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CHROMATOGRAPHY OF NON-IONIC ORGANIC COMPOUNDS ON ION-EXCHANGE RESINS

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It is well known that organic compounds need not carry ionic charges in order to be absorbed by ion-exchange resins. Resin polymers are organic solvents in their own right. Swollen with water or another liquid, the polymer network is like a sponge, freely penetrated by molecules from the surrounding solution. Non-Coulombic attractions often reinforce the ionic or Coulombic attraction between the fixed ions of the resin and organic counter-ions. This fact was recognized by Moore and Stein in their famous paper[1] on the cation-exchange chromatography of amino acids:

> "The rate of travel on a column of sulfonated polystyrene resin . . . is a result of the affinity of the resin for both the ionic and the non-ionic portions of the molecule."

It was known in the early days of ion-exchange chromatography that a cation-exchange resin, placed in a solution of aniline in water, would absorb considerably more aniline than corresponded to its cation-exchange capacity, and that strong-base anion-exchange resins could hold more than twice as much phenol as they could have held if only the phenolate anion were absorbed.

There are at least three ways in which an ion-exchange resin can hold uncharged molecules. One is by "solubilization" or "matrix affinity," the forces between absorbed molecules and the polymer matrix, and these forces, in turn, can be of different

kinds. A very important effect is pi-electron overlap, the strong binding of aromatic solutes to an aromatic polymer matrix like crosslinked polystyrene. Another effect, important in mixed solvents, comes from the fact that the solvent composition inside the resin is different from that outside. The more polar solvent component tends to be more abundant inside the resin, with respect to the less polar component, because the ions of the resin solvate polar molecules. In mixtures of water and alcohol, for example, the proportion of water to alcohol is higher inside the resin than outside; this was shown in a very important research by Rückert and Samuelson[2]. The resin therefore absorbs highly polar compounds, like sugars, from their solutions in aqueous alcohol. The third kind of force that causes uncharged organic molecules to be absorbed by ion-exchange resins is the association of these molecules with the counter-ions of the resin. The first attempts to absorb uncharged molecules on resins used this effect. Borate ions, for example, form cyclic complexes with 1,2-diols, including sugars and sugar alcohols. A strong-base anion-exchange resin loaded with borate ions might therefore be expected to absorb sugars, and Khym and Zill[3] found this to be the case. Likewise an anion-exchange resin carrying bisulfite ions could absorb aldehydes and ketones, which form charged bisulfite complexes[4]. Cation-exchange resins carrying ions of metals like copper and zinc, which form ammonia complexes, were found to absorb ammonia and amines from solutions[5]. This phenomenon is the basis of "ligand-exchange chromatography"[6,7].

Needless to say, more than one of these effects can operate at the same time. An aromatic amine, bound to a copper-loaded polystyrene-type resin, is bound all the more strongly because pi-electron overlap with the aromatic rings of the resin reinforces the coordination of the amino group with copper ions. And, as we have already noted, these effects may modify ionic attractions and repulsions.

We shall not attempt a comprehensive account of all kinds of interactions between organic compounds and ion-exchange resins. We

shall present a brief historical review, and then concentrate on work done since 1969, with emphasis on work in our own laboratory. Recent reviews of the chromatography of organic compounds on ion-exchange resins have been written by Jandera and Kuracek[8,9], Khym[10], Samuelson[11], Scott[12], Singhal[13] and Walton[14].

HISTORICAL OUTLINE

Sugars were perhaps the first non-ionic organic compounds to be separated from complex mixtures by chromatography on ion-exchange resins. As we have noted, the first separations were done on borate-loaded anion-exchange resins, using solutions of sodium or potassium borate as eluents. Soon it was found that borate was unnecessary. Sugars were absorbed by strong-base anion-exchange resins in the sulfate or chloride forms, and also by cation-exchange resins. The absorption was greatly increased by adding alcohol. This phenomenon was carefully investigated by Hŭckert and Samuelson[2], who found that in addition to the attraction of sugar to the excess water in the resin, which we have already noted, there was an attraction to the resin matrix itself, which was noticeable at low alcohol concentrations and in resins carrying poorly hydrated ions, like perchlorate.

Complex mixtures of carbohydrates are now routinely analyzed by chromatography on ion-exchange resins. Anion-exchange resins in the sulfate form are preferred, although cation-exchange resins may be used. Eluents are mixtures of ethyl alcohol and water containing some 70% to 90% of alcohol by weight. It is usual to use gradient elution with a steadily increasing proportion of water[10,15].

