*Journal of Chromatography*, 102 (1974) 57–67


CHROM. 7732

# LIQUID CHROMATOGRAPHY OF ORGANIC COMPOUNDS ON ION-EXCHANGE RESINS

HAROLD F. WALTON
*University of Colorado, Boulder, Colo. 80302 (U.S.A.)*

## SUMMARY

The use of ion-exchange resins for the chromatography of non-ionized organic compounds is reviewed, with special reference to work in the author's laboratory. Current work on ligand-exchange chromatography of drugs, alkaloids and amino sugars is reported, and an approach called "matrix-affinity chromatography" is described.

## INTRODUCTION

In the early days of ion-exchange chromatography, around 1950, it occurred to Samuelson that organic compounds could be made to bind to ion-exchange resins through combination with inorganic ions. Thus, anion-exchange resins carrying bisulfite ions would bind aldehydes through the formation of aldehyde-bisulfite ions[1]. Resins carrying borate ions would bind polyhydric alcohols and sugars as their cyclic, ionic borate complexes. Khym and Zill[2] separated sucrose, fructose and glucose by eluting them in this order from a short column of anion-exchange resin in the borate form, using potassium borate solution as the eluent.

Soon it was found that borate ions were not necessary; glucose would bind to a strong-base anion-exchange resin even in the absence of borate. In fact, it would bind to a cation-exchange resin too. The binding was greatly strengthened by adding alcohol. Rückert and Samuelson[3] studied this effect and related it to the fact that, placed in water–alcohol mixtures, ion-exchange resins absorb water in preference to alcohol. The sugars are displaced out of the alcohol-rich liquid phase and into the water-rich resin phase. Elaborate, highly efficient techniques were developed for the chromatographic separation of mono-, di- and polysaccharides, the preferred stationary phase being an anion-exchange resin in the sulfate form[4].

Simultaneously with the early researches of Samuelson and co-workers, Sargent and Rieman[5] were developing "salting-out chromatography", in which salts like ammonium sulfate, added to water, pushed polar organic compounds out of the aqueous phase into the resin phase, which, again, was relatively water-rich owing to salt exclusion by the Donnan equilibrium. Then Sherma and Rieman[6] pursued "solubilization chromatography", in which the ion-exchange resin, due to its own hydrocarbon-like character, absorbed and "dissolved" less polar organic compounds like

ethers, ketones and even aromatic hydrocarbons. These were eluted from the column by aqueous alcohol. The more alcohol was added to the solvent, the faster was the elution, for these solutes were more soluble in alcohol than in water.

Today, Rieman's principle of "solubilization chromatography" is widely used, and some current applications of this process will be described in this paper.

Another landmark in the application of ion-exchange resins to the separation of organic compounds was the discovery by Helfferich[7] of "ligand-exchange chromatography". Helfferich used a column of cation-exchange resin loaded with ions of copper or nickel, ions that formed complexes with ammonia and amines. Such a column was used to absorb the compound 1,3-diamino-2-propanol from a dilute solution. It was stripped from the column in concentrated form by passing a concentrated ammonia solution. The ligand-exchange principle was applied to chemical analysis by Tsuji[8], Shimomura *et al.*[9] and others[10,11].

This paper will review recent progress in the author's laboratory, primarily in ligand-exchange chromatography, but also in the use of metal-free cation-exchange resin columns for the chromatography of non-ionic organic compounds.

## LIGAND-EXCHANGE CHROMATOGRAPHY

In ligand-exchange chromatography a column of cation-exchange resin is used that carries ions of metals that form ammonia complexes, such as Cu(II), Ni(II), Zn(II), and Ag(I). Compounds that contain basic nitrogen atoms are absorbed on the column through coordination with the metal ions. They are displaced by passing solutions of ammonia, and the order of elution reflects their binding to the metal-loaded resin. Solutions of ammonia in alcohol–water mixtures may be used if the solutes are poorly soluble in water.

Ideally, the metal ions remain attached to the resin and do not move. In practice, aqueous ammonia solutions contain ammonium ions, and these ammonium ions displace the metal from the resin to some extent. As long as the metal-ion concentration in the effluent is small, say $10^{-4}$ *M* or less, this "bleeding" of metal ions can be tolerated, but if the concentration is higher, it is necessary to add metal salt to the influent. This introduces another variable; metal ions draw the complexing organic ligands out of the resin and into the solution, decreasing the retention volume.

