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# LIQUID CHROMATOGRAPHY OF XANTHINES, ANALGESIC DRUGS AND COFFEE

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## SUMMARY

A 4% cross-linked sulfonated polystyrene cation exchanger in the sodium or ammonium form is a useful stationary phase for the chromatography of xanthines, analgesic drugs and coffee. The eluent is aqueous ethanol with or without added buffers. The elution of weak acids and bases depends in a systematic way on the acidity of the resin and eluent, and this fact is used to improve separations.

## INTRODUCTION

It is common knowledge that ion-exchange resins of the polystyrene type act as solid solvents for organic compounds, especially those whose molecules have aromatic character. These resins can therefore serve as stationary phases for the liquid chromatography (LC) of such compounds. There are many references to the chromatography of analgesic drugs[1,2] and the ultraviolet (UV) absorbing constituents of body fluids[3,4] with buffer solutions as eluents, but there are only a few references to the use of ion-free solvents. Nomura *et al.*[5] studied the chromatography of substituted phenols and various aromatic compounds on polystyrene-type cation-exchange resins, using pure water as the eluent. They showed that carboxylic acids were retained much more strongly by hydrogen-form resins than by sodium-form resins, which would be expected if the undissociated acids were absorbed, while the acid anions were excluded from the resin. Caffeine, ethoxybenzamide and 4-isopropylantipyrine, which are weak bases, were separated on a cation-exchange resin with 2% cross-linking, with alcohol–water mixtures as eluents[6].

In our laboratory we have found that a 4% cross-linked sulfonated polystyrene cation-exchange resin is a good stationary phase for the chromatography of analgesic drugs and xanthines. We have used 25% ethanol as the eluent. We have studied the effect of the counter-ion of the resin and find systematic differences between sodium ions, ammonium ions and mixed ammonium and hydrogen counter-ions, which can be correlated with the acidic or basic properties of the solutes. Some solute mixtures are best resolved on the sodium-form resin, other mixtures on the ammonium-form resin. We have applied our findings in a preliminary way to the

chromatographic analysis of coffee and tea, and shown that in certain cases a buffered eluent is desirable.

## EXPERIMENTAL

The resin used throughout was Aminex 50W-X4, diameter 20–30 $\mu$m, supplied by Bio-Rad Labs., Richmond, Calif., U.S.A. This resin is of the sulfonated polystyrene type. The columns were of glass, 6 mm I.D., with PTFE plungers to contain the resin column. Two columns were used, one with a resin bed some 22 cm long and another with a bed 42–44 cm long. Both were jacketed and heated to 65° by circulating water. The solvent, made by mixing one volume of 96% ethanol with three volumes of water, was pumped by a variable-speed pulseless pump or by nitrogen pressure from a stainless-steel reservoir. Samples, 0.25 ml in the long columns and 0.02 ml in the short, were introduced by rotary sample-injection valves. Effluents were monitored by the absorption of UV light at 254 nm. The chromatographic equipment was supplied by Chromatronix, Berkeley, Calif., U.S.A.

A difficulty in working with the 4% cross-linked resin is its softness and its deformation under pressure. Raising the pressure above 200–400 p.s.i. (15–30 bars) caused the resin to collapse and stop the flow. The flow-rates were therefore limited. For the long column the flow-rate was 12 ml/h. Faster flow was possible with the short column, especially when buffer solutions were used; then the resin contracted and became more rigid. The columns were heated to 65° to reduce the solvent viscosity and increase the mechanical strength of the resin.

The pure compounds were obtained from commercial sources. They were injected as solutions in 25% ethanol, in the concentration range 0.01 to 1 mg/ml, the concentrations being chosen to give adequate recorder deflection at full-scale absorbances of 0.32–0.64.

The coffee samples were prepared by extracting ground roasted coffee with boiling water or by dissolving "instant" coffee in hot water at the concentration that one would use for drinking. This was too concentrated to inject directly, except for preparative separations (see below). The "beverage coffee" was diluted 1:10 or more before injection, adding enough ethanol to make the final alcohol concentration 25%. Several kinds of ground coffee and instant (freeze-dried) coffee were tested, including two dark-roasted coffees.