In 1957 and 1958, Rieman and his students described two procedures that they called salting-out chromatography and solubilization chromatography. The first is used with water-soluble compounds like the lower alcohols, esters, ethers, aldehydes and ketones[16,17]. They are absorbed from concentrated salt solutions, like 4M ammonium sulfate, into cation- or anion-

exchange resins (it makes little difference which type is used) and then eluted with aqueous salt solutions of decreasing concentration. The more hydrophilic substances are eluted first, like glycerol and ethyl alcohol, and more hydrophobic, less water-soluble substances like n-pentyl alcohol emerge later.

In "salting-out chromatography" the organic solute is "salted out" of the external solution into the resin, where the concentration of mobile ions is less, due to salt exclusion by the Donnan equilibrium. The activity of nonelectrolytes in aqueous salt solutions follows the relation:

$$\log (\text{activity coefficient}) = (\text{constant}) \times (\text{ionic strength})$$

and this relation interprets fairly well the effect of salt concentration on the absorption of organic solutes of low molecular weight by the ion-exchange resin.

"Solubilization chromatography"[18,19] is used with compounds that are sparingly soluble in water, like longer-chain alcohols, esters and ketones, phenols and aromatic hydrocarbons. Again the resin can be cation-exchanging or anion-exchanging, and the counter-ion is of little consequence. The compounds are absorbed from water mixed with just enough organic solvent to keep them in solution, then eluted with solvent mixtures containing increasing proportions of methanol, ethanol or acetic acid. The more hydrophilic compounds, or those of lower molecular weight, are eluted first, and the less water-soluble compounds come out later. For example, aromatic hydrocarbons are eluted in the order benzene, toluene, xylene, naphthalene, methylnaphthalene.

A combination of salting-out and solubilization chromatography was described in 1962 by Halmekoski and Hannikainen[20], who used solutions of potassium phosphate in aqueous methanol to separate acetanilide and three derivatives of p-aminophenol on a column of cation-exchange resin.

In 1961 Helfferich[6] described "ligand-exchange chromatography." In its original form, ligand-exchange chromatography was

used for the separation of compounds containing basic nitrogen atoms, and this is still its chief use. They are absorbed by a cation-exchange resin loaded with ions of metals that form amine complexes, and are eluted by solutions of ammonia. If necessary, small amounts of metal salts are added to the ammonia eluents to replace losses of metal ions from the resin. By 1970, amino acids were being separated by this technique, as well as a number of aliphatic amines, aromatic amines, hydrazines and alkanolamines[14]. A related technique is "argentation chromatography," in which compounds having ethylenic double bonds are absorbed from the liquid or gas phase by solid supports loaded with silver ions. Macroporous resins have been used to carry the silver ions, but it is much more usual to use silica gel impregnated with silver nitrate.

The term "ion-exclusion chromatography" is used today to describe a powerful new method of separating weak organic acids and bases. We recall that "ion exclusion" was first used to separate water-soluble organic nonelectrolytes from ionized salts and strong acids. Ionic compounds are excluded from anion- and cation-exchange resins by the Donnan equilibrium, and therefore emerge from the column ahead of the nonelectrolytes, which penetrate the resin beads and so have a longer residence time[21]. Ion exclusion is now being used to modify the absorption of weak acids and bases. Matrix-affinity pulls a weak acid into a cation-exchange resin, but, in so far as the acid can ionize, the Donnan equilibrium pushes it out of the resin. The degree of ionization, and hence the repulsive force, may be controlled by controlling the pH. This technique has been used by Singhal and Cohn[13] for the chromatography of nucleic acid derivatives. We shall note other applications.

We shall describe advances of the last five years under two general headings, ligand-exchange and matrix-affinity chromatography. The second heading will cover cases in which ion exclusion takes a part.

LIGAND-EXCHANGE CHROMATOGRAPHY

By "ligand-exchange chromatography" we mean chromatography on cation exchangers loaded with metals that can coordinate with the ligands used. Usually, but not always, the ligands are compounds with basic nitrogen atoms and the eluents are solutions of ammonia.

The concept of ligand-exchange chromatography implies that the metal ions remain fixed on the exchanger, while only the ligands attached to them change places. This is an idealization, because aqueous ammonia solutions contain ammonium ions, and these can displace metal ions from the exchanger by ordinary cation exchange. It is therefore usual to choose an exchanger with functional groups that hold the metal ions by coordination or chelation, like iminodiacetate groups in the chelating resin Dowex A-1 or carboxylate groups in acrylic-type resins like Bio-Rex 70. The familiar resins with functional sulfonate ions may still be used, however. There is more leakage of metal ions in such resins, because they are more easily displaced by ammonium ions, but if the leakage is not too great it can be compensated by adding metal salt to the aqueous ammonia influent. The concentration of metal ions in the influent then becomes a variable to be considered in chromatography, because metal-ligand complexes are now formed in the solution as well as in the resin. The competitive equilibria that operate in such cases have been discussed by Inczedy[22]. Advantages to using sulfonated polystyrene resins include their good mechanical resistance to pressure gradients as well as their high capacity for holding ligands, compared to chelating resins, where the ligand-binding capacity of the metal ions is greatly reduced by coordination with the resin's functional groups[23].

