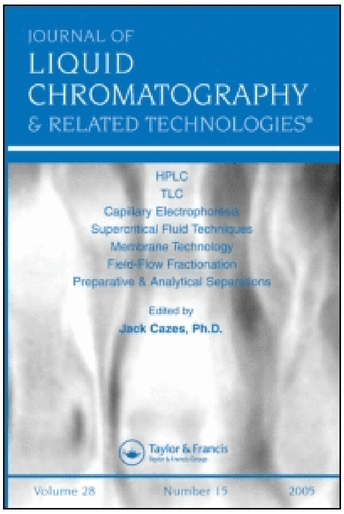

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MULTIFUNCTIONAL ZWITTERION-EXCHANGE STATIONARY PHASE FOR HPLC

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ABSTRACT

A zwitterion-exchange stationary phase (ZIXSP) was prepared for the separation of the mixture of organic bases, organic acids and amino acids by high performance liquid chromatography (HPLC). It was synthesized by the reaction of cyanuric chloride with sulfanilic acid and then bonded onto the silica gel on which the silane coupling agent was prebonded. The chromatographic behaviours of the prepared ZIXSP revealed that the capacity factors of each analyte varied with the pH value of the mobile phase. By applying the concept of acid-base equilibrium the correlation between the capacity factor on the ZIXSP and pH of the mobile phase could be explained reasonably. Based on our results, it is concluded that ZIXSP is a zwitterion exchanger that additionally provides π-π charge-transfer interaction and hydrophobic interaction functions.

INTRODUCTION

Knox[1,2] had found that the addition of zwitterionic pairing agents, i.e., 11-aminoundecanoic acid or 12-aminododecanoic acid, to the mobile phase could improve the efficiency of the C_{18} column (ODS Hypersil) for the separation of nucleotides. However, the addition of zwitterionic pairing agents to the mobile phase is not adapted to the preparative chromatography since it would contaminate the analytes. Later, Hartwick[3] prepared a zwitterion-exchange stationary phase (ZIXSP) by introducing both amino group and carboxylic acid group to the surface of silica gel. Since -COOH is a weak acid group and could not be dissociated into $-COO^-$ and H^+ at a lower pH value, the ZIXSP prepared by Hartwick would lose the properties of zwitterion-exchange at low pH value of mobile phase.

For separations of ionic organocompounds, contributions from forms of interaction other than ion exchange, such as H-bonding, π-π interaction or hydrophobic interaction, may be involved if the stationary phase provides the corresponding functional moiety.[4-6] The possibility of simultaneous separation of ionized/ionizable and non-ionic compounds with columns prepared by mixing together ion-exchange and reverse phase packing materials was confirmed by Wilson and co-workers.[7]

In our previous report,[8] multifunctional zwitterion phases containing aromatic moieties were prepared successfully and used to separate various weak organic acids and bases. In addition, we reported that the ion-exchanger which was synthesized through bonding aniline onto silica with 3-aminopropyltriethoxysilane and followed by sulfonation was an 1 : 1 mixed phase with 50% unsulfonated aniline-derived moiety. It was observed that this ion-exchanger was a zwitterion-exchanger that additionally provided hydrophobic-interaction and π-π charge-transfer interaction functions.

In our previous reports,[9,10] highly selective *s*-triazine-modified C_{18} phase and chiral phase were successfully prepared. The presence of an *s*-triazine ring in the bonded phase system not only led to a convenient reproducible synthetic way of introducing a desired organic moiety to the silica surface but also played an important role, possibly due to a π-π interaction with aromatic analytes, in the separation of aromatic hydrocarbons. Therefore, instead of the above process,[8] using *s*-triazine derivatization can be served as an alternative one for the preparation of an sulfonated ZIXSP.

In this study, we prepared a ZIXSP by introducing both sulfo- ($-SO_3H$) group and amino group through *s*-triazine derivative to the aminated silica gel. The chromatography behaviours of this ZIXSP with regard to the separation of the mixture of organic bases, organic acids and amino acids were explained by applying the concept of acid-base equilibrium.

Table 1

Characteristics of the Prepared Phase

	Elemental Analysis				Loading Capacity[a]	
	C/%	N/%	H/%	Cl/%[b]	mmol/g	μmol/m^2
BS	6.94	2.78	1.57		0.99	2.84
ZIXSP	9.83	4.35	1.61	1.03	0.28	0.80

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[a] Based on N%.

