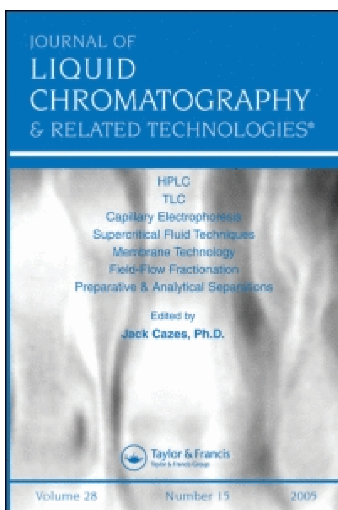
This article was downloaded by:
On: *25 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Ligand Exchange Chromatography of Free Amino Acids on Phosphated Zirconium Oxide Supports

J. A. Blackwell[a]; P. W. Carr[b]
[a] Specialty Adhesives and Chemicals Division Group Analytical Laboratory, 3M Company, Minnesota
[b] Chemistry Department and Institute for Advanced Studies in Bioprocess Technology, University of Minnesota, Minneapolis, Minnesota

To cite this Article Blackwell, J. A. and Carr, P. W.(1992) 'Ligand Exchange Chromatography of Free Amino Acids on Phosphated Zirconium Oxide Supports', Journal of Liquid Chromatography & Related Technologies, 15: 5, 727 — 751

To link to this Article: DOI: 10.1080/10826079208018831

URL: http://dx.doi.org/10.1080/10826079208018831

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(5), 727-751 (1992)

LIGAND EXCHANGE CHROMATOGRAPHY OF FREE AMINO ACIDS ON PHOSPHATED ZIRCONIUM OXIDE SUPPORTS

J. A. BLACKWELL[1] AND P. W. CARR[2]

[1]3M Company
Specialty Adhesives and Chemicals Division
Group Analytical Laboratory
236-2B-11, 3M Center
St. Paul, Minnesota 55144-1000
[2]Chemistry Department and
Institute for Advanced Studies in Bioprocess Technology
University of Minnesota
207 Pleasant Street, S.E.
Minneapolis, Minnesota 55455

Downloaded At: 09:31 25 January 2011

ABSTRACT

Phosphated zirconium oxide particles loaded with copper ions are a mechanically superior alternative to resin based supports for ligand exchange separations of amino acids. They show good stability under acidic and alkaline conditions and afford different selectivity than resin based exchangers. Acidic amino acids are more strongly retained on this phase than on silica or resin based supports due to interactions with the underlying zirconia surface. These interactions occur despite rigorous phosphation of the zirconia particle. The operational aspects of amino acid separations on this material are examined and compared to conventional resin based models.

INTRODUCTION

In 1977, Doury-Berthod, et al., presented the first detailed model of ligand exchange separations of amino acids using copper(II)

Copyright © 1992 by Marcel Dekker, Inc.

loaded ion exchangers [1,2]. Specifically, when using aqueous ammonia solutions with organic modifiers as the eluent, the retention of amino acids can be described by two equilibria. The first is an ion exchange equilibrium of the form:

$$2\ CuA(NH_3)_x^{+} + Cu(NH_3)_{4-e}^{+2} + eNH_3 = 2\ CuA(NH_3)_x^{+} + Cu(NH_3)_4^{+2} \qquad 1.$$

where A represents a singly charged amino acid anion and the species with bars are resin bound. The stoichiometry is dictated by the need to preserve electroneutrality in both phases. The other relevant equilibrium describes solution complex formation as follows:

$$Cu(NH_3)_4^{+2} + A^- = CuA(NH_3)_x^{+} + (4-x)NH_3 \qquad 2.$$

These equilibria assume that the major species present are restricted to NH_3, $Cu(NH_3)_4^{+2}$, $CuA(NH_3)_i^{+}$ and A^- in solution and $Cu(NH_3)_{4-e}^{+2}$ and $CuA(NH_3)_i^{+}$ within the resin. The assumption that other species and equilibrium are not significant contributors to the retention process is rather well established by their experimental results.

The major conclusion of the Doury-Berthod model of ligand exchange chromatography is that mixed ammonia/amino acid/copper(II) complexes exist in both the mobile and resin phases. However, the sequence of reactions by which the amino acid exchanges between the two phases is quite unclear. Their detailed data analysis supports the following conclusions. First, because the concentration of amino acid is low, only mono-amino acid complexes are formed ($Cu(AA)_1(NH_3)_j$; j = 0 to 3). Second, binuclear complexes ($Cu_2(AA)(NH_3)_i$) do not form in either phase. A very important experimental observation is that the retention of an amino acid is a complicated function of the mobile phase copper(II) concentration.

When the mobile phase concentration of ammonia is less than about 0.5M, retention of the amino acid decreases monotonically as the copper(II) concentration is increased. However, when the mobile

Downloaded At: 09:31 25 January 2011

phase ammonia concentration is greater than 0.5M, retention first increases as the copper(II) concentration is increased. The capacity factor then passes through a maximum and finally approaches a constant value at very high copper(II) concentrations.

The dependence of retention on the ammonia concentration is also rather complex. For the most part, retention of an amino acid decreases as the mobile phase concentration of ammonia is increased. The above results qualitatively support the idea that there is competition between ammonia and the amino acids for occupying the coordination sites on copper(II) in both the mobile and stationary phases.