If the resin is of the strong-acid polystyrene sulfonate type, only nickel ions are held sufficiently strongly to be useful in this type of chromatography. Resins with functional carboxyl groups hold metal ions much more strongly, and nickel, copper, zinc and cadmium ions can readily be used. The ligand-binding selectivity order varies with the metal ion, but in general, copper ions are the ions of choice because they form the most stable complexes (according to the Irving–Williams sequence).

Chelating resins hold metal ions very strongly, but they have the drawback that they use a large fraction of the ligand-binding capacity of the metals, so that the capacity available for chromatography is small. For most of the work described below we found carboxylic resins to be the most satisfactory, not only because they held the metal ions tightly, but also because commercial carboxylic resins are aliphatic-type, acrylate polymers, rather than styrene polymers, and do not absorb aromatic solutes so strongly that they cannot be eluted. Interactions between the solute molecules and the resin polymer backbone are very important, as will be seen.

Acrylate resins do have the disadvantage, however, that they are soft and cannot be used under high-pressure gradients.

*Selectivity orders*

Ligand-exchange selectivities of amines follow the rule that the binding is stronger, the less obstructed is the basic nitrogen atom. Primary amines are bound much more strongly than secondary, secondary amines are bound more strongly than tertiary. Substituents on nearby carbon atoms affect the binding, as is seen by comparing *n*-butylamine, isobutylamine, *sec.*-butylamine and *tert.*-butylamine[10]; distribution ratios between Dowex-50-Ni and 1.4 *M* ammonia are 21, 13, 11, and 9, respectively. Unsymmetrical dimethylhydrazine, monomethylhydrazine and hydrazine are eluted in that order, with hydrazine much more strongly bound than the others[9]. Similar sequences are found with aziridines and with ethanolamines, where the elution order is N-dimethylethanolamine, triethanolamine, diethanolamine, monoethanolamine[12], with monoethanolamine bound much more strongly than the others. These elution orders bear little relation to the base strengths of the amines.

The effects of various factors on elution orders are nicely seen with phenethylamine and its derivatives, which include the amphetamine drugs[13]. Some typical data are shown in Table I. Unsubstituted phenethylamine is held much more strongly than the other compounds. A methyl group on the carbon next to the amine nitrogen (in amphetamine) weakens the binding considerably, and a methyl group on the amine nitrogen itself (in metamphetamine) weakens it still more. The effect of the $\beta$-hydroxyl in ephedrine and norephedrine is to strengthen the binding, presumably through the formation of a chelate ring with –OH, –NH– and the metal ion.

Table II shows more data. Tryptamine, with two basic nitrogens, is bound much more strongly than phenethylamine, while mescaline is bound much less strongly, probably because the three methoxy groups make the substance more soluble in the mobile phase.

TABLE I

ELUTION VOLUMES OF PHENETHYLAMINE DERIVATIVES

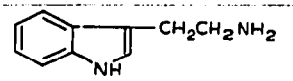
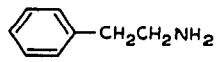
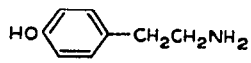
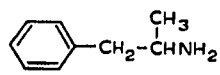
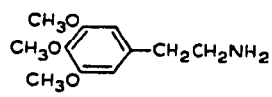
Bio-Rex 70 (methacrylate resin)-Cu, 0.10 *M* $NH_3$, 33% ethanol.

| *Compound* | *Molecular formula* | *Relative elution volume* |
|---|---|---|
| Phenethylamine | $C_6H_5-CH_2-CH_2-NH_2$ | 3.9 |
| Norephedrine | $C_6H_5-\underset{\displaystyle OH}{\underset{\displaystyle \vert}{CH}}-\underset{\displaystyle CH_3}{\underset{\displaystyle \vert}{CH}}-NH_2$ | 2.7 |
| Amphetamine | $C_6H_5-CH_2-\underset{\displaystyle CH_3}{\underset{\displaystyle \vert}{CH}}-NH_2$ | 2.15 |
| Ephedrine | $C_6H_5-\underset{\displaystyle OH}{\underset{\displaystyle \vert}{CH}}-\underset{\displaystyle CH_3}{\underset{\displaystyle \vert}{CH}}-NH-CH_3$ | 2.1 |
| Metamphetamine | $C_6H_5-CH_2-\underset{\displaystyle CH_3}{\underset{\displaystyle \vert}{CH}}-NH-CH_3$ | 1.75 |