The attractive feature of ligand-exchange chromatography is its selectivity; copper-loaded resins, for example, bind nitrogen bases as a class and organic sulfur compounds as a class. Another

attractive feature is that selectivity orders can be manipulated by changing the metal ion and the resin; this point will be illustrated later (see also ref. 23).

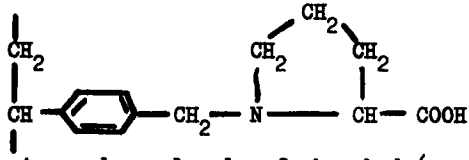
Separation of complex mixtures: trace collection. Metal-loaded resins have been used to absorb particular classes of compounds, like nitrogen bases and aromatic acids, from petroleum products[24] and from tobacco-smoke condensate[25]. Toluene was used as the solvent in the first case, methyl isobutyl ketone in the second case. The nonaqueous solvents would not penetrate a common gel-type (microporous) resin, so a macroporous resin was used in the first case, and a copper-loaded cellulosic exchanger carrying functional carboxyl ions in the second. The cellulosic exchanger was washed first with methyl isobutyl ketone to remove nonabsorbing substances, then with ethyl alcohol, then with alcohol containing ammonia. The nitrogen bases were removed by ammonia.

Alkyl and aryl sulfides and hydrosulfides were "filtered" from aromatic hydrocarbon mixtures by copper-loaded acrylate (Bio-Rex 70) resin; the sulfur compounds were selectively absorbed, then eluted as a group and further analyzed by gas chromatography[26]. A nickel-loaded chelating resin was used to collect amino acids from waste waters; basic and most of the neutral amino acids were retained, and later eluted with 5M ammonia, while the "acidic" amino acids, like glutamic acid, were not retained[27].

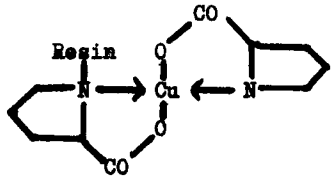
Amino acids, peptides. The main practical use of ligand-exchange chromatography has been in the analysis of amino acid mixtures and mixtures of amino acids and peptides. The Arikawa system of amino-acid analysis was described in patents[28]. The amino acids are absorbed on a column of sulfonated polystyrene resin of fine and uniform particle size, usually in the zinc form, then eluted, not with ammonia, but with acetate buffers that are about 0.001M in zinc ions. Elevated temperature is used. Modifications of this system that include amino sugars have been described[29,30]. Detection is with ninhydrin or a fluorescence-producing reagent.

A copper-loaded chelating resin was used to separate amino acids from peptides; amino acids were retained, but peptides were eluted by dilute aqueous ammonia as their colored copper complexes[31]. Other workers[32,33] used this system to separate amino acid-peptide mixtures into three fractions: first, acidic and neutral peptides and neutral amino acids, eluted with water; second, neutral amino acids and basic peptides, eluted by 1.5M ammonia; and third, basic amino acids and tryptophane (which is strongly absorbed because of its aromatic character), eluted by 6M ammonia.

Optical isomers of amino acids. A triumph of ligand-exchange chromatography has been the resolution of optical isomers of amino acids. Special resins are used, made by coupling chlormethylated polystyrene with optically active amino acids. These resins are then loaded with ions of copper or nickel. One such resin was described by Snyder, Angelici and Meck[34]. The optically active acid that was bonded to the resin was N-carboxymethyl-L-valine. Other resins were prepared by Davankov and coworkers, and described in an important series of papers[35-39]. The resin with bound proline will illustrate what happens: its structure is:



With a copper ion and a molecule of absorbed (or mobile) proline it forms a complex:



The absorbed proline may, in turn, be displaced from the copper ion by two molecules of water or ammonia. Now, the stability of the

complex depends on its stereochemistry. The complex formed from L-proline(fixed) and D-proline(mobile) is more stable than the one formed from L-proline(fixed) and L-proline(mobile), the difference in free energy being about 450 cal/mole. When a racemic mixture is absorbed, L-proline may be eluted by water or 0.1M ammonia, whereas 1-2M ammonia is needed to elute D-proline. The process is complicated by the strong tendency for one copper ion to form a bridge between two fixed proline residues in the resin, especially at low copper loadings, and for copper ions to leave the resin at high copper loadings and form amino acid complexes in the solution. At low copper loadings there is little discrimination between D- and L- forms. Davankov's group studied the effect of various experimental conditions, including the copper loading of the resin, the temperature, and also the nature of the fixed and mobile amino acids, which do not have to be alike.

