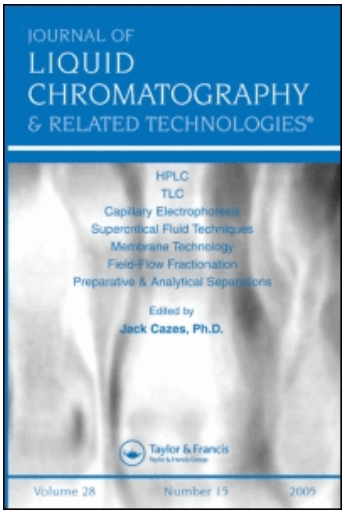


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Hydrophobic Interaction Chromatography of a Fosfomycin-Modified Zirconia Support for Some Basic Proteins

Hai-Bo He[a]; Qiong-Wei Yu[b]; Yu-Qi Feng[b]; Shi-Lu Da[b]
[a] Department of Chemistry, Shanghai University, Shanghai, P. R. China [b] Department of Chemistry, Wuhan University, Wuhan, P. R. China

**To cite this Article** He, Hai-Bo , Yu, Qiong-Wei , Feng, Yu-Qi and Da, Shi-Lu(2009) 'Hydrophobic Interaction Chromatography of a Fosfomycin-Modified Zirconia Support for Some Basic Proteins', Journal of Liquid Chromatography & Related Technologies, 32: 3, 468 — 482
**To link to this Article: DOI:** 10.1080/10826070802634745
**URL:** http://dx.doi.org/10.1080/10826070802634745

*Journal of Liquid Chromatography & Related Technologies*®, 32: 468–482, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802634745

# Hydrophobic Interaction Chromatography of a Fosfomycin-Modified Zirconia Support for Some Basic Proteins

**Hai-Bo He,[1] Qiong-Wei Yu,[2] Yu-Qi Feng,[2] and Shi-Lu Da[2]**

[1]Department of Chemistry, Shanghai University, Shanghai, P. R. China
[2]Department of Chemistry, Wuhan University, Wuhan, P. R. China



**Abstract:** In this study, we explored the use of fosfomycin modified microparticulate zirconia (FZ) as a novel hydrophobic interaction chromatography (HIC) stationary phase for proteins separation. The results showed that on the FZ stationary phase, the basic proteins such as lysozyme, ribonuclease-A, and a-chymotrypsin, presented a retention mechanism that well agreed with the theory of hydrophobic interaction. The undesirable cation exchange and ligand exchange interactions that usually resulted from the complex surface chemistry of zirconia matrix, could be effectively suppressed due to the phosphonate modification and moderately high salt concentration used in the mobile phases. A few basic proteins achieved good separations by the gradient elution mode with decreasing salt concentration, and high mass recoveries, as well as loading capacity for these proteins obtained on the FZ column. This demonstrates that the FZ stationary phase would be a complement for biocompatible HIC materials, especially for basic proteins, and allows zirconia based supports are more useful for bio-separation.

**Keywords:** Basic proteins, Fosfomycin, Hydrophobic interaction, Zirconia support

Correspondence: Yu-Qi Feng, Department of Chemistry, Wuhan University, Wuhan, P. R. China. E-mail: yqfeng@public.wh.hb.cn (Y.-Q. Feng) or hbhe2006@shu.edu.cn (H.-B. He)

## INTRODUCTION

Hydrophobic interaction chromatography (HIC) is an important separation mode for separating and purifying proteins, which is based on the hydrophobic properties of the biomolecular surface and their interactions with the non-polar ligands attached to a stationary matrix.[1,2] Owing to involvement of a weaker interaction than affinity, ion exchange, or reversed phase chromatography, HIC separation avoids the use of hazardous denaturing organic co-solvents, the structural damage to the proteins is assumed to be minimized, and the biological activity of the proteins is maintained. Therefore, HIC is widely employed to separate proteins in both preparative and analytical settings.[3] Furthermore, it has been shown that the rational design of downstream processing of a bimolecular normally requires an HIC stage.[4]

The typical bioprocess HIC media is based on the highly cross linked beaded agars matrices such as alkyl- or phenyl-sepharose.[5] Such soft matrices based HIC supports have a good biocompatibility as well as high bioactivity recoveries for proteins. Nonetheless, they have the common defects of poor mechanical strength, long run time, and poor separation efficiency in separations. Therefore, silica and polymer matrices with high mechanical strength are increasingly encouraged for preparation of HIC stationary phases.[6,7] Although polymers enjoy wide use in preparative applications of HIC matrices owing to affording good recovery and chemical stability in proteins separation, the development of optimized HIC methods remain troublesome, because polymers easily swell in organic solvent and high ionic strength solvents.[8] At the present time, silica occupies the dominant position among the matrix for biomolecular separations due to its ideal mechanical strength and pore structure. In addition, silica based HIC stationary phases generally exhibit better chromatographic efficiency compared with those shown by organic polymers; silica based stationary phases usually are not stable outside the pH range from 2 to approximately 8 and at temperatures higher than 40°C.[9] Silica itself is slightly soluble, even in neutral (pH 7) solutions, especially in the presence of phosphate, a common buffer used in biochemistry.[10] Moreover, the silanol interactions for basic solutes may generate irreproducible retention, severely tailed peaks, and low column efficiency.[11] The above deficiencies make the application of silica to be limited to a great extent. Therefore, the research into the development of a more appropriate HIC stationary phase seems very challenging.

