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CHROMATOGRAPHIC BEHAVIOUR OF AROMATIC AMINO ACIDS ON THIN LAYERS OF ANION AND CATION EXCHANGERS

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SUMMARY

The chromatographic behaviour of 29 aromatic amino acids (18 sulphonic, 9 carboxylic and 2 arsonic) on both cellulose and polystyrene-based anion and cation exchangers was studied. The influence of the protonation of the amino group on the selectivity of the polystyrene-based anion and cation exchangers towards such compounds was investigated, and the influence of the addition of ethanol to the aqueous eluent on the cation exchanger in the acid form was also studied.

INTRODUCTION

Ion-exchange chromatography on polystyrene-based ion exchangers has certain limitations when applied to the separation of substances that contain one or more aromatic rings owing to the strong adsorption by the matrix of the resin towards the hydrophobic portion of such molecules. It has been attempted to overcome this disadvantage by eluting with aqueous–organic solvents[1–3] and/or by using ion exchangers with matrices that differ from the polystyrene matrix[4,5].

As recent studies with some aromatic amines on Porapak Q columns[6] showed that their adsorption decreased as a result of protonation of the amino group, we thought it would be useful, on the basis of this characteristic, to study the behaviour of aromatic amino acids on anion exchangers. The behaviour of these compounds has also been studied on strong and weak cation exchangers.

EXPERIMENTAL

Solutions were obtained by dissolving the acids in water to give concentrations of 1–2 mg/ml. The compounds were detected by spraying with a solution of 5% *p*-dimethylaminobenzaldehyde in a 5:1 mixture of ethanol and glacial acetic acid.

Preparation of thin layers

Thin layers of AG 1-X4 (ClO_4^-), Dowex 50-X4 (H^+ and Na^+), CMCNa*, Rexyn 102 (Na^+) and alginic acid were prepared as described in previous papers[6,7]. With alginic acid, Carlo Erba cellulose was added because it gave a lower elution rate.

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* CMCNa = Sodium carboxymethylcellulose.

DEAE-cellulose (Cellex D) layers were prepared by mixing 6 g of exchanger in 40 ml of water. Cellex D (Bio-Rad Labs., Richmond, Calif., U.S.A.), like the above exchangers, was rinsed with water and methanol and then dried at room temperature.

The chromatographic measurements were carried out at 25°. The migration distance was 11 cm unless otherwise stated.

RESULTS AND DISCUSSION

Anion exchangers

AG 1-X4 (ClO₄⁻). This resin was used in the perchlorate form in order to obtain an exchanger with the smallest affinity for the aromatic acids. It is known that perchlorate is the ion most strongly retained by the resin in comparison with other anions with the same charge.

When eluting with sodium acetate, sodium perchlorate and buffer solutions (acetate buffer), most of the acids remained at the start and those which ran gave rise to elongated spots. With acidic eluents, however, a differentiated migration with the formation of compact spots was obtained, while with dilute solutions of mineral acids, the formation of a double front, the first of which is due to the acid, was observed. In order to eliminate this drawback, which was particularly evident when eluting with 0.01 M perchloric acid, we used solutions with a constant concentration of a weak acid and a variable concentration of a strong acid.

In Table I are given the R_F values of 29 aromatic amino acids on AG 1-X4 (ClO₄⁻) layers when eluting with 1 M acetic acid with perchloric acid concentrations between 0.01 and 0.5 M. As the concentration of the mineral acid in the eluent increased, for most compounds an increase in R_F value was observed, whereas those of 3,5-diaminobenzoic acid and, for perchloric acid concentrations $\geqslant$ 0.05 M, of p-amino-hippuric acid, did not change. An exception to this general behaviour was observed with m-aminobenzoic acid, 5-aminosalicylic acid, 3,4-diaminobenzoic acid and 4,4′-diaminodiphenylamine-2-sulphonic acid, whose R_F values decreased as the perchloric acid concentration increased.

