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HPLC separation of positional isomers on a dodecylamine-*N*,*N*-dimethylenephosphonic acid modified zirconia stationary phase

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Abstract

The chromatographic performance of a new zirconia stationary phase (DPZ) modified with dodecylamine-*N*,*N*-dimethylenephosphonic acid (DDPA) is studied by using positional isomers as probes. The DDPA modified zirconia via one phosphonic group has a polar inner-layer and a non-polar outer-layer on its surface. The alkyl chain of outer-layer provides the hydrophobic interaction, while the polar inner-layer that consists of an amine group and a free phosphonic group provided dipolar and ion-exchange/columbic repellent interaction sites. The effects of methanol content, ionic strength and pH of mobile phase on capacity factors of the solutes are studied in detail, and baseline separations of toluidine, nitroaniline, aminophenol, dihydroxybenzene, and nitrophenol isomers were achieved on the new zirconia stationary phase. In addition, retention mechanism of the isomers on the DDPA-modified zirconia stationary phase is also proposed.

Keywords: Dodecylamine-*N*,*N*-dimethylenephosphonic acid (DDPA); Modified zirconia; Chromatographic performance; Retention mechanism; Positional isomer

1. Introduction

Zirconia is one of the alternative materials for silica in HPLC due to its excellent thermal, chemical and mechanical stability [1]. Zirconia and modified zirconia stationary phases have been successfully used in HPLC. Polymer-coated zirconia can be used under very aggressive conditions [2]. Polybutadiene-coated zirconia (PBD-ZrO_2) [3–6] is the most studied zirconia stationary phase. It is stable even in 1 mol l^{-1} sodium hydroxide solution at 100 °C and can be used to separate proteins, peptides and small basic analytes. Due to the strong Lewis acid-base interaction, zirconia can be modified easily with a Lewis base, such as phosphonic acid [7–15]. Alkylphosphonic acid modified zirconia (APA-ZrO_2), which exhibited high stability in reversed-phase chromatographic conditions, were used to separate aromatic hydrocarbons, basic compounds, bases and nucleosides [8–11]. *N*,*N*,*N*′,*N*′-ethylenediaminetetramethylenephosphonic acid (EDTPA) is an organic phosphonate analog of EDTA. Zirconia modified with EDTPA can be used for separation of proteins [12–15].

Many disubstituted benzene compounds are environmental pollutants, drug metabolites or intermediates in chemical and pharmaceutical industries, so the separation of their positional isomers is very important. Since isomers usually possess similar physical and chemical properties, their separation is one of the most challenging areas of separation science. HPLC is one of the most popular techniques for positional isomer separation. ODS is the most used silica-based stationary phase in HPLC, but it is not very suitable for separation of positional isomers due to its "*simple*" retention mechanism. Therefore, stationary phases, especially that can provide various retention mechanisms [16–19], were prepared for separation of positional isomers. Zirconia and modified zirconia can also provide various retention mechanisms, and thus have a potential use for the purpose. For example, carbon-coated zirconia [20–22] has been successfully applied in the separation of positional isomers, but some isomers (such as *o*-dihydroxybenzene) cannot be eluted due

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to strong Lewis acid–base interaction and chelating effect [22].

In this work, a zirconia-based stationary phase (DPZ) which possess various retention mechanisms was prepared by modification of zirconia with dodecylamine-*N*,*N*-dimethylenephosphonic acid (DDPA). DDPA was adsorbed on zirconia via only one phosphonic group and blocked most of the Lewis acid sites of zirconia. The separations of positional isomers, including aminophenols, dihydroxybenzenes and nitrophenols, were achieved on the novel stationary phase.

2. Experimental

2.1. Chemicals reagent

Zirconia (10 μm) was prepared in our laboratory according to the method reported elsewhere [10]. The surface area of 29 $m^2\ g^{-1}$ and average pore diameter of 9 nm were measured by nitrogen adsorption/desorption isotherms on a model ST-03A specific surface area analyser (The Analytical Instrument Plant, Beijing, China).

