Koka, *Vestsi Akad. Navuk Belarusk. SSR. Ser. Khim. Navuk*, (1966) 35.
26 I. P. Koka and G. L. Starobinets, *Vestsi Akad. Navuk Belarusk. SSR. Ser. Khim. Navuk*, (1967) 27.
27 G. L. Starobinets and I. P. Koka, *Vestsi Akad. Navuk Belarusk. SSR. Ser. Khim. Navuk*, (1964) 16.
28 A. Schwarz and G. E. Boyd, *J. Phys. Chem.*, 69 (1965) 4268.
29 S. Katz and C. A. Burtis, *J. Chromatogr.*, 40 (1969) 270.
30 C. D. Scott, J. E. Attrill and N. G. Anderson, *Proc. Soc. Exp. Biol. Med.*, 125 (1967) 181.
31 C. D. Scott, *Clin. Chem.*, 14 (1968) 521.
32 C. D. Scott, R. L. Jolley, W. W. Pitt and W. F. Johnson, *Amer. J. Clin. Pathol.*, 53 (1970) 701.
33 C. D. Scott, F. Wayne and V. E. Walker, *Anal. Biochem.*, 32 (1969) 182.
34 C. A. Burtis and K. S. Warren, *Clin. Chem.*, 14 (1968) 290.
35 C. A. Burtis, W. C. Butts, W. T. Rainey and C. D. Scott, *Amer. J. Clin. Pathol.*, 53 (1970) 769.
36 C. A. Burtis, *J. Chromatogr.*, 52 (1970) 97.
37 C. D. Scott, J. M. Jansen and W. W. Pitt, *Amer. J. Clin. Pathol.*, 53 (1970) 739.
38 N. E. Skelly and W. B. Crummett, *Anal. Chem.*, 35 (1963) 1680.
39 E. W. Grant and W. W. Hilty, *J. Amer. Pharm. Ass.*, 42 (1953) 150.
40 M. Sjöström and A. Randell, *J. Amer. Pharm. Ass., Sci. Ed.*, 48 (1959) 445.
41 W. Kamp, *Pharm. Weekbl.*, 92 (1957) 1.
42 W. Kamp, *Pharm. Weekbl.*, 99 (1964) 1092.
43 S. M. Blaug and L. C. Zopf, *J. Amer. Pharm. Ass.*, 45 (1956) 9.
44 *Chromatronix Liquid Chromatography Application*, No. 11, Chromatronix, Berkeley, Calif., 1970.

