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Characterization of wide-pore reversed-phase columns for biopolymer separations
I. Single-parametric evaluation

Ágota Bede[a], Géza Rippel[b], László Szepesy[b,*], Henk A. Claessens[c]

[a]*Department of Chemical Technology, Technical University of Budapest, Budafoki u. 8, H-1521 Budapest, Hungary*

[b]*Department of Agricultural Chemical Technology, Technical University of Budapest, Budafoki u. 8, H-1521 Budapest, Hungary*

[c]*Laboratory of Instrumental Analysis, Eindhoven University of Technology, P.O.B. 513, NL-5600 MB Eindhoven, Netherlands*

Abstract

Nine prepacked wide-pore columns recommended for protein separation were studied. The stationary phases investigated were widely different with respect to the type of ligands. In order to characterize the columns, measurements were made with six different test mixtures containing two different homologous series, substituted benzenes and amino acids, peptides and proteins. The isopotential eluent compositions obtained for the homologous series revealed the polar–apolar nature of the stationary phases. The relative hydrophobicity calculated for the individual test compounds provided more thorough but still partial characterization. The involvement of substituted benzenes resulted in the most complete description. The characterization with amino acids, peptides and proteins furnished results similar to those obtained for smaller test compounds having low or intermediate polarity. The examination of the behaviour of proteins revealed that the retention is determined by the hydrophobicity of the stationary phases and the other characteristics affect the selectivity.

Keywords: Stationary phases, LC; Hydrophobicity; Proteins; Amino acids; Peptides; Benzenes

1. Introduction

In spite of the development of new stationary phases and improvements in other chromatographic techniques the method most often used is RP-HPLC, especially for the separation of biological materials. Although the application of RP-HPLC has decreased, according to the most recent survey [1], more than half of all separations in HPLC are performed with aqueous–organic eluents and RP columns. Since the introduction of RP phases in the early 1970s, the discussions of their properties and their characterization have been manifold and controversial. The widespread use of RP-HPLC resulted in the introduction of a variety of different RP packings by various manufacturers. Well over 100 different RP stationary phases are commercially available. The manufacturers' descriptions are poor and insufficient for characterizing the stationary phases. For this reason, it is difficult to compare

* Corresponding author.

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the stationary phases and to select the appropriate one for a defined separation problem.

There have been numerous reports on the characterization of RP packing materials using physical measurements, elemental analysis and various spectroscopic methods. These non-chromatographic techniques have been reviewed and discussed by several authors [2–5]. The other group of characterization techniques involve chromatographic characterization utilizing the information obtained from the retention behaviour of some selected compounds. Chromatographic characterization is the most desirable approach for chromatographers, in order to test one's own columns without using any expensive instrumentation.

Since the introduction of RP phases, a number of test compounds and test procedures have been suggested. The test compounds have to be selected so that they can show differences in the chromatographic properties of the packing materials such as hydrophobic properties, steric selectivities, the extent of silanol activity and ion-exchange properties.

In order to characterize RP columns, mostly non-polar compounds, e.g., benzene homologues, are used as test solutes. The use of homologous series has been extensively investigated [2–10] for the characterization of RP phases. Although non-polar test solutes give an insight only into hydrophobic properties, methods have been suggested to characterize hydrophobic and polar selectivity based on measurements with homologous series [11–13]. According to several researchers, it is generally the polar solutes that yield the most critical information concerning the surface characteristics of RP packings. Goldberg [14] used five different tests to compare RP columns. Engelhardt and co-workers [15–18] introduced several test mixtures to characterize hydrophobic, silanophilic and other polar interactions. Tanaka and co-workers [19–21] also elaborated detailed test procedures by using alkylbenzenes and different polar compounds in order to define the various interactions. To evaluate shape selectivity, Sander and Wise [22,23] introduced a selectivity test mixture containing polyaromatic hydrocarbons of different planarity. Several other test mixtures and test procedures have also been described, and good summaries can be found in reviews [24,25]. Recently, Valkó and Slégel [26] introduced a new chromatographic hydrophobicity index to characterize the hydrophobic character of compounds in RP-HPLC. This index can also be used to characterize the hydrophobicity of stationary phases [12].

In recent years, chemometric methods such as principal component analysis (PCA), cluster analysis (CA) and factor analysis (FA) have been successfully applied for the interpretation of chromatographic data and the classification of stationary phases [27–30].