## RESULTS AND DISCUSSION

### *Comparison of sodium-form and ammonium-form resins*

Table I compares the elution volumes of a number of xanthines and some other compounds of physiological interest and lists values of their ionization constants as acids. Fig. 1 shows chromatograms of a solution of the drug "Excedrin" on the two forms of the resin; from the practical, analytical point of view the ammonium form gives the better curve. Fig. 2 shows curves for a mixture of xanthine with four derivatives of xanthine; this time the sodium form of the resin is better. In both cases the elution order is changed by changing the counter-ion of the resin. These figures, and the data of Table I, were obtained with the long column, whose resin bed measured 43.0 cm in the ammonium form and 44.5 cm in the sodium form.

TABLE I

ELUTION VOLUMES, COUNTER-IONS AND ACID STRENGTHS

Column: diameter, 6 mm; heights, Na-resin 44.5 cm, $NH_4$-resin 43.0 cm; flow-rate, 12 ml/h; temperature, 65°; resin, Aminex 50W-X4, 20–30 $\mu$m. Void volume approx. 4 ml, taken as 4.0 ml and 3.85 ml in Na- and $NH_4$-resin columns, respectively; these numbers are in the same ratio as bed heights and were used to calculate $k'$. $pK_a$ values taken from Kortüm *et al.*[7], unless another reference is given; values are at 25°.

| *Compound* | $pK_a$ | *Elution volume (ml)* | | *Distribution ratio, k'* | |
|---|---|---|---|---|---|
| | | *Na-resin* | *$NH_4$-resin* | *Na-resin* | *$NH_4$-resin* |
| Acetylsalicylic acid | 3.5 | 4.0 | 3.85 | (0) | (0) |
| Uric acid | 5.4 | — | 4.2 | — | 0.09 |
| Salicylamide | 8.4 | 10.2 | 18.6 | 1.55 | 3.30 |
| 4-Hydroxyacetanilide (acetaminophen) | — | 12.5 | 13.4 | 2.12 | 2.48 |
| Xanthine | 7.53* | 4.6 | 6.0 | 0.15 | 0.56 |
| 1,3-Dimethylxanthine (theophylline) | 8.8 | 5.4 | 9.0 | 0.35 | 1.33 |
| 3,7-Dimethylxanthine (theobromine) | 10.0 | 9.7 | 10.0 | 1.42 | 1.60 |
| 6-Hydroxypurine (hypoxanthine) | 8.9* | 7.0 | 12.5 | 0.75 | 2.25 |
| 1,3,7-Trimethylxanthine (caffeine) | — | 11.7 | 10.8 | 1.92 | 1.80 |
| Pyridine-3-carboxylic acid (nicotinic acid) | 4.8** | 4.2 | 4.0 | 0.05 | 0.04 |
| N-Methylpyridine-3-carboxylic acid (trigonellin) | — | 15.0 | 15.8 | 2.62 | 3.10 |

* From ref. 8.
** From ref. 9.

