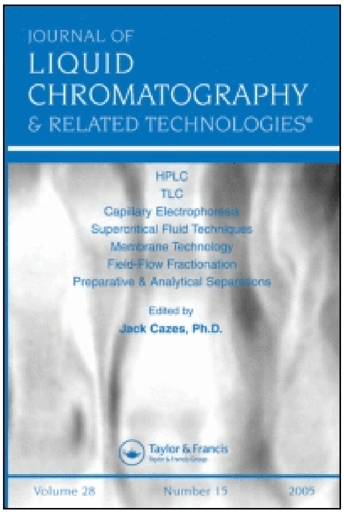

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RETENTION BEHAVIOR AND SIMULTANEOUS SEPARATION OF CARBOXYLIC AND AROMATIC ACIDS USING ION-EXCLUSION CHROMATOGRAPHY

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RETENTION BEHAVIOR AND SIMULTANEOUS SEPARATION OF CARBOXYLIC AND AROMATIC ACIDS USING ION-EXCLUSION CHROMATOGRAPHY

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ABSTRACT

Ion-exclusion chromatography using a high-capacity sulphonated polystyrene-divinylbenzene (PS-DVB) resin was evaluated for the separation of a range of carboxylic and aromatic acids. The parameters affecting the retention behaviour of these acids were examined in relation to their chemical structure. Results obtained on the ion-exclusion column for the separation of aromatic acids were similar to those obtained on a C_{18} column. The ion-exclusion column exhibited both the ion-exclusion effect for the separation of hydrophilic carboxylic acids and the hydrophobic interaction for the separation of aromatic acids, which offered the possibility of simultaneous separation of carboxylic and aromatic acids by careful selection of separation conditions such as column temperature and content of organic modifier.

INTRODUCTION

Ion-exclusion chromatography (IEC) using a polystyrene-divinylbenzene (PS-DVB) column has been widely used for the separation of carboxylic acids, carbohydrates and alcohols in biotechnology and food applications,[1] where separation is based on size exclusion effect, hydrophobic interactions, and the ion-exclusion effect.[2] In general, the retention behaviour of solutes depends on their first dissociation constant (pKa1) and their hydrophbicity,[2] as well as on the separation conditions such as content of organic modifier and column temperature. There are many applications of ion-exclusion chromatography using PS-DVB columns for separation of carboxylic acids. However, hydrophobic acids such as the higher aliphatic carboxylic acids and aromatic acids are strongly retained on the column due to hydrophobic interactions.[3-6] This problem is usually overcome by the additions of organic modifiers to the eluent. Such additions include organic modifier,[3] and low concentrations of polyalcohols or sugars.[5-6] Therefore, ion-exclusion chromatography with PS-DVB resins are mainly used for the separation of either hydrophilic or weakly hydrophobic acids.

An alternative is to use the inherent hydrophobic characteristics of the PS-DVB resin to separate hydrophobic acids such as aromatic acids. To date, there are few reports on the separation of hydrophobic acids using a PS-DVB column. Medved et al.[7] reported the ion-exclusion chromatographic separation of phenol carboxylic acids using a benzoic acid eluent with conductometric detection, but the resolution between solutes was poor due to their using the unsuitable conditions. More recently, Glod and Perez[8] have suggested that aromatic acidic compounds are characterized by longer retention times than predicted by the pure ion-exclusion mechanism, which resulted in hydrophobic interaction between solute and resin. However, this requires systematic studies on the retention of a range of solutes, including both carboxylic and aromatic acids.

In this paper, the retention behaviour of carboxylic and aromatic acids on the PS-DVB column were investigated systematically to examine the ion-exclusion and hydrophobic interaction characteristics. The result of this work should lead to new applications, e.g., simultaneous separation of carboxylic and aromatic acids. Therefore, we investigated the relationship between retention of solutes and their chemical structure, and parameters which determine solute retention, including the concentrations of sulfuric acid and organic modifier in the eluent, and column temperature, as well as comparison of the retention behaviour of aromatic acids on the C_{18} column.

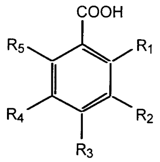
EXPERIMENTAL

All experiments utilized a fully automatic Waters Liquid chromatography system including an autosampler (717), a temperature controller (626), a UV detector (486) and computer-based Millennium[32] software. A Bio-Rad cation guard column and an HPX-87 organic acid column (300 x 7.8 mm I.D, Richmond, CA, USA) were used for separation of the test acids. The column was equilibrated for 60 min prior to use and 10 µL of standard solution was injected into the chromatographic system.