[b] By Carius method.[11]

EXPERIMENTAL

Chemicals

Silica gel (Nucleosil; pore size 100 Å, particle size 10 μm, surface area 350 m^2/g). was obtained from Macherey-Nagel and dried at 100 °C for 10 h. The chemicals used in the synthetic processes and analytes used in the chromatographic experiments were of reagent grade: N-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAPS, Tokyo Kasei); *p*-sulfanilic acid (Riedel-Dehaen); Cyanuric chloride (Fluka). The solvents used for HPLC were LC-grade acetonitrile and deionized water.

Preparation of ZIXSP

To a solution of pre-dried silica gel (3 g) in 150 mL of toluene. AEAPS (6 mL) was added and refluxed for 15 h. This bonded silica gel (BS. Figure1) was collected by filtration and washed with methanol, ether and then dried under vacuum in the presence of the drying agent, P_2O_5 The BS was characterized by elemental analysis (Table 1).

Sodium carbonate (0.02 mol) and *p*-sulfanilic acid (0.02 mol) were dissolved in 0-5 °C water (50 mL), then a solution of cyanuric chloride (0.02 mol) in acetone (10 mL) was added and stirred. Another portion of sodium carbonate (0.02 mol) in 20 mL water was dropply added within 30 min under 0-5 °C.

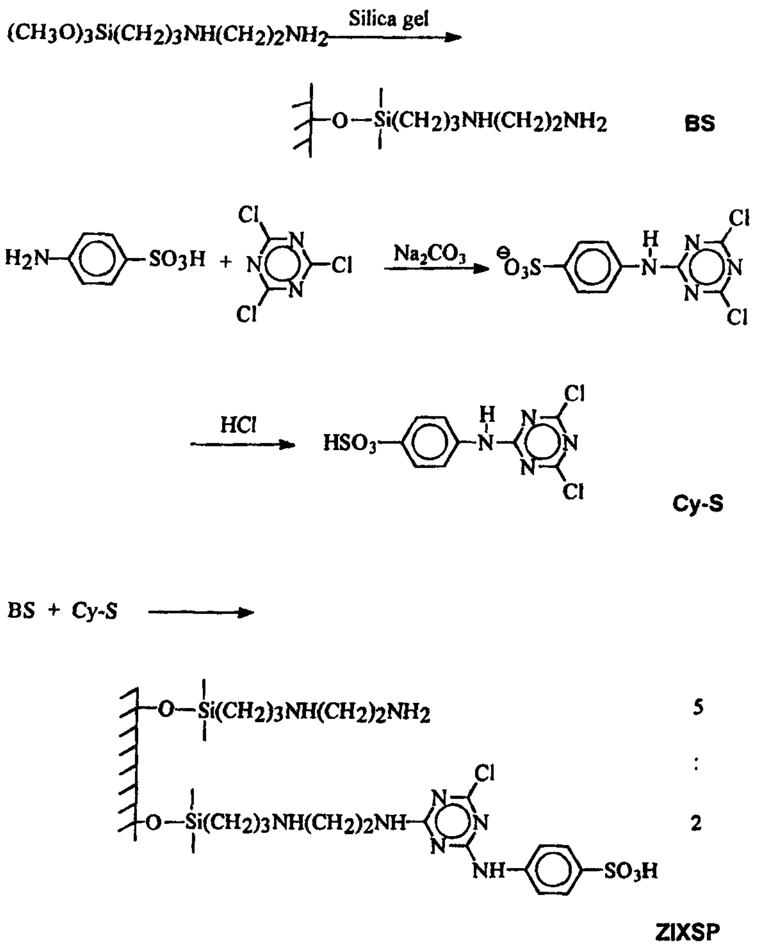

Figure 1. Preparation of zwitterion exchange stationary phase ZIXSP

After the pH of the reaction mixture was adjusted to 1-2 with diluted hydrochloric acid, the derivative of cyanuric chloride was collected by filtration, washed with water and dried over P_2O_5 under vacuum. The product (Cy-S, Figure 1) was characterized by IR and ^{1}HNMR.

BS (3 g) was suspended in 150 mL of dimethylsulfoxide (DMSO). Cy-S (0.003 mol) was added and stirred at 50 °C for 2 h, NaOH (0.12 g) was then added and reacted for a further 24 hours.

The reaction mixture was filtered and the product (ZIXSP, Figure1) was washed with water, acetone, methanol and ether. Characterization was done by elemental analysis (Table 1).