Aliphatic and aromatic amines, drugs and alkaloids. In our laboratory we have been systematically exploring the possibilities of ligand-exchange chromatography for the analysis of mixtures of amino compounds, especially compounds of biological interest. Reference 14 summarizes our work through 1970. Certain generalizations could be made at that time concerning elution orders and strengths of binding of amino compounds to metal-loaded resins. They are:

1. The binding strength depends greatly on the atomic environment of the basic nitrogen atom. Primary amines are bound much more strongly than secondary amines, secondary amines are bound more strongly than tertiary amines. Bulky atoms close to the amine nitrogen hinder the coordination to the metal ion; a molecule with $-CH_2-NH_2$ is bound more strongly than one with $-CH(CH_3)-NH_2$.

2. Binding is strengthened by the possibility of chelate ring formation. Thus, 1,2-diamines are bound much more strongly to a copper-loaded resin than are monoamines.

3. Aromatic amines, or amines with aromatic groups in their structure, are bound strongly by polystyrene-type metal-loaded resins, due to pi-electron binding.

4. Elution orders are affected by the nature of the exchanger as well as by the nature of the metal ion.

Our studies made since 1970 have confirmed the validity of these generalizations. The partition of solutes between solutions and metal-loaded resins is a complex phenomenon, however, and is only partly predictable.

We shall summarize our findings with different groups of compounds, and comment on points of interest.

(1) Alkanolamines[40]. A nickel-loaded acrylic-type resin, with functional carboxyl groups, was used. With 0.25M ammonia, the elution sequence and approximate volumes, in multiples of the bulk column volume, were as follows: Dimethylethanolamine, 1.3; triethanolamine, 1.5; diethanolamine, 2.6; monoethanolamine, 15.

These data show the "obstruction effect," the effect of placing carbon atoms next to the basic nitrogen, very nicely. Another influence that should be considered in any quantitative treatment is the ability of hydroxyl groups to coordinate with the metal ions in conjunction with the amino groups. Alone, hydroxyl groups coordinate very weakly; glycerol, for example, is scarcely retained at all by the nickel-loaded resin. Rather, it tends to form hydrogen bonds with the water outside the resin.

(2) Aziridines[40]. Ethyleneimine,  , and three substitued ethyleneimines were eluted from nickel-loaded and copper-loaded sulfonic and chelating resins. The unsubstituted ethyleneimine was held the most strongly. Substitution on one of the carbon atoms weakened the binding to the resin, and substitution on the nitrogen weakened it even more. The compound $C_2H_2N.C_2H_4OH$ was held more strongly than $C_2H_2N.C_2H_5$, however, pointing to the role of the hydroxyl group in coordinating with the metal (Ni or Cu).

(3) Amino sugars[41]. The amino sugars provide an ideal illustration of the advantages of ligand-exchange chromatography.

Their $-NH_2$ group is "hindered" in the sense that it is attached to a carbon atom which in its turn is attached to two other carbon atoms, but these carbons carry hydroxyl groups that can coordinate with the metal ion in concert with the amino group, and the facility with which coordination takes place depends on the stereochemical arrangement of these hydroxyl groups. Thus we found, first, that amino sugars are strongly held by copper- and nickel-loaded resins, and second, that there was a considerable difference between the three amino sugars tested. On copper-loaded Bio-Rex 70 (acrylic type resin with functional carboxyl groups) with 1.0M ammonia eluent, elution volumes were, in multiples of the bulk column volume: glucosamine, 3.9; galactosamine, 5.0; mannosamine, 8.4.

An acrylic-type resin was preferred to a polystyrene-type resin because the ligand binding was not as strong, and one could therefore use less concentrated ammonia solutions. Further, the pi-electron effect caused the polystyrene-sulfonate resin to bind tryptophane and histidine very strongly, and we wished to elute the amino sugars selectively with minimum interference from amino acids.

Ammonia displaces the amino sugars from the metal-loaded resin in the form of metal complexes, presumably uncharged complexes. The copper amino-sugar complexes strongly absorb ultraviolet light of 254 nm wavelength, making it possible to use ultraviolet detection and to see one microgram of eluted amino sugar with no difficulty. These complexes are no doubt analogous to the uncharged complex formed between copper ions and diethanolamine.

(4) Diamines and polyamines[42]. These compounds illustrate the versatility of ligand exchange; different metal ion-resin matrix combinations give different elution volumes, as is shown in Table I.