More recently, the zirconia spherical based protein separation media has gained considerable attention. As compared with the silica matrix, zirconia has superior mechanical, thermal, and chemical stability (pH 0–14). More importantly, it has been found that zirconia based phases are more preferable for the separation of basic solutes. By taking

advantage of the abundant Lewis acid sites on zirconia, Carr et al. prepared various zirconia based strong cation exchange stationary phases by chemisorption of strong Lewis bases such as fluoride,[12,13] phosphate,[14] and EDTPA (ethylenediamine-N, N′-tetramethylphosphonic acid)[15–17] on the zirconia matrices. Successively, a weak cation exchange zirconia based polymeric stationary phase was also prepared.[18] Both the zirconia based strong and weak cation exchangers are very useful for protein and bimolecular separations and offer a great potential for replacing polymer and silica based ion exchangers because of the excellent stabilities and biocompatibility of zirconia.

As a follow up study and a complement to a new family of zirconia based stationary phases, which offer great utility for biological compounds separation and purification, we developed a novel HIC stationary phase using zirconia as matrices. This new HIC phase was prepared by modification of zirconia via chemisorption of fosfomycin, which has both phosphonate and hydrophobic epoxy propane groups. The chromatographic characteristics were studied by examining the retention behavior of basic proteins. The separation of a few basic proteins was achieved by HIC with gradient elution mode. The effects of a number of process variables, including salt type and concentration, pH, and temperature were assessed. Protein recovery and column loading capacity were also investigated.



## EXPERIMENTAL

### Reagents and Chemicals

Microspherical zirconia was homemade[19] with a mean particle diameter of 5 μm, the specific surface area, pore volume, and pore diameter, which were determined by the nitrogen adsorption procedure, is $14\,m^2 \cdot g^{-1}$, $0.072\,mL \cdot g^{-1}$, and 18 nm, respectively. Fosfomycin was obtained from Northeastern Pharmaceutical Factory (Shengyang, China). Lysozyme (chicken egg white), ribonuclease A (bovine pancreas), a-chymotrypsin (bovine pancreas), cytochrome C (type VI from horse heart), a-chymotrypsinogen A (bovine pancreas), were obtained from Sigma and were used without further purification. Solutes were dissolved in distilled water at a concentration of $2–5\,mg \cdot mL^{-1}$. Other compounds and reagents were analytical reagent grade and were commercially available without further purification before use.

### Preparation of Fosfomycin Modified Zirconia (fz) Stationary Phase

The FZ stationary phase was prepared on the basis of chemisorption of fosfomycin to the Lewis acid sites on the zirconia surface. The

modification was conducted in an aggressive condition according to our previous work:[20] 7 g of zirconia particles was slurried in 60 mL of $0.1\,mol \cdot L^{-1}$ sodium fosfomycin solution, which was adjusted to pH 5 with diluted hydrochloric acid. The particles were sonicated under vacuum for 10 min and then refluxed with stirring at 100°C. After 12 h of refluxing, the fosfomycin modified zirconia particles were sequentially washed with distilled water, methanol, and acetone and dried under vacuum at 100°C overnight. The structure of the resulting FZ stationary phase was as depicted in Figure 1.

## Column Packing

The FZ particles were slurried in 2-propanol and packed into a 150 × 4.6 mm stainless column at a packing pressure of 5000 p.s.i and using pure methanol as the driving solvent. For comparison, an unmodified zirconia column was also packed in the same way.



## Apparatus

The isocratic HPLC system used in these studies consisted of a Elite P230 liquid chromatographic pump (Elite, Dalian, China), a FL2200 variable wavelength UV-detector (Fuli, Wenling, China), a Rheodyne 7125 injector system (sample loop 1 mL for column loading capacity measurement, and 20 μL for other analyses), attached Echrom 98 chromatographic data system (Elite, Dalian, China). Columns were thermostated using a ZW stable temperature controller column oven (Elite, Dalian, China). An Agilent 1100 liquid chromatograph with diode array detection (Agilent, USA) was used for gradient elution.

## Chromatography Measurements

The mobile phases used are described in the figure captions. All mobile phases were filtered through a G4 fritted glass funnel of 3–4 μm pore size

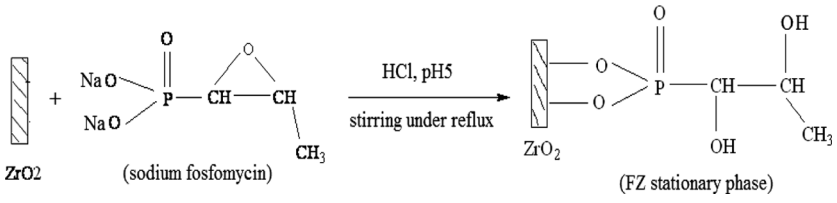


*Figure 1.* Scheme to prepare the FZ stationary phase.

and degassed prior to use. The flow rate of the mobile phase was 1.00 or $0.8\,mL \cdot min^{-1}$. The wavelength used for detection was 280 nm. The void volume was determined using distilled water. The retention factors of the proteins were determined in isocratic chromatographic runs. The peak area and peak width integration were processed using Elite 98 Chemstation software.

