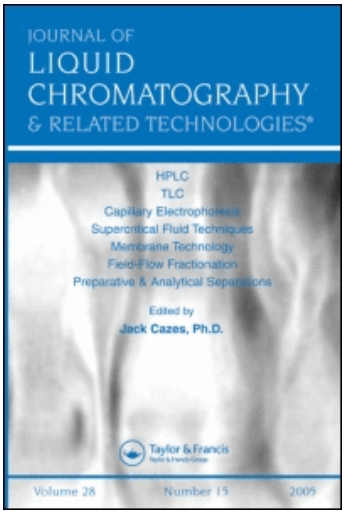

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CHROMATOGRAPHIC BEHAVIOR OF AROMATIC ACIDS ON MACROPOROUS ION-EXCHANGE RESIN*

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ABSTRACT

The chromatographic behaviour of aromatic acids on an anion exchange resin was examined in order to develop a high-speed separation procedure of urinary aromatic acids. The ion-exchange resin was a macro porous anion-exchange resin (5μm particle size). The eluent was a mixture of an ammonium acetate buffer, acetonitrile and octylsodium sulfate. The concentration effect of acetonitrile, ammonium acetate buffer, octylsodium sulfate and temperature effect were discussed. The application of the present chromatographic system to urine analysis was also described.

INTRODUCTION

The retention of aromatic acids on hydrophobic packings has been discussed in relation with the hydrophobicity and the dissociation constant of the solutes in eluents at low pH (1). Ionization of the solutes decreased their retention, and the pH where these acids have the half of their maximum retention corresponded

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to the dissociation constant (pKa) of the acids (2,3). On hydrophobic packings the prediction of their retention time, in a given pH eluent was possible by combining the use of their hydrophobicity expressed as log P and their dissociation constant (pKa). On the other hand, their chromatographic behaviour on ion-exchangers was very complicated due to the inductive effect on ion-ion interaction.

In the present paper the concentration effect of acetonitrile, ammonium acetate, hydrophobic ion and the temperature effect in different pH eluents were examined to find a way to control retention of aromatic acids on an ion-exchange resin. Furthermore this system was applied to urine analysis.

EXPERIMENTAL

A liquid chromatograph was assembled with a Waters model 6000A pump, a Rheodyne model 7125 injector, a Hitachi model 100-20 spectrophotometer with Altex model 155-01, 8μL flow cell, a Hitachi Perkin-Elmer model MPF-4 spectrofluorometer with Aminco model B18-63019, 20μL flow cell, a Brinkman model 2544 and a Linear Instrument model 916 recorders.

The column was 15 cm long, 4.1mm i.d., and packed with a 5μm macro porous strong anion exchange resin (Hitachi 3013N) by slurry method. Analytical grade chemicals were obtained from Eastman Kodak, Chem. Service and Fisher Sci. Inc.. The chemicals listed in Table I were injected into the column without further purification. The acetonitrile was glass distilled UV grade from

Burdick-Jackson and distilled water was treated through a MilliQ system (Millipore Limited).

RESULTS

In reversed-phase mode liquid chromatography, using an non-ionic organic porous polymers such as polystyrene or polymethacryl gels as the packing, the column efficiency was high with eluents rich in organic solvents containing surfactants, due to the excellent swelling of the packing. This high efficiency was also observed at high temperature due to the rapid mass transfer of solutes.

In the ion-exchange mode the pH effect was examined in 0.5M ammonium acetate buffer with 25% acetonitrile and 0.01M octylsodium sulfate at 60°C. The variations of the log k' with pH are shown in Fig. 1.

Generally these acids were separated in about pH 4.5 buffer solution. This pH value is close to their pKas. A change of 0.1 pH unit significantly influenced the retention of acids. At high pH, where the acids are under their ionized conjugate base form the retention became constant due to maximum ion-ion interaction and small pH change did not affect their retention. A mixture of 25% acetonitrile, 0.01M octylsodium sulfate, 0.5M ammonium acetate buffer was selected as the standard eluent and the other parameters were changed to improve the separation of the acids.