All of six sets of positional isomers (nitroanilines, toluidines, aminophenols, cresols, dihydroxybenzenes and nitrophenols) were obtained from commercial sources and were of reagent grade. The samples were dissolved in methanol, solution of aminophenols and dihydroxybenzenes were renewed every 6 h due to oxidization during storage.

The following solution were prepared: solution of ammonium molybdate, 1.3 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ was dissolved in 100 ml diluted sulfuric acid (137 ml/863 ml); solution of tin(II) chloride, 2.5 g $SnCl_2$ was dissolved in 100 ml glycerol; Phosphorous acid (50%), dodecylamine, formaldehyde (37%) are analytical reagents, obtained from Shanghai General Chemical Reagent Factory (Shanghai, China). Before use, distilled water was boiled for 15 min to remove dissolved carbon dioxide.

2.2. Preparation of columns

Dodecylamine-*N*,*N*-dimethylenephosphonic acid was synthesized by a Mannich-type reaction according to reference [23]. Briefly, 0.01 mol dodecylamine was added to 30 ml HCl (1:1), and then heated to 80 °C with stirring, after dodecylamine was dissolved, 0.25 mol phosphorous acid and 0.25 mol formaldehyde were added dropwise, the mixture was allowed to react at 80 °C for 4 h. The reaction was cooled and adjusted to pH 2 with pyridine, then the white product was filtered, washed with ice cold water and recrystallized from aqueous methanol to give a crystalline solid.

The modification procedure of zirconia with DDPA was improved on the basis of a method described in reference [2]: 7.0 g zirconia was placed in 250 ml round-bottomed flask and suspended in 150 ml 2-propanol containing 1.0 g DDPA. After 8 h at reflux, the particles were allowed to settle, and the DDPA solution was decanted, the particles were filtered and washed several times with hot 2-propanol to remove any physically adsorbed DDPA. DDPA modified zirconia (DPZ) was dried at 100 °C in a clean vacuum oven overnight, and slurry-packed into 15 cm × 4.6 mm stainless-steel HPLC column at 5500–6000 psi.

Surface coverage analysis of DPZ was characterized by elemental reanalysis, with Model-1106 elemental analyzer (Carlo Erba, Italy).

For the purposes of comparison, a commercially available conventional octadecyl bonded silica was also examined (5 μm, 100 Å, 15 cm × 4.6 mm, purchased from Elite chromatographic company, Dalian, China).

2.3. The coloration reaction of phosphate/phosphonate modified zirconia

It is well known that phosphate/phosphonate can react with ammonium molybdate $[(NH_4)_6Mo_7O_{24}{\cdot}4H_2O]$ and tin(II) chloride $[SnCl_2]$, giving a blue product (phosphomolybdic blue). In order to investigate the adsorption mode of DDPA on zirconia, various zirconia packings were tested, including: (A) native ZrO_2; (B) phosphate modified ZrO_2 [24]; (C) APA-ZrO_2 [11]; (D) zirconia dipped 48 h in a DDPA solution (at room temperature, 25 °C); (E) DPZ, freshly prepared; (F) DPZ, used 2 months in RP-HPLC (DDPA was not added to mobile phase). All of the zirconia packings were washed thoroughly with 2-propanol, methanol or water until no phosphate/phosphonate acid was detected in filtrate.

The testing process was performed as follows: 0.1 g of zirconia packings were put into 10 ml test tubes, respectively, and 5 ml methanol/water, 2 ml ammonium molybdate solution was added, after 5 min, 0.5 ml $SnCl_2$ solution was added.

2.4. Chromatography

All studies were carried out on an Elite IIhigh-performance liquid chromatography systems (Dalian, China), the data was processed using Echrom 98 ChemStation software (Dalian, China).

Methanol/water was used as mobile phase. Acetate or Tris buffers were used to control the pH of the mobile phase when investigating the effect of pH on the capacity factor of solutes. The pH values were measured in the aqueous buffer.

The detection wavelength was at 254 nm. Column dead time was measured with methanol as probe.