### Protein Recovery Studies

The protein mass recovery studied refer to a reported method by Carr et al.[20,24] The recovery of protein was measured by comparing the amount (peak area) of protein collected in a control experiment in which the column was replaced with a zero dead volume detector to that obtained with the column in place. The protein was eluted isocratically, and the ionic strength of the mobile phase was adjusted to ensure sufficient retention ($k > 1$) so that any disturbance at the dead time was not a problem in peak integration.



## RESULTS AND DISCUSSION

According to the elemental analysis results (C (%) 0.37), about 7.45 μmol of the fosfomycin was immobilized per square meter of zirconia surface. The Lewis acidity on the FZ stationary phase was evaluated by eluting some Lewis bases under a variety of typical reversed phase conditions. The strong Lewis bases such as the nitrophenol isomers and nitrobenzoic acids on the FZ column were eluted within a short time (<20 min) using methanol-water as mobile phases; while on the bare zirconia column using the same mobile phases, the ortho-nitrophenol were not eluted within 1 h. This is identical to the results reported earlier.[20] Apparently, the fosfomycin modification effectively shielded the sites responsible for strong interactions of hard Lewis bases with the zirconia's surface, and the short hydrophobic chain of the fosfomycin would impart the FZ stationary phase as a relatively weak hydrophobic property.

### Chromatography of Basic Proteins

The suitability of the FZ stationary phase for protein chromatography was investigated by eluting five model basic proteins (lysozyme, ribonuclease A, a-chymotrypsin, cytochrome C, and a-chymotrypsinogen A) and using ammonium sulfate aqueous solution as eluate in an linear gradient mode. The proteins are always eluted in the sequence of ribonuclease A, cytochrome C, a-chymotrypsin, lysozyme, and a-chymotrypsinogen

A, this is in accord with their hydrophobicity in general, implying that the hydrophobic interaction plays an important role in retenion mechanism. As displayed in Figure 2, a good separation between ribonuclease A and lysozyme (Figure 2 a), and cytochrome C and a-chymotrypsinogen A (Figure 2b) could be achieved by adjusting the gradient elution time.

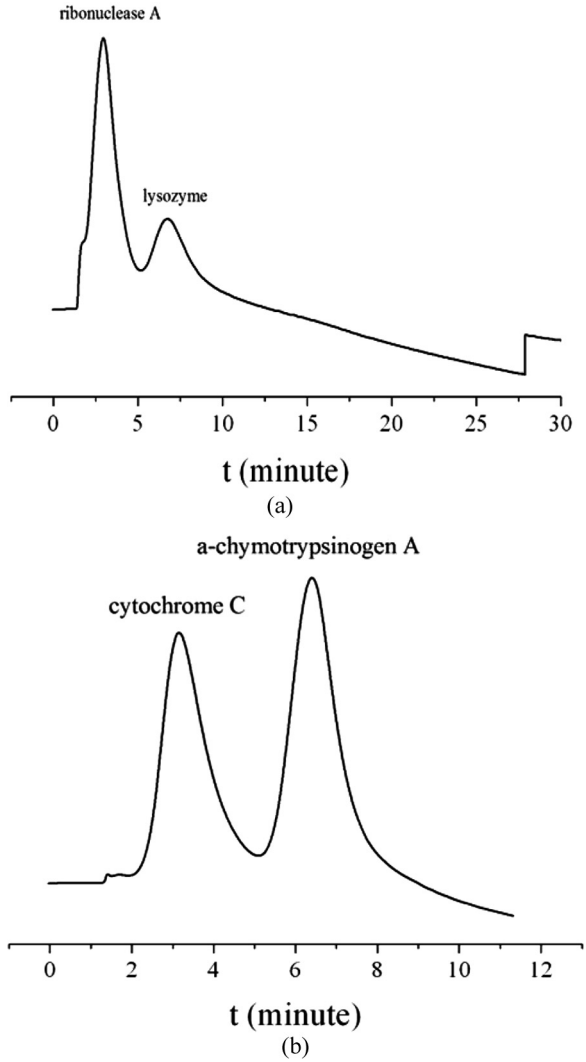


***Figure 2.*** Protein separations using the FZ column. A linear gradient of (3.0-0) $mol \cdot L^{-1}$ $(NH_4)_2SO_4$ in 20 $mmol \cdot L^{-1}$ NaAc at pH 7.1 was run in (a) 30 min, (b) 15 min. Flow rate was 1 $mL \cdot min^{-1}$ at 18°C ; detection at 280 nm.

The retention mechanism for three basic proteins on the FZ stationary phase was studied in detail under isocratic elution conditions.

## Effect of Salt

The main system characteristics affecting protein retention in HIC are concentration and type of salt.[21,22] In this study, ammonium sulphate aqueous solutions with a concentration between 0.2–2.0 $mol \cdot L^{-1}$ were used as the mobile phases to characterize the chromatographic behavior of lysozyme, ribonuclease A, a-chymotrypsinogen A, and cytochrome C on the FZ stationary phase. As shown in Figure 3, in the range of 0.2–1.0 $mol \cdot L^{-1}$ of ammonium sulphate in the mobile phase, the retention factors of four proteins decreased sharply when the concentration of ammonium sulphate in the eluate was increased, suggesting an ion exchange interaction between solutes and stationary phase at lower salt concentration. This is explainable because only a part phosphate would bind to zirconia via tridentate form even if the modification was conducted under refluxing conditions,[23,24] so the fosfomycin would act as the cation exchange interaction sites for these proteins with high p*I* values on the FZ phase.