TABLE II

ELUTION VOLUMES OF PHENETHYLAMINE DERIVATIVES

Bio-Rex 70-Ni (carboxylic), 33% ethanol, 0.1 *M* $NH_3$.

| *Compound* | *Structural formula* | *Relative elution volume* |
|---|---|---|
| Tryptamine |  | 5.2 |
| Phenethylamine |  | 2.8 |
| Tyramine |  | 2.75 |
| Amphetamine |  | 1.55 |
| Mescaline |  | 1.45 |

*Alkaloids*

It is logical to apply ligand-exchange chromatography to the alkaloids, but one difficulty is immediately evident. The alkaloids are all tertiary amines, which means they will be weakly bound. When we began work on alkaloids we used the commercial carboxylic resin, Bio-Rex 70 (Bio-Rad Labs., Richmond, Calif., U.S.A.), in its copper and nickel forms and found that the retention of alkaloids was slight, and separations were inefficient. Then we tested an experimental resin, Bio-Rex PC-20, in the copper form and found that it gave much better retention. We ordered more of this resin and the next batch we received was ineffective!

Seeking resins that would bind alkaloids, we took Poragel-PT, a highly polar resin made for liquid chromatography by Waters Ass. (Milford, Mass., U.S.A.), which carries ethylene glycol ester groups on a polystyrene base. We hydrolyzed this resin according to the suggestion of Freeman and Madamba[14] and produced a resin with functional carboxyl groups. This was converted to its copper form, and was found to be effective in binding alkaloids.

We measured the ammonia uptake of the copper-loaded resins, and found that the bound ammonia to total copper ratio in the resin, for a given ammonia concentration in the solution, was about 50% higher in the resins that showed good alkaloid retention than it was in the resins that showed poor alkaloid retention. Evidently the environment of the metal ion in the carboxylic resins is very dependent on subtle details of the polymer structure.

Fig. 1 shows a chromatogram of a mixture of three alkaloids, morphine, codeine and strychnine, on hydrolyzed Poragel-PT-Cu. The alkaloids were introduced

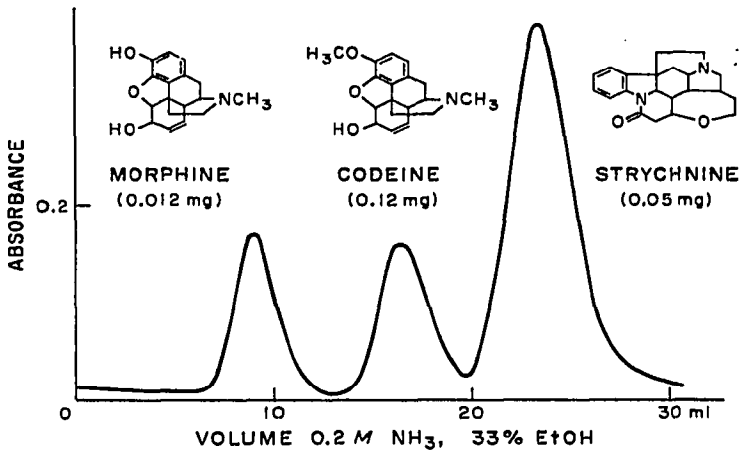



Fig. 1. Separation of alkaloids on Poragel-PT-Cu at 50°.

as their sulfate salts, and were converted to the free bases in the flowing stream of ammonia as soon as they entered the column; sulfate ions appeared in the effluent at one void volume. The solvent was 33% ethanol, not water, because the alkaloid bases are almost insoluble in water.