In the past decade, HPLC has become increasingly popular for the analysis and separation of biopolymers such as polypeptides, proteins and nucleic acids. In order to achieve rapid and high-resolution separations of biopolymers, a number of distinct criteria should be fulfilled by the stationary phases. For this reason, a new branch of bonded phases has been developed using wide-pore (30 nm or above) silicas and improved bonding chemistry in order to furnish a soft and homogeneous surface [31–34]. Although a general trend of elution order versus hydrophobicity is apparent, RP-HPLC of peptides and proteins exhibits many irregularities. For this reason, in addition to the test procedures suggested for the separation of low-molecular-mass solutes, the columns should be tested with some peptide and/or protein mixtures [31,34]. Unlike small molecules, the retentions of many proteins are not significantly altered by variations in the alkyl chain length bonded to the silica. On the other hand, cyano-, propyl- and diphenyl-bonded phases have shown slightly different selectivities compared with the generally used C_8 and C_{18} phases [35]. However, different wide-pore silicas packings have rarely been compared with each other.

In this study, we compared and evaluated a number of wide-pore RP columns containing various ligands (TRIF, CN, C_3, C_4, C_8 and C_{18}) and obtained from different manufacturers. In addition to the standard test solutes suggested for narrow-pore packings, measurements with

selected amino acids, peptides and proteins were also carried out. Evaluation of the columns was accomplished by using single parametric methods by selecting some descriptors suggested in the literature to characterize stationary phases for RP-HPLC. In a subsequent paper multiparametric evaluation and comparison of the columns investigated will be presented.

2. Experimental

2.1. Materials

The test components used for characterization were all of analytical-reagent grade and were obtained from different sources. Small molecules in test mixture 1 = benzene homologues: benzene (B), toluene (T), ethylbenzene (EB) and propylbenzene (PB); test mixture 2 = *n*-alkyl *p*-hydroxybenzoates: methyl (ME), ethyl (EE), *n*-propyl (PE) and *n*-butyl *p*-hydroxybenzoate (BE); and test mixture 3 = other compounds: aniline (A), nitrobenzene (N), N,N-dimethylaniline (N), phenol (Ph), chlorobenzene (CB), acenaphthene (AC) and fluorene (FL). These compounds are referred to as standard test compounds. In addition, selected amino acids and peptides were used to characterize the columns: amino acids, γ-benzyl-L-glutamate (BGlu), γ-benzyl-L-aspartate (BAsp), DL-tryptophan (Trp), DL-phenylalanine (PhA) and DL-tyrosine (Tyr); peptides, tryptophanylalanine (TA), tryptophanylleucine (TL), phenylalanylglycine (PG), phenylalanylleucine (PL) and phenylalanylglycylglycine (PGG). The large molecules used were the proteins: ovalbumin (OVA), β-lactoglobulin (LAC), cytochrome *c* (CYT), lysozyme (LYS), α-chymotrypsinogen A (CHY) and ribonuclease A (RNA).

2.2. Columns

The characteristics of the columns used are listed in Table 1.

2.3. Chromatography

A Merck–Hitachi (Merck, Darmstadt, Germany) fully automated chromatograph was used, consisting of an L-4250 UV–Vis detector, L-6200 programmable pumps and a Rheodyne (Cotati, CA, USA) injector with a 10-μl loop. System control, data acquisition and evaluation were performed with HPLC Manager D-6000 software (Merck) running on an IBM PC AT-compatible computer.

Measurements for small molecules were made under isocratic conditions in water–acetonitrile mobile phase of various compositions. The composition of the mobile phase was always adapted to the components, i.e., it was varied so as to obtain 5–7 retention values in the range $0.2 < k < 10$ for all the test components on all the

Table 1
Characteristics of the columns

Column	Source	Support material	Ligand type	Dimensions (mm × mm I.D.)	Particle size (μm)	Pore size (Å)	Abbreviation
Zorbax SB 300 TRIF	Rockland Technologies (Newport, DE, USA)	Silica	Trifluoroacetamide	150 × 4.6	5.0	300	Z-TFA
Zorbax SB 300 CN	Rockland Technologies	Silica	CN	150 × 4.6	5.0	300	Z-CN
Zorbax SB 300 C_3	Rockland Technologies	Silica	C_3	150 × 4.6	5.0	300	Z-C3
Zorbax SB 300 C_8	Rockland Technologies	Silica	C_8	150 × 4.6	5.0	300	Z-C8
Aquapore Butyl	Applied Biosystems (San Jose, CA, US)	Silica	C_4	100 × 4.6	7.0	300	A-C4
Aquapore OD-300	Applied Biosystems	Silica	C_{18}	100 × 4.6	7.0	300	A-C18
Synchropak C_4	Synchrom (Linden, IN, USA)	Silica	C_4	100 × 4.6	6.5	300	S-C4
Synchropak RP-P C_{18}	Synchrom	Silica	C_8	100 × 4.6	6.5	300	S-C18
Synchropak RP-C_4	Synchrom	Silica	C_4	250 × 4.1	6.5	300	S-C4L