The behaviour of the acids whose R_F values are $\geqslant$ 0.8 in 1 M acetic acid, can be explained only with difficulty, as an increase in the perchloric acid concentration should cause the R_F values either to increase or to remain constant according to whether the acid and basic functional groups are only partly or completely protonated in 1 M acetic acid. The addition of perchloric acid seems, therefore, to cause a decrease in the solubility of these acids in the mobile phase owing to the increased ionic strength of the eluent. This assumption seemed to be confirmed by the analogous behaviour of several organic acids (including the above four acids) when eluting with 1 M acetic acid alone or with mixtures of 1 M acetic acid and sodium perchlorate or chloride, that is, in the case of eluents with the same proton activity and different ionic strength.

Many separations can be predicted on the basis of the R_F values. The following mixtures of isomers were successfully separated: orthanilic, sulphanilic and methanilic acids; m-aminobenzoic and o-aminobenzoic (or p-aminobenzoic) acids; o-arsanilic and p-arsanilic acids; 4-aminosalicylic and 5-aminosalicylic acids; and 6-amino-m-toluenesulphonic and 4-amino-m-toluenesulphonic acids.

Interesting separations can also be obtained of mixtures of compounds that contain two aromatic rings (4,4′-diaminodiphenylamine-2-sulphonic and 4,4′-diamino-

TABLE I

R_F VALUES OF AROMATIC ACIDS ON THIN LAYERS OF AG 1-X4 (ClO_4^-)

Acetic acid + perchloric acid solutions as eluents; acetic acid concentration 1 M.

Substance	Concentration of $HClO_4$ (M)			
	0.01	0.05	0.1	0.5
Orthanilic acid	0.01	0.12	0.25	0.62
Sulphanilic acid	0.09	0.53	0.69	0.80
Methanilic acid	0.21	0.66	0.74	0.82
o-Aminobenzoic acid	0.28	0.55	0.65	0.80
p-Aminobenzoic acid	0.29	0.59	0.68	0.80
m-Aminobenzoic acid	0.88	0.88	0.86	0.83
o-Arsanilic acid	0.35	0.49	0.52	0.70
p-Arsanilic acid	0.65	0.70	0.81	0.92
4-Aminosalicylic acid	0.06	0.21	0.26	0.31
5-Aminosalicylic acid	0.81	0.83	0.82	0.74
p-Aminohippuric acid	0.69	0.88	0.88	0.88
6-Amino-m-toluenesulphonic acid	0.01	0.26	0.41	0.65
4-Amino-m-toluenesulphonic acid	0.04	0.43	0.62	0.78
4-Amino-2-chloro-m-toluenesulphonic acid	0.00	0.00	0.04	0.39
3-Amino-4-chlorobenzenesulphonic acid	0.00	0.00	0.06	0.47
2-Aminophenol-4-sulphonic acid	0.35	0.67	0.70	0.82
3-Amino-4-methoxy-benzenesulphonic acid	0.19	0.66	0.76	0.83
2,4-Dimethylaniline-5-sulphonic acid	0.33	0.66	0.69	0.80
3,4-Diaminobenzoic acid	0.81	0.80	0.79	0.72
3,5-Diaminobenzoic acid	0.84	0.88	0.88	0.89
2,4-Diaminobenzenesulphonic acid	0.15	0.50	0.60	0.80
2,5-Diaminobenzenesulphonic acid	0.69	0.72	0.77	0.80
4-Aminodiphenylamine-2-sulphonic acid	0.03	0.04	0.06	e.s.[*]
4,4'-Diaminodiphenylamine-2-sulphonic acid	0.88	0.88	0.87	0.86
4,4'-Diaminodiphenyl-3,3'-dicarboxylic acid	0.00	0.01	0.05	0.19
4,4'-Diaminodiphenyl-3-sulphonic acid	0.02	0.11	e.s.[*]	e.s.[*]
4,4'-Diaminodiphenyl-2,2'-disulphonic acid	0.20	0.78	0.82	0.94
1-Naphthylamine-4-sulphonic acid	0.00	0.02	0.07	e.s.[*]
1-Naphthylamine-7-sulphonic acid	0.01	0.02	0.07	e.s.[*]