Acetonitrile concentration effect. It is well known that when hydrophobic interactions are important and hold solutes on

TABLE I

pH Effect

	Compound	log P	pKa	log k'					
				pH=2.7	4.0	5.0	6.0	7.12	8.44
1	Uric acid	0.12	3.89	-0.617	-0.533	-0.017	0.167	0.222	0.614
2	4-Hydroxy-3-methoxyphenylacetic acid	1.19	4.29*	-0.078	0.049	0.287	0.302	0.291	0.272
3	Hippuric acid	1.21	3.80	0.039	0.385	0.424	0.354	0.341	0.319
4	4-Hydroxy-3-methoxymandelic acid	0.81	3.25*	-0.167	0.340	0.450	0.393	0.383	0.359
5	4-Hydroxy-3-methoxybenzoic acid	1.29	4.48*	0.150	0.250	0.484	0.534	0.544	0.523
6	Phenylacetic acid	1.92	4.25	0.243	0.242	0.583	0.573	0.564	0.551

7	Mandelic acid	1.21	3.85	0.153	0.625	0.766	0.699	0.687	0.677
8	3,4-Dihydroxyphenylacetic acid	0.97	4.20*	-0.015	0.276	0.725	0.804	0.813	0.804
9	Benzoic acid	1.92	4.19	0.389	0.751	0.848	0.840	0.832	0.824
10	3-Indoleacetic acid	1.85	4.75	0.520	0.651	0.888	0.946	0.933	0.937
11	3,4-Dihydroxymandelic acid	0.18	3.50*	-0.039	0.714	0.948	0.940	0.951	0.960
12	3,4-Dihydroxybenzoic acid	0.98	-4.48*	0.232	0.502	1.001	1.103	1.115	1.178
13	2-Hydroxyhippuric acid	1.47	-3.58*	0.328	0.830	0.933	0.933	1.056	1.366
14	2-Naphthoic acid	2.81	4.17	0.942	1.103	1.310	1.302	1.292	1.294

* Experimental conditions. Column: Hitachi 3013N, 5μm macro porous ion-exchange resin packed in 15 cm long, 4 mm i.d., eluent: 0.5M ammonium acetate buffer with 25% acetonitrile and 0.01M octylsodium sulfate, temperature: 60°C, flow rate: 1 mL/min. Log P and pKa were obtained from ref. 1. The pKa (*) were obtained from ref. 8.

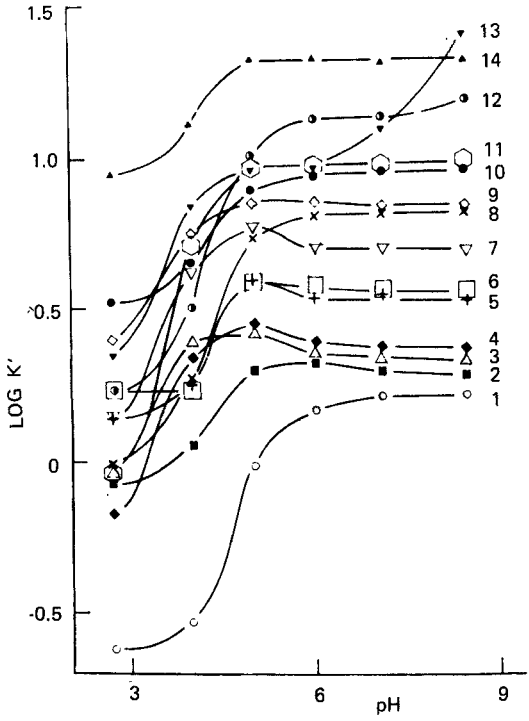

Fig. 1 pH effect
The chromatographic conditions are similar to those in Table I and the numbers next to symbols are those in Table I.

hydrophobic packings, an increase of the concentration of the organic modifier decreases the retention. Conversely, an increase of the organic modifier concentration increases the retention of solutes which are retained by ion-ion interactions. This effect was examined in an eluent at pH 8.4 and the results obtained are shown in Fig. 2.