3. Results and discussion

3.1. Adsorption mode of DPZ

The results of coloration reaction are given in Table 1. Among all the zirconia packings, only zirconia treated with

Table 1
The coloration reaction of different zirconia packings

Packings	Color
Native zirconia	White
Phosphate modified zirconia	White
APA modified zirconia	White
Zirconia dipped in DDPA solution	Blue
DPZ (freshly prepared)	Blue
DPZ (used months)	Blue

DDPA gave a blue product, indicating that there are free phosphonic groups on the surface of zirconia. Since the modified zirconia has been washed thoroughly, the free phosphonic groups should not come from the physical adsorption of DDPA on zirconia, but from chemical adsorption. Zirconia that was dipped in a DDPA solution for 48 h (at room temperature) also has a free phosphonic group, though the coverage of this zirconia is only 0.64 μmol m^{-2} (N% = 0.0260%, C% = 0.3169%), far smaller than the coverage of DPZ (3.3 μmol m^{-2}, N% = 0.1342%, C% = 1.6636%). This result can only be interpreted as DDPA adsorbed on zirconia only with one phosphonic group, as shown in Fig. 1.

The structure of DPZ is quite different from ODS and APA-ZrO_2 [11], there is a zwitterion layer that consists of an amine group and a phosphonic group on the surface of DPZ. Obviously, this polar inner-layer would play an important role in the retention and selectivity of positional isomers with different polar groups.

3.2. Effect of mobile phase on capacity factors of positional isomers

3.2.1. Effect of methanol content

Nitrophenols are the strongest acids among the six sets of isomers, and toluidines are the strongest bases. The effect of the methanol content on the capacity factors of their isomers on DPZ is shown in Fig. 2.

The capacity factors of nitrophenols decrease linearly with the increasing of methanol content, which indicated the major interaction between nitrophenols and DPZ is hydrophobic interaction. However, the capacity factors of toluidines do not decrease linearly at the whole range from 50 to 90%, especially capacity factors of *p*-toluidine, which even show slight increase from 80 to 90%. This situation is quite different from that on traditional zirconia stationary phase, such as APA-ZrO_2 [9], which indicated that there are strong secondary interactions between toludines and DPZ besides hydrophobic interaction provided by the alkylchain.

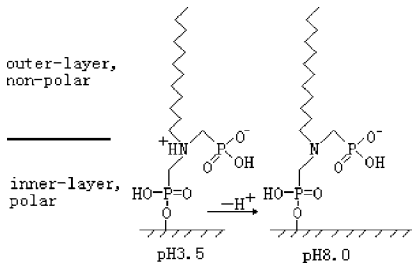

Fig. 1. The adsorption mode of DDPA on zirconia.

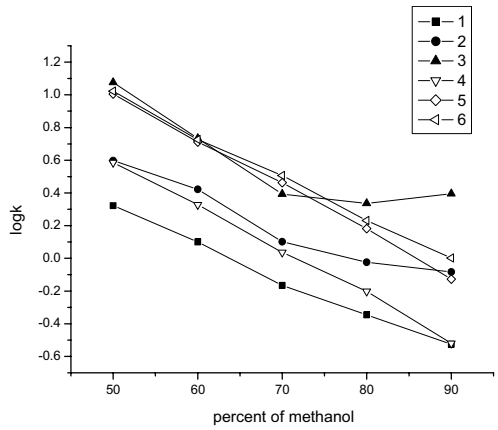

Fig. 2. Effect of the methanol content of mobile phase on capacity factors of nitrophenols and toluidines. Solutes: (1) *o*-toluidine; (2) *m*-toluidine; (3) *p*-toluidine; (4) *o*-nitrophenol; (5) *m*-nitrophenol; (6) *p*-nitrophenol.

3.2.2. Effect of ionic strength

The effect of ionic strength on capacity factors of anilines at pH 3.5 was shown in Fig. 3. Capacity factors decrease with the increasing of ionic strength, mainly due to the decreasing of ionic interaction between protonated anilines and dissociated phosphonic group at pH 3.5. The strength of ionic interaction agrees with the basicity of solutes: the greater the basicity, the stronger the ionic interaction. So the capacity factors of toluidines decrease sharply, while capacity factors of nitroanilines decrease slightly, because toluidines are the strongest bases and nitroanilines are the weakest one among the substituted anilines.