[*] e.s. = elongated spot.

diphenyl-3-sulphonic acids; and 4,4'-diaminodiphenyl-2,2'-disulphonic and 4,4'-diaminodiphenyl-3,3'-dicarboxylic acids), although such compounds, with the exception of 4,4'-diaminodiphenylamine-2-sulphonic acid and 4,4'-diaminodiphenyl-2,2'-disulphonic acid, gave rise to elongated spots as the perchloric acid concentration increased.

Cellex D (ClO_4^-). On this exchanger, sodium perchlorate solutions with a concentration between 0.02 and 0.5 M were used as eluents. It was not possible to use acidic eluents as the acetic acid solutions rise irregularly for a distance of a few centimetres.

With mineral acid solutions, a regular migration of the eluent was obtained; however, the formation of a double front was observed. When eluting with 0.05 M perchloric acid, for instance, the first front had $R_F = 0.33$; this R_F value was the same for almost all of the aromatic acids. With respect to the AG 1-X4 layers, Cellex D has a smaller selectivity for the different compounds, with the exception of those that

contain two aromatic rings or a chlorine atom in the molecule. The R_F values were very similar in most instances, with an average difference of less than $\pm$ 0.05 unit. From an analytical point of view, therefore, this exchanger is not very suitable, even if the compactness of the spots may favour some separations. For example, consider the separation between 3-amino-4-chlorobenzenesulphonic and 4-amino-2-chloro-*m*-toluenesulphonic acids, and that between 1-naphthylamino-4-sulphonic and 1-naphthylamino-7-sulphonic acids, whose R_F values differ by 0.15 and 0.14 unit, respectively, as shown by the data in Table II.

TABLE II

SEPARATIONS OBTAINED ON THIN LAYERS OF CELLEX D (ClO_4^-)

Elution with 0.1 M NaClO$_4$.

Substance	R_F value
m-Aminobenzoic acid*	0.73
4- or 5-aminosalicylic acid	0.42
3-Amino-4-chlorobenzenesulphonic acid	0.62
4-Amino-2-chloro-*m*-toluenesulphonic acid	0.47
1-Naphthylamine-4-sulphonic acid	0.35
1-Naphthylamine-7-sulphonic acid	0.21
4,4'-Diaminodiphenyl-2,2'-disulphonic acid	0.68
4-Aminodiphenylamine-2-sulphonic acid	0.37
4,4'-Diaminodiphenyl-3-sulphonic acid	0.15
4,4'-Diaminodiphenyl-3,3'-dicarboxylic acid	0.03

* *m*-Aminobenzoic acid can be replaced with *p*- and *o*-aminobenzoic acid, *p*-, *m*- and *o*-aminobenzenesulphonic acid, *p*-aminohippuric acid (R_F range 0.70–0.75).

In Table II are also reported two other separations, the more important of which is that concerning the four acids that contain two aromatic rings. It is useful to note that this separation cannot be obtained on thin layers of AG 1-X4.