The separation of these alkaloids is satisfactory, but the bands are undesirably broad, even though the column was run at 50° to raise diffusion rates. Table III lists elution volumes found with a number of alkaloids, and it is clear that, potentially, ligand-exchange chromatography could be an effective means of analysis of alkaloid mixtures if we could get narrower bands and better resolution. This work is still in progress, and details will be published later*. One difficulty must, however, be pointed

TABLE III

ELUTION SEQUENCE OF ALKALOIDS ON Cu- OR Ni-LOADED, HYDROLYZED PORAGEL-PT

Multiples of bulk column volume, 0.06 *M* $NH_3$, 33% ethanol.

| *Compound* | *Relative elution volume* |
|---|---|
| Morphine | 1.0 |
| Ethyl morphine | 2.2 |
| Codeine (methyl morphine) | 2.3 |
| Papaverine | 3.8 |
| Strychnine | 4.0 |
| Cocaine | 4.5 |
| Atropine | 5.0 |
| Narcotine | 6.8 |
| Nicotine | 8.0 |
| Methadone | 8.0 |

* A preliminary report was presented at the American Chemical Society meeting in Chicago, August 1973.

out. Ligand-exchange chromatography is done in strongly alkaline solution. Some alkaloids hydrolyze rapidly under these conditions, while others are oxidized. Oxidation can be prevented, but hydrolysis cannot. Cocaine, for example, which has two ester groups in its molecule, hydrolyzes to a small but noticeable extent on the column, even when the solution is prepared just before injection. In 44 h, a solution of cocaine in 0.1 *M* ammonia hydrolyzes completely to ammonium benzoate. The same problem arises with heroin, which hydrolyzes to morphine.

*Amino sugars*

The aminohexoses, glucosamine, galactosamine and mannosamine, are strongly held on metal-loaded resins in spite of their "obstructed" amino groups. It is presumed that chelate rings are formed through the hydroxyl groups of the sugars. The sugars themselves —glucose, galactose and mannose— are not held at all, and elute at one void volume. Ligand exchange, therefore, separates amino sugars very effectively from other sugars.

We were surprised to find how great was the effect of the stereochemistry. Table IV shows data for a nickel-loaded resin. For copper-loaded resins the order of elution was the same, but there was more difference between mannosamine and galactosamine, less between galactosamine and glucosamine.

TABLE IV

ELUTION VOLUMES OF AMINO SUGARS

Bio-Rex 70-Ni (carboxylic), 0.68 *M* $NH_3$.

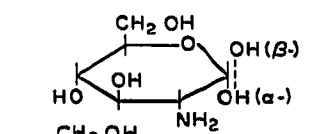
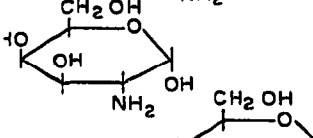
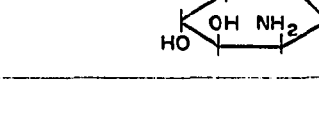
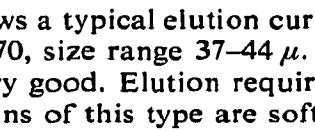
| *Compound* | *Structure* | *Relative elution volume* |
|---|---|---|
| Glucose (at void volume) |  | 1.00 |
| Glucosamine |  | 3.05 |
| Galactosamine |  | 4.30 |
| Mannosamine |  | 5.10 |

Fig. 2 shows a typical elution curve. The resin was of the acrylic type, copper-loaded Bio-Rex 70, size range 37–44 $\mu$. The theoretical-plate height was about 0.25 mm, which is very good. Elution required 50 min, which is rather slow, but, as was noted above, resins of this type are soft and will not stand high-pressure gradients.

The ordinate in Fig. 2 is UV absorbance. Amino sugars do not absorb in the UV; however, they remove copper from the resin, and the copper complexes absorb at 254 nm. They absorb more strongly than the copper-ammonia ions themselves, and this method of detection is very sensitive. Moreover, it is free from the artifacts