One of the problems preventing further detailed examination of the ligand exchange process is the physical limitation of the resin based supports used in the afforementioned studies. The mechanical shortcomings of derivatized resins in high performance liquid chromatography are well known. The problems of resin swelling and compaction are exacerbated by the osmotic pressure changes incurred when metal ions are loaded onto or stripped from the resin. To overcome these critical problems, inorganic based ion exchangers have been explored as supports for ligand exchange chromatography. Foucault, et al., investigated copper(II) loaded silica as a support for the ligand exchange chromatography of amino acids and peptides [3-6]. They recognized that the major limitations to the separation efficiency resulted from the slow kinetics of ligand exchange and slow diffusion of ligands within the resin particles. If mass transport limitations can be eliminated, then under these ideal circumstances, band widths will be limited by the chemical rate of ligand exchange. They sought to reach this limit of efficiency by improving the diffusion characteristics of the stationary phase by using silica particles. Impressive gains in efficiency were obtained using silica based supports in place of resin based supports. Efficiency was kept high by raising the ammonia content of the eluent to more than 0.12 molar.

However, silica is notoriously soluble in alkaline solutions. Foucault offset this effect by virtue of the copper(II) silicate

Downloaded At: 09:31 25 January 2011

coating formed on the silica particle. This cladding was made more resistant to attack by the addition of 1 ppm of copper(II) to the mobile phase. The presence of organic modifiers in the mobile phase also suppressed the silica's solubility. Guyon, et al., found that the solubility of silica decreased drastically as the organic modifier content of the solvent was increased [7]. Further protection was provided by the use of a sacrificial copper saturated silica pre-column. However, despite the many protective measures, the column had a limited useful lifetime (about two weeks of continuous operation). Addition of copper(II) also allows spectrophotometric observation of the amino acid complexes.

Chemically stable porous microparticulate zirconium oxide, coated with a layer of zirconium phosphate, should be ideal for examining the effect of organic modifiers and temperature. Thermal effects were not explored in either resin based systems or silica based systems due to the adverse effects of high temperatures on the supports. The operational parameters for the use of phosphated zirconium oxide particles in ligand exchange chromatography are the subject of this investigation.

Downloaded At: 09:31 25 January 2011

EXPERIMENTAL

Chemicals

Amino acids were reagent grade or better and were obtained from commercial sources. Morpholinoethanesulfonic acid (MES) was obtained from Sigma (St.Louis, MO). Copper(II) chloride dihydrate, ammonium hydroxide and dibasic potassium phosphate trihydrate were obtained from Mallinckrodt (Paris, KY). Sodium hydroxide was obtained as a 50% solution from Curtin Matheson Scientific (Houston, TX). Hydrochloric acid, sodium chloride, nitric acid and HPLC grade tetrahydrofuran, isopropanol and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ). Acetic acid was obtained from EM Science (Cherry Hill, NJ). Phosphoric acid and disodium ethylenediaminetetraacetic acid were obtained from J.T. Baker (Phillipsburg, NJ). All chemicals were reagent grade or better.

The water used in these studies was prepared by passing house deionized water through a Barnsted NanoPure water system with an additional organic-free cartridge and a 0.2 µm final filter. The water was subsequently boiled for five minutes then cooled to room temperature immediately prior to use to remove carbon dioxide.

Chromatographic Supports

The porous zirconium oxide particles were provided by the Ceramic Technology Center of the 3M Company and were described earlier [8-19]. The particles used in this investigation had a nominal diameter of 5.3 µm ± 1.3 µm, an average pore diameter of 308 Å by mercury porosimetry and an average B.E.T. surface area of 32.5 square meters per gram. The particles were initially pretreated in order to remove as many of the manufacturing impurities as possible [14-19].

Zirconium phosphate particles were synthesized by a variation of the method used by Schafer [9-11]. Previous work by Weber showed that phosphonates adsorb to a greater extent on zirconium oxide at lower pH [20]. The phosphation method of Schafer was, therefore, modified to take advantage of this potentially more effective phosphation chemistry [9,11]. We used hydrochloric acid instead of potassium chloride to synthesize the zirconium phosphate particles. Use of a strongly acidic medium converts the phosphate coated product to the acid form. This serves as a common starting condition since the ionic form of the zirconium phosphate has a strong effect on its ion exchange properties [21].

Ten grams of acid/base pretreated particles were suspended in 100 mL of 1 M hydrochloric acid which contained 0.1 M phosphoric acid in an acid washed 250 mL round bottomed glass flask. The particles were ultrasonicated under vacuum (ca. 5 torr) for fifteen minutes. The solution was then refluxed for three hours at a vigorous boil. The boiling action precluded the need for stirring, however, every fifteen minutes the flask was vigorously agitated by

Downloaded At: 09:31 25 January 2011

hand to prevent sedimentation. After boiling, the particles were allowed to cool and were then washed twice with 100 mL aliquots of freshly boiled water. Two washes with 100 mL aliquots of HPLC grade isopropanol completed the rinsing process. During rinsing, a fair number of fines were observed and were decanted after a five minute settling period. After the reflux treatment, the particles looked like white paste. However, following the isopropanol washes, the particles became a free flowing powder. Microscopic examination showed that the particles did not aggregate or fuse.

Chromatographic columns were prepared in 50 mm by 4.6 mm column blanks using 1/4" Parker end fittings. Titanium screens with 2 μm mesh were used instead of frits to minimize any potential extraneous metal ion contamination from the frits. Columns were packed by the upward slurry technique using isopropanol as the solvent. Packing pressure was 4500 psi (300 atm.). Following the packing procedure, all columns were flushed with freshly boiled water to displace all of the packing solvent prior to introducing the buffers.

Downloaded At: 09:31 25 January 2011

Chromatographic Systems

Chromatographic studies were carried out on two systems. The first system consisted of a Hewlett Packard (Avondale, PA) Model 1090M liquid chromatograph with a DR5 ternary solvent delivery system and a diode array detector. The optional expanded pH range kit as well as ultrahigh molecular weight polyethylene piston seals (UPC-10) obtained from Bal Seal Engineering (Santa Ana, CA) were installed. Data were processed using a Hewlett Packard 9000/Series 300 computer outfitted with ChemStation software.