This table is not a good guide to chromatography, for it does not show relative volumes nor plate heights. Moreover, elution volumes

TABLE I

ELUTION SEQUENCES OF DI- AND POLYAMINES

Cu		Zn		NH_4
Sulfonic	Carboxylic	Sulfonic	Carboxylic	Cellex-CM
Spd	Spm	C_3	Spm	C_3
Spm	Spd	C_4	C_3	C_4
C_6	C_4	Spd	Spd	Spd
C_3	C_5	Spm	C_4	C_5
C_2	C_6	C_5	C_5	Spm
—	C_3	C_2	C_6	C_6
—	—	C_6	—	—

Spd = spermidine, $H_2NC_3H_6NHC_4H_8NH_2$

Spm = spermine, $H_2NC_3H_6NHC_4H_8NHC_3H_6NH_2$

C_2 = 1,2-propanediamine; C_3 = 1,3-propanediamine

C_4 = putrescine, 1,4-butanediamine

C_5 = cadaverine, 1,5-pentanediamine; C_6 = 1,6-hexanediamine

depend on ammonia concentrations in a way that is different for different compounds, depending on the number of ammonia molecules that replace one ligand molecule in the resin-metal combination. For example, the corrected elution volume of the 1,3-diamine on copper-loaded polystyrene sulfonate resin depends inversely on the square of the ammonia concentration, indicating that one ligand molecule displaces two ammonia molecules. The sequences in Table I are those for the ammonia concentrations likely to be used in chromatography, that is those that give elution volumes between 1 and 10 times the bulk column volume. As is customary, these concentrations are higher, the more tightly the metal is covalently

bound to the exchanger, and the greater the capacity of the exchanger for the metal. Thus, cellulose carboxylate exchanger requires the lowest ammonia concentrations, then the carboxylic resin, and polystyrene sulfonate resin requires the greatest ammonia concentration. If polystyrene chelating resin had been used, our experience with other solutes indicates that a lower ammonia concentration would be needed than with sulfonate resin: see ref. 23.

Commenting on elution orders, we see the high stability of the 1,3-diamine chelate of copper, compared to that of zinc; the 1,2-diamines are bound so strongly by copper-loaded resins that once absorbed, it is almost impossible to elute them with ammonia. With other diamines the binding strength increases with chain length, a common feature of the selectivity of resins for short-chain aliphatic molecules and ions, which reflects, perhaps, the increasing repulsion of the hydrogen-bonded water molecules outside the resin. The diamine-polyamine sequence is different for copper and zinc, and a surprising inversion of selectivity is found between spermine and spermidine as one changes from a polystyrene resin to an acrylic resin.

Our best chromatographic separations were obtained with copper-loaded Bio-Rex 70 and with zinc-loaded Aminex A-7 (polystyrene sulfonate, 9 microns diameter). This zinc-loaded resin holds its metal ions relatively weakly, and we had to add a fairly high concentration of zinc salt to the influent, between 0.001 and 0.002 molar. The zinc-ion concentration affects the elution volumes, because metal-amine complexes are now formed in the solution as well as in the resin, and the elution peaks were shifted with respect to one another. This effect can be used to advantage, and by adjusting the zinc-ion concentration we were able to get very good separation of the peaks for the physiologically important compounds putrescine, spermidine, spermine and cadaverine (eluted in that order): see Table II. These compounds were eluted with no interference from amino acids or histidine.

TABLE II

ELUTION OF DIAMINES: EFFECT OF TEMPERATURE AND ZINC-ION CONCENTRATION

Temperature	20°	55°	55°
Zn conc., mg/l	50	50	115
Elution volumes:			
1,3-diaminopropane	16.5	16.5	—
1,4-diaminobutane	21	19.3	16.5
Spermidine	24	26.3	20.2
Spermine	30	32.5	23.1
1,5-diaminopentane	40	33.7	27.7

Column: Aminex A-7, 0.63 cm x 24 cm

Ammonia concentration, 5.5M

(5) Phenethylamine and related drugs[43,44]. Because these compounds carry aromatic rings they are very strongly absorbed by polystyrene-type resins, both as molecules and as cations, and give broad chromatographic bands with bad tailing. We found it necessary to use the acrylate resin, Bio-Rex 70. Copper was the best counter-ion. The free amine bases are sparingly soluble in water, and ethyl alcohol was therefore added to the eluents. Also, the free bases are oxidized by air at a rate sufficient to cause trouble unless precautions are taken to minimize contact with the air. The sensitivity of some organic bases to oxidation or decomposition in alkaline solution is one of the drawbacks of ligand-exchange chromatography with ammonia eluents.