Influence of complex formation on the chromatographic behaviour of the aromatic acids. In the separation of aliphatic acids on polystyrene-based anion exchangers, Samuelson and co-workers[8-10] have shown the advantages that result from the formation of complexes between the metal ions in the eluent and the compounds to be separated. Bearing in mind the possibilities of the formation of complexes between the aromatic amino acids and divalent ions (Cu(II), Zn(II) and Mg(II)), we investigated the chromatographic behaviour of the acids when eluting with solutions of divalent ions. On AG 1-X4 layers, Cu(II) gave the best results as a ligand agent. In particular, as the chromatograms in Fig. 1 show, it was possible to obtain the separation of the isomers of aminobenzoic acid by eluting with copper(II) perchlorate of concentration 0.25 M in 0.5 M acetate buffer solution. It should be noted that this separation was not possible either on AG 1-X4 layers by eluting with acetic plus perchloric acid solutions (see Table I) or on Cellex D layers by eluting with sodium perchlorate or buffer solutions. It should be noted, however, that when eluting with Cu(II) solutions, the spots were generally elongated even if the elongation may be due, almost entirely, to the pH of the eluent.
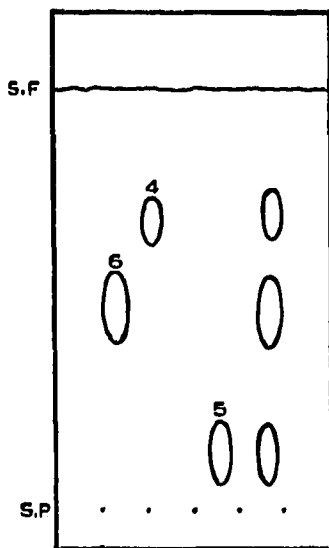
Fig. 1. Thin-layer chromatogram of aminobenzoic acids on AG1-X4 (ClO₄⁻). Eluent: 0.5 M acetic acid buffer + 0.25 M Cu(ClO₄)₂. Spots: 4, o-aminobenzoic acid; 5, p-aminobenzoic acid; 6, m-aminobenzoic acid.

From a comparison between the sequence of R_F values obtained when eluting with Cu(II) solutions and that relative to mineral acids, it was noted that, of the isomers of aminobenzoic acid, the *ortho*-form was the least retained because, as could be predicted from the position of the amino substituent in the ring, it gave rise to the most stable complex with the metal ion. This characteristic can be used for analytical purposes when the *ortho*-form, in the absence of complexing agent, is retained less than, the same as or a little more than the other isomers. When, on the contrary, the *ortho*-form is retained so strongly that its separation from the other isomers is possible without the use of a complexing agent, as with the two arsanilic acids, the addition of Cu(II) ions to the eluent may render the separation impossible, because the R_F values become closer as the concentration of the eluent increases. At a Cu(II) concentration of 0.25 M, o- and p-arsanilic acids can no longer be separated.

On Cellex D layers, the use of Cu(II) solutions as eluent was unsatisfactory owing to the formation of a double front due to the strong adsorption by the exchanger of Cu(II) ions.

Cation exchangers

In order to obtain a complete picture of the chromatographic behaviour of aromatic amino acids, we also considered polystyrene-based (Dowex 50-X4), cellulose-based (CMCNa and alginic acid) and paraffin-based (Rexyn 102) cation exchangers, in both the acid and sodium salt forms.