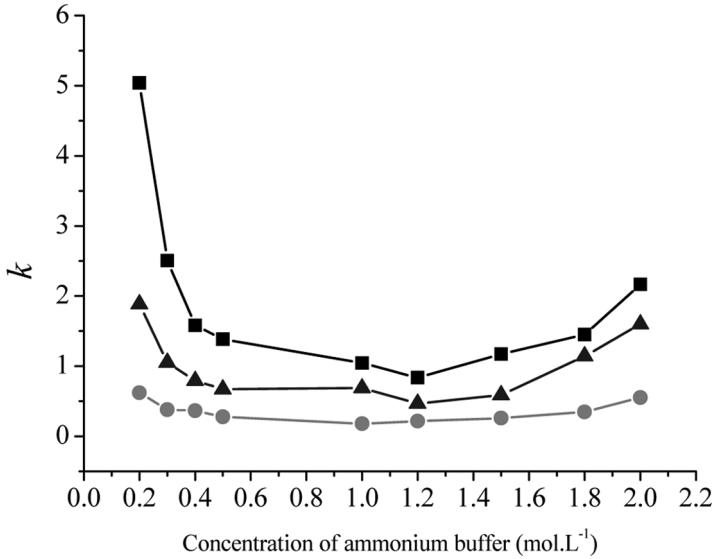


***Figure 3.*** Concentration effect of salt in mobile phase on proteins using the FZ column. Solutes: (■)lysozyme; (●)ribonuclease A; (▲)a-chymotrypsin. Experimental conditions: isocratic elution with 20 $mmol.L^{-1}$ NaAc + variable concentration of $(NH_4)_2SO_4$ for pH 8.1; 18°C; 1 $mL \cdot min^{-1}$; detection at 280 nm.

However, we note that at higher salt concentration, increasing the amount of ammonium sulphate in the eluate increased the retention time of all of the same proteins (as shown in Figures 3 and 5). This is typical hydrophobic interaction behavior. Moreover, Figure 4 depicts the plots of the logarithm of retention factors for lysozyme and ribonuclease A against the concentration of salt concentration with good regression coefficients; this further corroborates the solvophobic theory for proteins retention in HIC.[25]

As can be seen from Figure 6, the retention of proteins was also influenced by the type of salt. The proteins are eluted more easily from the FZ stationary phase when using ammonium sulfate as compared to ammonium acetate and ammonium chloride, this is a familiar phenomenon usually occurring in HIC.[21]

## Effect of pH



Since the extreme basic or acidic conditions would denature significant amounts of proteins, a moderate pH mobile phase should be strongly recommended in protein separations in order to keep their bioactivities. The mixtures of sodium acetate and ammonium sulfate between pH 5.1–8.1 were used in our study of the effect of the mobile phase pH. As shown in Figure 5, a monotonic decrease in retention with increasing pH

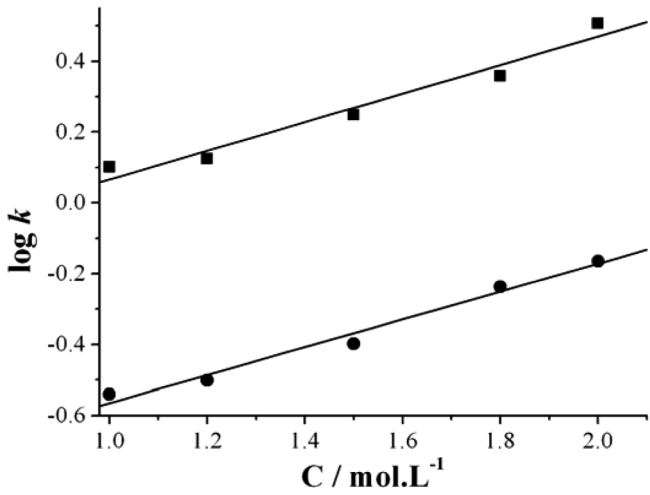


***Figure 4.*** Plot of log $k$ versus C on the FZ column. (■) lysozym: log $k = -0.338 + 0.404\,C$, $r = 0.981 \pm 0.038$; (●) ribonuclease A: log $k = -0.959 + 0.394\,C$, $r = 0.991 \pm 0.023$. Experimental conditions: isocratic elution with 20 mmol · $L^{-1}$ NaAc + (1.0–2.0) mol · $L^{-1}$ $(NH_4)_2SO_4$ for pH 7.1; 18°C; 1 mL · $min^{-1}$; detection at 280 nm.