Table III shows some elution volumes. It illustrates structural effects very well, namely, (a) the effect of methyl

TABLE III

ELUTION VOLUMES ON BIO-REX 70

(Multiples of bulk column volume; eluent, 0.1M NH_3 in 33% ethanol)

Counter-ion:		Cu	Ni
Compound:			
Tryptamine		—	5.2
Phenethylamine	$C_6H_5CH_2CH_2NH_2$	3.9	2.8
Tyramine	$HO.C_6H_4CH_2CH_2NH_2$	—	2.75
Norephedrine	$C_6H_5CHOH.CH_2NHCH_3$	2.7	—
Amphetamine	$C_6H_5CH_2CH(CH_3)NH_2$	2.15	1.55
Ephedrine	$C_6H_5CHOH.CH(CH_3)NHCH_3$	2.1	—
Metamphetamine	$C_6H_5CH_2CH(CH_3)NHCH_3$	1.75	—
Mescaline	$(CH_3O)_3C_6H_2CH_2CH_2NH_2$	—	1.45

groups on or near the basic nitrogen atom, which decrease the retention; (b) the effect of hydroxyl groups two carbons removed from the amine group, which increase the retention, presumably by forming chelate rings; (c) the effect of the methoxy groups in the 3,4,5-positions of mescaline, which make the molecule more hydrophilic than the parent phenethylamine. The single 4-hydroxy group in tyramine has little effect.

(6) Alkaloids[45]. Alkaloid bases are bound only weakly by metal-loaded resins, because they are generally secondary or tertiary amines, and the basic nitrogen atoms are protected from coordination with metal ions. In fact, there are only a few resins whose polymer matrix is sufficiently open to allow large, weakly-bound alkaloid molecules to enter and coordinate with the metal ions. Those resins that do bind alkaloids have a greater

capacity for binding ammonia than those that do not. Once again, polystyrene-based resins are inappropriate because of their strong affinity to alkaloids having aromatic structures, and the broad elution bands that result.

The best cation-exchange resin for alkaloids chromatography was made from the commercial product Poragel-PT (Waters Associates) by hydrolyzing the ester groups that this resin contains, and turning it into an ion exchanger with functional carboxyl groups. Copper was the best counter-ion. With 0.06M ammonia in 33% ethyl alcohol, these elution volumes, in multiples of the bulk column volume, were found:

Morphine	1.0	Cocaine	4.5
Nicotine	2.0	Atropine	5.0
Codeine (methyl morphine)	2.25	Narcotine	6.8
		Quinine	9
Ethyl morphine	2.5	Reserpine	14
Papaverine	3.0	Cinchonine	16
Strychnine	3.5		

Band widths were rather poor, reflecting the slow diffusion of large molecules in and out of the resin. Nevertheless, a chromatogram of opium extract showed five distinct peaks, four of which were identified.

We have found that hydrolyzed Poragel PT in its ammonium form retains alkaloids almost as well as, and in some cases better than the copper-loaded resin. It may be that ligand exchange plays only a minor role in our alkaloids chromatography, and that the main effect is the solubility of the alkaloid bases in the resin matrix.

(8) Miscellaneous ligands. Theophylline, guanine, xanthine, adenine and caffeine were eluted in that order from a copper-loaded chelating resin, with 1M ammonia as eluent[45]; this paper lists a number of other derivatives of xanthine and presents a method for the quantitative determination of caffeine in beverages. The nucleic acids, DNA[46] and RNA[47], were separated into fractions by

absorption on an aluminum-loaded cation-exchange resin, eluting with various buffers. A Fe(III)-loaded macroporous resin, eluted with anhydrous methanol, separated butyl alcohol, ethylene glycol, glycerol, mannitol and sorbitol, which emerged in that order[48]. Resins carrying ions of Fe(III), Ti(IV) and Hg(II) separated isomeric hydroxybenzoic[49] and hydroxynaphthoic[50] acids, with water or ethanol as the eluents. A polymer incorporating Hg(II) bound sulfhydryl groups and was used to fractionate an enzyme[51].

MATRIX-AFFINITY CHROMATOGRAPHY

Matrix-solute interactions are all-pervasive in ion-exchange chromatography, and it is hard to draw hard-and-fast categories. Separations of complex multicomponent mixtures, like urine[12,52], always involve the absorption of neutral molecules or of acids and bases that are far too weak to be absorbed in their ionic forms. One of the first papers describing the deliberate use of an ion-exchange resin to separate nonionic organic compounds was that of Wu and McCready[53], reporting the separation of four isomeric butyl alcohols on a potassium-form sulfonated polystyrene cation exchanger, using water as the eluent. Tertiary butyl alcohol, or 2-methyl-2-propanol, emerged first, then 2-butanol, then 2-methyl-1-propanol, and finally 1-butanol. The most compact molecule, the one that would least disrupt the hydrogen-bonded structure of water, was released first, and the most extended molecule, which would most disrupt the water structure, came out last. This same sequence was found in the ligand-exchange chromatography of primary butylamines[14,23], where it could be attributed to decreasing obstruction of the amino group, but one should never forget that the distribution of a solute between a solvent and an absorbent depends on interactions in the solvent, as well as those in the absorbent.