Table 1

pKa_1 and Chemical Structure of the Test Organic Acids

Solute	pKa_1	Chemical Structure
HNO_3	-1.5	HNO_3
Oxalic	1.27	HOOC-COOH
Citric	3.13	$(HOOC{=}CH_2)_2C(OH)COOH$
Tartaric	3.04	$HOOCCH_2CH(OH)CH(OH)COOH$
Malic	3.46	$HOOCCH_2CH(OH)COOH$
Succinic	4.21	$HOOCCH_2CH_2COOH$
Glutaric	4.34	$HOOC(CH_2)_3COOH$
Fumaric	3.03	HOOCCH=CHCOOH
Pyruvic	2.49	$H_3CCOCOOH$
Formic	3.75	HCCOH
Acetic	4.76	H_3CCOOH



		R1	R2	R3	R4	R5
Benzoic	4.19	H	H	H	H	H
4-Hydroxybenzoic	4.48	H	H	OH	H	H
2,5-dihydroxybenzoic	2.97	OH	H	H	OH	H
3,4-dihydroxybenzoic	4.48	H	OH	OH	H	H
Gallic	4.41	H	OH	OH	OH	H
Phthalic	2.95	COOH	H	H	H	H
Trimesic	3.10	COOH	H	COOH	H	H
Pyromellitic	1.80	COOH	H	COOH	COOH	H
Phenol	9.70	C_6H_5OH				

Standard solutions of carboxylic and aromatic acids were prepared using analytical grade chemicals without further purification. Standards of aliphatic and aromatic acids were prepared daily from a 10 mM stock solution in Milli-Q water and diluted to the required concentrations before use. The mobile phase required for ion-exclusion chromatography was prepared by dissolution of an appropriate concentration of sulfuric acid and acetonitrile in Milli-Q water. All eluents were filtered through a Millipore 0.45 µm membrane filter and degassed in an ultrasonic bath prior to use.

RESULTS AND DISCUSSION

Retention Behaviour of the Test Acids and Their Chemical Structures

The relationship between retention and chemical structure of the test acids on the PS-DVB resins were examined to determine if their retention was governed by the ion-exclusion effect or hydrophobic interactions. A range of organic acids were selected as the target solutes, including both hydrophilic carboxylic acids (mono- and di- carboxylic acids) and hydrophobic aromatic acids (benzylcarboxylic and phenolcarboxylic acids). The pKa of solutes together with their molecular structure[9] are listed in Table 1.

The retention of the carboxylic acids was investigated using 5 mM H_2SO_4 as the eluent. In ion-exclusion chromatography, it is well known that solute retention is mainly determined by pKa_1 because of the ion exclusion effect.[1-2] Partly ionized solutes are eluted at retention volumes intermediate between V_o (complete ion-exclusion) and $V_o + V_i$ (complete permeation). The relationship between the retention volume of carboxylic acids and their pKa_1 is shown in Fig. 1, where (a) represents the mono-carboxylic acids and (b) the di-carboxylic acids. It can be seen from Fig. 1 that the retention volumes for the carboxylic acids fall between V_o (3.95 mL, HNO_3) and $V_o + V_i$ (34.59 mL, phenol). The relationships are S-shaped, which is characteristic of the ion-exclusion effect.[1-2] Clearly, ion-exclusion is the primary mechanism for the retention of mono- and di-carboxylic acids on the PV-DVB resins. However, a poor correlation was observed between retention volume and pKa, indicating that other factors influence retention such as size exclusion and hydrophobic absorption.[2]

Fig. 2(a) shows the relationship between the retention volume of benzene carboxylic acids with various numbers of -COOH and their pKa. As can be seen, the retention volumes for benzylcarboxylic acids - phthalic (17.5 mL), trimellic (12.49 mL) and pyromellitic acid (6.43 mL) - were between V_o (3.95 mL) and $V_o + V_i$ (34.59 mL). The retention volume depended on pKa, indicating that, with the exception of benzoic acid (57.90 mL), the retention mechanism of benzene carboxylic acids on the (PS-DVB) column was based on the ion-exclusion effect.