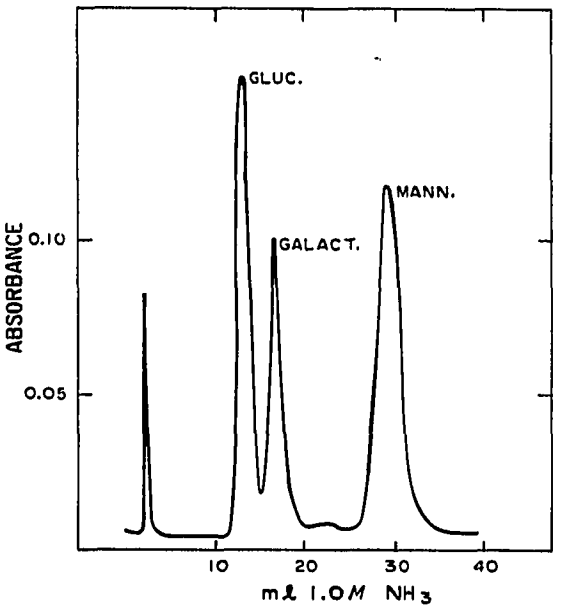



Fig. 2. Separation of amino sugars on Bio-Rex 70-Cu, 37–44 $\mu$. Column, 6 mm × 20 cm. Flow-rate, 0.7 ml/min; influent was $1 \times 10^{-4}$ *M* in $CuSO_4$; absorbance at 254 nm. Weight of each sugar introduced was 50 $\mu$g as the hydrochloride.

that appear in refractive index curves. The sharp peak at one void volume is due to the conversion of the amino sugar chlorides to ammonium chloride, which displaces a little copper from the resin. To maintain the copper loading of the resin we added $10^{-4}$ *M* copper sulfate to the ammonia influent. Under these circumstances the peak area was proportional to the amount of amino sugar injected.

Amino sugars often occur together with amino acids, and we therefore studied the elution of several pure amino acids, as well as a standard mixture of twenty amino acids supplied by the Bio-Rad Labs. Most amino acids eluted between one and two void volumes, with 1 *M* ammonia as the eluent. Those that were more strongly retained were the basic amino acids lysine and histidine. In addition, tryptophan was retained strongly by hydrolyzed Poragel-PT-Cu, which has a polystyrene matrix, but not by Bio-Rex 70, which is an acrylic polymer. Fig. 3 shows an elution curve with Bio-Rex 70. With this resin lysine elutes just after glucosamine, but the two peaks can be distinguished, and moreover the intensity of the lysine peak is much less than the peak given by an equal weight of glucosamine. With hydrolyzed Poragel-PT at 60°, lysine elutes just after one void volume, while tryptophan elutes midway between galactosamine and mannosamine. It is thus possible, by careful work and using the appropriate resin, to distinguish amino sugars clearly from accompanying amino acids.

For a "real" analysis we took crab shells and hydrolyzed them with 6 *M* hydrochloric acid, and injected the hydrolyzate after evaporating most of the excess acid. We found a large peak at the void volume, two smaller peaks between one and two void volumes, then a well-isolated peak for glucosamine, followed by the histidine peak. The resin was Bio-Rex 70.

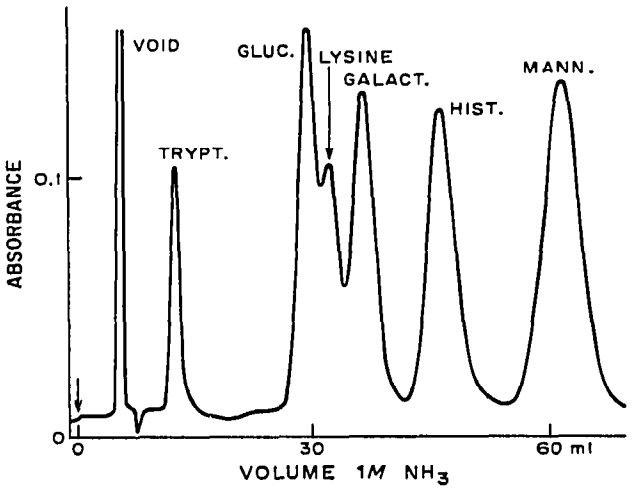



Fig. 3. Separation of amino sugars and amino acids on Bio-Rex 70-Cu, 37–44 μ. Column, same as in Fig. 2: flow-rate, 0.3 ml/min. Quantities introduced (μg): tryptophan, 6; glucosamine·HCl, 40; lysine·HCl, 160; galactosamine·HCl, 60; histidine, 80; mannosamine·HCl, 50. Absorbance measured at 254 nm; influent $10^{-4}$ *M* in $CuSO_4$.

This is a preliminary report. The work is in the process of development and will be the subject of a later publication.

## MATRIX-AFFINITY CHROMATOGRAPHY: ANALGESIC DRUGS AND XANTHINES

Working with aromatic solutes in ion-exchange chromatography one is constantly aware that these solutes are strongly held by resins having a polystyrene matrix. This affinity is superimposed on other interactions, such as metal–ligand interactions. It is, for example, impossible to perform ligand-exchange chromatography of amphetamines on resins like Dowex-50. Broad bands result, with considerable tailing.