The second system consisted of an Altex 110A isocratic pump (Fullerton, CA) with a Rheodyne (Cotati, CA) 7120 injector valve. The detector was a Perkin Elmer (Norwalk, CT) LC-15 fixed wavelength detector with a 230 nm filter. For high pH eluents, the piston seals were replaced with unfilled Teflon piston seals obtained from Beckman Instruments (San Ramon, CA). Both systems were outfitted

with a 50 x 4.6 mm column filled with 10-20 μm zirconia particles. This precolumn was placed before the injection valve to scavenge any metal ion contaminants in the buffer.

Copper(II) Ion Loading

The phosphated zirconium oxide supports were loaded with copper (II) by passing a solution of 1 mM copper(II) chloride/1 M ammonia in 50% acetonitrile through the column at 1.0 mL/min. The loading was performed at 21°C and was monitored at 254 nm for the copper(II) breakthrough. Using this procedure, the total loading capacity was determined to be 4.5 $\mu mol/m^2$. Eluent was passed through the column at 0.5 mL/min until the baseline became constant.

Downloaded At: 09:31 25 January 2011

RESULTS AND DISCUSSION

Elution Sequence of Amino Acids

Three organic modifiers were examined to assess their effect on the retention of amino acids. The results are shown in Table I. In accord with previous findings [1-7], basic amino acids are very strongly retained. However, the elution order differs from that of Doury-Berthod [1,2] and Foucault [3-6] in that acidic amino acids are well retained and many neutral amino acids elute before the acidic amino acids. On silica and resin based supports, acidic amino acids are weakly retained and always elute before the neutral amino acids [1-6]. The fact that silica, like zirconia, contains no organic moieties and is very polar argues against both hydrophobicity and dipolarity as the source of the change in retention sequence observed on zirconium phosphate.

The complexes between neutral amino acids and copper(II) are positively charged at the pH of this experiment. According to the Doury-Berthod model of ligand exchange chromatography, retention of such complexes will be greater than that of acidic amino acids because the positively charged complexes will be retained by both ion exchange and ligand exchange. The complexes with acidic amino acids can be retained only by ligand exchange. However, the predictions of this model do not agree with our experimental

TABLE 1

Organic Modifier Effects on Amino Acid Retention

Capacity Factors[a]

Amino Acid	AcCN	THF	k'_{THF}/k'_{AcCN}	MeOH	k'_{MeOH}/k'_{AcCN}
phenylalanine	0.98	0.73	0.75	1.49	1.52
tryptophan	1.25	0.76	0.61	2.79	2.23
methionine	1.30	1.23	0.95	2.14	1.65
leucine	1.37	1.25	0.91	2.35	1.71
tyrosine	1.53	1.65	1.07	2.57	1.08
valine	1.64	1.71	1.04	2.37	1.44
isoleucine	1.71	1.32	0.77	2.05	1.20
glutamic acid	1.88	2.66	1.41	3.34	1.78
aspartic acid	2.22	3.05	1.37	4.49	2.02
glutamine	2.68	2.55	0.95	3.38	1.26
threonine	2.82	3.13	1.11	5.06	1.79
alanine	3.16	3.19	1.01	3.91	1.23
asparagine	3.50	3.39	0.94	5.80	1.66
glycine	4.83	5.07	1.05	6.51	1.35
serine	7.04	6.98	0.99	11.31	1.61
lysine	7.73	7.31	0.95	11.21	1.45
proline	10.83	2.68	0.25	2.53	0.23
histidine	33.09	20.35	0.62	37.08	1.12
arginine	eno	eno	--	eno	--
cysteine	eno	eno	--	eno	--

a. Eluent contained 1.0M aqueous ammonia in 50% water and 50% organic modifier. Flow was 0.5 mL/min at 45°C. Injections were 10 μL of a 12mM solution of the amino acid in the starting buffer. eno = elution not observed.

Downloaded At: 09:31 25 January 2011

results. Tridentate amino acids were less retained than bidentate amino acids. This indicates that, under these conditions, ligand exchange may be less important to retention than is ion exchange.

This significant difference in elution sequence suggests that an additional retention mechanism may be operating. Under the elution conditions used here, the acidic amino acids form uncharged

complexes with copper(II). Since there is no organic component to the support, there is no possibility for hydrophobic retention. Dipolar interactions may cause the high relative retention of the acidic amino acids, but these dipolar forces are also expected with the other amino acid complexes.

The possibility exists that ligand exchange takes place at the Lewis acid sites present on the underlying zirconium oxide surface [14-19]. Based on prior studies of retention of simple benzoic acid derivatives on zirconia [16-19], we are convinced that surface Lewis acid sites (Zr(IV)) play a very major role in retaining Lewis base solutes. While many of these sites are covered with phosphate by our pretreatment, the NMR studies of Schafer [9-11] suggest that, in the absence of phosphate in the mobile phase, there are still many available Lewis acid sites on the surface. Phosphate would bind tightly to such sites, but would be exchangeable to a certain extent with other hard Lewis bases. This is consistent with enhanced retention of those amino acids that have hard Lewis base side chains (e.g. carboxylic and alcoholic groups).

Downloaded At: 09:31 25 January 2011

Basic amino acids form positively charged complexes with copper(II). Histidine and lysine may be singly or doubly charged. These amino acids should be strongly retained since both ligand and ion exchange are possible. However, our inability to elute arginine and cysteine under these conditions suggests a strong interaction with the modified surface since simple electrostatic attractions do not account for the very strong retention of these species.