45 W. Kamp and J. A. Fresen, *Pharm. Weekbl.*, 94 (1959) 33.
46 W. Kamp and C. P. C. Post, *Pharm. Weekbl.*, 96 (1961) 179.
47 K. Tsuda and T. Matsumoto, *J. Pharm. Soc. Jap.*, 67 (1947) 238.
48 K. Tsuda and T. Matsumoto, *J. Pharm. Soc. Jap.*, 67 (1947) 236.
49 S. M. Rosenthal and C. W. Tabor, *J. Pharmacol. Exp. Ther.*, 116 (1956) 131.
50 H. Tabor, S. M. Rosenthal and C. W. Tabor, *J. Biol. Chem.*, 233 (1958) 907.
51 S. Holder and H. J. Bremer, *J. Chromatogr.*, 25 (1966) 48.
52 J. R. Parrish, *J. Chromatogr.*, 18 (1965) 535.
53 S. Friedman, J. E. McFarlane, P. K. Bhattacharyya and G. Fraenkel, *Arch. Biochem. Biophys.*, 59 (1955) 484.
54 L. O. Pilgeram, E. M. Gal, E. N. Sassendrath and D. M. Greenberg, *J. Biol. Chem.*, 204 (1953) 367.
55 D. D. Christianson, J. S. Wall, R. J. Dimler and F. R. Senti, *Anal. Chem.*, 32 (1960) 874.
56 H. Wetterqvist and T. White, *Scand. J. Clin. Lab. Invest.*, 25 (1970) 325.
57 F. Saccani, *Boll. Chim. Farm.*, 109 (1970) 271.
58 F. Saccani and C. Neri, *Boll. Chim. Farm.*, 109 (1970) 275.
59 F. Saccani and C. Neri, *Boll. Chim. Farm.*, 109 (1970) 344.
60 J. I. Jones, *J. Chem. Soc.*, (1950) 1392.
61 T. Seki and S. Morimoto, *Nippon Kagaku Zasshi*, 77 (1956) 1124.
62 J. Churáček and P. Jandera, *J. Chromatogr.*, 53 (1970) 69.
63 A. Berggren, C. O. Björling and B. Willman-Johnson, *Acta Chem. Scand.*, 12 (1958) 1521.
64 A. Berggren and C. O. Björling, *Acta Chem. Scand.*, 11 (1957) 179.
65 K. Montgomery, P. Jennings and M. Weinswig, *J. Pharm. Sci.*, 56 (1967) 141.
66 K. Montgomery, P. Jennings and M. Weinswig, *J. Pharm. Sci.*, 56 (1967) 393.
67 D. J. Smith, *J. Ass. Offic. Anal. Chem.*, 52 (1969) 847.
68 J. Hunt and M. I. Blake, *J. Pharm. Sci.*, 59 (1970) 683.
69 M. I. Blake and J. Hunt, *Talanta*, 17 (1970) 876.
70 W. Reiter, *Arzneim.-Forsch.*, 19 (1969) 1751.
71 J. R. Wilson and M. D. Nutting, *Anal. Chem.*, 35 (1963) 144.
72 S. L. Gibbon and J. L. Way, *J. Chromatogr.*, 26 (1967) 202.
73 K. Blau, *Biochem. J.*, 80 (1961) 193.
74 T. L. Perry and W. A. Schroeder, *J. Chromatogr.*, 12 (1963) 358.
75 D. R. Morris, K. L. Koffron and C. J. Okstein, *Anal. Biochem.*, 30 (1969) 449.
76 R. A. Wall, *J. Chromatogr.*, 37 (1968) 549.
77 M. Umezu and J. Yamamoto, *Hakko Kagaku Zasshi*, 47 (1969) 257.
78 N. Kirschner and C. McGoodall, *J. Biol. Chem.*, 226 (1957) 207.
79 H. Watanabe, *Bunseki Kagaku (Jap. Anal.)*, 10 (1961) 271.
80 H. Watanabe, *Bunseki Kagaku (Jap. Anal.)*, 11 (1962) 233.
81 H. Watanabe, *Nippon Kagaku Zasshi*, 83 (1962) 54.
82 H. Watanabe and T. Suzuki, *Bunseki Kagaku (Jap. Anal.)*, 17 (1968) 1264.
83 H. Watanabe and T. Suzuki, *Bunseki Kagaku (Jap. Anal.)*, 18 (1969) 739.
84 T. L. Perry, W. A. Schroeder, B. J. Lukon and E. H. Buckley, *J. Chromatogr.*, 15 (1964) 258.
85 H. B. F. Dixon, *J. Chromatogr.*, 24 (1966) 199.
86 Y. Yoshino, H. Kinoshita and H. Sugiyama, *Nippon Kagaku Zasshi*, 86 (1965) 405.
87 H. Hatano, K. Sumizu, S. Rokushika and F. Murakami, *Seikagaku*, 38 (1966) 505.
88 M. Yoshioka, A. Ohara, H. Kondo and H. Kanazawa, *Chem. Pharm. Bull.*, 17 (1969) 1276.
89 H. Hatano, K. Sumizu, S. Rokushika and F. Murakami, *Anal. Biochem.*, 35 (1970) 377.
90 J. Inczédy, *Proceedings of the Third Analytical Chemistry Conference, Budapest, 1970*, p. 21.
91 R. Bandere, I. Lazdinsh, I. V. Smorodina and A. A. Avots, *Latv. PSR Zinat. Akad. Vestis, Kim. Ser.*, No. 3 (1970) 292.
92 T. A. Wheaton and I. Stewart, *Anal. Biochem.*, 12 (1965) 585.
93 R. Sargent and W. Rieman, *Anal. Chim. Acta*, 17 (1957) 408.
94 W. Kamp, *Pharm. Weekbl.*, 94 (1959) 521.
95 M. Blake and B. Carlstedt, *J. Pharm. Sci.*, 55 (1966) 1462.
96 J. Halmekoski, in O. Hanc (Editor), *Sci. Pharm., Proc., 25th, 1965*, Butterworths, London, 1966, pp. 2, 111.
97 J. Halmekoski and J. Jokisalo, *Farm. Aikak.*, 77 (1968) 255.

98 H. P. Gregor, G. K. Hoeschele, J. Potenza, A. G. Tsuk, R. Feinland, M. Shida and P. Teyssié, *J. Amer. Chem. Soc.*, 87 (1965) 5525.