It was logical to exploit the matrix–solute affinity as a basis for chromatography in its own right. We therefore used cation-exchange resins in their ammonium and sodium forms, as well as anion-exchange resins, with water–alcohol mixtures, electrolyte-free, as eluents, and we obtained good chromatographic separations of non-ionized but polar aromatic compounds like ethyl benzoate and the drugs phenacetin (*p*-ethoxyacetanilide) and caffeine. The question then arises: Why use an ion-exchange resin at all? Why not use a styrene–divinylbenzene copolymer with no ionic groups? The answer is that the ions become solvated and make the resins permeable. One might achieve permeability by using a macroporous non-ionic resin, and such resins have, indeed, been used to absorb aromatic solutes[15]. For chromatography they have the drawback that the binding zones are not homogeneous. We have found that gel-type resins of small and uniform particle size, and particularly resins of low crosslinking, act as highly uniform, highly permeable stationary phases and can give theoretical-plate heights of 0.1 mm and less.

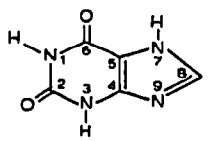
Our work in this area has been published[16,17], and what follows here is a summary and commentary.

*Effects of counter-ions, crosslinking, solvent and pH*

Working with phenacetin and caffeine as solutes and cation-exchange resins carrying as counter-ions Li, Na, K, and $NH_4$, we found that the counter-ion had little effect on the elution volumes but a marked effect on the band width. Ammonium ions gave the sharpest bands, sodium ions the broadest. Later, however, when we learned how to get really narrow bands, we decided that the differences in band width were not significant. The difference between ammonium and sodium ions became important when the solute was a weak acid. The ionization of very weak acids within the resin, which is quite appreciable in a sodium-form resin, is suppressed in the more acidic ammonium-form resin, with a corresponding increase in the elution volumes: see Table V. This effect is related to the Donnan exclusion of co-ions, in this case the anions of the weak acids. It is important for acids having p$K$ values around 7–8. Salicylamide, for instance, with $pK_a = 8.4$, had an elution volume (corrected) on an ammonium-form resin which was more than double that on the corresponding sodium-form resin. Acids that are much stronger, like acetylsalicylic acid, are excluded from the cation-exchange resin completely.

TABLE V

ACIDITY AND ELUTION VOLUMES OF XANTHINE DERIVATIVES:



4% crosslinking, 25% ethanol.

| *Compound* | *p$K_a$* | *k'* | | *Ratio* |
|---|---|---|---|---|
| | | *Na resin* | *$NH_4$ resin* | |
| Uric acid (8-OH) | 5.4 | 0 | 0.1 | — |
| Xanthine | 7.5 | 0.15 | 0.56 | 3.7 |
| Theophylline (1,3-$CH_3$) | 8.8 | 0.35 | 1.33 | 3.8 |
| Hypoxanthine | 8.9 | 0.75 | 2.25 | 3.0 |
| Theobromine (3,7-$CH_3$) | 10.0 | 1.42 | 1.60 | 1.13 |
| Caffeine (1,3,7-$CH_3$) | 14.0 | 1.9 | 1.8 | 0.95 |

Crosslinking affects the elution volumes of different solutes in different ways, and part of this effect may be due to Donnan equilibrium. Salicylamide is retained much more strongly by 8% than by 4% crosslinked resin (both in the ammonium form), and the reason may be that the higher ammonium ion concentration in the 8% resin represses the ionization more.

Singhal[18] and Singhal and Cohn[19], working with nucleic acid derivatives on ion-exchange columns, have found that very weak acids may be eluted from a cation-exchange resin at volumes greater than the void volume but smaller than the total volume of water in the column, as measured by injecting a trace of radioactive tritiated water. They call this effect "ion-exclusion chromatography". Elution volumes can, of course, be manipulated by changing the pH of the eluting buffer. We believe we have an example of ion-exclusion chromatography in our analysis of coffee, noted below.

It remains to mention the effect of solvent composition, that is, the ratio of alcohol to water. The more alcohol, the lower the elution volumes, and the effect is considerable. It would be easy to perform solvent-gradient elution if this were desired.