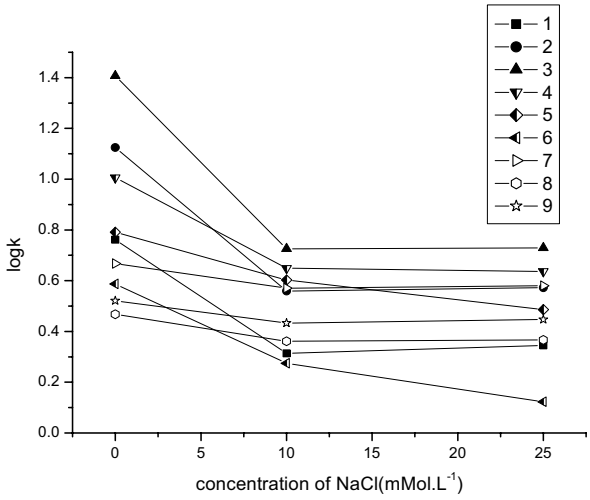

Fig. 3. Effect of the ionic strength of mobile phase on capacity factors of (1) *o*-toluidine; (2) *m*-toluidine; (3) *p*-toluidine; (4) *o*-aminophenol; (5) *m*-aminophenol; (6) *p*-aminophenol; (7) *o*-nitroaniline; (8) *m*-nitroaniline; (9) *p*-nitroaniline. Mobile phase: methanol/buffer (50/50, v/v), pH 3.5, 10 mmol l^{-1}HAc, flow rate: 1.0 ml min^{-1}.