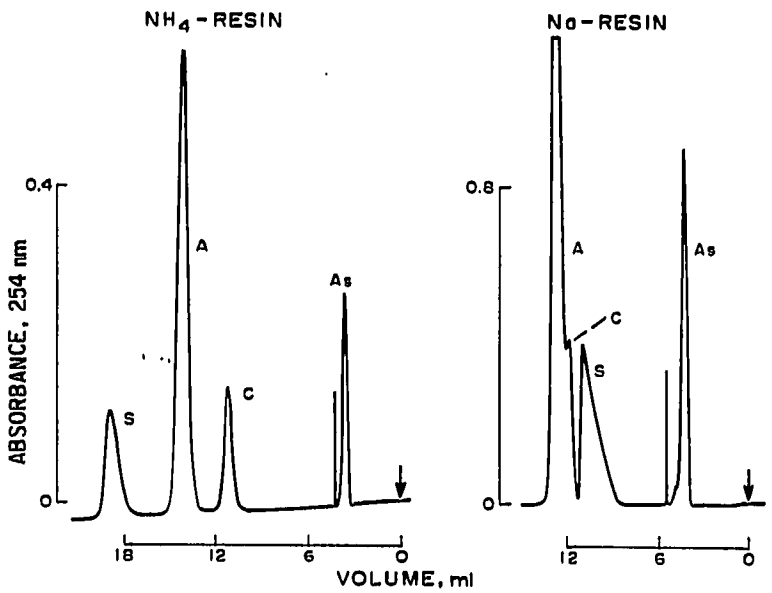


Fig. 1. Chromatogram of Excedrin. Quantity introduced, 35 $\mu$g. Columns, 0.6 cm × 43 cm (ammonium resin) and 0.6 × 44.5 cm (sodium resin); temperature, 65°; flow-rate, 12.0 ml/h; solvent, 25% alcohol. As = Aspirin; C = caffeine; A = acetaminophen (4-hydroxyacetanilide); S = salicylamide. The aspirin peak was registered at double the sensitivity of the other peaks.

The void volume, estimated from the minimum elution volumes of apparently non-absorbed solutes, was close to 4.0 ml, or 32% of the bulk bed volume. The plate number, calculated from the peak width of acetaminophen (Fig. 1) or caffeine (Fig. 2) was 2500–3000.

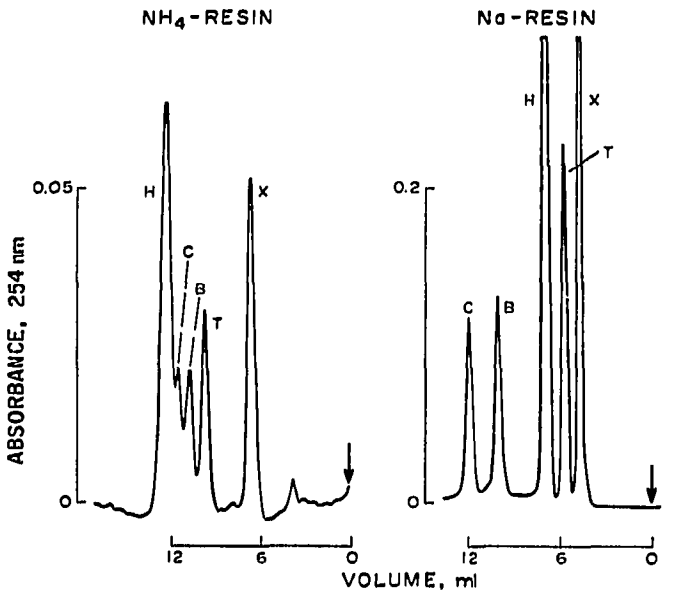


Fig. 2. Chromatogram of xanthines. Same column and flow conditions as Fig. 1. X = Xanthine (3.5 μg); T = theophylline (2.5 μg); B = theobromine (2.5 μg); C = caffeine (2.5 μg); H = hypoxanthine (1.25 μg). The small peak at one void volume with $NH_4$-resin is an impurity in the xanthine.

Table I shows clearly the effect of acid strength. The strongest acids (uric, nicotinic, acetylsalicylic) are retained weakly or not at all. The anions of the acids are repelled by the fixed ions of the cation-exchange resin, and their concentration within the resin has to be very low. The resin polymer acts as a solvent for the undissociated, uncharged acids but the concentration of uncharged acid within the resin can only be appreciable if the acid is very weak. The weakest acids (caffeine, theobromine, trigonellin, acetaminophen) are held almost equally by the sodium and ammonium forms of the resin. Acids of intermediate strength, with p$K$ values between 7 and 9, are without exception held more strongly by the ammonium resin than by the sodium resin. The explanation must be that the acidity of the ammonium ion stabilizes the uncharged acid molecules.