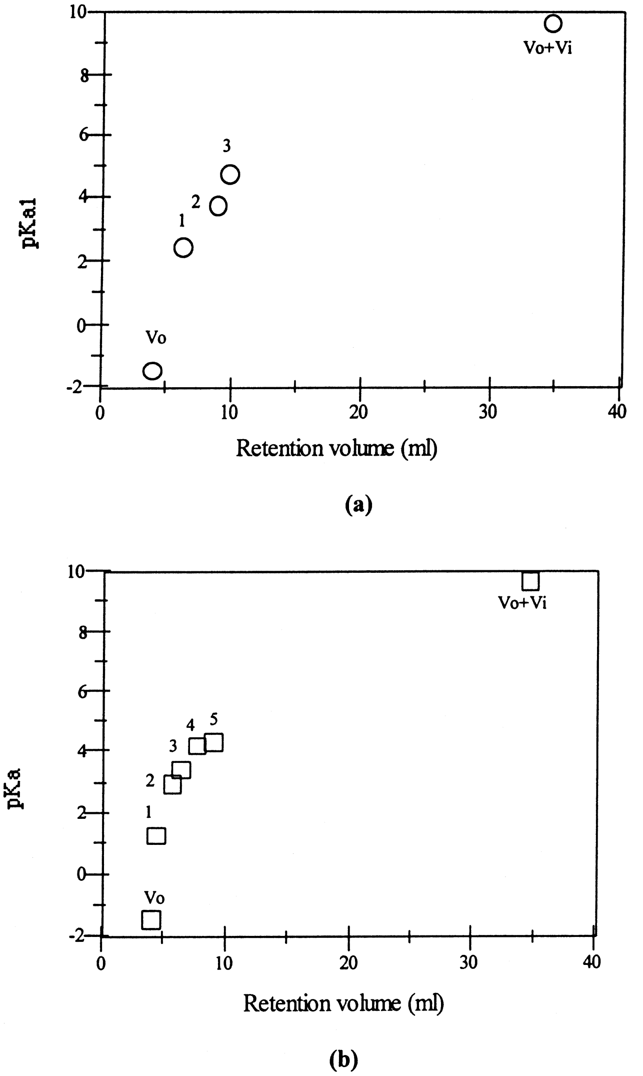


Figure 1. The relationship between retention volume and pKa1. (a) Mono-carboxylic acids. V_O: HNO_3, V_{O+Vi}: phenol, (1) pyruvic, (2) formic, (2) acetic (b) di-carboxylic acids. (1) oxalic, (2) tartaric, (3) malic, (4) succinic, (5) glutaric. Conditions: 5 mM H_2SO_4 in the eluent, flow rate: 0.6 mL/min, injected volume: 10 µL, the column temperature: 40°C.

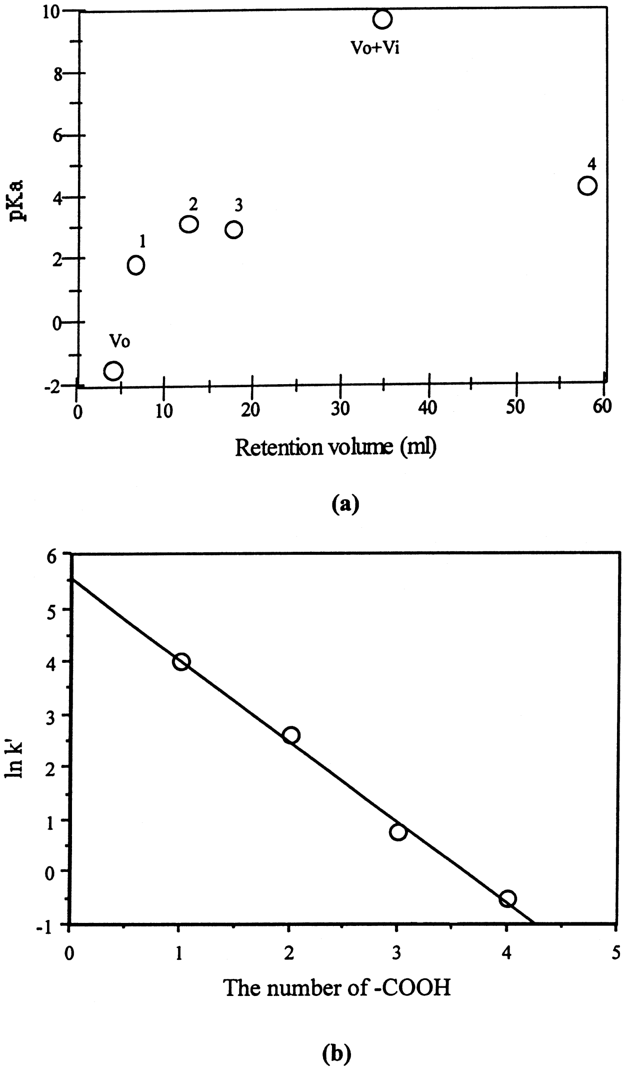


Figure 2. (a) The relationship between retention volume of benzenecarboxylic acids and their pKa_1. (b) The dependence of the lnk' on the number of -COOH group. (1) pyromellitic, (2) trimellic, (3) phthalic, (4) benzoic. Conditions as described in Fig. 1.

Similar results were observed by Ohta et al. in their studies of the ion-exclusion chromatographic separation of aromatic acids performed on TSK gel columns.[10] However, retention volumes for benzene carboxylic acids were greater than those of carboxylic acids despite their similar pKa. e.g., phthalic and tartaric acid both contain two -COOH groups and their pKa are 2.91 and 2.96, respectively, but the phthalic acid retention volume (17.50 mL) is much larger than that of tartaric acid (5.35 mL). Such differences in retention volume can be attributed to, in addition to an ion-exclusion effect, a hydrophobic interaction between benzylcarboxylic acids and the resin network. Fig. 2(b) illustrates the relationship between ln k' of benzylcarboxylic acids and the number of -COOH groups in their structure.