columns. The hold-up time was measured at all compositions with an aqueous solution of sodium nitrite.

Measurements for proteins were made under gradient conditions at two different gradient times (t_G = 15 and 45 min) and at pH 2 in water–acetonitrile mobile phase. The data obtained under gradient conditions were evaluated according to the linear solvent strength (LSS) model of gradient elution with a program written in C and run on the computer used as a part of the chromatographic system [12]. All the measurements were repeated at least twice and the average values were used for the calculations.

3. Results and discussion

The retention profile of components under reversed-phase liquid chromatographic (RPLC) conditions can be given as

$$\ln k = \ln k_w - Sx \quad (1)$$

where x is the organic content of the eluent and S and $\ln k_w$ are the slope and the intercept of the profile, respectively, characteristic not only of the components but also of the phase system (stationary and mobile phases) used. This equation gives a good approximation within a limited range (generally $0.2 < x < 0.8$) of eluent composition.

For homologous series, more specific profiles were derived [12]. According to the model of Bidlingmeyer et al. [13], the retention of homologous series can be described as

$$\ln k = c_0 + c_1(c_2 - x)(c_3 - n_c) \quad (2)$$

where n_c is the incremental carbon number and c_1–c_3 are constants. The organic content indicated by c_2 is called the isopotential eluent composition. The individual retention profiles described by Eq. 1 converge to this point and in this eluent ($x = c_2$) the retention behaviour of the components is independent of n_c, i.e., the phase system cannot differentiate the members of homologous series. It was presumed that the isopotential eluent composition (x_{ip}) could be a measure of the overall polarity or strength of the stationary phases [13], because the higher is x_{ip} the lower is the polarity of the stationary phases or, generally, the lower is the retention of the components at an identical eluent composition. Earlier we found that the order of column strength furnished by x_{ip} corresponds well with that accomplished from the ligand type and column geometry even if a wide diversity of columns is considered [12].

The results obtained for the columns investigated are shown in Fig. 1. It is immediately seen that the above statement is not or only partly true for this set of stationary phases. The order indicated by the apolar test mixture 1 is different from that expected from the ligand type. For example, S-C18 seems to be much weaker than Z-C8 and S-C4L and is in the same range as S-C4, Z-C3 and Z-CN. The most striking difference is between S-C4L and S-C4. As far as we know, these columns were filled with the same packing material and the only difference was in the column size. The results indicate that other properties of these columns must be also dissimilar.

The two test mixtures exhibit different behaviour. Our earlier results indicated that x_{ip} values calculated for benzene homologues reflected the apolar/hydrophobic nature and that obtained for *n*-alkyl *p*-hydroxybenzoates indicated the polar character of the stationary phases [12]. In this case, the pattern of test mixture 2

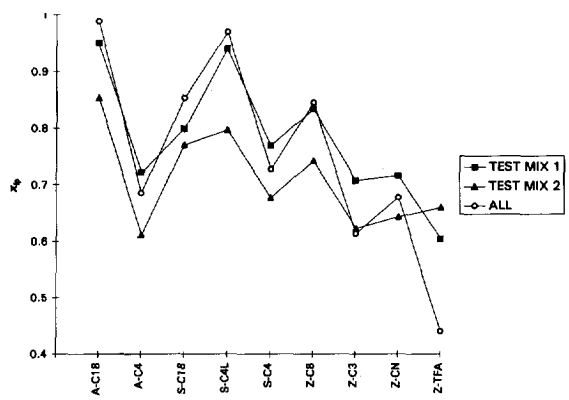

Fig. 1. Isopotential eluent composition determined for the different columns with various test mixtures. ■ = test mixture 1; ▲ = test mixture 2; ○ = all components.

does not correspond to that of test mixture 1: neither the order of columns nor the relative differences among them are the same. Note the unique behaviour of Z-TFA. On this column the x_{ip} value of the more polar test mixture 2 is higher than that of test mixture 1. This is a clear sign that besides hydrophobic interactions presumed to be the dominant effect on the retention process in RPLC, another effect(s) having a polar nature must be involved. If this were not true, the substitution of the molecular residue of the homologous series should result in regular shifts of the retention of components, and therefore the x_{ip} values of the test mixtures should show similar patterns.