Effect of Mobile Phase Modifier on Retention

Some physical properties of the organic modifiers used here are given in Table II. The methanol modified eluent is clearly the weakest eluent. Acetonitrile and tetrahydrofuran are similar in eluotropic strength, but showed a few minor differences in selectivity. Tetrahydrofuran and methanol showed similar selectivities, especially towards proline, glutamine and isoleucine, but showed large difference in eluotropic strength. Inspection of the relevant physical parameters, which we expect to cause

TABLE 2

Physical Parameters for Selected Solvents

Property	Acetonitrile	Methanol	Tetrahydrofuran
dielectric constant	37.5	32.70	7.58
dipole moment	3.92	1.70	1.75
α (hydrogen bond donating ability)	0.19	0.93	0.00
β (hydrogen bond accepting ability)	0.31	0.62	0.55

Downloaded At: 09:31 25 January 2011

differences in eluotropic strength, does not show any striking correlation with the results.

Previous investigations on the effect of organic solvent on ligand exchange processes suggest that the major difference between these solvents lies in the solvation energies of the various intermediates [22]. Ligand exchange rate constants decreased significantly when the dielectric constant of the media was lowered. The overall effect is to decrease the separation efficiency, but the increased retention of the amino acids cannot be due to a decrease in rates. Most likely, the greater retention is due to the inability of the organically modified mobile phases to solubilize the charged amino acids and their copper complexes.

The elution orders show that no major changes in selectivity were observed with the three solvents. That is, the neutral amino acids are least retained, followed by the acidic amino aoids, with the basic amino acids being the most retained. However, some notable selectivity changes include the increased retention of

tryptophan and the decreased retention of isoleucine in methanol and changes in the retention of proline. Other minor changes in selectivity were observed, but for the most part, the elution order was conserved.

Based on solute efficiency, tetrahydrofuran appears to be the most suitable modifier for ligand exchange chromatography, however, there are a number of technical problems involved with its use. First, ammonia is not fully miscible with aqueous tetrahydrofuran. At high organic content, phase separation takes place, thereby limiting the range of available eluotropic strength. Second, tetrahydrofuran is not entirely stable in basic solutions. Acetonitrile was used for the remaining studies.

Downloaded At: 09:31 25 January 2011

Effect of Ammonia Concentration on Retention

The concentration of organic modifier in the mobile phase was varied to establish its effect on retention (see Table III). At 0.1M ammonia, increasing the acetonitrile concentration increased the retention of all amino acids. The effect is greatest for glutamic and aspartic acids, threonine and asparagine. Similarly, in 1M ammonia, the retention of all amino acids except proline increased upon an increase in the concentration of acetonitrile. Once again, the largest effect was observed with glutamic and aspartic acids, threonine and asparagine. The effect of ammonia is more complicated than the effect of acetonitrile. In 75% acetonitrile, an increase in ammonia concentration from 0.1 to 1.0M increased the retention of all amino acids. In contrast, in 50% acetonitrile, seven amino acids showed increased retention when the ammonia concentration was increased, while all the others showed decreased retention. The reason for these selectivity reversals is not readily apparent. Arginine and cysteine did not elute under any of the condition employed in this study.

The elution order at low ammonia concentrations changed significantly, relative to that at high ammonia concentration. It is apparent that changes in both the amount of organic modifier and ammonia concentration have dramatic effects on elution. Doury-

TABLE 3

Effect of Organic Modifier Concentration on Amino Acid Retention Capacity Factors[a]

Amino Acid	0.1 M NH_3 50% AcCN	0.1 M NH_3 75% AcCN	1.0 M NH_3 50% AcCN	1.0 M NH_3 75% AcCN
proline	0.93	1.49	10.83	9.23
isoleucine	0.98	1.56	1.71	5.03
phenylalanine	1.27	1.83	0.98	3.43
valine	1.32	2.44	1.64	6.50
glutamic acid	1.49	7.44	1.88	28.35
aspartic acid	1.81	6.89	2.22	24.91
leucine	1.99	2.58	1.37	4.55
methionine	2.12	2.64	1.30	5.26
tryptophan	2.25	2.37	1.25	4.41
threonine	2.86	10.02	2.82	18.98
alanine	2.92	5.53	3.16	15.59
glutamine	3.27	8.91	2.68	23.28
tyrosine	3.83	8.99	1.53	14.85
glycine	5.43	13.14	4.83	29.41
asparagine	6.94	23.87	3.50	28.55
serine	7.11	27.60	7.04	50.60
histidine	20.66	eno	33.09	eno
lysine	eno	eno	7.73	39.75
arginine	eno	eno	eno	eno
cysteine	eno	eno	eno	eno

a. Flow was 0.5 mL/min at 45°C. Injections were 10 μL of a 12mM solution of the amino acid in the starting buffer. eno = elution not observed.

Downloaded At: 09:31 25 January 2011

Berthod suggested that at high ammonia concentrations, ligand exchange is less important than ion exchange. Conversely, at low ammonia concentrations, both processes contribute substantially. Consequently, changes in selectivity with ammonia concentration are not surprising.

The selectivity differences are evident in the ratio of capacity factors under various elution conditions (Table IV). Glutamic acid, aspartic acid, threonine, glutamine, tyrosine, asparagine and serine are more strongly affected by the ammonia concentration than by the amount of organic modifier. Increases in organic modifier concentration have the common effect of simply increasing capacity factors.

At 50% acetonitrile, the higher concentration of ammonia was a stronger eluent. This is likely due to the higher concentration of ammonium ion which can displace species held by ion exchange. If ligand substitution were the driving force for the effect of the ammonia concentration, then the same effect should be observed at the higher organic modifier concentration. The opposite is observed, however, indicating that the ion exchange process is more significant at higher ammonia concentration and lower organic modifier content.

At 75% acetonitrile, retention of all the amino acids was greater in 1M ammonia than in 0.1M ammonia. This is reasonable since higher ammonia concentrations shift the balance from ligand exchange to ion exchange. Retention increases, since displacement of an ion from the phase is not very favorable when a high percentage of organic modifier is present.