99 A. G. Tsuk and H. P. Gregor, *J. Amer. Chem. Soc.*, 87 (1965) 5534.
100 A. G. Tsuk and H. P. Gregor, *J. Amer. Chem. Soc.*, 87 (1965) 5538.
101 B. K. G. Theng, E. Vansant and J. B. Vytterhoeven, *Trans. Faraday Soc.*, 64 (1968) 3370.
102 C. O. Björling and A. Berggren, *Acta Chem. Scand.*, 9 (1955) 567.
103 R. T. Conner and G. J. Straub, *Ind. Eng. Chem., Anal. Ed.*, 13 (1941) 385.
104 R. C. Rebertus, *Anal. Chem.*, 38 (1966) 1089.
105 S. Romano, *J. Chem. Educ.*, 47 (1970) 478.
106 D. Aures, R. Hakanson and L. Spolter, *Z. Anal. Chem.*, 243 (1968) 483.
107 J. R. Broich, M. M. De Mayo and L. A. Dal Cortivo, *J. Chromatogr.*, 33 (1968) 526.
108 D. Cozzi, P. G. Desideri, L. Lepri and G. Ciantelli, *J. Chromatogr.*, 35 (1968) 396.
109 L. Lepri, P. G. Desideri, V. Coas and D. Cozzi, *J. Chromatogr.*, 49 (1970) 239.
110 L. D. Metcalfe, *Anal. Chem.*, 32 (1960) 70.
111 C. McMartin and J. Winter, *J. Chromatogr.*, 41 (1969) 188.
112 C. McMartin, P. Simpson and N. Thorpe, *J. Chromatogr.*, 43 (1969) 72.
113 G. Tortolani, P. Bianchini and V. Mantovani, *Farmaco, Ed. Prat.*, 25 (1970) 772.
114 R. J. Merrils and J. P. Farrier, *Anal. Biochem.*, 21 (1967) 475.
115 R. J. Merrils and J. Offerman, *Biochem. J.*, 99 (1966) 538.
116 *820 Liquid Chromatographic Methods, DuPont Prospectus 820 M5*, DuPont, Wilmington, Del., March 1970.
117 J. J. Kirkland, *J. Chromatogr. Sci.*, 7 (1969) 361.
118 R. A. Henry and J. A. Schmit, *Chromatographia*, 3 (1970) 116.
119 *Chromatronix Liquid Chromatography Application*, No. 13, Chromatronix, Berkeley, Calif., 1970.
120 M. W. Anders and J. P. Latorre, *Anal. Chem.*, 42 (1970) 1430.
121 *Chromatronix Liquid Chromatography Application*, No. 10, Chromatronix, Berkeley, Calif., 1970.