*Applications*

Refs. 16 and 17 describe applications of "matrix-affinity chromatography", as we may call it, to mixtures of analgesic drugs, xanthines, and coffee. Primarily we used electrolyte-free aqueous alcohol as the eluent, and we showed that we could change elution orders by changing from the ammonium form of the resin to the sodium form. For some applications the ammonium form was better, for others the sodium form. Best results were obtained with a sulfonated polystyrene resin of 4% cross-linking and a particle diameter of 20–30 $\mu$, supplied by Bio-Rad Labs.

Electrolyte-free eluents have the advantage that it is easy to remove the solvent for preparative purposes or for mass spectrographic examination. One could, if one wished, use the resin bed as an internal buffer of any desired pH by equilibrating it beforehand with the proper solution. However, the resin would have to be regenerated, or reconditioned, at frequent intervals during use if one passed acid solutes that exchanged ions with the resin. A better practice, for most purposes, is to use a buffered eluent. We used a formic acid–ammonium formate buffer in 25% ethanol for the analysis of the UV-absorbing constituents of coffee, with excellent results. Adding electrolyte to the eluent causes the resin to shrink and become more rigid, an important advantage with resins of low crosslinking.

Using a glass column of 0.6 × 14 cm and a formate buffer of pH 3.65 at 60° we found six distinct peaks, three of which —caffeine, caffeic acid and trigonellin— were identified. With a longer column we could do better. We believe that this technique, which is really the same as Rieman's "solubilization chromatography" done with modern equipment, is a very powerful one for the examination of water-soluble and alcohol-soluble constituents of natural products.

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation, currently under Grant No. GP-37779X and a travel grant, GP-43414. I am very grateful to the Foundation for its support, and also to James Navratil, Eduardo Murgia and Jerry Harder, students at the University of Colorado, some of whose work is reported here.

REFERENCES

1 G. Gabrielson and O. Samuelson, *Sv. Kem. Tidskr.*, 62 (1950) 214; 64 (1952) 150.
2 J. X. Khym and L. P. Zill, *J. Amer. Chem. Soc.*, 73 (1951) 2399.
3 H. Rückert and O. Samuelson, *Acta Chem. Scand.*, 11 (1957) 303, 315.
4 L. I. Larsson and O. Samuelson, *Acta Chem. Scand.*, 19 (1965) 1357.
5 R. Sargent and W. Rieman, *J. Phys. Chem.*, 61 (1957) 354.
6 J. Sherma and W. Rieman, *Anal. Chim. Acta*, 20 (1959) 357.
7 F. Helfferich, *Nature (London)*, 189 (1961) 1001.
8 A. Tsuji, *Nippon Kagaku Zasshi (J. Chem. Soc. Jap., Pure Chem. Sect.)*, 81 (1960) 847, 1090.
9 K. Shimomura, L. Dickson and H. F. Walton, *Anal. Chim. Acta*, 37 (1967) 102.
10 H. F. Walton, in J. A. Marinsky and Y. Marcus (Editors), *Ion Exchange and Solvent Extraction*, Vol. 4, Marcel Dekker, New York, 1973, Ch. 4.

11 J. C. Wolford, J. A. Dean and G. Goldstein, *J. Chromatogr.*, 62 (1971) 148.
12 K. Shimomura, Tong-Jung Hsu and H. F. Walton, *Anal. Chem.*, 45 (1973) 501.
13 C. M. de Hernandez and H. F. Walton, *Anal. Chem.*, 44 (1972) 890.
14 D. H. Freeman and A. Madamba, private communication.
15 A. K. Burnham, G. V. Calder, J. S. Fritz, G. A. Junk, H. J. Svec and R. Willis, *Anal. Chem.*, 44 (1972) 139.
16 P. Larson, E. Murgia, Tong-Jung Hsu and H. F. Walton, *Anal. Chem.*, 45 (1973) 2306.
17 E. Murgia, P. Richards and H. F. Walton, *J. Chromatogr.*, 87 (1973) 523.
18 R. P. Singhal, *Arch. Biochem. Biophys.*, 152 (1972) 800.
19 R. P. Singhal and W. E. Cohn, *Biochemistry*, 12 (1973) 1532.