At 50% acetonitrile, the same generalization is true, however, a number of amino acids depart from the expected behavior. A number of non-polar amino acids (Asn, Gln, Gly, Leu, Met, Phe, Trp, Tyr) are less retained at higher ammonia concentrations. This behavior cannot be explained by the proposed retention equilibria and may be a consequence of the surface Lewis acid sites.

Downloaded At: 09:31 25 January 2011

TABLE 4

Effect of Organic Modifier Concentration on Amino Acid Retention Capacity Factor Ratios[a]

Amino Acid	0.1M NH_3 $k'_{75\%AcCN}$ / $k'_{50\%AcCN}$	1.0M NH_3 $k'_{75\%AcCN}$ / $k'_{50\%AcCN}$	50% AcCN $k'_{1.0M\ NH3}$ / $k'_{0.1M\ NH3}$	75% AcCN $k'_{1.0M\ NH3}$ / $k'_{0.1M\ NH3}$
tryptophan	1.05	3.53	0.56	1.86
methionine	1.25	4.05	0.61	1.99
leucine	1.30	3.32	0.69	1.76
phenylalanine	1.44	3.50	0.77	1.87
isoleucine	1.59	2.94	1.74	3.22
proline	1.60	0.85	11.65	6.19
valine	1.85	3.96	1.24	2.66
alanine	1.89	4.93	1.08	2.82
tyrosine	2.35	9.71	0.40	1.65
glycine	2.42	6.09	0.89	2.24
glutamine	2.72	8.69	0.82	2.61
asparagine	3.44	8.16	0.50	1.20
threonine	3.50	6.73	0.99	1.89
aspartic acid	3.81	11.22	1.23	3.62
serine	3.88	7.19	0.99	1.83
glutamic acid	4.99	15.08	1.26	3.81
lysine	--	5.14	--	--
histidine	--	--	1.60	--
arginine	--	--	--	--
cysteine	--	--	--	--

a. conditions as in Table 3.

Downloaded At: 09:31 25 January 2011

Effect of Ionic Strength

The role of ammonia concentration on the elution of amino acids was further studied by varying the ammonia concentrations while maintaining constant ionic strength. The influence of an organic modifier was eliminated by use of a purely aqueous eluent. The results of this study are summarized in Table V.

TABLE 5

Effect of Ammonia/Ammonium Chloride Concentration at Constant Ionic Strength

Capacity Factors[b]

Eluent[a]	Tryptophan	Phenylalanine	Alanine	Tyrosine
0.50 M NaCl	eno	eno	eno	eno
0.49 M NaCl 0.05 M NH_3 0.05 M NH_4Cl	3.55 (147)	3.60 (105)	5.87 (51)	7.52 (372)
0.30 M NaCl 0.20 M NH_3 0.20 M NH_4Cl	0.73 (41)	0.67 (34)	1.10 (52)	1.41 (162)
0.50 M NH_3 0.50 M NH_4Cl	0.36 (22)	0.27 (18)	0.46 (21)	0.67 (97)

a. All eluents contain 0.25mM $CuCl_2$ at pH 9.2. Flow was 0.5 mL/min at 45°C. eno = elution not observed.
b. values in parenthesis are experimentally determined reduced plate heights.

Downloaded At: 09:31 25 January 2011

The amino acids did not elute when the ammonia/ammonium chloride concentration was zero. This indicates that retention is not entirely due to ion exchange since 0.5M sodium chloride should readily elute at least some of the ionically bound solutes. Increasing the ammonia/ammonium concentration to 50mM caused elution in a reasonable range of capacity factors. However, the efficiencies were extremely poor, as indicated by the very high reduced plate heights (see Table V). Use of 200mM NH_3/NH_4^+ buffer decreased the capacity factors and improved the efficiency. At the highest eluent concentration, the reduced plate heights were quite typical of those observed in ligand exchange chromatography.

The observed decrease in capacity factors as the displacing ligand concentration was increased is due to a shift in the ligand

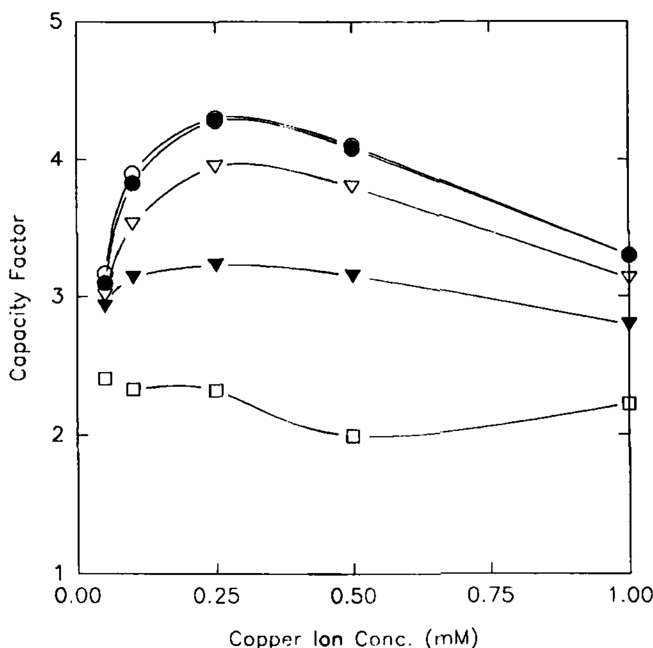


Figure 1. Alanine retention as a function of solution copper ion concentration for a fixed amount of injected solute. Alanine concentrations were: (○) 0.01mM; (●) 0.10mM; (▽) 1.00mM; (▼) 10.0mM; and (□) 100mM with 10 μL injection volumes. Eluent was 1M ammonia in 50% acetonitrile in addition to the copper(II) chloride. Temperature was 45°C with a flow rate of 0.5 mL/min. Detection was at 254 nm. Column dimensions were 50 x 4.6 mm.