This regularity is further contradicted by the results obtained for the joint data (designated "all" in Fig. 1). The absolute values and their positions relative to that of the individual test mixtures seems to be unique for all stationary phases. This means that the behaviour of the two test mixtures changes significantly from column to column, and it is characteristic of the stationary phases. The contribution of polar interactions is profound on some of the columns investigated, which must also be taken into account in the characterization.

Since x_{ip} is defined for a set of components, it suppresses the differences between the individual members of the set. Another disadvantage of x_{ip} is its restricted applicability, namely, it can be applied only for homologous series. Recently, a new parameter termed the hydrophobicity index was suggested [26], which can be calculated as

$$x^* = \ln k_w / S \quad (3)$$

where $\ln k_w$ and S are as in Eq. 1. (Originally the hydrophobicity parameter was designated φ_0, but it could be misleading when the retention profiles of components are determined under gradient conditions. Instead of the subscript zero we suggested the use of the superscript asterisk [36].) Note that x^* corresponds to that eluent composition where $\ln k = 0$ (cf., Eq. 1), which means that when using this eluent the molar concentrations of the related compound are identical in the stationary and mobile phases.

It was presumed that x^* is a measure of the hydrophobicity of the components, which is independent of the phase system applied [26]. Our earlier results indicated that this parameter is a sensitive indicator of the quality of the phase system in RPLC and also in hydrophobic interaction chromatography (HIC), and the relative values calculated for different pairs of components reveal the selectivity [36].

The results obtained for the test mixtures examined above are shown in Fig. 2. The order of columns indicated by x^* calculated for test mixture 1 seems to be more realistic than that of x_{ip} for the same set. The strength of the stationary phases decrease with increasing hydrophobicity of the ligand, which agrees with expectations. The patterns for BE and PE on the Aquapore and Synchropak columns are very similar to that for test mixture 1. This similarity is also valid for the Zorbax columns but here the x^* values of BE and PE are shifted. However, the order of the columns is the same as obtained for test mixture 1.

On the other hand, compared with Fig. 1 there is no or almost no difference between S-C4L and S-C4. This corresponds to our preliminary knowledge but seems to conflict with the results outlined above. The explanation of this apparent contradiction is the different retention behaviours of the test components on these columns. The retention profiles are much steeper on S-C4 than on S-C4L but the intercepts on the x-axis (x^*) are almost the same, and therefore the x_{ip}

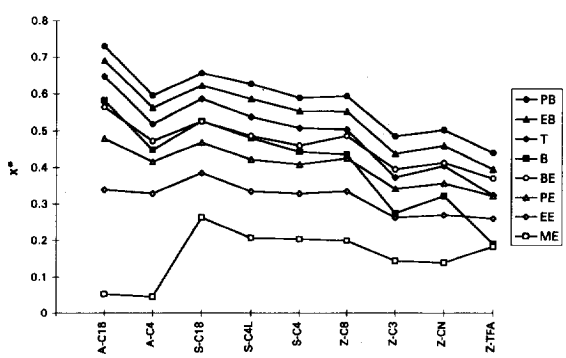

Fig. 2. Relative hydrophobicity of the columns determined from the retention of the homologous series investigated.

values calculated for S-C4L must be lower than those for S-C4. Note that this is also true for A-C18 and A-C4 as regards test mixture 2, but to a lesser extent, for almost all pairs of columns.

EE and ME show different patterns to BE and PE. On the basis of the results obtained for ME, the least polar column is A-C18, EE shows intermediate polarity and PE and BE exhibit the highest values for this column. The relative position of A-C4 changes similarly but the values obtained for Z-TFA show just the opposite tendency. It is likely that the retention of BE and PE is affected rather by the hydrophobicity of the columns, the effect of which is less for EE and negligible for ME. For the latter compounds the contribution of polar interaction(s) is greater.

However, the results detailed above seem to be unsuitable for complete characterization of the stationary phases. Either the set of test components selected or the parameters calculated are incomplete, i.e., the picture displayed by these characteristics is oversimplified.