Dowex 50-X4 (Na⁺), CMCNa and Rexyn 102 (Na⁺). In Fig. 2 are reported some separations obtained on Dowex 50-X4 (Na⁺) layers when eluting with 0.5 M acetate buffer. It is interesting to note that on this exchanger the acids that contain a sulphonic or arsonic group (or two sulphonic groups if the molecule contains two aromatic rings) were the least retained ($R_F > 0.90$). An exception is 4-amino-2-chloro-
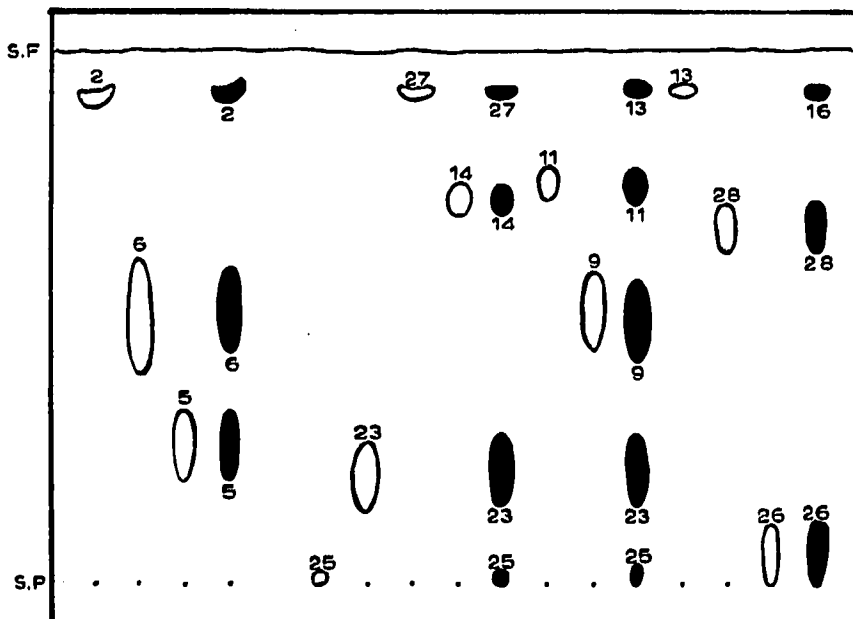
Fig. 2. Thin-layer chromatogram of aromatic acids on Dowex 50-X4 (Na⁺). Migration distance 14 cm. Eluent: 0.5 M acetic acid buffer. Spots: 2, sulphanilic acid; 5, p-aminobenzoic acid; 6, m-aminobenzoic acid; 9, 4-aminosalicylic acid; 11, p-aminohippuric acid; 13, 4-amino-m-toluenesulphonic acid; 14, 4-amino-2-chloro-m-toluenesulphonic acid; 16, 2-aminophenol-4-sulphonic acid; 23, 4-aminodiphenylamine-2-sulphonic acid; 25, 4,4′-diaminodiphenyl-3,3′-dicarboxylic acid; 26, 4,4′-diaminodiphenyl-3-sulphonic acid; 27, 4,4′-diaminodiphenyl-2,2′-disulphonic acid; 28, 1-naphthylamine-4-sulphonic acid.

m-toluenesulphonic acid, which was retained more strongly (R_F = 0.72), probably owing to the presence of a chlorine atom in the molecule[6].

CMCNa and Rexyn 102 (Na⁺) layers did not give satisfactory results because on these exchangers, when eluting with 0.1 M acetate buffer solution, the aromatic amino acids had R_F values ⩾ 0.80, with the exception of 4,4′-diaminodihpenyl-3,3′-dicarboxylic and 4,4′-diaminodiphenyl-3-sulphonic acids.

Alginic acid. The three aminobenzoic acids, the two arsanilic acids and sulphanilic acid have already been studied on this exchanger[11], so we extended our study to the series of the aromatic amino acids. Acetic acid (1 M) and acetic and perchloric acid mixtures were used as eluents in order to decrease the pH gradient along the layer. On alginic acid, compact spots were obtained also for acids with two aromatic rings in the molecule, unlike the result on Dowex 50-X4 (Na⁺).

As regards the separations that can be obtained on this layer, in Table III are reported those of acids with two aromatic rings; their separation on polystyrene-based exchangers was more difficult owing to strong adsorption, which led to the formation of elongated spots.

Dowex 50-X4 (H⁺). No appreciable differences were observed in comparison with Dowex 50-X4 (Na⁺). In particular, those acids which ran virtually with the solvent front on the exchanger in the sodium form, showed very high R_F values (⩾ 0.72)

TABLE III

SEPARATIONS OBTAINED ON THIN LAYERS OF ALGINIC ACID

Substance	Eluent	R_F value
1-Naphthylamine-7-sulphonic acid	1 M AcOH	0.66
1-Naphthylamine-4-sulphonic acid		0.84
4,4'-Diaminodiphenyl-3,3'-dicarboxylic acid	1 M AcOH	0.00
4,4'-Diaminodiphenyl-3-sulphonic acid		0.13
4,4'-Diaminodiphenyl-2,2'-disulphonic acid		0.78
3,5-Diaminobenzoic acid	1 M AcOH	0.29
3,4-Diaminobenzoic acid	0.1 M HClO$_4$	0.42
4,4'-Diaminodiphenyl-3,3'-dicarboxylic acid	1 M AcOH	0.19
4,4'-Diaminodiphenylamine-2-sulphonic acid	0.1 M HClO$_4$	0.41
4-Aminodiphenylamine-2-sulphonic acid		0.68

on the exchanger in the acid form also for concentrations of hydrochloric acid in the eluent lower than that reported in Table IV (column 1).