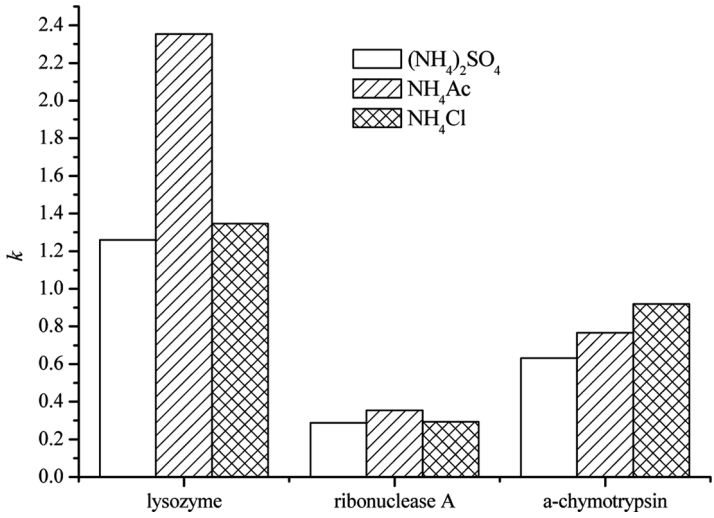


***Figure 5.*** pH effect on protein (a) lysozym, (b) ribonuclease A and (c) a-chymotrypsin, retentions on the FZ column. Experimental conditions: isocratic elution with 20 mmol · $L^{-1}$ NaAc + (1.0–2.0) mol · $L^{-1}$ $(NH_4)_2SO_4$ for (■) pH 8.1, (●) pH 7.1, (▲) pH 6.1, and (▼) pH 5.1; 18°C; 1 mL · $min^{-1}$; detection at 280 nm.



values of mobile phase was observed for all proteins studied. Srinivasan et al.[26,27] believed that the pH effect for proteins retention mainly attributed to the electrostatic interactions between the stationary phase and proteins. Obviously, the higher pH of the mobile phase may deprotonate the proteins. A less positive protein will result in a decrease on the cation exchange interaction between the proteins and the FZ stationary phase. Interestingly, as exhibited in Figure 7, when ammonium sulfate with lower concentration was used as the eluting salt, with the exception that lysozyme reached a maximum retention of about pH 5.3, the retention of other proteins obviously decreased with increasing mobile phase pH. This implies that eluting salts with higher concentration should be employed to minimize the undesirable effects such as cation exchange and ligand exchange interactions between proteins and the FZ stationary phase. The above results may be advantageous for selectivity improvement in separations with the FZ stationary phase.

## Effect of Temperature

It has been found that proteins undergo conformational changes when the temperature is increased; leading to the amino acids on the inner surface of proteins becoming exposed.[28] This will enhance the hydrophobic force between protein and stationary phase, resulting in longer

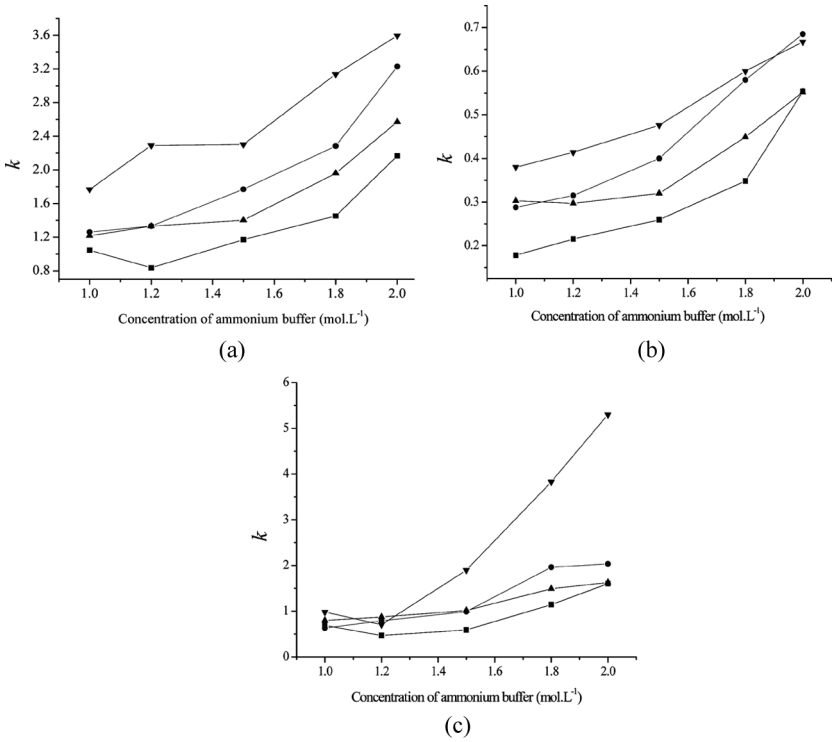


***Figure 6.*** Effect of salt type on protein retentions on the FZ column. Experimental conditions: isocratic elution with $20\,mmol \cdot L^{-1}$ NaAc + $1.0\,mmol \cdot L^{-1}$ various ammonium salt for pH 7.1; $1\,mL \cdot min^{-1}$; detection at 280 nm.

retention time. It is an important phenomenon that distinguishes the hydrophobic interaction chromatography from other chromatography methods.[29,30] According to Horvath et al.,[31] the thermodynamic behavior of proteins on HIC should be expressed as the following nonlinear Van't Hoff equation

$$\ln k = a + b/T + c/T^2 + \ln\varphi$$

Where k is the solutes retention factors, $\varphi$ is the column phase ratio, T is column temperature. The Van't Hoff plots shown in Figure 8 exhibits a positive slope and an ideal correlation ($r > 0.973$), illustrating a typical HIC character for proteins separation with the FZ stationary phase.