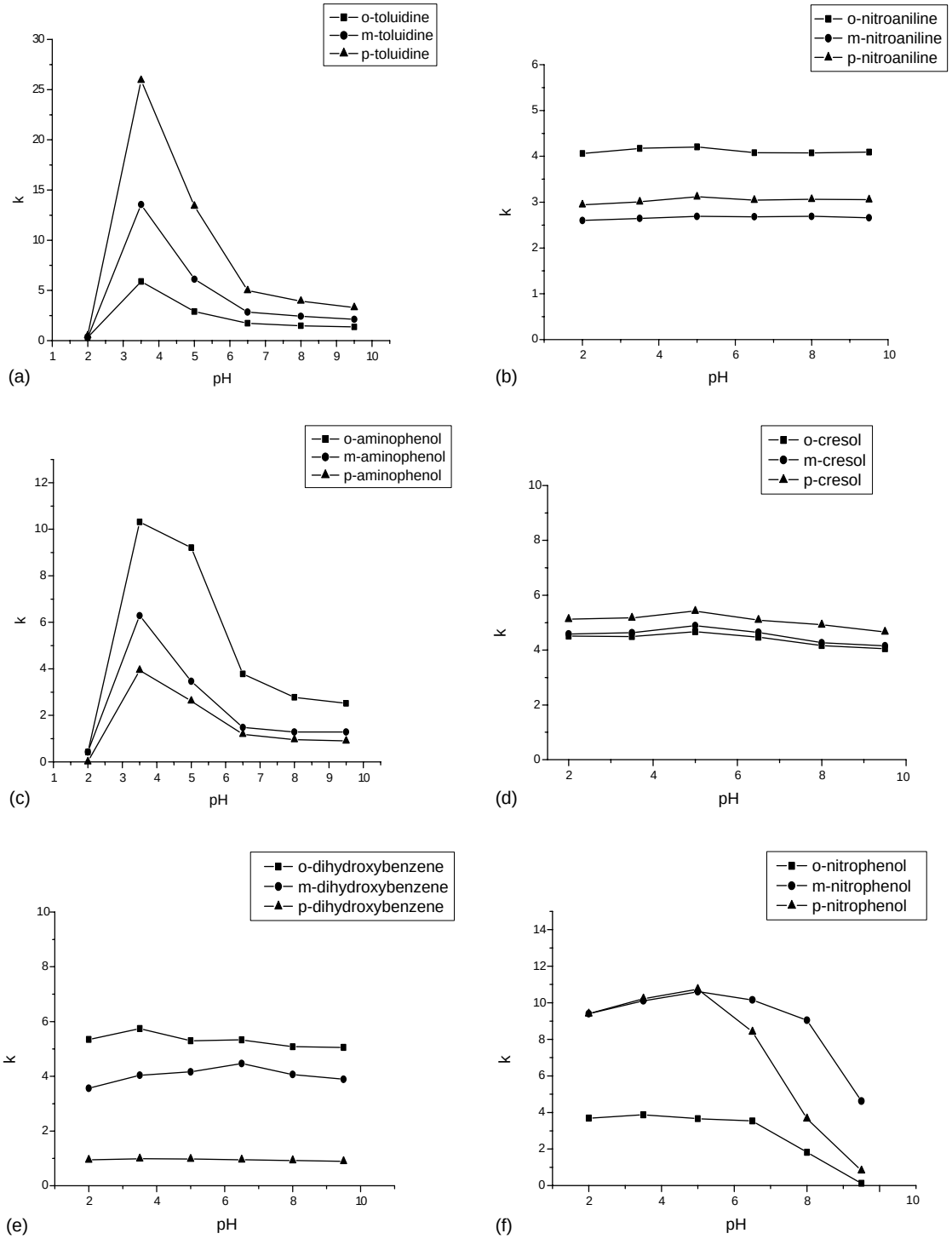

Fig. 4. Effect of mobile phase pH on capacity factors of isomers: (a) toluidine; (b) nitroaniline; (c) aminophenol; (d) cresol; (e) dihydroxybenzene; (f) nitrophenol. Mobile phase: methanol/buffer (50/50), 10 mmol l^{-1} HAc for pH 2.0, 3.5, 5.0, 6.5; 10 mmol l^{-1} Tris for pH 8.0, 9.5, flow rate: 1.0 ml min^{-1}.

3.2.3. *Effect of mobile phase pH*

For the separation of ionizable solutes, the variation of mobile phase pH can lead to extreme changes in the retention and selectivity, because the degree of ionization varies with the pH of the mobile phase. As for DPZ, the surface charge of stationary phase is dependent on the pH of the mobile phase. So the effect of pH on capacity factors of positional isomers was investigated in detail, and the results obtained were shown in Fig. 4.

The maximal capacity factors of toluidines and aminophenols at pH 3.5 is due to the strongest ionic interaction at pH 3.5, which can be ascribed to the increase in dissociation of

phosphonic group at pH > 3.5 and increase in protonation of these amines at pH < 3.5.

Capacity factors of nitrophenols change a little at pH 2.0–5.0, but at pH 6.5–9.5, capacity factors decrease due to the coulombic repellent interaction between ionized nitrophenols and dissociated phosphonic group.

Capacity factors of nitroanilines are nearly constant at pH 2.0–9.5 because their basicity is much less than that of aniline (pK_b of *p*-nitroaniline is 14.26, almost neutral compounds), so nitroanilines aren't ionized and always exist in neutral form. Cresols and dihydroxybenzenes have less acidity than phenol, their capacity factors only slightly decreased at pH > 5.0.

3.2.4. Retention data and elution order on DPZ and ODS

Separations of positional isomers on DPZ were compared with those on ODS. The capacity factors and elution order of isomers at pH 8.0 and pH 3.5 were listed in Table 2. Capacity factors of all isomers on DPZ are greater than on ODS (except *o*-toluidine at pH 8.0). Selectivity of most isomers is also better on DPZ than on ODS. The capacity factors and selectivity of toluidines on APA-ZrO_2 are smaller than that on DPZ; however, most of phenols were severely tailed and even could not be eluted on APA-ZrO_2 [25].