The lnk' inversely ($y = 5.55 - 1.53x$, $r^2 = 0.995$) related to the number of -COOH groups. This is probably due to their effect on polarity, leading to reduced hydrophobic interaction between solute and PS-DVB resin network.[11] It is concluded that mixed ion-exclusion and hydrophobic interaction[8] govern the retention of benzylcarboxylic acids.

Phenolcarboxylic acids with varying numbers of -OH group were selected as target compounds to further examine the mixed ion-exclusion and hydrophobic interaction mechanism. Retention volumes for the phenolcarboxylic acids, their pKa and number of -OH groups are: gallic (16.14 mL, pKa: 4.41, -OH: 3), 3,4- dihydroxybenzoic (27.62 mL, pKa: 4.48, -OH: 2), 2,5-dihydroxybenzoic (38.88 mL, pKa: 2.97, -OH: 2), 4-hydroxybenzoic (44.90 mL, pKa: 4.48, -OH: 1), and benzoic acid (57.90 mL, pKa: 4.19, -OH: 0). The dependence of the retention volume on pKa is shown in Fig 3(a). Retention volumes of gallic and 3,4-dihydroxybenzoic acids were between V_o (3.95 mL) and V_o+V_i (34.59 mL) but volumes did not depend on pKa. Retention volumes of other phenolcarboxylic acids were greater than those expected on the basis of a pure ion-exclusion effect despite having similar pKa value.[8] In this case, consideration of only the ion exclusion effect cannot predict the dependence of retention volume on pKa.

Retention volumes mainly depend on solute polarity. For example, solute polarity increased with the number of -OH groups, leading to a decrease in retention volume. The dependence of lnk' on the number of -OH groups is shown in Fig. 3(b) for conditions including: (1) a 5 mM H_2SO_4 eluent; (2) a 5mM H_2SO_4 + 7.5% ACN eluent. In both cases, lnk' decreased as the number of -OH groups increased. However, lnk' decreased more rapidly when an organic modifier was added to the eluent ($\ln k' = 2.97 - 0.16x$, $r^2 = 0.997$) than without the modifier ($\ln k' = 2.26 - 0.53x$, $r^2 = 0.994$). Retention volumes decreased with increasing solute polarity and with the addition of an organic modifier, which was a typical hydrophobic characteristic. Therefore, we suggest that the retention of phenocarboxylic acids in this case was mainly governed by hydrophobic interactions.

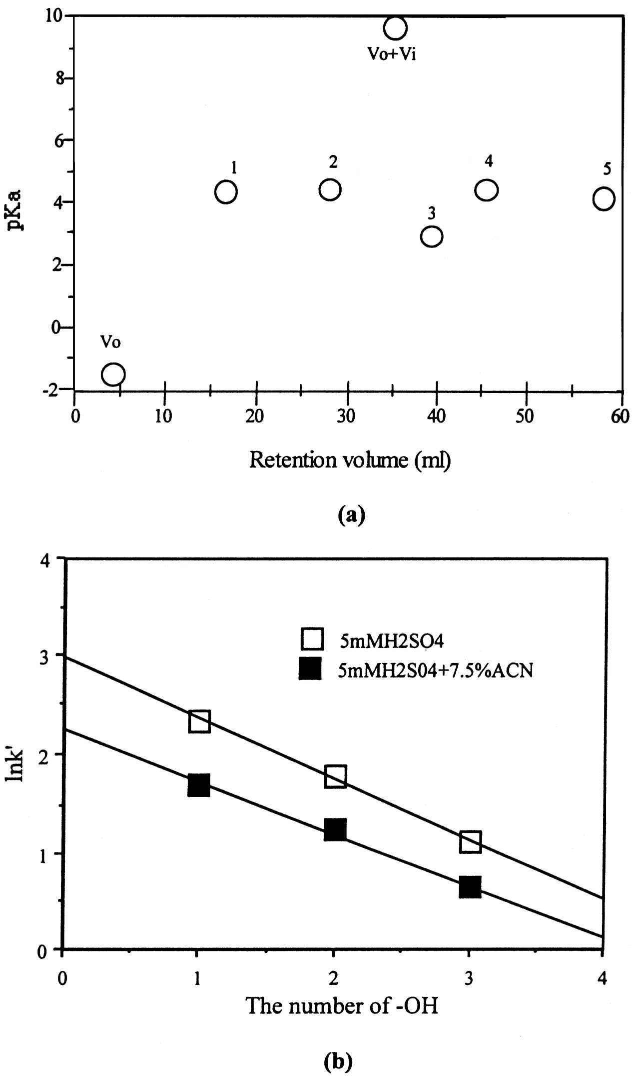
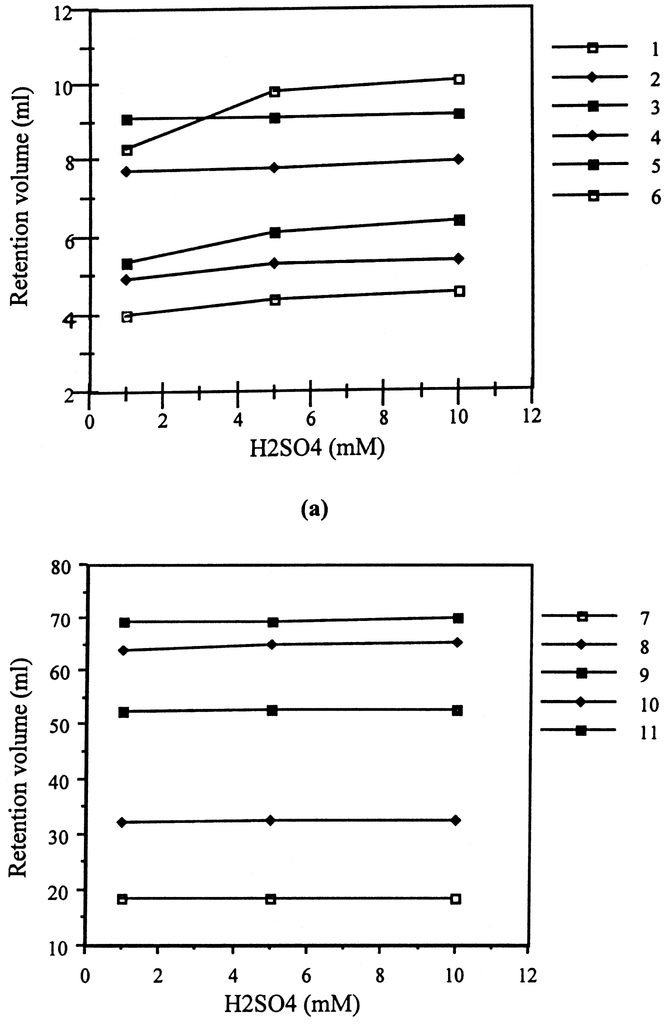