The retention of polar acids, for which ion-ion interactions are strong, like 3,4-dihydroxy-phenylacetic, 3,4-dihydroxy-

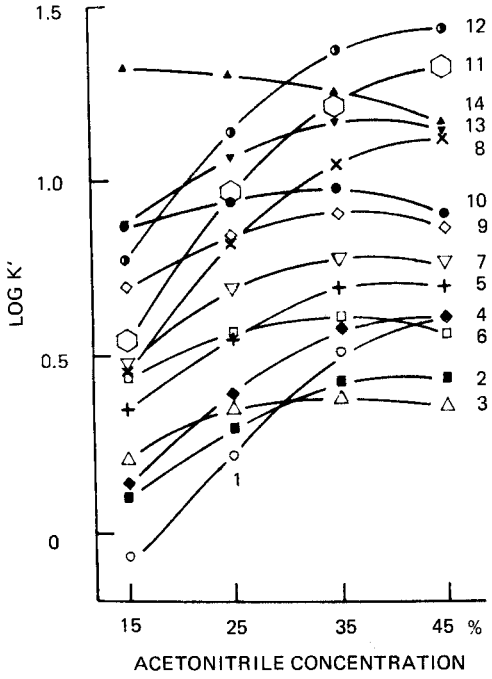

Fig. 2 Acetonitrile concentration effect
The chromatographic conditions are similar to those in Table I and the numbers next to symbols ar those in Table I.

mandelic and 3,4-dihydroxy-benzoic acids increases with acetonitrile concentration. Conversely, the retention of naphtoic acid whose hydrophobicity is high was shortened by addition of acetonitrile. This means, that even if naphtoic acid is completely ionized in this eluent, the hydrophobicity cannot be neglected to discuss its retention. The chromatographic behaviour of the other acids was intermediate between these groups. This phenomenon could be seriously enhanced by the hydrophobicity and ion-exchange capacity of packings. This is a specific character of a

polystyrene- divinylbenzene ion-exchange resin, and would not be observed on ion-exchange cellulose which shows little matrix effect.

Temperature effect. In reversed-phase mode, the retention of solutes was shortened at higher temperature and the tendency was reversed in an ion-exchange mode liquid chromatography. This phenomenon was clearly observed in this system. At pH 2.7 where these acids are almost under their molecular form, the retention was decreased by increasing the column temperature. The results obtained are shown in Fig. 3.

This tendency was weaker for mandelic acids which were only partly ionized in acetonitrile mixtures.

At pH 8.4, the retention of the acids increased at high temperature, except for naphthoic and indoleacetic acids due to their strong hydrophobicity. The results obtained are shown in Fig. 4.

The influence was very significant for polar acids (dihydroxybenzene- carboxylic acids), therefore the selectivity of retention for polar and non-polar acids can be effectively controlled in an isocratic eluent.

Salting-out effect. An interesting phenomenon was observed when the ammonium acetate concentration effect was measured, namely the chromatographic behaviour of dihydroxy- benzenecarboxylic acids. Whereas change in acetonitrile concentration affects very effectively the control of the retention of dihy-

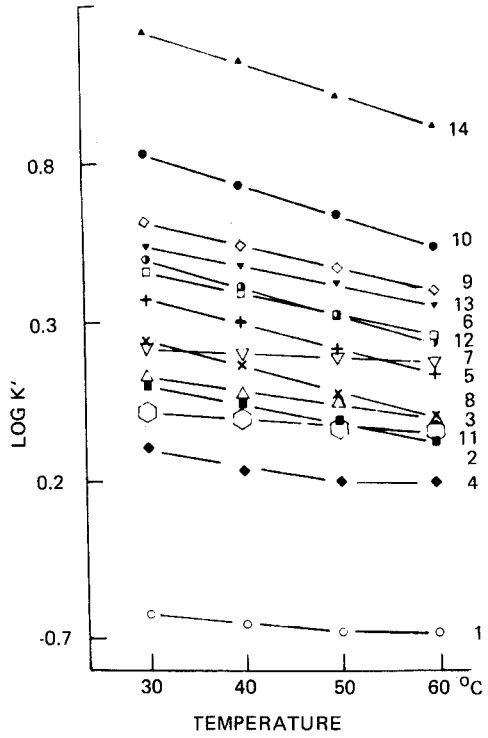

Fig. 3 Temperature effect in pH 2.7 eluent
The chromatographic conditions are similar to those in Table I and the numbers next to symbols ar those in Table I.

droxybenzenecarboxylic acids (Fig.2), the ammonium acetate concentration does not significantly affect their retention. The results obtained are shown in Fig. 5.