Absorption of acids on cation-exchange resins. Anions are excluded from cation-exchange resins; therefore, acids that are

absorbed by cation-exchange resins must be absorbed in their non-ionized, molecular form. Lactic, formic, acetic, propionic and butyric acid are eluted in that order by water from a 12% crosslinked cation-exchange resin[54]. Aromatic acids are, of course, bound more strongly than aliphatic acids by polystyrene resins, and salicylic, benzoic, phthalic, isophthalic and terephthalic acids were separated chromatographically, again with water as the eluent[56]. A significant feature of the elution peaks shown in this paper is that they show reverse tailing; the ultraviolet absorption shown by the detector rises relatively slowly, then drops abruptly back to the base line. Peaks of this shape are associated with nonlinear absorption isotherms in which the binding becomes stronger with increasing loading of the absorbent. The proportion of undissociated acid, of course, rises with rising concentration. This effect is not found with phenols, which are acids so weak that their ionization is almost negligible[56].

Chromatography of 38 substituted benzenes, including phenols and nitrophenols, on a cation-exchange resin with water as the eluent was studied by Nomura et al.[57]; the isomeric hydroxybenzoic acids were separated on such a resin, using acidified salt solutions to "salt out" the acids into the resin[58].

Polar-substituted benzenes, analgesic drugs, xanthines. One of the earliest applications of modern high-speed liquid chromatography was to the analysis of mixtures of analgesic drugs. On a pellicular anion-exchange resin, Stevenson and Burtis[59] eluted caffeine, acetylsalicylic acid, 4-hydroxyacetanilide, phenacetin (4-ethoxyacetanilide) and salicylamide in this order, using an alkaline buffer. Ion exchange plays a part here, but the elution order bears little relation to acid strengths. In general, polystyrene-type resins tend to absorb para-disubstituted benzenes especially strongly, but it is hard to generalize about the interactions. In our laboratory we have studied the chromatography of a number of polar-substituted benzenes (esters, phenol ethers, amides, etc.) on anion- and cation-exchange resins of different

types[60]; anion-exchange resins are better "solvents," that is, they retain these compounds more strongly than cation-exchange resins, but they give broader bands, indicating slower diffusion within the resin. We found that the counter-ion affected the band width more than it did the position of the elution band. Of the singly-charged cations used, ammonium and potassium ions gave the narrowest bands and sodium the broadest.

The influence of the counter-ion in this type of chromatography has generally been overlooked and needs more study.

From cation-exchange resins aspirin, caffeine and phenacetin are eluted in this order, using alcohol-water mixtures. One might have expected caffeine to be eluted last, since it is a base, but it is such a weak base (pK_b about 14) that this quality has no effect.

Studying analgesic drugs and the xanthines[61], we found, first, that by using a 4% crosslinked polystyrene sulfonic resin of small, uniform particle size we could get very sharp elution bands, even with sodium as the counter-ion, and second, that certain compounds were retained much more strongly by the ammonium-form resin than by the sodium-form resin. The compounds in question were salicylamide, 4-hydroxyacetanilide, hypoxanthine and theophylline; see Table IV. These compounds are weak acids, with pK_a about 8. It seemed that in the ammonium-form resin, which would be weakly acidic, they existed simply as the undissociated acids, but that in the sodium-form resin, some ionization occurred, causing partial exclusion. This interpretation was corroborated by the strong reverse tailing of the salicylamide peak in the sodium-form resin (see the discussion above).

The eluent in these cases was aqueous alcohol, electrolyte-free. If buffered eluents were used the peaks of these weak acids could be moved to lower volumes by raising the pH, and to higher volumes by lowering it. A similar observation was made by Singhal and Cohn[13,62] for nucleic acid derivatives.

TABLE IV

ELUTION OF WEAK ACIDS; COUNTER-ION EFFECT

Compound	pK_a	Partition ratio, k'	
		Na-resin	NH_4-resin
Uric acid	5.4	0.1	0.1
Salicylamide	8.4	1.55	3.30
4-Hydroxyacetanilide	—	2.12	2.48
Xanthine	7.5	0.15	0.56
Theophylline	8.8	0.35	1.33
Hypoxanthine	8.9	0.75	2.25
Theobromine	10.0	1.42	1.60
Caffeine	—	1.92	1.80

Resin, Aminex 50W-x4, eluent, 25% ethanol.
(See reference 61)

The power to manipulate elution volumes by changing the pH was put to use in the chromatography of ultraviolet-absorbing constituents of coffee. Using a 14-cm column of 4% crosslinked resin and a formate buffer of pH 3.65 at 65°, percolated coffee gave five peaks and freeze-dried coffee six, three of which were identified as being due to caffeine, caffeic acid and trigonellin. The proportions of these constituents depended in a characteristic way on the type of coffee and the degree of roasting. Again the peaks were very narrow, corresponding to a theoretical-plate height of 0.2 mm.