## Protein Recovery and Column Loading Capacity

In this study, the protein recovery on the FZ phase was studied as described before. The mobile phases were chosen to give reasonable

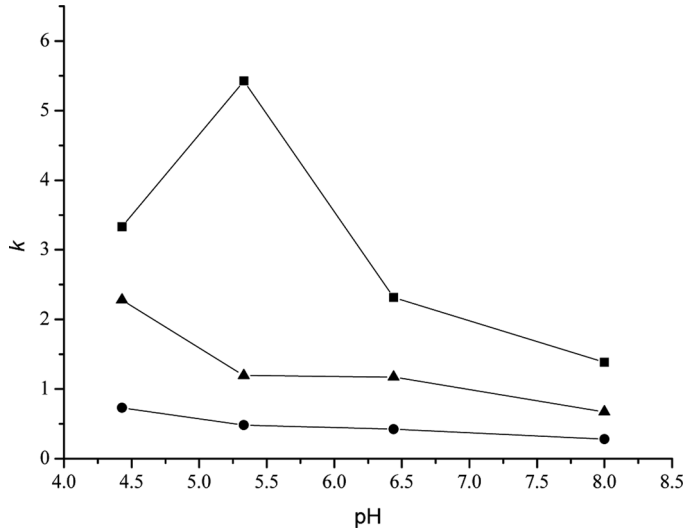


***Figure 7.*** pH effect on protein retentions on the FZ column. Experimental conditions: isocratic elution with 20 mmol · $L^{-1}$ NaAc + 0.5 mol · $L^{-1}$ $(NH_4)_2SO_4$ for (■) lysozyme, (●) ribonuclease A and (▲) a-chymotrypsin; 18°C; 1 mL · $min^{-1}$; detection at 280 nm. 1 mL · $min^{-1}$; detection at 280 nm.

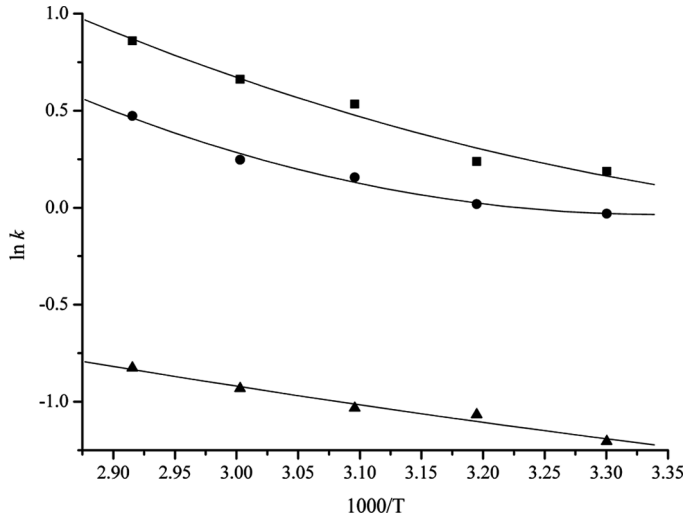


***Figure 8.*** Effect of temperature on protein retentions on the FZ column. Experimental conditions: isocratic elution with 20 mmol · $L^{-1}$ NaAc 1.0 mol · $L^{-1}$ $(NH_4)_2SO_4$ at pH 7.1 for (■) Lys (r = 0.973), (▲) α-Chy (r = 0.989) and (▼) RNase-A (r = 0.976); 1 mL · $min^{-1}$; detection at 280 nm.

***Table 1.*** Proteins recovery study on the FZ column[a]

| Proteins | mobile phase (pH 7.1) | t(min) | % recovery ± s.d. (n = 3)[b] |
|---|---|---|---|
| lysozym | 1.0 mol · $L^{-1}$ $(NH_4)_2SO_4$ + 20 mmol · $L^{-1}$ NaAc | 2.96 | 97.6 ± 2.3 |
| ribonuclease A | 2.5 mol · $L^{-1}$ $(NH_4)_2SO_4$ + 20 m mol · $L^{-1}$ NaAc | 3.53 | 99.2 ± 1.0 |
| a-chymotrypsin | 1.8 mol · $L^{-1}$ $(NH_4)_2SO_4$ + 20 m mol · $L^{-1}$ NaAc | 4.50 | 95.4 ± 3.6 |

[a]Experimental conditions: 1 mL · $min^{-1}$; 18°C; detection at 280 nm.
[b]See Experimental section 2.6 for calculation.

retention factors and, therefore, accurate peak integration for quantitative analysis. As shown in Table 1, the FZ stationary phase gave good recovery ratios for all the basic proteins tested, with the average recovery being higher than 90%, indicating that the undesirable irreversible adsorption between proteins and the FZ phase is well suppressed under such conditions. This means that there is less chance of column fouling