Figure 3. (a) The relationship between retention volume of phenolcarboxylic acids and their pKa_1. (b) The dependence of the ln k' on the number of -OH group. (1) gallic, (2) 3,4-dihydroxybenzoic, (3) 2,5-dihydroxybenzoic, (4) 4-hydroxybenzoic, (5)benzoic. Conditions as described in Fig. 1.



(a)

(b)

Figure 4. The effect of the concentration of sulfuric acid in the eluent on the retention of the test acids. (1) oxalic, (2) citric, (3) pyruvic, (4) succinic, (5) T-aconitic, (6) fumaric, (7) gallic, (8) 2.5-dihydroxybenzoic, (9) 4-hydroxybenzoic, (10) benzoic, (11) vanillic, (12) coumaric acids. Other conditions as described in Fig 1.

On the basis of these results mentioned, we conclude that simultaneous separation of carboxylic and aromatic acids on the PS-DVB column can be obtained by the selection of separation conditions, including addition of an organic modifier to eluent, adjustment of pH and column temperature, which utilize both ion-exclusion and hydrophobic effects.

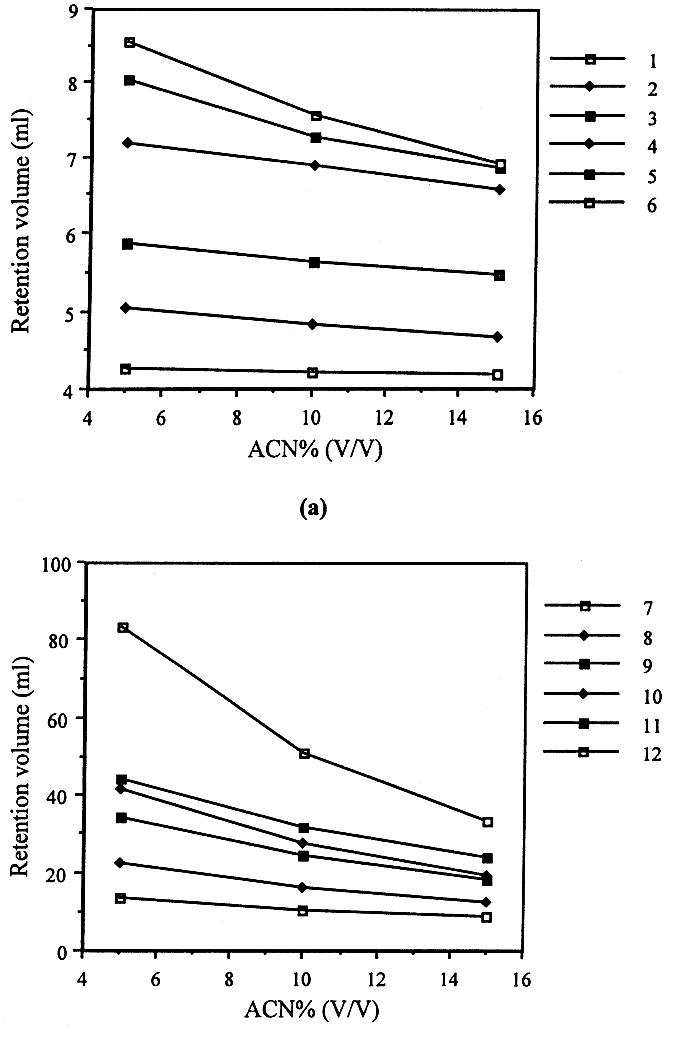
Effect of Separation Conditions on Retention Behaviour

