

## Performance of 2-Amino Tetraphenyl Porphyrin as Stationary Phase in RP-HPLC of Amino Acids

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**ABSTRACT:** The search for new stationary phases has been one of the predominant concerns in high performance liquid chromatography (HPLC) in order to achieve better resolutions, longer column lives, and reduce the time of analysis. A chromatographic packing for separation of underivatized amino acids (AAs) were prepared by dynamically coating 2-amino tetraphenyl porphyrin (atpp) on a C-18 reversed-phase packing and its properties were examined. The retention characteristics of 20 AAs forming the building blocks of proteins were investigated on the atpp coated C-18 column at pH 7. Results obtained seem to confirm a mixed mechanism of retention involving the hydrophobic interaction between the aromatic porphyrin macrocycle and some of the AAs,  $\pi$ - $\pi$  interaction between the  $\pi$ -electrons of the porphyrin macrocycle and the  $\pi$ -electrons of analyte, and the hydrogen bonding interactions between AAs and the porphyrin nitrogens.

**KEY WORDS:** Amino acids, Stationary phase, Amino tetraphenyl porphyrin (atpp), Reverse phase, RP-HPLC Analysis, Hydrophilic, Hydrophobic, H-bonding,  $\pi$ - $\pi$  interaction

### INTRODUCTION

Because of its ease and high efficiency the reversed phase high-performance liquid chromatography (RP-HPLC) has found extensive applications in separation and analyses of both hydrophobic and hydrophilic substances. Recent developments, including microbore column and gradient elution techniques, seem to surpass the conventional ion-exchange chromatography in separating free and derivatized AAs, polypeptides and proteins [1].

According to solvophobic theory in this type of chromatography, the increase in retention time or capacity

factor can either be correlated with the increase in hydrophobicities of the solute and the stationary phase or the decrease in polarity of the mobile phase [2-4].

The concept of immobilized metal ion affinity chromatography, first proposed by Porath *et al.* in 1975, has found many applications especially in the separation of peptides and proteins.

In this method separation is based on selective coordination interactions between the metallic center (eg.  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ), that is immobilized onto the stationary phase, and the solute.

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Metal complexes with iminodiacetate, nitrilotriacetate or (tris- (carboxymethyl) ethylenediamine) are covalently attached to the silica based stationary phase. Analyses of retention of AAs allow successful prediction of peptides retention on immobilized metal ion affinity columns [5].

The essential disadvantage of this method, however, is the leaching of metal ions from the stationary phase, leading to contamination of separated peptides and proteins, making it inapplicable in preparative separations. Although the column can be regenerated by refurbishing it at intervals with fresh solution containing metal ions, the immobilized metal ion affinity chromatography has in practice found limited applications. An additional disadvantage of this method is the necessity to use high salt concentrations in order to decrease ionic interactions with unmetallated chelate.

In recent years Meyerhoff, *et al.* have pioneered stationary phases based on tetraphenyl porphyrin (tpp) or protoporphyrin IX immobilized onto amino propyl silica gel through peptide bonds. The phases prepared in this way are modified with different metal ions like  $\text{Fe}^{3+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{In}^{3+}$ ,  $\text{Sn}^{4+}$  [5]. Various applications of these phases have been reported, eg. determination of various classes of organic substances, polycyclic aromatic hydrocarbons, and fullerenes. The  $\pi$ - $\pi$  interaction between the  $\pi$ -electrons of the porphyrin macrocycle and the  $\pi$ -electrons of analyte has been suggested. The role of metallic center is especially important in separation of the carboxylic and sulphonic acids, amines, and certain AAs and peptides. The specific interaction between the aromatic ring of analyte and the porphyrin macrocycle has been pointed out [5-9].