In order to gain a better insight, a third test mixture containing substituted benzenes and two polyaromatic hydrocarbons was also applied. The parameters calculated for this set are shown in Fig. 3. Benzoic acid was not included because it had no retention on most of the columns, except for A-C4 and S-C4L. On Z-CN it eluted also after the dead time (t_m), but the variation of retention with the eluent composition was within the error of measurement. Note that this behaviour of benzoic acid cannot be correlated with any of the parameters detailed above.

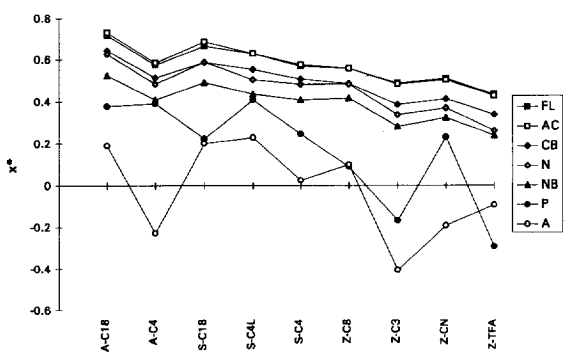

Fig. 3. Relative hydrophobicity of the columns determined from the retention of the polar and polyaromatic test compounds.

In some instances the parameters calculated are fictitious as they are lower than zero. This means that in these instances the ln k_w values of the components are lower than zero. However, the values are applicable for comparison.

The order of columns is the same for FL, AC, CB, N and NB, which is very similar to that obtained for test mixture 1. The values for FL and AC indicate no shape selectivity; the differences on A-C18 and S-C18 are not sufficient for complete resolution of these components. The substituted benzenes show slight variations from column to column. This indicates that for these compounds the hydrophobic interaction plays a dominant role in the retention.

Larger differences and very dissimilar patterns are displayed by P and A. These components are routinely applied for measuring the silanol activity of stationary phases, usually their relative retention and/or the tailing of peaks being examined. However, the picture is more complex, since the absolute and also the relative values obtained for these compounds seem to be characteristic of the stationary phases.

On S-C18 the order and position of compounds is "normal", i.e., the value for A being slightly lower than that for P. However, compared with other columns, the absolute values seem to be high. Related to S-C18, A-C4 shows much lower activity towards A but much higher activity towards P. The "A-activity" of A-C18 and S-C4L is about the same as that of S-C18, but their "P-activity" is higher. S-C4 and Z-CN show "P-activity" similar to that of S-C18 but lower and different "A-activity". Note that the patterns for A and P are not parallel, so their difference and relative position must also be characteristic of the stationary phases. For example, the order of these compounds is inverted to a lesser extent on Z-C8 and significantly on Z-TFA compared with the others.

Since the above results are affected by the hydrophobicity of the columns, the values obtained for substituted benzenes were related to that of T. The results are shown in Fig. 4. These values strengthen the conclusions drawn for CB,

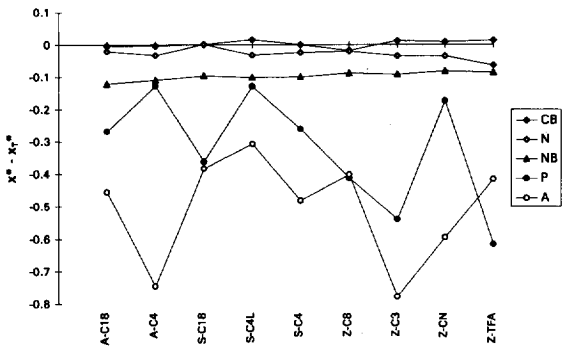

Fig. 4. Relative hydrophobicity of the columns compared with that of toluene determined from the retention of the polar test compounds.

N and NB. It is clearly seen that the chloride, nitro and dimethylamino substitution affects only the overall polarity of these compounds but does not contribute specifically to the retention process on these columns. However, the values for N decrease and those for NB increase slightly from S-C18 to Z-TFA. It is also interesting that the pattern for N is not parallel to that for A but shows an opposite tendency, and rather it is similar to that for BE and PE. It seems that the dimethylamino group acts as a polar and not as a basic substituent.