In general the organic modifier concentration effect is clearly observed on the chromatographic behaviour of non-ionic compounds and the salt concentration effect is observed on the behaviour of ionic compounds. The later is called salting-out (or-in) effect. In the present system a 0.5M solution of ammo-

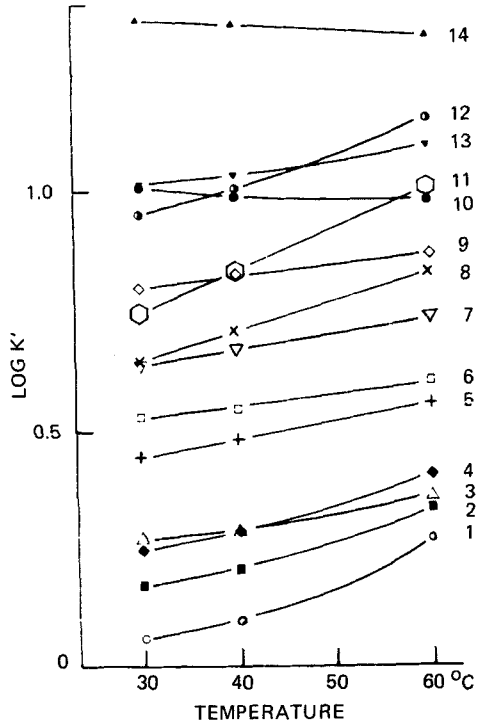

Fig. 4 Temperature effect in pH 8.4 eluent
For chromatographic conditions see Table I. The numbers next to symbols are those in Table I.

nium acetate was however, not strong enough to elute dihydroxybenzenecarboxylic acids from the column. The salting-out effect could be easily observed by using a strong electrolyte like sodium phosphate.

Hydrophobic ion concentration effect. In reversed-phase mode liquid chromatography, the addition of surfactants to the eluent decreases the retention of non-ionic compounds (4), therefore it is expected that anionic surfactant could also control

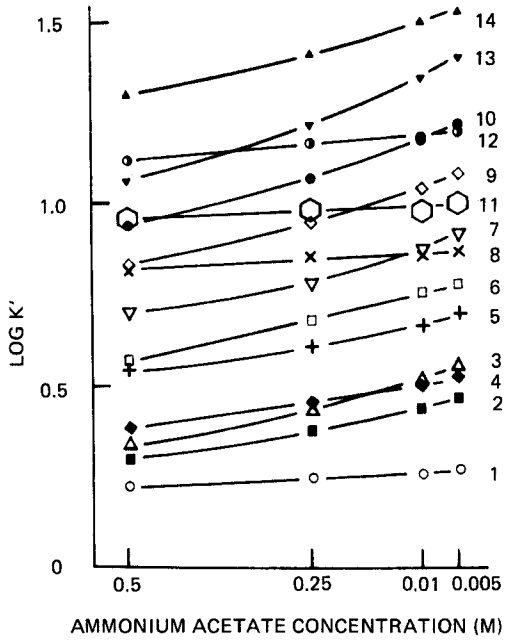

Fig. 5 Salting-out effect
Chromatographic conditions see Table I. The numbers next to symbols are those in Table I.

the retention of hydrophobic compounds on an anion-exchange resin (1).

The addition of a hydrophobic ion in an eluent decreases the ion-exchange capacity and the hydrophocity of the ion-exchange resin. The behaviour of monohydroxy- and dihydroxy- benzenecarboxylic acids were different at pH 8.4. The ammonium acetate concentration change affects the retention of the former, whereas the concentration of octylsodium sulfate affects the retention of the latter. The results are shown in Fig. 6.