In IEC, the retention of carboxylic acids depends mainly on the degree of ionization of the solute. This is determined mainly by the eluent pH and the nature and concentration of organic modifier in the eluent.[1-2] The hydrophobic interaction is strongly governed by the concentration of organic modifier and column temperature.[12] Eluent pH, organic modifiers and column temperature were further examined in order to characterize both ion-exclusion and hydrophobic interaction behaviours for carboxylic and aromatic acids on the PS-DVB column.

Fig. 4 shows the dependence of retention on the concentration of sulfuric acid in the eluent, where (a) and (b) correspond to the carboxylic and aromatic acids, respectively. The retention of carboxylic acids was dependent on sulfuric acid concentration whereas that of aromatic acids was not. The retention volume of carboxylic acids increased with the concentration of sulfuric acid due to a decrease in ionization as the eluent pH decreased.[13] Aromatic acid retention was independent of sulfuric acid concentration due to their presence as neutral species. The greater retention volumes for aromatic acids was due to hydrophobic interactions between the aromatic acids and the resin network.[2,8]

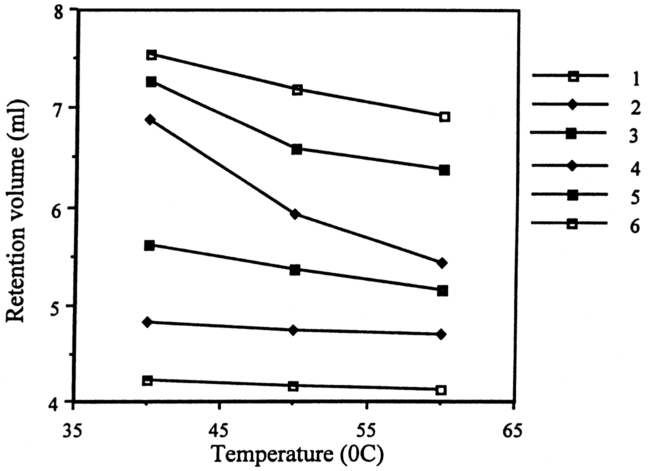
The effect of an organic modifier on retention was studied by increasing volumes of acetonitrile (ACN) to 15% (V/V). As shown in Fig. 5 (a) and (b), the presence of ACN affects both carboxylic and aromatic acid retention. As the ACN content in the eluent increased, carboxylic and aromatic acid retention decreased. This effect was greater for aromatic acids and lesser for carboxylic acids. For example, when the ACN content was raised from 5% to 15%, the retention volume of the hydrophobic coumaric acid was reduced by 46.90 mL, whereas that of the hydrophilic citric acid only decreased 0.20 mL. Increasing content of organic modifier in the eluent significantly reduced solute retention, suggesting that aromatic acids would be retained on the PS-DVB column by hydrophobic interactions.[8]

The influence of the temperature on the solute retention was studied by varying column temperatures from 40 to 60°C using 5 mM H_2SO_4 + 10 ACN% as the eluent. Fig. 6 shows the dependence of retention volume on column temperature. Retention volumes decreased as the temperature increased. For example, as the temperature was increased from 40 to 60°C, the furilic retention volume decreased from 58.17 to 37.70 mL without sacrificing chromatographic

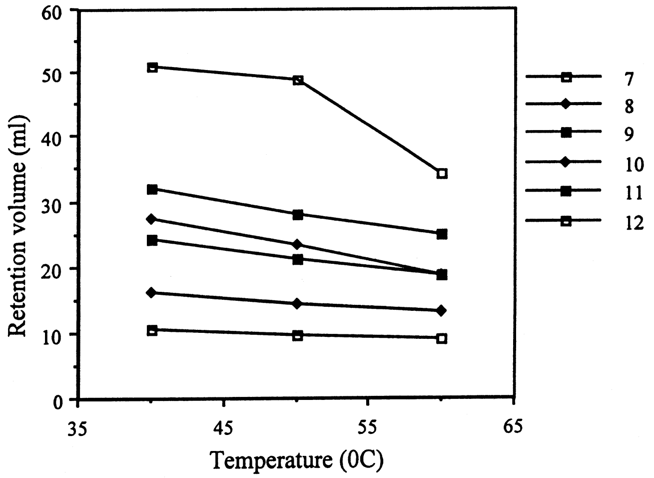


(b)

Figure 5. The effect of the concentration of acetonitrile in the eluent on the retention of the test acids. Conditions: 5 mM H_2SO_4 + ACN in the eluent at 50°C. Others as described in Fig. 1.