122 R. H. Stokes and H. F. Walton, *J. Amer. Chem. Soc.*, 76 (1954) 3327.
123 F. Helfferich, *Nature (London)*, 189 (1961) 1001.
124 F. Helfferich, *J. Amer. Chem. Soc.*, 84 (1962) 3237.
125 F. Helfferich, *J. Amer. Chem. Soc.*, 84 (1962) 3242.
126 J. J. Latterell and H. F. Walton, *Anal. Chim. Acta*, 32 (1965) 101.
127 H. F. Walton and J. J. Latterell, *Analytical Chemistry 1962, Symposium Volume*, Elsevier, Amsterdam, 1963, p. 356.
128 A. G. Hill, R. Sedgley and H. F. Walton, *Anal. Chim. Acta*, 33 (1965) 84.
129 K. Shimomura, L. Dickson, and H. F. Walton, *Anal. Chim. Acta*, 37 (1967) 102.
130 S. Kamata, K. Inoue and N. Ishibashi, *Denki Kagaku*, 33 (1965) 207.
131 Bak Chong Min, *Daehan Hwahak Hwoejee*, 11 (1967) 56.
132 W. Funasaka, K. Fujimura and S. Kuriyama, *Bunseki Kagaku (Jap. Anal.)*, 18 (1969) 19.
133 R. A. A. Muzzarelli, A. F. Martelli and O. Tubertini, *Analyst (London)*, 94 (1969) 616.
134 P. V. Webster, J. N. Wilson and M. C. Franks, *Anal. Chim. Acta*, 38 (1967) 193.
135 L. M. Rinaldini, *Anal. Biochem.*, 36 (1970) 352.
136 M. Katayama and S. Funahashi, *J. Chromatogr.*, 32 (1968) 777.
137 A. M. Luly and K. Sakodynsky, *J. Chromatogr.*, 19 (1965) 624.
138 E. Soczewinski and M. Rojowska, *J. Chromatogr.*, 27 (1967) 206.
139 L. D. Coussio, G. B. Marini-Bettòlo and V. Moscatelli, *J. Chromatogr.*, 11 (1963) 238.
140 W. Manikowski, *Ann. Acad. Med. Lodz*, 9 (1967) 227.
141 H. V. Street, *Clin. Chim. Acta*, 7 (1962) 226.
142 H. V. Street and S. K. Niyogi, *Nature (London)*, 190 (1961) 1199.
143 H. V. Street and S. K. Niyogi, *Nature (London)*, 190 (1961) 537.
144 H. V. Street and S. K. Niyogi, *Nature (London)*, 190 (1961) 718.
145 H. V. Street, *Acta Pharmacol. Toxicol.*, 18 (1961) 281.
146 H. V. Street, *Clin. Chim. Acta*, 7 (1962) 107.
147 H. V. Street, *J. Chromatogr.*, 7 (1962) 64.
148 V. P. Dole, Wan Kyun Kim and I. Eglitis, *Psychopharmacol. Bull.*, 3 (1966) 45.
149 V. P. Dole, Wan Kyun Kim and I. Eglitis, *J. Amer. Med. Ass.*, 198 (1966) 115.
150 V. P. Dole, Wan Kyun Kim and I. Eglitis, *J. Amer. Med. Ass.*, 198 (1966) 349.

151 R. Hüttenrauch and L. Klotz, *Experientia*, 19 (1963) 95.
152 L. Klotz and R. Hüttenrauch, *Arch. Pharm. (Weinheim)*, 296 (1963) 721.
153 D. Cozzi, P. G. Desideri, L. Lepri and V. Coas, *J. Chromatogr.*, 43 (1969) 463.
154 M. Takimoto and T. Yatsuo, *Kogyo Kagaku Zasshi*, 63 (1960) 1938.
155 M. Takimoto, *Kogyo Kagaku Zasshi*, 64 (1961) 1234.
156 M. Takimoto, *Kogyo Kagaku Zasshi*, 64 (1961) 1452.
157 M. Takimoto, *Kogyo Kagaku Zasshi*, 64 (1961) 1456.
158 M. Furukawa, T. Takeuchi, T. Murayama, M. Maruyama and B. Tomita, *Kogyo Kagaku Zasshi*, 71 (1968) 1154.
159 T. G. Bryan and A. L. Gorske, *J. Chromatogr.*, 34 (1968) 67.
160 N. Nomura, D. I. Shiho, K. Ohsuga and M. Yamada, *J. Chromatogr.*, 42 (1969) 226.
161 S. D. Farlin, G. T. Schelling and U. S. Garrigus, *J. Anim. Sci.*, 26 (1967) 1205.
162 D. J. Durzan, *Can. J. Biochem.*, 47 (1969) 657.
163 E. N. Boitsov, Yu. I. Mushkin and V. M. Karlik, *Zavod. Lab.*, 35 (1969) 790.
164 E. Masuda and Y. Ito, *J. Chromatogr.*, 31 (1967) 650.
165 S. O. Tsaregorodtseva, *Lab. Delo*, (1969) 59.
166 E. Lewandowski, *Anal. Chim. Acta*, 23 (1960) 317.
167 W. Kemula and S. Brzozowski, *Rocz. Chem.* 35 (1961) 711.
168 W. Kemula, *Proc. Symp. Electrode Processes, 1966*, p. 32.
169 W. Kemula, *Chem. Anal. (Warsaw)*, 13 (1968) 1013.
170 H. D. Spitz, H. L. Rothbart and W. Rieman, *Talanta*, 12 (1965) 395.
171 W. Kemula and D. Sybilska, *Anal. Chim. Acta*, 38 (1967) 97.