The elution peaks of certain acids were unsymmetrical; note the salicylamide peak in the sodium resin, Fig. 1. The uric acid peak had a similar shape. The slow rise in concentration, followed by a rapid drop as the solute leaves the column, indicates that the absorption by the resin becomes stronger as the solute concentration increases. The fraction of a weak acid that is associated rises with its concentration. The effect is less marked with the ammonium-form resin because the hydrogen-ion concentration within the resin is more nearly constant.

*Effect of hydrogen ions*

Experience with the chromatography of coffee (see below) led us to make experiments with buffered eluents containing ammonium ions and hydrogen ions. Three such eluents were used: (1) 0.10 *M* ammonium formate, 0.15 *M* formic acid, pH 3.65; (2) 0.075 *M* ammonium sulfate, plus sulfuric acid to pH 2.8; (3) 0.20 *M* ammonium chloride, 0.05 *M* hydrochloric acid, pH 2.3. All contained ethanol and water in the proportion 1:3. The resin column was brought to equilibrium with each eluent before samples were injected. The selectivity quotient for $NH_4^+$ against $H^+$ in this resin is about 2, so the $NH_4^+ : H^+$ ratio in the resin should be twice what it is in the solution.

Results are shown in Table II. Raising the acidity raises the strength of binding to the resin, probably because the bases form cations. It is clear that different mixtures have different optimum pH values for their separation.

TABLE II

EFFECT OF ACIDITY ON ELUTION

Column distribution ratio, $k'$ = (corrected elution volume)/(void volume); column, diameter 6 mm, length 22 cm; packing, Aminex 50W-X4, 20–30 $\mu$m; temperature, 65°; flow-rate varied, 20–40 ml/h; $pK_b$ values are for the uncharged molecules[8,9]; that for trigonellin was measured roughly in our laboratory.

| *Compound* | $pK_b$ | *Column distribution ratio, k', for* | | | |
|---|---|---|---|---|---|
| | | *$NH_4$-resin, aqueous alcohol* | *Ammonium formate, pH 3.6* | *Ammonium sulfate, pH 2.8* | *$NH_4Cl$ + HCl, pH 1.3* |
| Caffeine | 14 | 2.1 | 1.9 | 2.1 | 3.3 |
| Trigonellin | (11.5) | 3.2 | 3.8 | 5.4 | 22.5 |
| Nicotinic acid | 12.0 | 0.0 | 3.0 | 4.5 | 22.0 |
| Hypoxanthine | 12.0 | 1.0 | — | 2.8 | 14.5 |

*Chromatography of coffee*

Typical curves obtained with aqueous alcohol eluent and no buffer are shown in Figs. 3–6. They were run on a 22-cm column at 65°, with flow-rates near 20 ml/h. There are three major peaks. The first is at least partially due to tannin, for the effluent is colored brown. The second was identified as caffeine by the UV absorption spectrum and by the addition of pure caffeine, which heightened the peak without producing a new one. The third was identified by collecting a couple of milligrams of the solute, using a preparative-scale column, examining the UV and infrared (IR) spectra and comparing these spectra and the elution volumes with those of pure trigonellin.

Coffee is known to contain trigonellin[10–12] and it has been shown that this substance is converted to nicotinic acid during roasting[10,13]. Nicotinic acid is also present in raw coffee[12]. Our chromatograms of dark-roasted coffees (see Fig. 5) showed that these contained less than half as much trigonellin, compared to caffeine, as the light-roasted coffee commonly sold in the U.S.A.