Downloaded At: 09:31 25 January 2011

exchange equilibrium towards the free amino acids. Since the amino acid/copper complexes are the retained species, this leads to a decrease in retention.

Effect of Copper Ion Concentration

Figure 1 shows the effect of varying the mobile phase copper ion concentration. The capacity factors are at a maximum at a copper concentration of 0.25mM. The capacity factor decreased precipitously as the copper ion concentration was decreased from 0.25mM and decreased slowly as the copper ion concentration was increased above 0.25mM.

These observations agree with the experimental findings of Doury-Berthod [1,2] in that a maximum in the capacity factors is obtained when the solution copper ion concentration is equal to $[NH_3]^{4-x}/K$. This is the point where the equilibria between ligand and ion exchange are balanced. Based on the assumption that the mobile phase ammonia concentration is close to 1M, the formation constant for the ligand exchange reaction is estimated to be approximately $10^{3.6}$. Using literature values for the first formation constant of alanine with copper(II) [23] and the β values for ammonia and copper(II) [24] the ligand exchange equilibrium constant for the exchange of two ammonia ligands for an alanine ligand is about $10^{2.9}$. This value is in good agreement with the experimentally determined value when factors such as differences in solvent dielectric strength are taken into account.

Downloaded At: 09:31 25 January 2011

The rapid decrease in the capacity factors as the solution copper ion concentration was decreased below 0.25mM is due mainly to a shift in the ligand exchange equilibrium. At low copper ion concentrations, there are fewer mixed complexes formed. These complexes are the species that are retained in the ion exchange mode. A high total ligand concentration favors the formation of monodentate complexes by shifting the ligand exchange equilibria. This is the same result obtained when the copper ion concentration is decreased since the relative concentration of ligands increases. Conversely, as the copper ion concentration was increased, the relative ligand concentration decreased and the ligand exchange equilibrium shifted to favor the bidentate ligand. An additional cause of the dramatic decrease in capacity factor at low copper concentration is the decrease in the amount of copper complex bound to the support. This will decrease the capacity of the exchanger.

At copper concentrations above 0.25mM, the capacity factors decreased slowly as the copper(II) concentration is increased. This is due the shift of the ligand exchange equilibrium towards the formation of copper/amino acid complexes. These complexes are strongly retained by the exchanger, but as the copper ion concentration increases, more tetraammine complexes are formed which

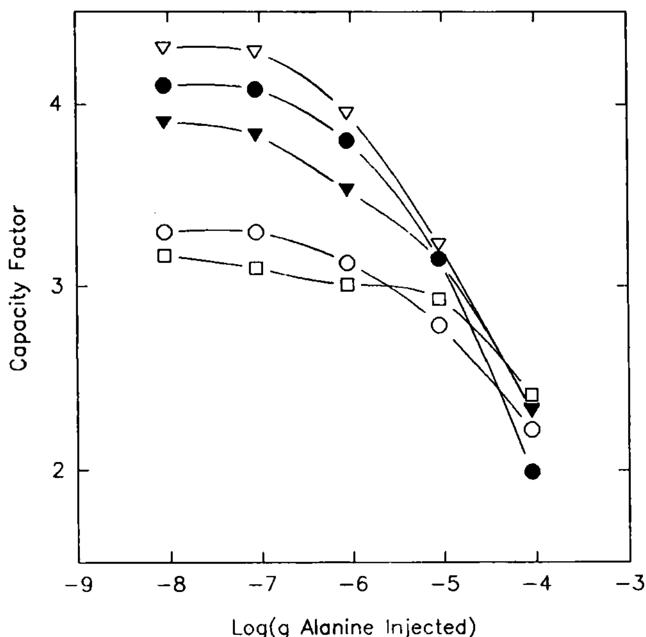


Figure 2. Loading capacity as a function of solution copper ion concentration. (□) 0.05mM; (▼) 0.10mM; (▽) 0.25mM; (●) 0.50mM; and (○) 1.00mM copper(II) chloride. Eluent also contained 1M ammonia in 50% acetonitrile. Flow rate was 0.5 mL/min at 45°C with detection at 254 nm.

Downloaded At: 09:31 25 January 2011

can displace the ion exchangeable complexes. This causes a decrease in capacity factor for those amino acids which are complexed.

Loading study data for alanine on the copper loaded phase are shown in Figure 2. It is evident that the loading capacity of the system is very dependent upon the amount of copper(II) in solution. At 0.25mM copper ion, the maximum capacity factors are obtained, but the loading capacity is lowest. At this copper ion concentration, the linear loading (as determined by a decrease in capacity factor of 10% from the plateau value) is approximately two orders of magnitude. This degree of linearity is rather poor, but it is

TABLE 6

Temperature Effects on Amino Acid Retention[a]

amino acid	capacity factors 25°C	60°C
proline	0.68	0.93
isoleucine	0.69	0.98
phenylalanine	0.96	1.27
valine	1.01	1.32
leucine	1.42	1.99
methionine	1.51	2.12
tryptophan	1.75	2.25
glutamic acid	2.38	1.49
alanine	2.47	2.92
aspartic acid	3.11	1.81
glutamine	3.53	3.27
threonine	4.09	2.86
glycine	5.00	5.43
tyrosine	5.29	3.83
asparagine	6.79	6.94
serine	8.77	7.11
histidine	16.18	20.66
lysine	eno	eno
arginine	eno	eno
cysteine	eno	eno

a. Isocratic elution at 0.3 mL/min with 0.1M ammonia in 50% acetonitrile. Injection loads were typically 1 mol of amino acid in the eluent.