Chromatographic Studies

The chromatographic studies were carried out with a Waters liquid chromatographic system which consisted of a M-510 solvent-delivery system, a U6K injector and Spectroflow 757 variable-wavelength UV detector. Stainless-steel column (300 mm x 4 mm I.D.) was packed by the balanced density slurry method.

Results and Discussion

In this study, AEAPS which provides possible cation-exchange sites was used as a coupling agent. The AEAPS-derived silica phase (BS) then reacted with the *s*-triazine derivative of sulfanilic acid (Cy-S) to give the expected ZIXSP (Figure 1). Both the BS and ZIXSP were characterized by elemental analysis, and the data are shown in Table 1. It demonstrated that each gram of BS contained about 0.99 mmol of AEAPS, and each gram of ZIXSP contained about 0.28 mmol of Cy-S. Therefore, about seven molecules of AEAPS bonded on the silica gel was further bonded with two molecules of Cy-S.

Effective separation of the mixture of organic bases, organic acids and amino acids on the ZIXSP by HPLC was observed: Representative chromatogram is shown in Figure 2. The effect of the pH of mobile phase on the values of k' (capacity factor) for various analytes on this ZIXSP is shown in Figure 3. It was observed that the relationship between the capacity factor of analyte and the pH of mobile phase in the range pH 3 to 5 depended upon the nature of analytes.

For the organic bases, aniline and benzylamine, the k' values decreased as the pH of mobile phase increased. However, for the weak organic acids, 1-naphthylacetic acid and benzoic acid, the capacity factors increased before pH 5 and decreased after pH 5, as the pH of mobile phase increased. Interestingly, the k' of a strong organic acid, *p*-toluenesulfonic acid, decreased rapidly with increasing the pH value of mobile phase, while that of zwitterion compounds, phenylalanine, leucine and alanine, had a volcano at pH about 4.

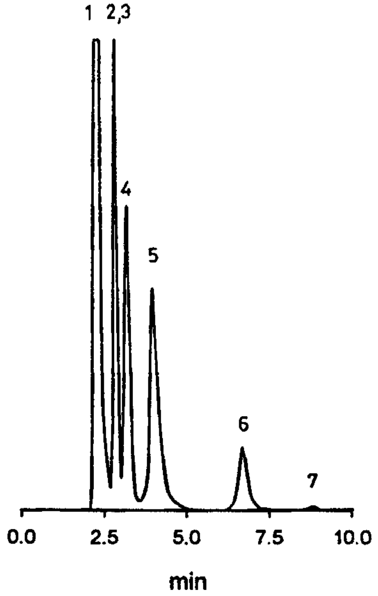

Figure 2. Chromatogram for the separation of organic bases, organic acids and amino acids on the ZIXSP. Mobile phase: CH_3CN-H_2O (2 : 1), [KH_2PO_4]: 1 m*M*, pH 7.0, flow rate: 1.1 mL/min, UV detection, room temperature. analytes: (1) *p*-toluenesulfonic acid, (2) benzylamine, (3) aniline, (4) benzoic acid, (5) 1-naphthylaectic acid, (6) phenylalanine, (7) aniline.

The result with respect to the effects of pH (Figure 3) of the mobile phase on the capacity factor revealed the possibly available contributions of zwitterion-exchange, π-π charge-transfer, and hydrophobic interactions by this ZIXSP to the separation process.

ZIXSP Behaves as a Cation Exchanger in the Separation of Amines

The correlation between the capacity factor on the ZIXSP and pH of the mobile phase can be explained by applying the concept of acid-base equilibrium, which was also applied by Horvath et al. for separating ionogenic substances on an alkyl phase.[12]