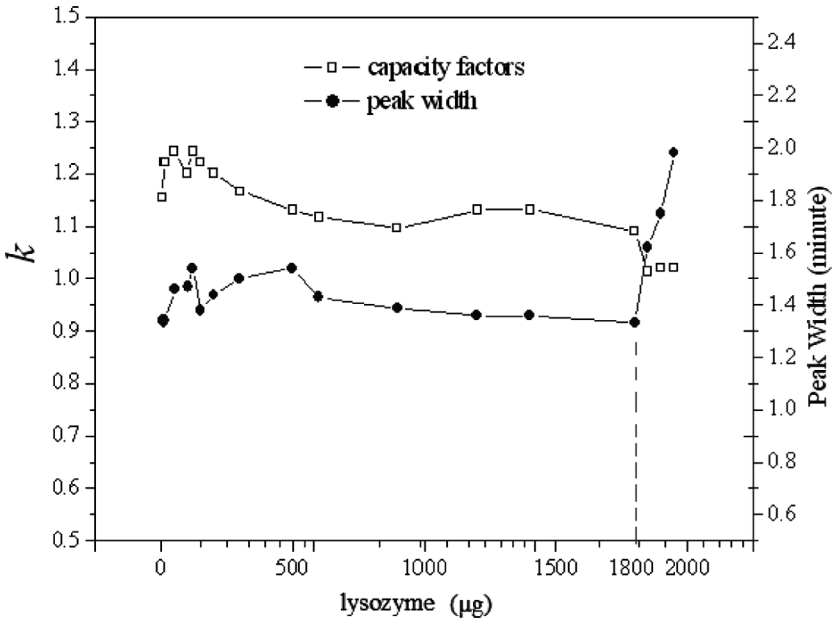


***Figure 9.*** Study of loading capacity of the FZ column. Experimental conditions: isocratic elution in 20 mmol · $L^{-1}$ NaAC + 1.0 mol · $L^{-1}$ $(NH_4)_2SO_4$ mobile phase at pH 7.1; 1 mL · $min^{-1}$; 18°C; detection at 280 nm.

in analytical chromatography, which is also favorable for preparative and industrial scale purification of proteins.

As studied by Blackwell et al.,[12] when the sample injection quantities are within column loading capacity, the retention time and the peak width will remain constant. Overloading the column will cause a decrease in retention time and an increase in peak width. In this study, we chose lysozyme as the probe to investigate the column performance with increasing sample injection quantities. The results in Figure 9 showed that an increase in sample load had negative effects especially on peak width. Based on the amount of sample, which cause a 10% increase in peak width, the loading capacity of the FZ column was estimated to $722\,\mu g \cdot mL^{-1}$ of blank column volume. This demonstrates that zirconia is modified by fosfomycin as potential materials for bioseparations in preparative scale. It should be pointed out, that columns with higher loading capacity would be very promising using zirconia with large pore structures. Further work is in progress in our laboratory.



## CONCLUSIONS

A novel hydrophobic interaction chromatography stationary phase has been produced by modification of zirconia with fosfomycin via Lewis acid-base interaction. This new HIC material demonstrates typical hydrophobic properties. Basic proteins are retained on this new HIC phase predominantly by their hydrophobic characteristics. The undesirable cation exchange and ligand exchange interaction are effectively suppressed in the presence of moderately high concentration eluting salts. A few proteins are well separated with gradient elution by HIC. The material also shows high loading capacity and desirable mass recovery for proteins, demonstrating the potential as a useful HIC media for biomolecules separation.

## ACKNOWLEDGMENT

The authors gratefully acknowledge National Nature Science Foundation of China (Grant: 20475040), the Excellent Young Teachers Program of MOE, P.R.C.

## REFERENCES

1. Belew, M.; Yafang, M.; Bin, L.; Berglof, J.; Janson, J.-C. Purification of recombinant hepatitis B surface antigan produced by transformed Chinese hamster ovary(CHO) cell line grown in cuhure. Bioseparation **1991**, *1*, 397–408.
2. Jennissen, H.P. Hydrophobic interaction chromatography: the critical hydrophobicity approach. Int. J. Bio-Chromatogr. **2000**, *5* (2), 131–163.