Fig. 3 was obtained just after the column had been regenerated by passing an ammonium chloride solution; Fig. 4 was obtained after a number of coffee samples had been run. Always the trigonellin peak drifted to higher elution volumes as more

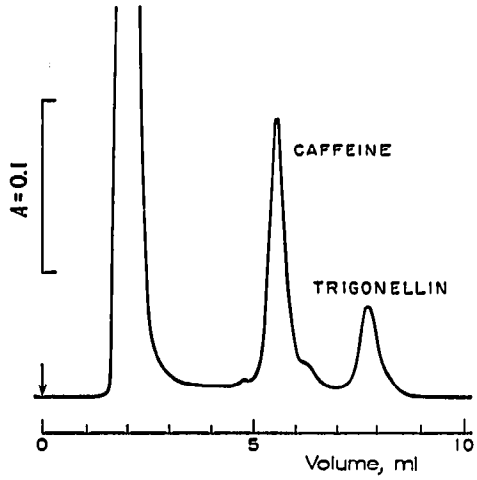


Fig. 3. Chromatogram of freeze-dried coffee. Column freshly regenerated with ammonium chloride. See text for details.

and more coffee was passed. It was this observation that led us to make the tests described under *Effect of hydrogen ions*. Presumably, acid constituents of coffee (whose pH is normally close to 6) replace some of the ammonium ions in the column by hydrogen ions.

Fig. 6 shows decaffeinated coffee (Sanka). A chromatogram of this coffee on the 45-cm column showed more detail near the caffeine peak. Chromatograms of tea, not shown, had a caffeine peak and a large "tannin" peak at one void volume, but no trigonellin or other major peaks.

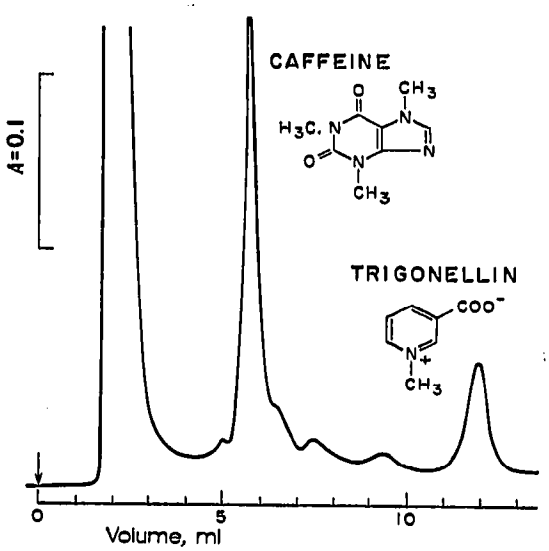


Fig. 4. Chromatogram of freeze-dried coffee, same product as Fig. 3. The column had been in use for some time and not regenerated. See text for details.

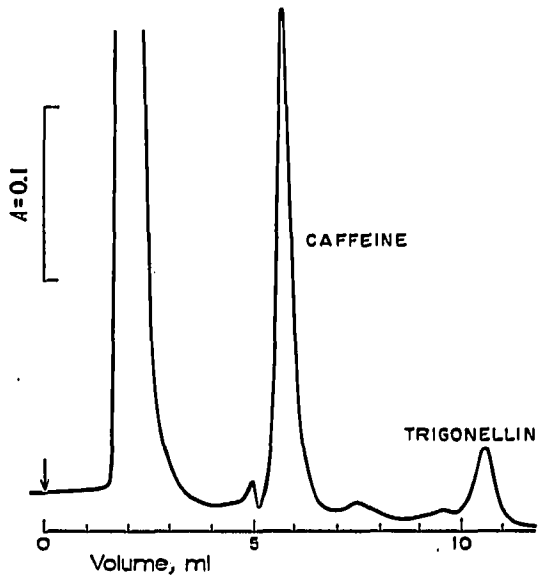


Fig. 5. Chromatogram of dark-roasted Venezuelan coffee. Note small trigonellin peak. See text for details.