(a)



(b)

Figure 6. The effect of the column temperature on the retention of the test acids. Conditions: 5 mM H_2SO_4+10% ACN as the eluent. Other conditions as described in Fig. 1.

resolution. Generally there was a marked decrease in retention volumes of aromatic acids indicating that hydrophobic interactions were improved by enchancing the rate of mass transfer. Increased temperatures also increased the efficiency of the separation.[14-15] Retention of carboxylic acids decreased slightly with increasing temperature owing to small changes in pKa.[16]

Fig.7 shows a typical chromatogram for the separation of 17 carboxylic and aromatic acids obtained using an eluent containing 5 mM H_2SO_4 and 7.5% ACN at 50°C. Symmetrical peaks and good resolution were obtained for the separation of mono-, di- and tri-carboxylic acids, as well as benzylcarboxylic and phenolcarboxylic acids. Classification of the acids and their order of elution was: Carboxylic acids - oxalic, citric, tartaric, malic, succinic, fumaric, formic and acetic acids; Aromatic acids - gallic, phthalic, 3,4-dihydroxybenzoic, 2,5-dihydroxybenzoic, 4-hydroxybenzoic, vanillic, benzoic, coumaric and ferulic acids.

The Retention Behaviour of Phenolcarboxylic Acid on C_{18} Column

In order to examine the hydrophobic interaction between aromatic acids and the PS-DVB resin network, the above experiment was repeated on a single-mode hydrophobic column. A C_{18} column and an eluent containing 5 mM H_3PO_4 + 7.5% ACN at 50°C was used to separate seven aromatic acids. A typical chromatogram is shown in Fig. 8. The separation of aromatic acids was in the order: gallic, 3,4-dihydeoxybenzoic, 4-hydroxybenzoic + 2,5-dihydroxybenzoic, vanillic, coumaric, benzoic and ferulic acids.

The eluted order of aromatic acids on the reversed-phase column was similar to that on the ion-exclusion column. Retention volume decreased with increasing molecular polarity. These findings supported the retention of aromatic acids by hydrophobic interaction with ion-exclusion (PS-DVB) columns.

CONCLUSION

This study of retention behaviour indicates that a variety of mechanisms are involved in the separation of carboxylic and aromatic acid on PS-DVB column. Retention behaviour also depends on the chemical properties of the solute. Separation of hydrophilic solutes is governed largely by the ion-exclusion effect; whereas hydrophobic solutes are governed by hydrophobic interaction mechanisms.

This was demonstrated by the dramatic influence of the solute structure, eluent pH, organic modifier and column temperature on capacity factors. This was also supported by comparison with the reversed-phase separation of the