Aromatic hydrocarbons and compounds of low polarity. Funasaka and his colleagues have studied in great detail the absorption of mono- and disubstituted benzenes on ion-exchange resins in a number of nonaqueous solvents[63,64]. They found that monosubstituted benzenes were absorbed with increasing strength in

the substituent sequence CH_3 - Cl - F - CN - NO_2, which is the order of increasing pi-electron density. The hydrogen-bonding character of the solvent affected the binding, and of six resins tested, a pyridinium resin was the strongest absorbent.

Pyridinium resins should perhaps not be classified as ion exchangers, though they do act as anion exchangers in acid solutions. In neutral and nonaqueous solvents they act as electron donors and absorb solutes according to their electron-accepting power. The absorptive properties of a resin prepared by polymerizing 2-methyl-5-vinylpyridine have been investigated by Freeman[65,66] and interpreted in terms of the three-way interactions resin-solvent, resin-solute and solute-solvent.

In his pioneer work on "solubilization chromatography," Rieman[18,19] showed that aromatic hydrocarbons could be separated by chromatography on anion- or cation-exchange resins. Because of current interest in polynuclear hydrocarbons in the environment, we have begun to explore the possibility of analyzing hydrocarbon mixtures in this way[67,68]. We are interested in resins as selective filters for trace amounts of dissolved hydrocarbons in natural waters as well as their use as stationary phases in liquid chromatography. Comparing polystyrene-type cation and anion-exchange resins, we find, as we did with polar aromatic solutes, that anion-exchange resins show stronger retention, but cation-exchange resins give narrower elution bands and are more promising for chromatography. Most of our work has been done with 4% crosslinked Aminex polystyrene sulfonate resin of 20-30 microns diameter.

The resin counter-ion is important. Between Na and NH_4 there is not much to choose, but the calcium-form resin gives almost double the retention volume of the sodium-form resin for the solutes phenanthrene and pyrene; moreover, the theoretical-plate height is appreciably smaller. A possible explanation is that one doubly-charged calcium ion takes up less space in the resin than two singly-charged sodium ions, and therefore leaves more space

for absorbed hydrocarbon molecules. The iron(III)-form resin seemed slightly better than the calcium(II) form, and a series of chromatographic tests was made with the iron-form resin.

The solvent must contain water to swell the resin and make it permeable, and it must contain an organic constituent to dissolve the aromatic hydrocarbons. Acetonitrile-water mixtures containing between 20 and 40% of acetonitrile were used, the lower proportion of acetonitrile being used with lower-molecular weight hydrocarbons like the xylenes, indene and biphenyl. The advantage of acetonitrile is its low viscosity, which favors narrow bands. If the resin counter-ion was Fe(III), the eluent was made about 0.01M in nitric acid to avoid hydrolysis. The temperature was 60°.

Very good chromatographic curves were obtained with hydrocarbons up to pyrene. Compounds with larger molecules, like chrysene and benzo(a)pyrene, gave unduly broad bands. Chlorinated and brominated derivatives were retained much more strongly than the unsubstituted hydrocarbons, in agreement with the experience of Funasaka[64].

For the liquid chromatographic analysis of polynuclear aromatic hydrocarbons, the method of choice today is probably to use a C-18 bonded packing on microparticulate silica. We have compared our resin columns with a commercial pre-packed column of this type. The bonded packing gives a faster analysis, some 20 minutes compared to 90 - 120 minutes with a resin column, and it performs better with compounds of higher molecular weight. However, our column gives more symmetrical peaks and better resolution, and permits much higher loading. If one had to look for small amounts of one hydrocarbon in the presence of much larger amounts of other, similar hydrocarbons, we think that our method would be superior. More testing is needed, however.

We have used short columns of calcium-loaded resin to filter polynuclear aromatic hydrocarbons out of solutions in water having concentrations of 1 ppm and less. Retention is very efficient, and the gel-type resin has the advantage, compared to the macroporous

non-ionic styrene-divinylbenzene polymers now used for this purpose[69], that, being ionic and carrying fixed negative charges, it does not absorb the humic acids that make up most of the dissolved organic material in many natural surface waters. Unlike polynuclear aromatic compounds and chlorinated aromatics, humic acids are not toxic and are of little environmental concern.

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