The elution order of *o*-, *m*-, *p*-isomers on DPZ is quite different from that on ODS. All of the *o*-isomers have larger capacity factor than *m*-, *p*-isomers on ODS at pH 3.50. However, no general rule of the elution order of isomers on DPZ was observed because of its complicated retention mechanisms.The elution of toluidines ($o < m < p$) is in order of increasing of basicity ($pK_b = 9.56, 9.28, 8.90$ for *o*-, *m*-, *p*-toluidine, respectively), because the main interaction for separation of toluidines is ionic interaction. *p*-Toluidine, which is the strongest bases, eluted at last due to the greatest ionic interaction.

Although aminophenols have similar basicity to toluidines ($pK_b = 9.28, 9.83, 8.50$ for *o*-, *m*-, *p*-aminophenol, respectively), the elution order of aminophenols ($p < m < o$) does not correlate with the basicity. *p*-Aminophenol eluted firstly due to the greater hydrophilicity and the *o*-aminophenol eluted last due to chelating effect with zirconia. The elution order of dihydroxybenzene ($p < m < o$) can be explained similarly [22].

Table 2
Retention data and elution order of isomers on DPZ and ODS

Isomers	DPZ					ODS				
	k		*α*		Order	*k*		*α*		Order
	pH 3.5	pH 8.0	pH 3.5	pH 8.0		pH 3.5	pH 8.0	pH 3.5	pH 8.0	
Toluidine										
o	5.88	1.48 (1.66)[a]			$o < m < p$	1.51	1.79	1.40		$p < m < o$
m	13.55	2.41 (2.02)[a]	2.30	1.64 (1.21)[a]		1.46	1.92	1.35	1.07	$(o < m \approx p)$[b]
p	25.92	3.93 (2.30)[a]	4.41	2.66 (1.38)[a]		1.08	1.90		1.06	
Nitroaniline										
o	4.73	3.87	1.58	1.51	$m < p < o$	2.43	2.27	2.27	2.28	$p < m < o$
m	3.00	2.56				1.50	1.40	1.40	1.40	
p	3.38	3.09	1.13	1.21		1.07	1.00			
Aminophenol										
o	10.31	2.78	2.62	2.89	$p < m < o$	0.29	0.44	15.33	4.44	$p < m < o$
m	6.29	1.28	1.60	1.33		0.18	0.20	9.67	2.03	
p	3.94	0.96				0.02	0.10			
Cresol										
o	4.63	4.16			$o < m < p$	2.98	3.04	1.09	1.15	$p \approx m < o$
m	4.63	4.27	1.03	1.02		2.74	2.67	1.00	1.01	
p	5.17	4.92	1.15	1.18		2.74	2.64			
Dihydroxybenzene										
o	5.74	5.08	5.79	5.51	$p < m < o$	0.62	0.58	3.40	3.44	$p < m < o$
m	4.04	4.06	4.07	4.41		0.34	0.32	1.90	1.89	
p	0.99	0.92				0.18	0.17			
Nitrophenol										
o	3.88	1.82			$o < m < p$ $(o < p < m)$[b]	3.51	1.34	1.52	1.92	$p < m < o$ $(p < o < m)$[b]
m	10.10	9.05	2.61	4.97		2.61	2.16	1.13	3.08	
p	10.22	3.66	2.64	2.01		2.32	0.70			

k: capacity factor; *α*: relative retention; mobile phase: methanol/buffer (50/50); buffer: 10 mmol l^{-1} HAc for pH 3.5; 10 mmol l^{-1} Tris for pH 8.0.

[a] Retention data on APA-ZrO_2, mobile phase: methanol/buffer (30/70); 10 mmol l^{-1}Tris, pH 8.0, from ref. [25].

[b] Elution order of isomers at pH 8.0.

The elution order of nitrophenols change from *o* < *m* < *p* at pH 3.5 to *o* < *p* < *m* at pH 8.0. Acidity of *p*-nitrophenol (pK_a = 7.15) is greater than that of *m*-nitrophenol (pK_a = 8.39), so the ionization degree of *p*-nitrophenol is higher than *m*-nitrophenol at pH 8.0. Because of higher degree of ionization, the hydrophobic interaction decreased and coulombic repellent interaction between anionic solutes and dissociated phosphonic group increased, so the capacity factor of *p*-nitrophenol decreases dramatically.