3. Lienqueo, M.E.; Mahn, A.; Salgado, J.C.; Asenjo, J.A. Current insights on protein behaviour in hydrophobic interaction chromatography. J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. **2007**, *849* (1–2), 53–68.
4. Asenjo, J.A.; Andrews, B.A. Is there a rational method to purify proteins? From expert systems to proteomics. J. Mol. Recognit. **2004**, *17* (3), 236–247.
5. Hjerten, S.; Rosengren, J.; Pahlman, S. Hydrophobic interaction chromatography. The synthesis and the use of some alkyl and aryl derivatives of agarose. J. Chromatogr. **1994**, *101*, 281–288.
6. Conder, J.R.; Hayek, B.O. Adsorption and desorption kinetics of bovine serum albumin in ion exchange and hydrophobic interaction chromatography on silica matrices. Biochem. Engine. J. **2000**, *6* (3), 225–232.
7. Gong, B.; Wang, L.; Wang, C.; Geng, X. Preparation of hydrophobic interaction chromatographic packings based on monodisperse poly(glycidyl-methacrylate-*co*-ethylenedimethacrylate) beads and their application. J. Chromatogr. A. **2004**, *1022*, 33–39.
8. Arshady, R. Beaded polymer supports and gels. II. Physicochemical criteria and functionalization. J. Chromatogr. **1991**, *586* (2), 199–219.
9. Kirkland, J.J.; van Straten, M.A.; Claessens, H.A. High pH mobile phase effects on silica-based reversed-phase HPLC columns. J. Chromatogr. A. **1995**, *691*, 3–19.
10. Glajch, J.L.; Kirkland, J.J.; Koehler, J. Effect of column degradation on the reversed-phase high-performance liquid chromatographic separation of peptides and proteins. J. Chromatogr. **1987**, *384*, 81–90.
11. Vervoort, R.J.; Debets, A.J.; Claessens, H.A.; Cramers, C.A.; de Jong, G.I. Optimisation and characterisation of silica-based reversed-phase liquid chromatographic systems for the analysis of basic pharmaceuticals. J. Chromatogr. A. **2000**, *897*, 1–22.
12. Gruen, M.; Kurganov, A.A.; Schacht, S.; Schueth, F.; Unger, K.K. Comparison of an ordered mesoporous aluminosilicate, silica, alumina, titania and zirconia in normal-phase high-performance liquid chromatography. J. Chromatogr. A. **1996**, *740* (1), 1–9.
13. Kurganov, A.A.; Trudinger, U.; Isaeva, T.; Unger, K.K. Native and modified alumina, zirconia, titania in normal and reverse-phase high performance liquid chromatography. Chromatographia **1996**, *42* (3/4), 217–222.
14. Feng, Y.Q.; Fu, H.J.; Zhang, Q.H.; Da, S.L.; Zhang, Y.J. Retention behavior of basic compounds on alkylphosphonate-modified magnesia-zirconia composite stationary phase in HPLC. Chromatographia. **2000**, *52* (3–4), 165–168.
15. Yang, X.Q.; Dai, J.; Carr, P.W. Analysis and critical comparison of the reversed-phase and ion-exchange contributions to retention on polybutadiene coated zirconia and octadecyl silane bonded silica phases. J. Chromatogr. A. **2003**, *996*, 13–31.
16. He, H.B.; Feng, Y.Q.; Da, S.-L.; Hu, Z.-X.; Qu, L. High-performance liquid chromatography of some basic drugs on a n-octadecylphosphonic acid modified magnesia-zirconia stationary phase. J. Sep. Sci. **2005**, *28*, 1577–1586.
17. Blackwell, J.A.; Carr, P.W. Fluoride-modified zirconium oxide as a biocompatible stationary phase for high-performance liquid chromatography. J. Chromatogr. **1991**, *549*, 59–75.

18. Mullick, A.; Griffith, C.M.; Flickinger, M.C. Expanded and packed bed albumin adsorption on fluoride modified zirconia. Biochem. Biotechnol. **1998**, *60* (3), 333–340.
19. Schafer, W.A.; Carr, P.W. Chromatographic characterization of a phosphate- modified zirconia support for biochromatographic applications. J. Chromatogr. **1991**, *587*, 149–160.
20. Clausen, A.M.; Carr, P.W. Chromatographic characterization of phosphonate analog EDTA-modified zirconia support for biochromatographic applications. Anal. Chem. **1998**, *70*, 378–385.
21. Subramanian, A.; Sarkar, S. Interaction of immunoglobulin G with N, N, N′, N′-ethylenediaminetetramethylenephosphonic acid-modified zirconia. J. Chromatogr. A **2003**, *989*, 131–138.
22. Subramanian, A.; Sarkar, S. Use of a modified zirconia support in the separation of immunoproteins. J. Chromatogr. A. **2002**, *944*, 179–187.
23. Hu, Y.; Carr, P.W. Synthesis and characterization of new zirconia-based polymeric cation-exchange stationary phases for high-performance liquid chromatography of proteins. Anal. Chem. **1998**, *70*, 1934–1942.
24. Zhang, Q.H.; Feng, Y.Q.; Da, S.-L. Preparation of zirconia for high-performance using sol-gel technology. Chin. J. Chromatogr. **1999**, *17* (3), 284–286.
25. He, H.B.; Zhang, W.N.; Da, S.-L.; Feng, Y.Q. Preparation and characterization of a magnesia–zirconia stationary phase modified with $\beta$-cyclodextrin for reversed-phase high-performance liquid chromatography. Anal. Chim. Acta **2004**, *513*, 481–492.
26. Melander, W.; Horvath, C. Salt effects on hydrophobic interactions in precipitation and chromatography of proteins: an interpretation of the lyotropic series. Arch. Biochem. Biophys. **1977**, *183*, 200–215.
27. Haimer, E.; Tscheliessnig, A.; Hahn, R.; Jungbauer, A. Hydrophobic interaction chromatography of proteins IV Kinetics of protein spreading. J. Chromatogr. A **2007**, *1139*, 84–94.
28. He, H.B.; Feng, Y.Q.; Da, S.-L.; Wang, Z.H. An investigation on the cation-exchange and Lewis-base interactions of a n-octadecylphosphonic acid-modified magnesia-zirconia stationary phase. Anal. Chim. Acta **2005**, *551*, 213–221.
29. Melander, W.R.; Corradini, D.; Horvath, C. Salt-mediated retention of proteins in hydrophobic-interaction chromatography: Application of solvophobic theory. J. Chromatogr. **1984**, *317*, 67–85.
30. Srinivasan, R.; Ruckenstein, E. Role of physical forces in hydrophobic interaction chromatography. Sep. Purif. Methods. **1980**, *9*, 267–370.
31. Xia, F.; Nagrath, D.; Cramer, S.M. Effect of pH changes on water release values in hydrophobic interaction chromatographic systems. J. Chromatogr. A. **2005**, *1079* (1–2), 229–235.

Received March 31, 2008
Accepted January 22, 2009
Manuscript 6340