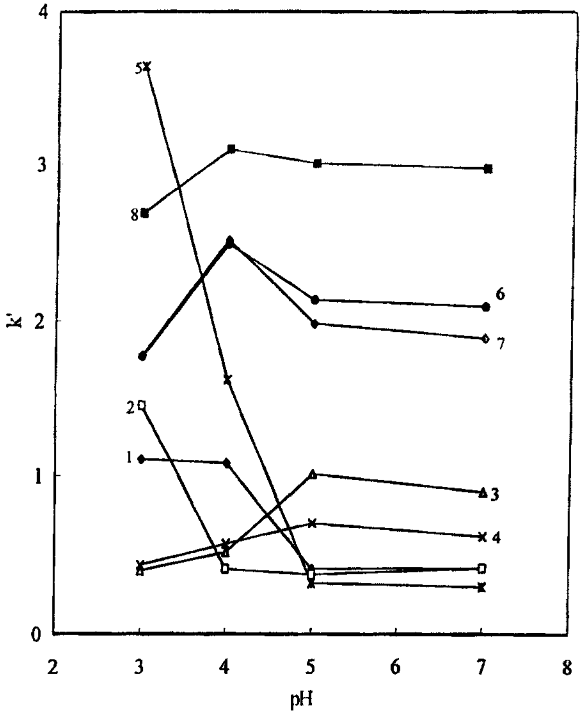

Figure 3. Effect of the pH of mobile phase on the values of k' for (1) aniline, (2) benzylamine, (3) 1-naphthylacetic acid, (4) benzoic acid, (5) *p*-toluenesulfonic acid, (6) phenylalanine, (7) leucine, (8) alanine on home-made ZIXSP. Mobile phase: CH_3CN-H_2O (2:1), [KH_2PO_4]: 1 m*M*, flow rate: 1.1 mL/min.

When the pH of mobile phase is less than 7, the organic base (B) will be protonated as eq. (1).

$$B + H^+ \overset{K_b}{\rightleftharpoons} HB^+ \tag{1}$$

Suppose HB^+ as it interacted with the stationary phase, would replace a counter ion (C^+) from the stationary phase as eq. (2).

$$HB^+_m + C^+_s \overset{K_c}{\rightleftharpoons} HB^+_s + C^+_m \tag{2}$$

The subscript characters of s or m, indicates the species which is in the stationary phase or in the mobile phase, respectively. In ion-exchange chromatography, the retention of an analyte on the organic stationary phase bearing ionic functional groups can be predicted as the result of two processes: (a) the distribution of an analyte between an aqueous mobile phase and the organic stationary phase and (b) the reaction of an analyte with the ionic sites of the stationary phase (i.e. ion exchange). Then, the capacity factor k' can be presented as eq. (3).

$$k' = \frac{V_s}{V_m} \times \frac{[B]_s + [BH^+]_s}{[B]_m + [BH^+]_m}$$

$$= \frac{V_s}{V_m} \times \frac{D_B + K_b[H^+]_m K_c \frac{[C^+]_s}{[C^+]_m}}{1 + K_b[H^+]_m} \qquad (3)$$

where V_s is the volume of stationary phase within the column, V_m is the total volume of solvent within the column and D_B is the distribution coefficient of the unprotonated base between stationary phase and mobile phase ($[B]_s/[B]_m$).

In eq. (3), in the range pH 3 to 7, $K_b[H^+]_m$ could be neglected for aniline ($K_b = 4.2 \times 10^{-10}$) and benzylamine ($K_b = 0.2$). Since $[C^+]_S$ is about equal to the total exchange capacity[13] for the stationary phase, and $[C^+]_m \fallingdotseq [KH_2PO_4] = 1$ m*M*, eq. (3) would be simplified as:

$$k' = \frac{V_s}{V_m}(D_B + C[H^+]_m) \qquad (4)$$

where C is a constant equal to $K_bK_c[C^+]_s[C^+]_m^{-1}$

If we are not concerned with the dependence of the D_B value on the pH of the mobile phase, according to eq. (4), the k' values of aniline and benzylamine would be linearly proportional to $[H^+]$ in the mobile phase. While Figure 3 exhibited that the k' values of aniline and benzylamine decreased with increasing the pH of the mobile phase in the range of pH 3 to 5 and remained constant as pH≥5. The chromatographic results indicated that beside the contribution of cation exchange, the distribution of the unprotonated organic bases between the stationary phase and the mobile phase is important. The interactions between the unprotonated organic bases and stationary phase may be due to the π-π charge-transfer provided by the phenyl and/or *s*-triazine ring moiety and the hydrophobic character of this stationary phase. The roles of these interactions, compared to the function of cation exchange, became predominate interactions when the pH of the mobile phase increased.