Trojanowicz *et al.* [5] have recently reported the reduction of retention times for di- and tri-peptides by addition of modifiers to the eluent. They have also investigated the retention of 18 AAs on silica stationary phase with either immobilized unmetallated tpp or its Zn(II) and Cu(II) metallated forms. Xiao *et al.* have presented the HPLC results of amino acids separated on propylsilica metallated with  $\text{Fe}^{3+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{In}^{3+}$ ,  $\text{Sn}^{4+}$ . Davankov *et al.* [10], have prepared a reversed packing

by dynamically coating the conventional reversed phase packings with N-alkyl-AAs, and succeeded in the optical resolution of AAs by ligand exchange chromatography. They have also shown that such sorbents were relatively stable and reproducible. Shinbo *et al.* [11] applied this procedure in the separation of racemic amino acids by the use of chiral crown ether coated packing. They showed that the resulting packing separated many alkali metal ions efficiently and had a good stability.

We hereby report the results of our investigations on analytical performance of a stationary phase prepared by dynamically coating atpp on a reversed-phase packing against the 20 AAs commonly found in proteins.

## EXPERIMENTAL

### Materials and reagents

AAs in the L-form, were purchased from Merck (Darmstadt, Germany) and Sigma (St. Louis, Mo, U.S.A). Standard solution of each AA was prepared daily by dissolving appropriate amounts (1-5mg) of the solid reagent in the eluent solution. Dilute solution of sodium hydroxide of analytical reagent grade was used to adjust the pH of the HPLC water in the mobile phase.

### Apparatus and chromatographic conditions

A liquid chromatograph (LC-6A, Shimadzu, Kyoto, Japan), equipped with a variable wavelength UV-visible detector (SPD-6AV Shimadzu, Japan), injection valve with 10 $\mu$ L loop, was used for measurement of the retention times. The stationary phase was a C18 Partisil P10 ODS packed in a stainless-steel tube (25cm x 4mm ID). The samples were dissolved separately in a small portion of the mobile phase, and the minimal concentration of each AA required for detection was injected. HPLC measurements were carried out under isocratic conditions with UV detection at 210 nm and a flow rate 1.0 mL.min<sup>-1</sup>.

### Capacity factors and separation coefficients

The capacity factor ( $k$ ) was calculated from  $(t_x - t_0)/t_0$ , where  $t_x$  and  $t_0$  respectively are the average retention time of the sample and that of a least adsorbable AA. Separation coefficients were calculated from  $\alpha = k_1/k_2$ ,

where  $k_1$  and  $k_2$  respectively are capacity factors under conditions 1 and 2.

### Preparation of the modified packing

The reversed phase column was coated with atpp by passing a solution (0.25 gr/L) of the porphyrin compound in dioxane-water (1:1) through the column (flow rate 1 mL.min<sup>-1</sup>). An effective coating was obtained when the effluent was colored and had the same composition as the feed solution. Experiments showed that such a coating is quite stable and reproducible between pH 2 to 8 against the aqueous mobile phases.

## RESULTS AND DISCUSSIONS

### C18 column

#### Nonpolar side chains

As can be seen from Fig. (1), capacity factor,  $k$ , of AAs with nonpolar side chains increase as the number of carbon atoms of the side chain increase. The difference in  $k$  values between Leu and Ile shows that the further the methyl terminal group in the side chain, the better it seems to participate in the hydrophobic interactions with the column. The presence of S in Met side chain does not seem to influence the hydrophobicity of the side chain. Notably the cyclization of the side chain in Pro has led to a reduction in the side chain hydrophobicity. This is because the rigid ring shows a less hydrophobic interaction. The opposite is true for Phe and Trp, which have aromatic rings in their side chains. This effect is even stronger in Trp due to its extra 5 membered ring.