Acids that have an $R_F \leqslant 0.48$ when eluting with 2 M hydrochloric acid gave rise to spots which became more elongated as the acid concentration exceeded 2 M. Typical behaviour is that of 4-amino-2-chloro-m-toluenesulphonic acid, which was less retained when eluting with 1 M acetic acid ($R_F = 0.82$) than with 0.5 M hydrochloric acid ($R_F = 0.55$). This behavior is probably due to the increasing protonation of the amino group as the pH decreased. As the hydrochloric acid concentration increased, the R_F value of 4-amino-2-chloro-m-toluene sulphonic acid did not change until the concentration reached 2 M. The same behaviour was shown by the two naphthylaminosulphonic acids and by 4-aminodiphenylamine-2-sulphonic acid, and it can be explained by assuming that the ion-exchange reaction becomes more irreversible owing to the strong adsorption towards such compounds during an ion-exchange process.

Use of water–ethanol mixtures as eluents

Pietrzyk[12] observed that compounds that contain basic groups (the amino group in this work) bind the cation exchangers in the acid form according to the following relationship:

$$R-SO_3^- \; H^+ + B = R-SO_3^- \cdot BH^+ \tag{1}$$

The equilibrium of eqn. 1 is strongly influenced by the type of the solvent and, with water–ethanol mixtures, may be displaced towards the left when the concentration of the more basic component (*i.e.*, water) is increased. This principle has recently been used for the column separation of nitro- and chloroanilines, sulpha drugs and purines[13]. As the results achieved seemed to be promising, we considered it useful to study the chromatographic behaviour of the aromatic amino acids on alginic acid and Dowex 50-X4 (H$^+$), eluting with water–ethanol mixtures in different proportions.

On alginic acid, no satisfactory results were obtained because, owing to the high elution rate, the spots were greatly elongated. On Dowex 50-X4 (H$^+$) layers, however, the rate of elution with the aqueous–organic eluent is much less than that of aqueous solutions so that compact spots are obtained with an elution time of about 4 h. The results are reported in Table IV (columns 2, 3 and 4).

TABLE IV

R_F VALUES OF AROMATIC ACIDS ON THIN LAYERS OF DOWEX 50-X4 (H⁺)

Eluents: 1, 2 N hydrochloric acid; 2, ethanol–water (9:1); 3, ethanol–water (4:1); 4, ethanol–water (1:1).

Substance	Eluent			
	1	2	3	4
Orthanilic acid	0.83	0.67	0.90	0.90
Sulfanilic acid	0.87	0.43	0.70	0.84
Methanilic acid	0.87	0.42·	0.66	0.82
o-Aminobenzoic acid	0.29	0.05	0.08	0.03
p-Aminobenzoic acid	0.21	0.00	0.01	0.03
m-Aminobenzoic acid	0.29	0.00	0.00	0.00
o-Arsanilic acid	0.47	0.39	0.38	0.24
p-Arsanilic acid	0.38	0.00	0.00	0.00
4-Aminosalicylic acid	0.18	0.11	0.09	0.06
5-Aminosalicylic acid	0.22	0.00	0.00	0.00
p-Aminohippuric acid	0.42	0.00	0.00	0.00
6-Amino-m-toluenesulphonic acid	0.72	0.70	0.88	0.89
4-Amino-m-toluenesulphonic acid	0.87	0.55	0.67	0.79
4-Amino-2-chloro-m-toluenesulphonic acid	0.56	0.82	0.92	0.92
3-Amino-4-chlorobenzenesulphonic acid	0.83	0.82	0.92	0.92
2-Aminophenol-4-sulphonic acid	0.86	0.39	0.62	0.83
3-Amino-4-methoxybenzenesulphonic acid	0.86	0.40	0.64	0.83
2,4-Dimethylaniline-5-sulphonic acid	0.86	0.56	0.75	0.80
3,4-Diaminobenzoic acid	0.14	0.00	0.00	0.00
3,5-Diaminobenzoic acid	0.25	0.00	0.00	0.00
2,4-Diaminobenzenesulphonic acid	0.78	0.12	0.23	0.39
2,5-Diaminobenzenesulphonic acid	0.78	0.07	0.10	0.21
4-Aminodiphenylamine-2-sulphonic acid	0.29	0.53	0.80	0.69
4,4'-Diaminodiphenylamine-2-sulphonic acid	0.21	0.02	0.01	0.01
4,4'-Diaminodiphenyl-3,3'-dicarboxylic acid	0.01	0.00	0.00	0.00
4,4'-Diaminodiphenyl-3-sulphonic acid	0.13	0.01	0.02	0.02
4,4'-Diaminodiphenyl-2,2'-disulphonic acid	0.87	0.02	0.15	0.73
1-Naphthylamine-4-sulphonic acid	c.s.*	0.63	0.86	0.89
1-Naphthylamine-7-sulphonic acid	0.48	0.52	0.75	0.79