Figs. 7, 8 and 9 show curves obtained with the ammonium formate buffer, pH 3.65 (see *Effect of hydrogen ions*). The column is shorter than before, partly because it had been repacked with fresh resin, partly because the resin contracts in the buffer solutions. New peaks are now seen. The identities of peaks I and II are unknown. Peak III was certainly caffeine and peak V trigonellin; these were identified

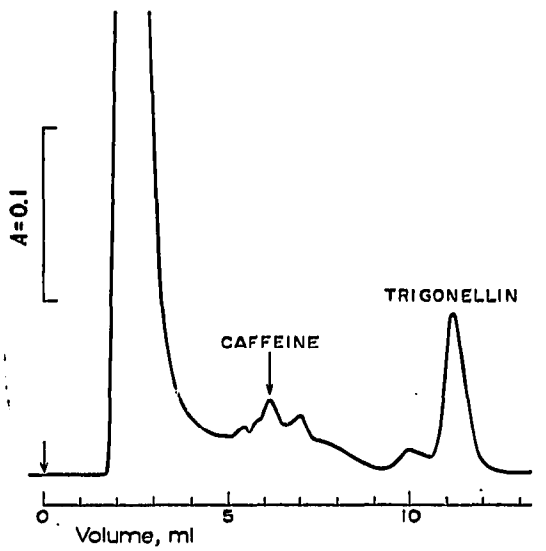


Fig. 6. Chromatogram of decaffeinated coffee. See text for details.

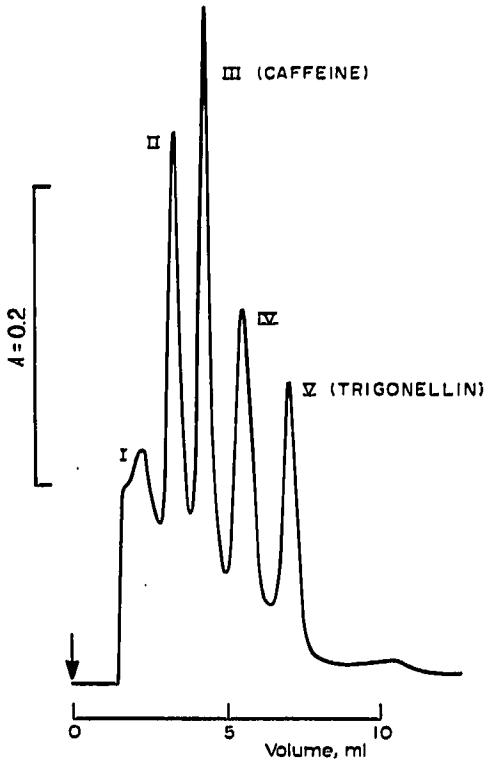


Fig. 7. Chromatogram of percolated coffee. Eluent, ammonium formate, pH 3.65 (see text); column length, 14 cm; flow-rate, 45 ml/h; temperature, 65°.

by their UV spectra and elution volumes, which were identical with those of the pure substances. Peak IV had an elution volume close to that of nicotinic acid, and when nicotinic acid was added to the coffee before injection, this peak became higher and retained its symmetry. There was no indication of a new peak, yet the UV absorption spectrum of this fraction was quite different from that of nicotinic acid. It had a maximum at 326 nm, a shoulder at 298 nm, and a minimum at 267 nm. Nicotinic acid has a sharp maximum at 261 nm. The compound 3,4-dihydroxycinnamic acid, or caffeic acid, which is known to occur in green and roasted coffee, has absorption maxima at 324 and 297 nm[14].

Eluted with ammonium sulfate of pH 2.8 (see *Effect of hydrogen ions*), coffee gave a chromatogram with the same peaks as Fig. 7, but with peaks II, IV and V displaced to higher volumes. Peaks II and III are now very close together, while III, IV and V are more widely separated. The column distribution ratios, $k'$, were as follows:

*pH 2.8:* 0.25, 1.75, 2.0, 4.0, 5.5

*pH 3.65:* 0.5, 1.2, 1.9, 2.7, 3.7

Elution at pH 1.3 gave a non-descript wavy pattern with the only distinctive peak being that of caffeine. Tests with pure compounds gave the elution volumes shown in Table II.