#### Polar side chains

Fig. (1) shows that  $k$  values of these AAs are smaller than those with nonpolar groups. The presence of polar groups (CO-NH<sub>2</sub>, SH, OH) lower their hydrophobicity. It is thus anticipated that Thr should be better retained than Ser whereas in fact the opposite is observed. This may be attributed to the possibility of intramolecular hydrogen bonds in Ser and to some extent in Cys, leading to their increased hydrophobicities compared to Thr. In the latter case the steric hindrance and the less electron donation of the methyl group in the side chain lowers the chance for intramolecular H bonding thus making it more hydro-

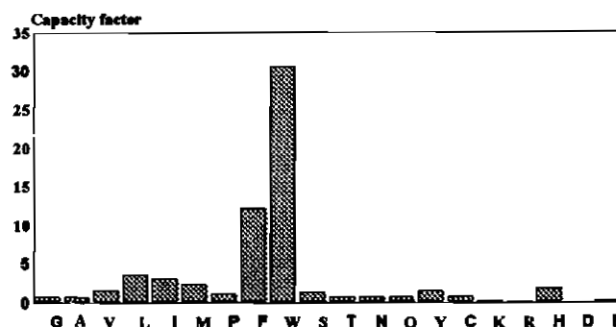


Fig. 1: Capacity factors ( $k$ ) of amino acids on C18-column at pH 7 of mobile phase

philic. The larger chain in Gln compared to Asn causes its better retention while the presence of an aromatic ring in Tyr contributes a further hydrophobicity. The SH group in Cys contributes some polarity but the chance of intramolecular H bonding is dim, thereby a lower hydrophobicity is expected.

#### Charged polar side chains

As expected the presence of electrical charges leads to profound effects and as a result the corresponding AAs have lower  $k$  values. Only in His the presence of a 5-membered aromatic ring contributes some hydrophobicity. The presence of more polar groups in Arg leads to a decrease in its  $k$  value compared to Lys whereas an increase in the carbon atoms in Glu side chain improves its retention compared to Asp.

#### atpp coated C18 column

Considering the molecular structure of 2-amino tetraphenyl porphyrin, the possibility of a number of interactions with the mobile phase, is expected namely; a) hydrophobic interactions between AAs and atpp, b) H-bonding between the amino or carboxyl groups of AAs and the N-atoms of the porphyrin macrocycle or the NH<sub>2</sub> groups of its, surrounding phenyl rings, c)  $\pi$ - $\pi$  interactions between aromatic AAs and the phenyl rings of atpp. Obviously any one or combinations of such effects may play a role in each case.

#### Nonpolar side chains

As can be seen from Fig. (2), Gly has a higher  $k$  value than Ala, which may be due to the smaller size of former

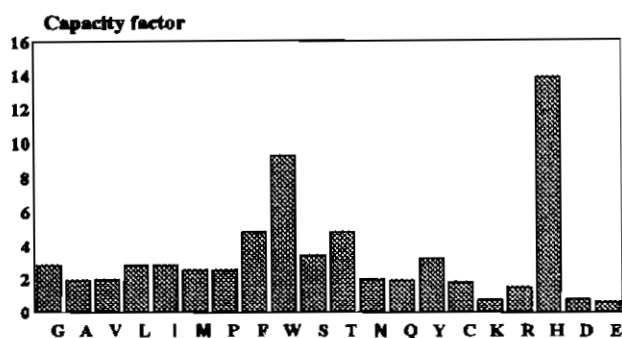


Fig. 2: Capacity factors ( $k$ ) of amino acids on atpp coated C18-column at pH 7 of mobile phase.

and its better fit inside the porphyrin macrocycle. For the rest of AAs the  $k$  values, as expected, run parallel with their hydrophobicities.

#### Polar side chains

In this family AAs having an OH group on their side chains i.e. Ser, Thr, and Tyr, which may form H-bonds with atpp show remarkable retentions. Despite its longer hydrocarbon chain Tyr exhibits a substantial retention due probably to its  $\pi$ - $\pi$  interaction with atpp. The notable retention of Thr and Ser possibly arises from their inability in forming intramolecular hydrogen bonds, allowing their intermolecular H-bonds with atpp. The lower  $k$  values for Cys compared to Ser show that despite their similar structures, the SH group in Cys can not be as effective as the OH group in Ser. Both Asn and Gln exhibit similar retentions, though less than Ser, Thr and Tyr, implying that the amide group can not be as effective in H-bonding as the OH group.