* c.s. = elongated spot.

The compounds can be divided into three groups according to their chromatographic behaviour. The first group is formed by the compounds that remain at the origin because, as they are strongly retained by the resin, they are not displaced by the water. o-Aminobenzoic acid, 4-aminosalicylic acid and, for ethanol concentrations < 80%, 4-aminodiphenylamine-2-sulphonic acid belong in the second group, the R_F values of which decrease as the concentration of water in the eluent increases. The behaviour of such acids is clearly due to a decrease of their solubility in the eluent as the ethanol concentration decreases. The third group is formed by the acids whose R_F values increase with increasing water concentration in the eluent, according to the shifting of the equilibrium towards the formation of the free base (see eqn. 1).

There are many separations that can be predicted on the basis of the R_F values, and we effected the following: orthanilic and sulphanilic (or methanilic) acids: o-arsanilic and o-aminobenzoic (or m- and p-aminobenzoic) acids; o- and p-arsanilic

acids; 6- and 4-amino-*m*-toluenesulphonic acids; 3-amino-4-methoxybenzenesulphonic and 2,4-dimethylaniline-5-sulphonic acids; and 2,4- and 2,5-diaminobenzenesulphonic and 4-aminodiphenylamine-2-sulphonic acids.

CONCLUSIONS

From the results obtained on the chromatographic behaviour of aromatic amino acids on polystyrene-based anion and cation exchangers under the same elution conditions, it is possible to draw some interesting conclusions about the role in the retention process played by the protonation of the amino group.

On anion exchangers, in particular, the separation of the three aminobenzenesulphonic acids on the basis of the difference in the degree of protonation of the amino group is possible with mixtures of 1 M acetic acid and 0.05 M perchloric acid (orthanilic acid, pK_a = 2.48; methanilic acid, pK_a = 3.24; sulphanilic acid, pK_a = 3.75)[14]. With the same eluent on Dowex 50-X4 (H^+), it is not possible to effect such a separation. As the only difference between the two cases is the direct sharing of the $-NH_3^+$ group in the ion-exchange process on the cation exchanger and the decrease in the affinity of the hydrophobic portion of the molecule towards the exchanger matrix on the anion exchanger, it follows that in order to increase the differences in the chromatographic behaviour of the aromatic amino acids, it is better to influence the degree of protonation of the functional group that is not directly involved in the ion-exchange process.

As regards the parameters that determine the selectivity in the ion-exchange process, that is, interactions of the side-chain of the organic ion with the solvent and the exchanger matrix, the latter (*i.e.*, interactions with the exchanger matrix) seems more likely to occur owing to the different behaviour of the aromatic amino acids on the cation exchanger compared with that on the anion exchanger under the same elution conditions.

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