ZIXSP Behaves as an Anion Exchanger in the Separation of Organic Acids

Suppose the organic acid in the mobile phase is in an equilibrium state as eq. (5)

$$AH \overset{K_a}{\rightleftharpoons} A^- + H^+ \qquad (5)$$

and A^- as it interacted with the stationary phase, would replace a counter ion (C^-) from the stationary phase as eq. (6).

$$A^-_m + C^-_s \overset{K_c'}{\rightleftharpoons} A^-_s + C^-_m \qquad (6)$$

Similarly, the capacity factor k' can be shown as:

$$k' = \frac{V_s}{V_m} \times \frac{[AH]_s + \left[A^-\right]_s}{[AH]_m + \left[A^-\right]_m}$$

$$= \frac{V_s}{V_m} \times \frac{D_{AH} + K'_c \dfrac{K_a}{\left[H^+\right]_m} \dfrac{[C^-]_s}{[C^-]_m}}{1 + \dfrac{K_a}{\left[H^+\right]_m}} \qquad (7)$$

Where $D_{AH} = \frac{[AH]_s}{[AH]_m}$. Since $[C^-]_S$ is about equal to the total anion exchange capacity[7] for the stationary phase, and $[C^-_m] \fallingdotseq 0.001\ M$ in pH range 3 to 7, eq. (7) would be simplified as:

$$k' = \frac{V_s}{V_m} \times \frac{D_{AH} + \dfrac{C'}{\left[H^+\right]_m}}{1 + \dfrac{K_a}{\left[H^+\right]_m}} \qquad (8)$$

where C' is a constant equal to $K_a K_c'[C^-]_s[C^-]_m{}^{-1}$.

In the case of weak acid, such as 1-naphthylacetic acid ($K_a = 5.8 \times 10^{-5}$)and benzoic acid ($K_a = 6.3 \times 10^{-5}$), the term of $K_a[H^+{}_m]^{-1}$ can be neglected at lower pH and eq. (8) could be simplified as:

$$k' = \frac{V_s}{V_m} \times \left(D_{AH} + \frac{C'}{\left[H^+\right]_m} \right) \qquad (9)$$

However, since the value of $K_a[H^+{}_m]^{-1}$ would become quite larger than 1 with increasing the pH of the mobile phase in the range of pH 5 to 7 for 1-naphthylacetic acid and benzoic acid. Eq. (8) could be changed into:

$$k' = \frac{V_s}{V_m} \frac{D_{AH}\left[H^+\right]_m + C'}{K_a} \qquad (10)$$

Indeed, it revealed that the k' of 1-naphthylacetic acid and benzoic acid increased with the pH of mobile phase before pH 5 (coincide with eq (9)) and tended to decrease with the pH of mobile phase after pH 5 (coincide with eq. (10)).

For the strong acid such as *p*-toluenesulfonic acid ($K_a = 0.020$), the value of K_a is large and in the whole investigated pH range of mobile phase $K_a[H^+{}_m]^{-1}$ is quite larger than 1. Therefore, eq. (8) could be simplified reasonably as eq. (10). Figure 3 also revealed that the k' of *p*-toluenesulfonic acid decreased rapidly with the pH of the mobile phase in the pH range 3 to 5 and leveled off in the pH range 5 to 7.

Since D_{AH} represented the distribution coefficient between the stationary phase and the mobile phase of undissociated organic acid, D_{AH} not being zero, implied that the additional factor(s) of undissociated organic acid in the stationary phase had contributed to the retention mechanism for this ZIXSP towards the separation of these acids. This factor(s) may be due to the π-π charge-transfer and the hydrophobic interactions between the acid and the phenyl and/or *s*-triazine ring moiety of this stationary.

ZIXSP Behaves as a Zwitterion Exchanger in the Separate of Amino Acids

In the pH range 3 to 7, the amino acids, phenylalanine, leucine, and alanine, were mostly in the form of zwitterions. Therefore, the COO^- and H_3N^+ groups of amino acid would interact with the amino and sulfo- moieties of the stationary phase respectively. The force of the quadrupolar charge interaction[1,2] among each of the three amino acids and this ZIXSP was stronger than that of the dipolar charge interaction between the simple organic acid or organic base and this ZIXSP. This resulted in that the k' of these amino acids were apparently higher than that of the other five compounds as shown in Figure 3. The occurrence of a volcano at about pH 4 of the mobile phase for the k' of phenylalanine, leucine and alanine may result from the formation of this quadrupolar charge interaction occurred most efficiently at this pH range.

CONCLUSIONS

The present results indicate that a multifunctional ZIXSP containing a *s*-triazine derived aromatic moiety can be successfully prepared and used to separate various organic acids and bases. The chromatographic behaviour of the phases shows that the effective selectivity of this ZIXSP is due to the sulfo- ($-SO_3H$) group for the cation-exchange ability, the amino group(s) for the anion-exchange ability, the *s*-triazine as well as phenyl rings for the charge-transfer, and the organic moiety for the hydrophobic character.

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