#### Charged polar side chains

In this family, His which has a five membered aromatic ring and participates in  $\pi$ - $\pi$  as well as hydrophobic interactions exhibits the highest  $k$  value. Whereas, in the case of Lys and Arg the increase in their number of amino groups and thereby their H-bonding, causes their improved retention. On the other hand Arg may participate in  $\pi$ - $\pi$  interactions with atpp. Asp and Glu possess acidic side groups and exist as anions at pH 7, and despite their ability to form H-bonds reveal similar

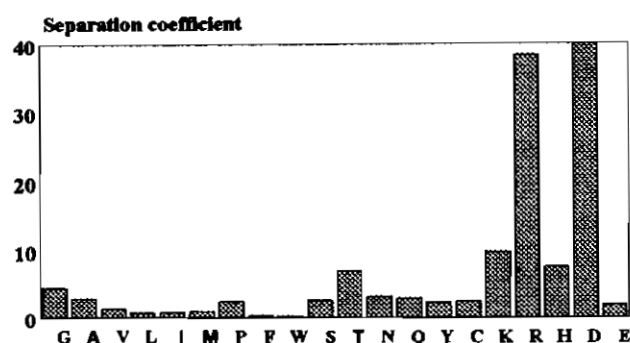


Fig. 3: Separation coefficient ( $\alpha$ ) of amino acids at pH 7 of mobile phase.

but low  $k$  values, due to their diminished hydrophobic interactions.

#### Comparison of C18 and atpp coated C18 column

For this purpose use is made of the separation factor,  $\alpha = k_{\text{atpp}}/k_{\text{C18}}$ , at pH 7, Fig. (3).

#### Nonpolar side chains

As can be seen,  $\alpha > 1$  for Gly, Ala, Val and Pro whereas  $\alpha < 1$  for Leu, Ile, Phe and Trp, and  $\alpha = 1$  for Met. These show that the more hydrophilic family prefers the atpp and the more hydrophobic one prefer the C18 column, i.e.  $\alpha$  decreases with hydrophobicity.

#### Polar side chains

In this case  $\alpha$  is higher than the previous class, i.e. the presence of polar side chains further improves the interaction of these AAs with the atpp column. This is particularly remarkable for Thr, which does not form intramolecular H-bond, and thus in contrast to Ser has a higher  $\alpha$  value.

#### Charged polar side chains

As expected in this case the  $\alpha$  values are even higher, the highest being those of Lys, Arg and His, that belong to basic AAs, and exist as protonated species at pH 7. The value of  $\alpha$  tends to increase with the number of nitrogen atoms in the side chain. However in the case of His the participation of one of such nitrogen atom in aromaticity lowers its  $\alpha$  value to that of Lys. The other

two AAs (Asp and Glu) possess anionic side chains at pH 7 and can enter into H-bond and  $\pi$ - $\pi$  interactions with atpp.

## CONCLUSIONS

Dynamic coating of commonly available HPLC columns is a truly convenient and inexpensive method for modifying and improving their performance. Besides its operational and economic advantages, unexpectedly it allows under proper conditions the recovery of and reuse of the initial C18 substrate columns. Results obtained show that atpp coated C18 column displays remarkable resolutions specifically against polar and charged polar AAs. It is readily possible to resolve 12 out of 20 common AAs under isocratic conditions. Altogether it is found that improved resolutions (compared to C18) are achieved as the number of charged or the H-bonding (intermolecular) groups in the side chain increase. On the other hand it is found that atpp seems to have better interactions with the  $\text{NH}_2$  group than with OH or COOH groups. The higher the hydrophobicity of the side chain, the lower the affinity of AA towards atpp.

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## Figure captions

Amino acids	Symbole
Glycine	G
Alanine	A
Valine	V
Leucine	L
Nonpolar Isoleucine	I
Side chain Methionine	M
Proline	P

Phenylalanine	F
Tryptophan	W
Serine	S
Threonine	T
Uncharged Asparagine	N
Polar Glutamine	Q
Side chain Tyrosine	Y
Cysteine	C
Lysine	K
Charged Arginine	R
Polar Histidine	H
Side chain Aspartic acid	D
Glutamic acid	E

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