Increasing concentration of the hydrophobic ion decreases the retention of hydroxybenzenecarboxylic acids, but this was

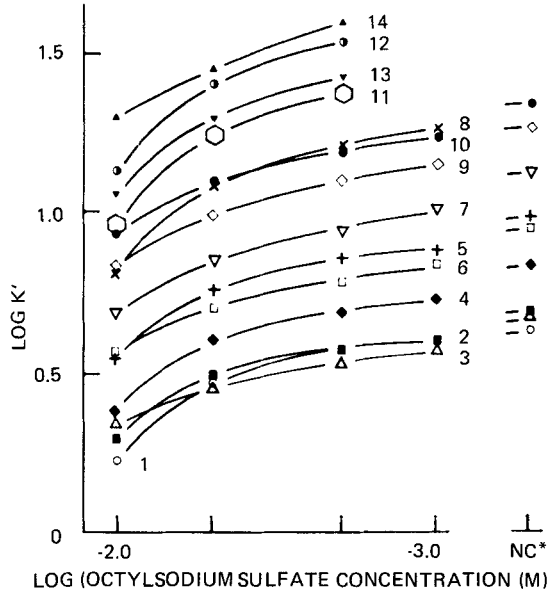

Fig. 6 Hydrophobic ion concentration effect
For chromatographic conditions see Table I and the numbers next to symbols are those in Table I. NC*: No octyl sodium sulfate in this eluent.

less effective for naphthoic acid. This phenomenon may be explained by the fact that the hydrophobic ion covered the ion-exchange group, then indirectly controlled the ion-exchange capacity.

Urine analysis. The present chromatographic system was used for urine analysis. The eluent was selected to obtain a good separation of acids which have a longer retention time than hippuric acid which is a major metabolite in urine and easily separated in ion-exchange chromatography (6). Examples of chromatograms obtained are shown in Figs. 7 and 8, and the capacity ratios are given in Table II.

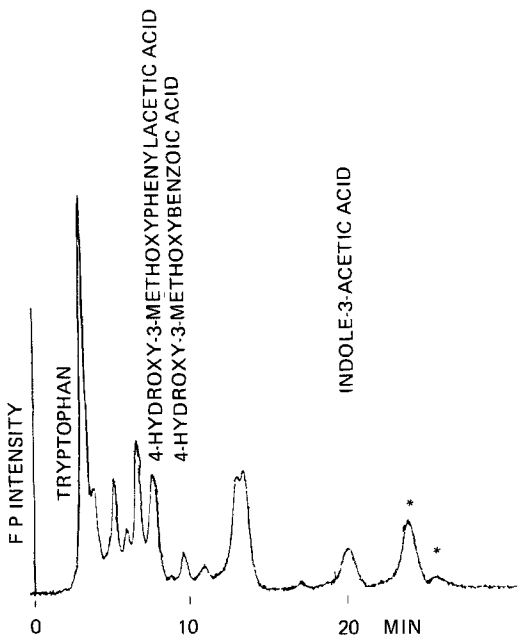

Fig. 7 Chromatograms of a new-born baby's urine.
The chromatographic conditions are in Table II. The flow rate was 0.5 mL/min.

Previously, in a reversed-phase mode with gradient elution, about 20 peaks of fluorescent compounds were observed, and in an ion-exchange mode with gradient elution, eight peaks were observed after hippuric acid (7). In the present system eight major peaks were observed and therefore, this chromatographic system should be useful to analyze fluorescent acids which have strong retention on an ion-exchange resin.

In Fig. 7, a peak was identified as indole-3-acetic acid in 10μL of a new-born baby's urine. In Figs. 8-A and B, one example of chromatograms obtained with different detectors is shown, A)