Downloaded At: 09:31 25 January 2011

expected since the maximum represents a balance between two equilibria. Increasing or decreasing the amount of copper in solution increases the linear loading capacity of the system by shifting the equilibrium balance towards one or the other equilibrium process.

Temperature Effects

Two different temperatures were evaluated as to their effect on retention (see Table VI). All but six amino acids were more

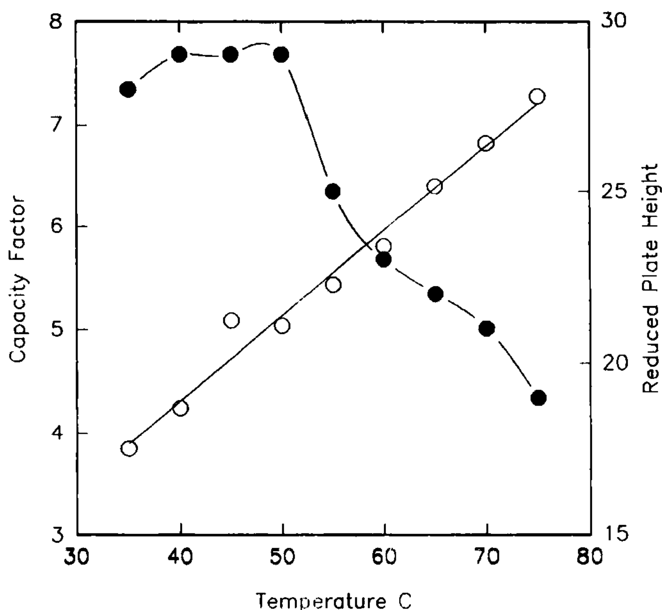


Figure 3. Capacity factor stability and separation efficiency as a function of operating temperature for alanine. (O) capacity factor; (●) reduced plate height. Eluent was 1M ammonia in 50% acetonitrile containing 0.25mM copper(II) chloride. Flow rate was 0.5 mL/min with detection at 254 nm.

Downloaded At: 09:31 25 January 2011

retained at the higher temperature. The exceptions are mainly those amino acids capable of forming tridentate chelates with copper(II).

The effect of temperature on retention was studied in more detail using a single amino acid. The operating temperature was varied from 35°C to 75°C in 5°C increments (see Figure 3). As the temperature was increased, the capacity factor increased regularly. The increase in retention with temperature indicates that the solute binding process is endothermic and thus the favorable free energy must be due to an entropy increase upon solute sorption. The reduced plate heights, however, did not follow a simple pattern. From 35°C to 50°C, the reduced plate height remained relatively constant at about 29. Above 50°C, the reduced plate height

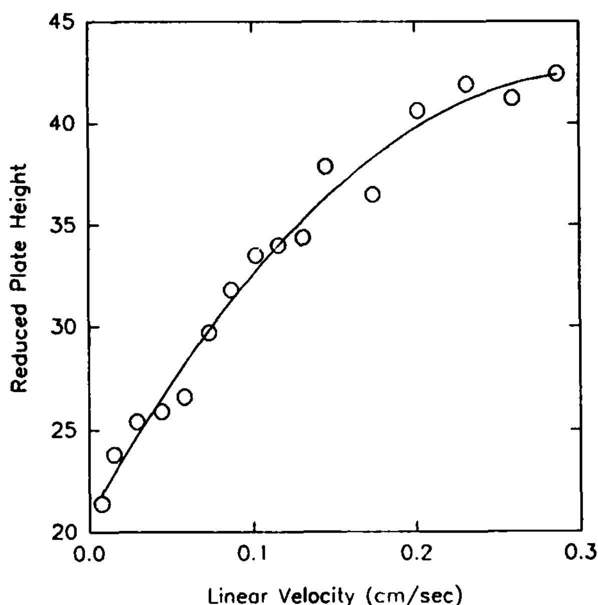


Figure 4. Van Deemter plot for alanine on copper loaded zirconium phosphate. Eluent was 1M ammonia in 50% acetonitrile containing 0.25mM copper(II) chloride. Operating temperature was 45°C with detection at 254 nm.

Downloaded At: 09:31 25 January 2011

decreased rather rapidly as the temperature was further increased. This behavior suggests that a series of steps take place upon binding. In any case, the behavior is not consistent with band broadening controlled by mass transport processes such as convection and diffusion.

Flow Rate Effects

The reduced plate heights change dramatically with flow rate as shown in Figure 4. The very steep slope strongly indicates the presence of a very slow step which most likely is dissociation of the amino acid from copper. Reduced plate heights for bidentate amino acids range from 63 for methionine to 128 for asparagine. Tridentate amino acids had even higher reduced plate heights (e.g.

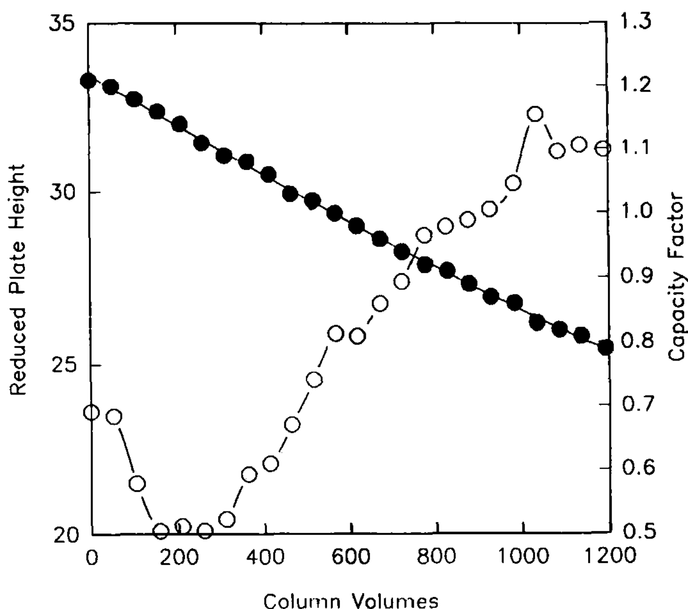


Figure 5. Phase stability of copper(II) loaded zirconium phosphate. (●) capacity factor; (O) reduced plate height for tryptophan in 50% acetonitrile containing 1M ammonia. Flow rate was 0.5 mL/min at 45°C. Detection was at 254 nm.