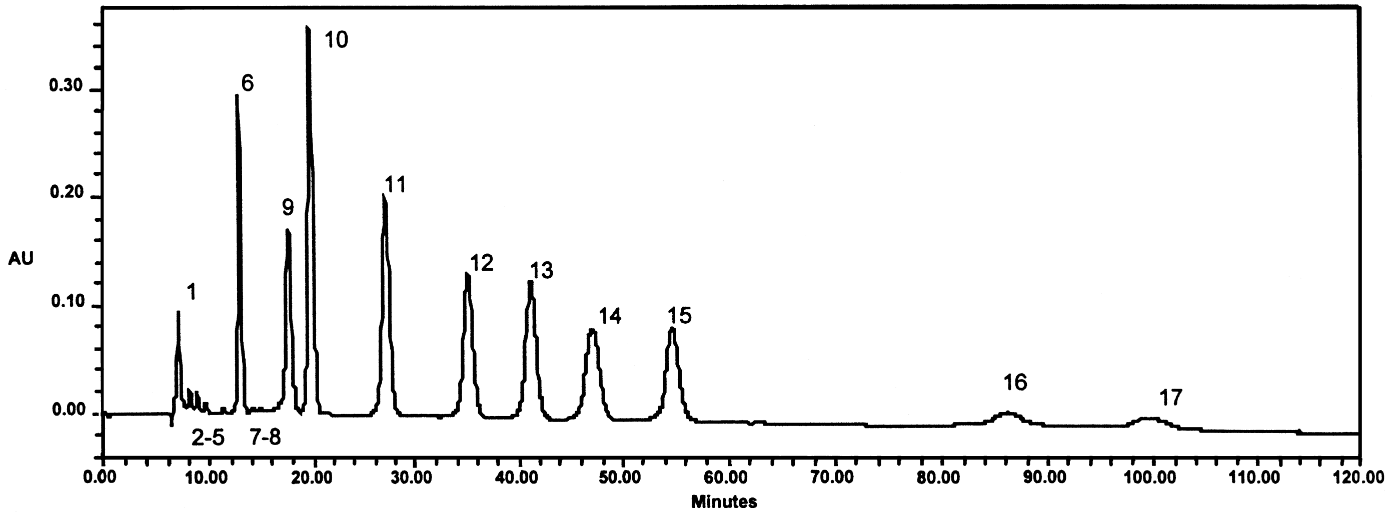


Figure 7. Chromatogram showing separation of carboxylic and aromatic acids on ion-exclusion column. (1) oxalic (0.7 mM), (2) citric (0.7mM), (3) tartaric (0.7 mM), (4) malic (0.7 mM), (5) succinic (0.7mM), (6) fumaric (0.7 mM), (7) formic (0.7 mM), (8) acetic (0.7mM) (9) gallic (0.26 mM), (10) phthalic (0.0.26 mM), (11). 3,4-dihydroxybenzoic (0.14 mM), (12) 2,5-dihydroxybenzoic (0.26 mM), (13) 4-hydroxybenzoic (0.26 mM), (14) benzoic (0.26 mM), (15) vanillic (0.14 mM), (16) coumaric (0.14mM), (17) ferulic acid (0.14 mM). Eluent : 5 mM $H2SO_4$ + 7.5% ACN at 50°C. Other conditions as described in Fig.1.

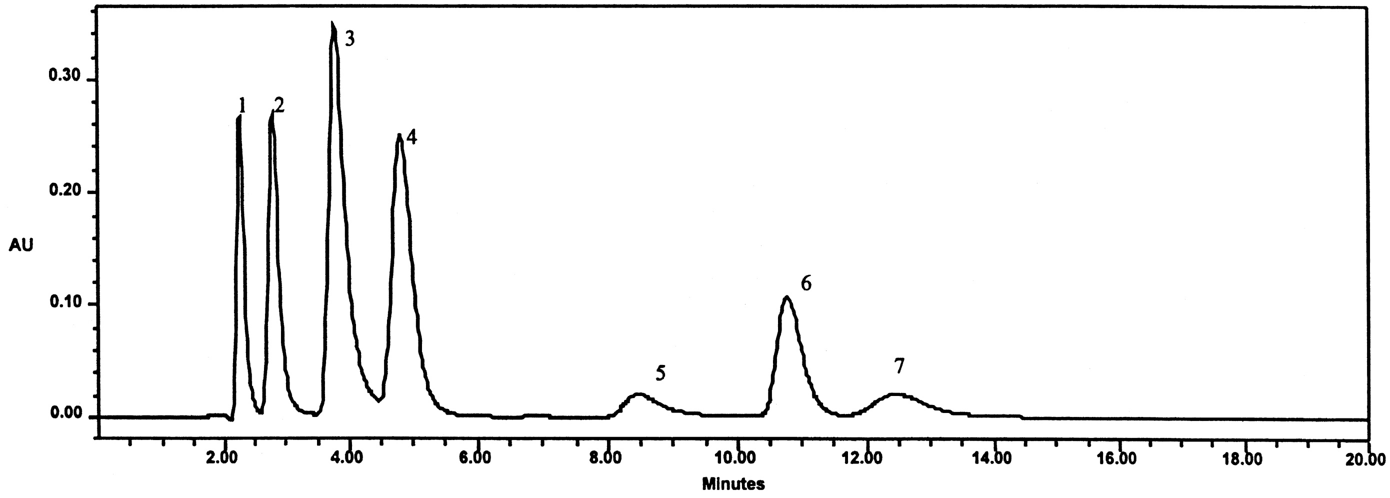


Figure 8. Chromatogram showing separation of aromatic acids on C_{18} column. (1) gallic (0.25 mM), (2) 3,4-dihydroxybenzoic (0.125 mM), (3) 4-hydroxybenzoic (0.25 mM) + 2.5-dihydroxybenzoic (0.25 mM), (4) vanillic (0.125 mM) , (5) coumaric (0.125 mM), (6)benzoic (0.25 mM), and (7)ferulic acid (0.125 mM). Conditions as described in Fig.7.

aromatic acids. In general, retention of test solutes on the PS-DVB column was determined by a combination of both ion-exclusion and hydrophobic interaction mechanisms.

The results presented here will provide the foundation for the development of new applications of the PS-DVB column such as simultaneous separation of carboxylic and aromatic acids in real samples.

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