After the transformation, the comparison of A and P reveals larger differences amongst the columns. The order of columns and the measure of their specific activity towards these components are also changed. (Note that the difference between the points for A and P is affected by the different scaling of Figs. 3 and 4 and not by the transformation.) Here we postulate that these compounds reflect the acid–base character of the stationary phases. This is supported by the fact that the behaviour of benzoic acid correlates well with the pattern for P, i.e., retention could have been achieved only on those columns which exhibit the highest "P-activity". The columns having lower activity are not "strong" enough to retain the fully ionized benzoic acid.

According to this specification, A-C4, S-C4L and Z-CN have high and Z-TFA and Z-C3 have low activity towards acidic components. Furthermore, S-C4L, S-C18, Z-C8 and Z-TFA have high and A-C4 and Z-C3 have low activity towards basic components.

On the other hand, the pattern for A is very similar to that for x_{ip} obtained for test mixture 2, which indicates that the polar interactions should also be taken into account. Therefore, the transformation of A using N or BE or PE instead of T could be more relevant. However, a rational selection of test components and the identification of the factors affecting their retention need multivariate data analysis, which will be reported elsewhere. Nevertheless, the results detailed above demonstrate that the polar–apolar characterization using homologous series does not provide a complete description of the nature of stationary phases. Besides, the acid–base character of the packings could also be revealed. The selection of compounds must be appropriate for testing these features also.

Since the columns investigated are recommended for protein separations, we examined the utility of the above characterizations for this purpose. Three further test mixtures containing amino acids, peptides and proteins were also applied. The components were analysed under conditions recommended for protein separation, i.e., acidic eluents were used (see Experimental).

The x^* values obtained for the three test mixtures are presented in Figs. 5–7. Note that Fig. 7 does not include A-C18, because on this column the protein separations gave very poor

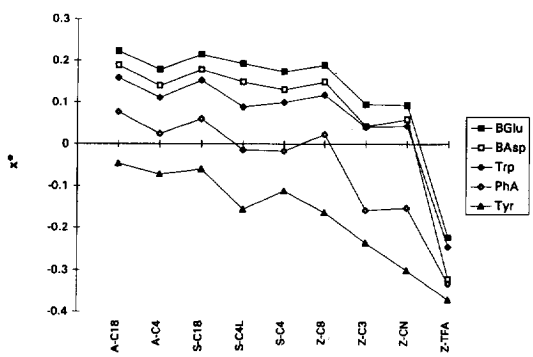

Fig. 5. Relative hydrophobicity of the columns determined from the retention of the amino acids investigated.

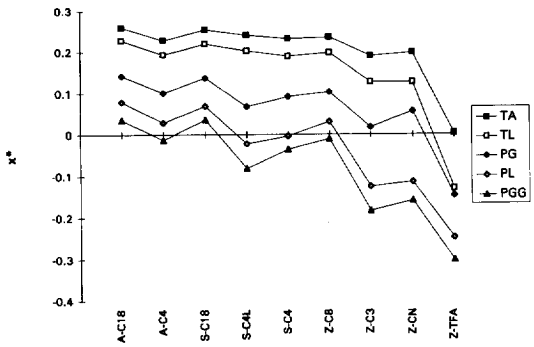

Fig. 6. Relative hydrophobicity of the columns determined from the retention of the peptides investigated.

results. The peak tailing was serious, the loss of activity was also very high and the reproducibility of retention was inadequate for evaluation. In most instances the relative error of the parameters of Eq. 1 calculated from the gradient data was estimated to be higher than 50%. Furthermore, this column needed extremely long conditioning when the aqueous constituent of eluent was changed (from water to TFA solution or the reverse). A long equilibration with the new eluent improved the reproducibility but the error of parameter estimation remained high. However, this process did not enhance significantly the kinetic parameters of the column. It is interesting that these effects were observable with the smaller test compounds, but their rates were acceptable even for the peptides. The causes of these phenomena could have been completely revealed. However, the results were very similar to those obtained on S-C18.

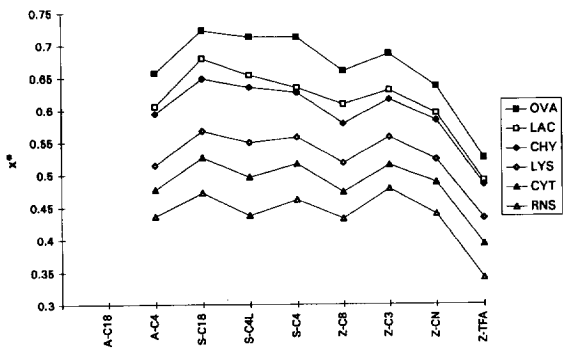

Fig. 7. Relative hydrophobicity of the columns determined from the retention of the proteins investigated.