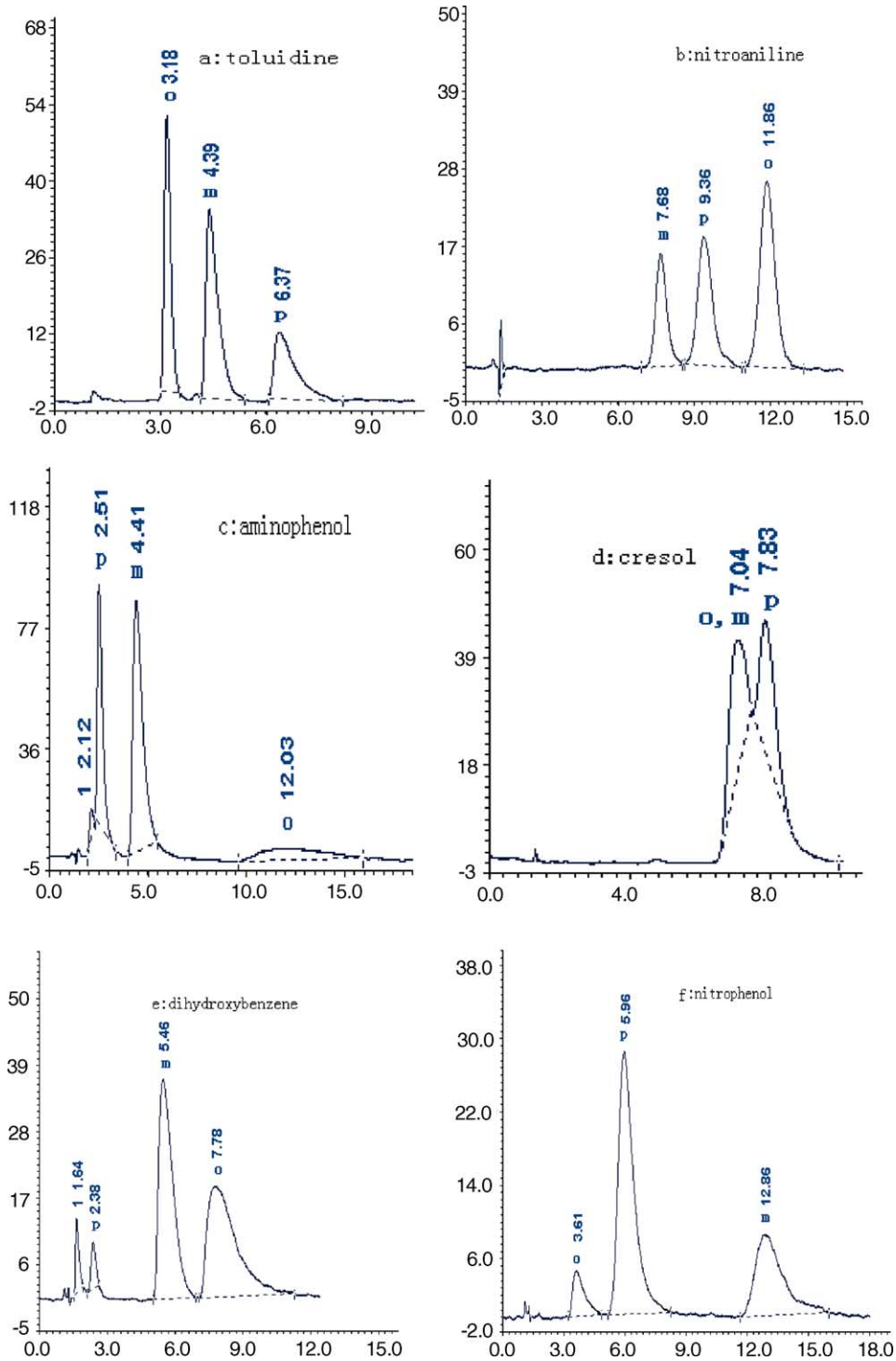

Fig. 5. Separation of mixture of isomers: (a) toluidines; (d) cresols; (e) dihydroxybenzenes and (f) nitrophenols (methanol/buffer (50/50), pH 8.0, 10 mmol l^{-1}Tris); (b) nitroanilines (methanol/buffer (30/80), pH 8.0, 10 mmol l^{-1}Tris); (c) aminophenols (methanol/buffer (25/75), pH 3.5, 10 mmol l^{-1} HAc, 10 mmol l^{-1}NaCl). The first peak of (c) and (e) were assigned to the oxidation product of *p*-isomer of aminophenol and dihydroxybenzene, respectively, verified by the chromatograms of pure crystals of isomers.

The elution order of nitroanilines and cresols on DPZ may be a composite result from various retention interactions. The slow elution of *o*-nitroanilines may be ascribed to the greater hydrophobic interaction provided by intramolecular hydrogen bonding. Dipolar interaction also contribute significantly to capacity factors on DPZ, because capacity factors of nitroaniline on DPZ is greater than on ODS though ODS has greater coverage and longer alkyl chain (C_{18} versus C_{12}).

3.3. Chromatography of separation of mixture of isomers

Separations of the mixture of positional isomers on DPZ are shown in Fig. 5. Toluidines, aminophenols, nitroanilines and dihydroxybenzenes can be separated on baseline at range of pH 3.5–9.5, but ionic strength must be increased to diminish the ionic interaction when toluidines and aminophenols are separated at pH 3.50. Nitrophenols can only be completely separated at pH 6.5–8.0. Separation of cresol isomers, as shown in Fig. 5(d), is not satisfactory, because the difference of polarity and acidity between cresols is too small. This result suggests that DPZ is not very suitable for separation of the less polar or non-polar isomers.