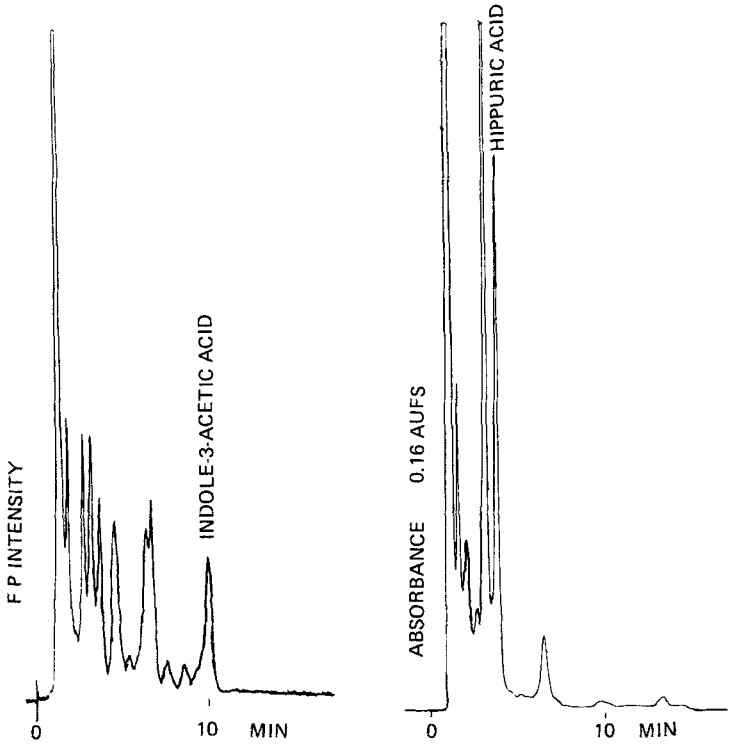

Fig. 8 Chromatograms of a child's urine.
A) Fluorescence detector and B) Ultra-violet detector. the chromatographic conditions are those in Table II.

was a chromatogram obtained with a fluorescence detector. This chromatogram was simpler than that obtained with an ultra-violet detector (chromatogram B) and some biological important compounds were selectively delected. One sample was analyzed within 15 minutes without pretreatment and without gradient elution. The two peaks labelled (*) in Fig. 7 might be protein. After filtration through a 0.45μm filter or passing through an alumina column, these strong fluorescent peaks disappeared.

Table II Retention time and selective detection

Compound	capacity ratio k'	
	a)	b)
5-Hydroxytryptophan	0.28	-
Tryptophan	0.28	-
Hippuric acid	-	2.15
4-Hydroxy-3-methoxyphenylacetic acid	2.30	-
Uric acid	-	2.65
4-Hydroxy-3-methoxycinnamic acid	-	2.88
4-Hydroxy-3-methoxymandelic acid	3.16	-
Phenylacetic acid	-	3.44
4-Hydroxy-3-methoxybenzoic acid	-	3.97
Mandelic acid	-	4.91
Benzoic acid	-	6.14
Indole-3-propionic acid	6.65	-
Indole-3-acetic acid	7.52	-
3,4-Dihydroxyphenylacetic acid	9.20	-
2-Hydroxyhippuric acid	-	10.91
3,4-Dihydroxymandelic acid	13.10	-
2-Naphthoic acid	-	14.40
3,4-Dihydroxybenzoic acid	-	19.58

Experimental conditions. Column: Hitachi 3013N, 5 µm macro porous ion-exchange resin packed in a 15 cm long, 4 mm i.d., eluent: 0.5M ammonium acetate buffer with 35% acetonitrile and 0.01M octylsodium sulfate, temperature: 60°C, flow rate: 1 mL/min.

a) Fluorescence detector (Excitation wavelength: 280 nm, Emission wavelength: 315 nm).

b) UV absorption detector (254 nm).

CONCLUSION

The retention mechanism of aromatic acids on an anion-exchange resin is a combination of hydrophobic and ion-ion interactions. The determining interaction depends upon the structure of solutes and packings. The retention of a highly hydrophobic acid like naphthoic acid was significantly influenced by the concentration of the organic modifier.

An interesting chromatographic behaviour was found for dihydroxybenzenecarboxylic acids. The retention of these acids increased in high pH eluents at high temperature probably due to strong ion-ion interactions. Increasing the ammonium acetate concentration did not significantly affect the retention of these acids, however the retention was strongly decreased by addition of octylsodium sulfate.

The monohydroxybenzenecarboxylic acids follow a common tendency in ion-exchange chromatography: salting-out, temperature, pH and acetonitrile concentration effects were observed.

The theoretical plate number of this column was about 3000 per 15cm long at a 1 mL/min flow rate. This performance and its chemical stability could be very useful in a clinical laboratory.

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