After the completion of measurements, it was immediately seen that the amino acids and peptides acted very similarly. The orders of columns in Figs. 5 and 6 are almost the same. Further, it is the same as that obtained with test mixture 1 (especially with B) and it is very similar to that for compounds having intermediate polarity such as BE, PE and N. This indicates that mainly the apolar segments of these components take part in the retention process, and the polar and ionic parts play a secondary role. In other words, the retention of these components is affected mainly by the hydrophobicity of the columns investigated.

In some instances, the relative positions of columns depend on the nature of test compounds. Z-TFA seems to be much weaker for these test mixtures than for the former sets, but to a lesser extent this is also true for all the other columns. The apparent strength of S-C4L changes with the polarity of the components. The more polar (less hydrophobic) is the test compound used, the lower is the apparent column strength indicated by that compound. This means that the polarity of columns is not negligible. However, the difference between S-C4L and S-C4 is much smaller than that shown by the x_{ip} values (cf., Fig. 1).

The results obtained for proteins are shown in Fig. 7. The order of columns is almost the same as for amino acids and for peptides. Z-C3 seems to be stronger and Z-TFA is weaker than for the smallest test compounds. The dependence of the apparent strength of S-C4L on the hydrophobicity of compounds is also similar to that obtained for amino acids and peptides.

In most instances, the changes in the differences between the retentions of the components seem to be more significant than the differences between the apparent strengths of the columns. From a practical point of view, this means that these columns differ mainly in their selectivity and not in their strength. The selectivities of the columns measured by Δx^* are shown in Fig. 8. It is seen that the differences in peak pairs vary

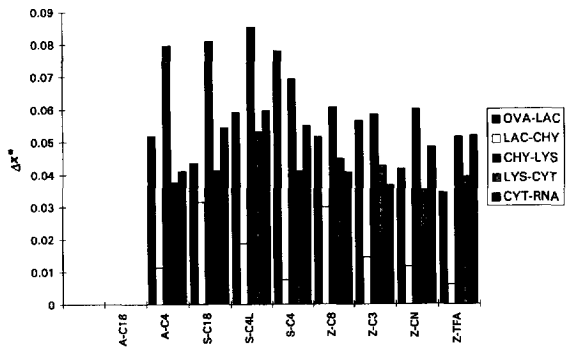

Fig. 8. Selectivities of the columns investigated for the separation of different pairs of proteins.

from column to column and they follow unique patterns.

Since the apparent strengths of columns obtained for the proteins are very similar to those obtained for the smaller test compounds having low or intermediate polarity, it is very likely that the hydrophobicity of the columns is the dominant factor affecting the retention of proteins. However, the differences in the selectivity of the stationary phases indicate the effect of other features. However, the patterns of selectivity do not show an acceptable correlation with any of the characteristics revealed above. Presumably all of them play some role but their contribution depends on the features of proteins having highly heterogeneous surfaces.

4. Conclusions

Nine commercially available wide-pore columns recommended for protein separation were studied. The stationary phases differed widely concerning the type of ligands. Six different test mixtures containing two different homologous series, substituted benzenes and amino acids, peptides and proteins, were chromatographed with acetonitrile–water as the eluent.

The values of the isopotential eluent composition calculated for the homologous series and the relative hydrophobicities calculated for the members of the homologous series revealed the polar–apolar nature of the stationary phases but the characterization seemed to be partial and sometimes ambiguous.

When the results for substituted benzenes were also taken into account, the acid–base features of the stationary phases could have been clearly characterized. In most instances these characteristics seemed to be relevant and were significantly different from those indicated by polar non-ionic compounds.

The characterization with amino acids, peptides and proteins provided analogous results; this set of components behaved very similarly. The comparison of these results with those for the smaller test compounds revealed that the retention of these components is affected mainly by the hydrophobicity of the stationary phases investigated.

A more thorough examination of the results obtained for the proteins showed that the main difference between the columns is in their selectivity and not in their strength. The latter is determined mainly by the hydrophobicity of the stationary phases but in the former other characteristics must also contribute since it changes significantly from column to column. On the basis of the single parametric evaluation of the results presented, an unambiguous classification of the columns investigated cannot be accomplished. In a subsequent paper we shall present the characterization and classification of the columns based on multiparametric evaluation.

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