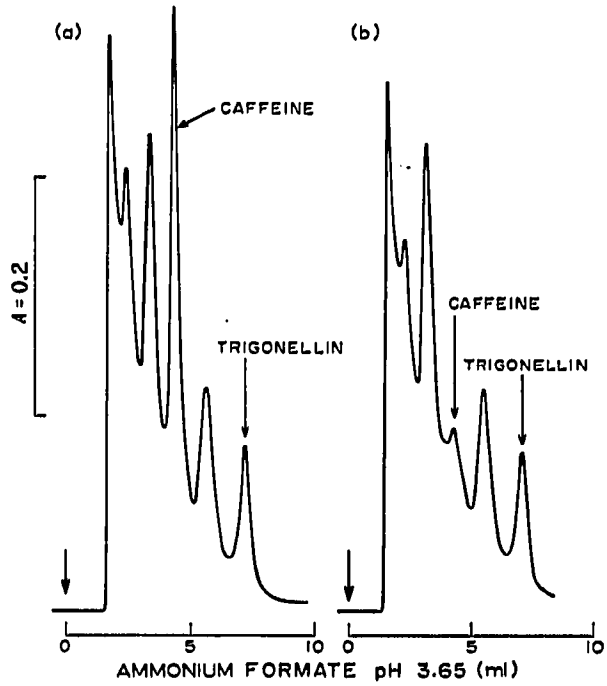


Fig. 8. Chromatograms of (a) freeze-dried and (b) decaffeinated coffee. Same conditions as Fig. 7.

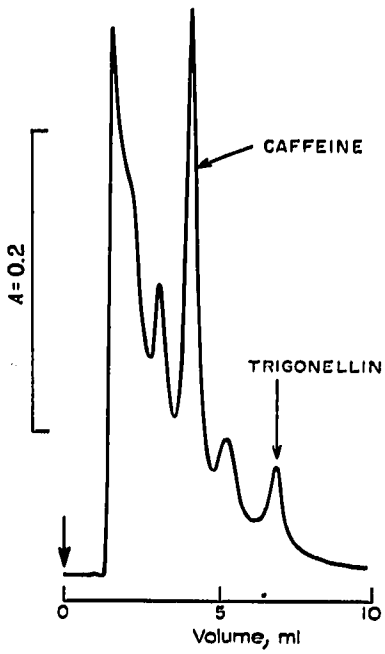


Fig. 9. Chromatogram of dark-roasted Venezuelan coffee. Same conditions as Fig. 7.

The LC of coffee was investigated by Wolford *et al.*[15], using a copper-loaded chelating resin and aqueous ammonia eluent. Their curves resemble our Fig. 2, except that caffeine emerged as the last peak. Their second peak is unidentified, but was probably trigonellin. These workers measured elution volumes of a large number of xanthines and purine derivatives. Comparing our data with theirs, we see that both systems give good chromatography, but the elution orders are different. In our case, acid–base behaviour was superimposed on the affinity of the resin matrix for undissociated aromatic compounds. In their case, the ability of the basic solutes to coordinate with copper ions was superimposed on the affinity of the resin matrix, which also was cross-linked polystyrene, for undissociated organic compounds. The two chromatographic systems thus complement one another for practical analytical use.

## CONCLUSION

We have shown that a 4% cross-linked cation-exchange resin is a versatile stationary phase for the chromatography of UV absorbing organic compounds[16]. The eluent can be electrolyte-free 25% ethanol, or it may contain ammonium salts and acids for buffering purposes. The counter-ion can be $Na^+$ or $NH_4^+$. Solutes of weakly acidic character, with $pK_a$ values greater than 8, are affected greatly by the acidity of the resin, and the use of buffered eluents is desirable in these cases.

The method has been tested by examining the dissolved constituents of boiled coffee. The presence of caffeine, trigonellin and caffeic acid has been demonstrated. Using a formate buffer of pH 3.65, five or six major peaks are obtained with minor peaks that could be characterized at higher resolution. Differences between different kinds of coffee are noticeable. For the quantitative measurement of caffeine and trigonellin an electrolyte-free eluent, with the resin in the ammonium form, would be preferable.

## ACKNOWLEDGEMENT

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