The peak shapes also indicate the various retention mechanisms. The tailing peaks of toluidines are observed which provide another evidence of cation exchange interaction. The lower column efficiency of phenols indicate the Lewis acid–base interaction between phenols and residual Lewis acid sites.

3.4. Stability of the stationary phase

The stability of the stationary phase is also investigated, the repeatability of retention have been examined by using toluene as probe. A plot of the capacity factor of toluene against the volume of mobile phase at pH 9.50 is shown in Fig. 6. The result indicates that the DPZ is stable in routine chromatographic analysis.

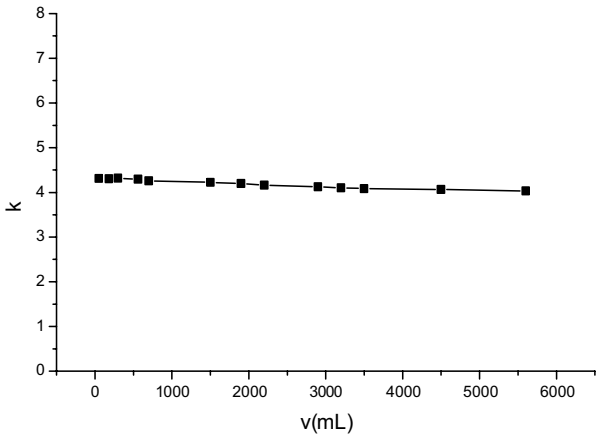

Fig. 6. Investigation of stability of DPZ stationary phase. Mobile phase: methanol/buffer (50/50, v/v), 10 mmol l^{-1} Tris, pH 9.50. Test solutes: toluene.

4. Conclusion

A new stationary phase (DPZ) was prepared by modification of zirconia with dodecylamine-*N*,*N*-dimethylenephosphonic acid. Although separation of acids (such as phenols, carboxylic acids) on zirconia stationary phase has been troublesome all the time due to the Lewis acid–base interaction, baseline separations of isomers, including aminophenols, dihydroxybenzenes, and nitrophenols, were achieved in this work. The adsorption mode of DDPA on zirconia was confirmed by coloration reaction to be via only one phosphonic group binding to the Lewis acid site of zirconia. Various retention mechanisms contributing to the retention of analytes on DPZ were proposed according to the effect of methanol content, ionic strength, and pH of mobile phase on capacity factors of isomers.

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