Downloaded At: 09:31 25 January 2011

histidine has a reduced plate height of 496). The very large differences in plate heights for species with approximately the same capacity factors reinforce our belief that band widths are limited by chemical kinetic effects.

Phase Stability

Separations on resin based exchangers require the addition of copper(II) to the mobile phase to maintain saturation and, therefore, reproducibility of the exchanger. Copper(II) is generally more tightly bound by amino acids than by the exchanger and is, therefore stripped from the column. The rate at which retentive phase is lost by this mechanism is shown in Figure 5. The capacity factor begins to decrease immediately when copper(II) is

removed from the eluent. This loss continued until the study was stopped at approximately 2500 column volumes of eluent.

CONCLUSION

The elution order for amino acids on phosphated zirconium oxide is quite different than that observed on silica or resin based supports. While basic amino acids remain the most highly retained class of amino acids, neutral amino acids are the least retained class of amino acids on phosphated zirconium oxide. On conventional supports, acidic amino acids are the least retained class due to the net charge on the amino acid/copper(II) complex preventing any ion exchange contribution to retention.

On the phosphated phase, acidic amino acids show anomalously high retention, compared to neutral amino acids. This increased retention is accounted for not by differences in electrostatic interactions, but by additional ligand exchange interactions between the carboxylate groups of acidic amino acids and Lewis acid sites on the underlying zirconium oxide particle. The anomalous retention of other amino acids, such as arginine and cysteine, may also be due to interactions with Lewis acid sites on the particle surface.

These Lewis acid sites develop when some of the phosphate molecules coordinated to the Lewis acid sites are displaced by amino acids which form more thermodynamically or kinetically stable complexes than phosphate. Amino acids bound to the Lewis acid sites can then be displaced by ammonia in much the same manner as their copper(II) bound counterparts. The net result would be a separation process which can be described by the Doury-Berthod model, but with the added effect of a second ligand exchange process with differing thermodynamic and kinetic properties than the copper(II) process. The band profile reflects this heterogeneity in ligand exchange sites via the chromatographic efficiencies observed here.

ACKNOWLEDGEMENTS

The authors would like to thank the National Institutes of Health and the National Science Foundation for supporting this

Downloaded At: 09:31 25 January 2011

program. JAB also acknowledges financial support from the Leading Edge Academic Program at 3M.

REFERENCES

1) M. Doury-Berthod, C. Poitrenaud and B. Tremillon, J. Chromatogr. 131: 73-90 (1977).

2) M. Doury-Berthod, C. Poitrenaud and B. Tremillon, J. Chromatogr. 179: 37-61 (1979).

3) M. Caude and A. Foucault, Anal. Chem. 51: 459-461 (1979).

4) A. Foucault, M. Caude and L. Oliveros, J. Chromatogr. 185: 345-360 (1979).

5) F. Guyon, A. Foucault and M. Caude, J. Chromatogr. 186: 677-682 (1979).

6) A. Foucault and R. Rosset, J. Chromatogr. 317: 41-54 (1984).

7) F. Guyon, L. Chardonnet, M. Caude and R. Rosset, Chromatographia 20: 30-34 (1985).

8) M.P. Rigney, Ph.D. Thesis, Univ. of Minnesota, 1988.

9) W.A. Schafer, MS Thesis, Univ. of Minnesota, 1990.

10) W.A. Schafer, E.F. Funkenbusch, K.A. Parson and P.W. Carr, J. Chromatogr. (in press).

11) W.A. Schafer and P.W. Carr, J. Chromatogr. (in press).

12) M.P. Rigney, T.P. Weber and P.W. Carr, J. Chromatogr. 484: 273-291 (1989).

13) M.P. Rigney, E.F. Funkenbusch and P.W. Carr, J. Chromatogr. 499: 291-304 (1990).

14) J.A. Blackwell and P.W. Carr, J. Chromatogr. 549: 43-57 (1991).

15) J.A. Blackwell and P.W. Carr, J. Chromatogr. 549: 59-75 (1991).

16) J.A. Blackwell, Ph.D. Thesis, Univ. of Minnesota, 1991.

17) J.A. Blackwell and P.W. Carr, J. Liq. Chrom. 14: 2875-2889 (1991).

Downloaded At: 09:31 25 January 2011

18) J.A. Blackwell and P.W. Carr, Anal. Chem. (submitted August 1991).

19) J.A. Blackwell and P.W. Carr, Anal. Chem. (submitted August 1991).

20) T.P. Weber, Ph.D. Thesis, Univ. of Minnesota, 1991.

21) S. Allulli, A. LaGinestra, M.A. Massucci, M. Pelliccioni and N. Tomassini, Inorg. Nucl. Chem. Letters 10: 337-341 (1974).

22) A. McAuley and J. Hill, Quart. Rev. 23: 18-36 (1969).

23) A.E. Martell and R.M. Smith, "Critical Stability Constants", Plenum Press, New York, 1974.

24) L.G. Sillen and A.E. Martell, "Stability Constants of Metal Ion Complexes", The Chemical Society, London, 1964.

Downloaded At: 09:31 25 January 2011