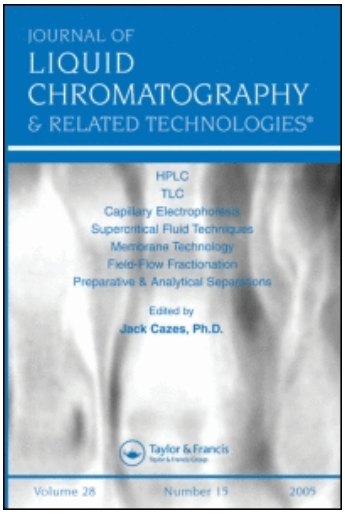

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Characterisation of Wide-Pore Reversed Phase Columns for Biopolymer Separations. II. Multiparametric Evaluation

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To cite this Article Rippel, G. , Bacsur, Gy. , Bede, Á. , Sándi, Á. and Szepesy, L.(1997) 'Characterisation of Wide-Pore Reversed Phase Columns for Biopolymer Separations. II. Multiparametric Evaluation', Journal of Liquid Chromatography & Related Technologies, 20: 11, 1667 — 1687
To link to this Article: DOI: 10.1080/10826079708006325
URL: http://dx.doi.org/10.1080/10826079708006325

J. LIQ. CHROM. & REL. TECHNOL., 20(11), 1667–1687 (1997)

CHARACTERISATION OF WIDE-PORE REVERSED PHASE COLUMNS FOR BIOPOLYMER SEPARATIONS. II. MULTIPARAMETRIC EVALUATION

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ABSTRACT

Nine commercially available silica based, wide-pore columns for protein separation were tested and compared using some low molecular weight aromatic test compounds recommended in the literature. From the retention values obtained under identical mobile phase conditions the strength of stationary phases could have been well characterised by spectral mapping analysis (SMA). However, inconsistency with experience was observed with respect to the polar-neutral and polar-ionic nature of packings. After equalising the mobile phase conditions, the confounding effect of the different column strength could have been eliminated and the selectivity differences amongst the stationary phases could have been revealed.

From the retention of the most relevant test compounds selected from the biplots obtained by SMA a set of single characteristics were constructed, providing a simple evaluating scheme applicable also for stationary phases of widely different type.

INTRODUCTION

In the past few years, numerous methods have been described in the literature to characterise RP-HPLC columns.[1-3] Attention was solely devoted to narrow-pore (NP) stationary phases, probably the most commonly used type of reversed phase packing materials. In spite of great efforts in column characterisation, none of the proposed testing procedures has been generally accepted. The existing methods usually involve isocratic measurements of previously selected compounds with various chemical properties.[1-4,6,10] Computed from the isocratic retention times, retention factor (k') and selectivity (α) are the two basic input data for column characterization.

However, these standard testing procedures are not applicable for wide-pore (WP) columns that are primarily developed for reversed phase separation of biopolymers. A WP column has weaker hydrophobic character than the corresponding NP media due to the lower surface area. Therefore, isocratic runs with the proposed mobile phase strength do not give meaningful data since most of the test components are eluted at or near to the hold-up time of column, i.e. without significant retention. To characterise these specific RP columns, lower mobile phase elution strength and multivariate evaluation of chromatographic data is required.

The endeavour to find chemicals that exclusively measure one certain physico-chemical property of the stationary phase, i.e. hydrophobic strength, hydrophobic selectivity, steric selectivity, acidic and basic activity constitutes the subject of many papers.[1-7,10-11] Multiparametric methods have been successfully applied to reveal the capability of a certain column to engage in different interactions during the chromatographic process (for a review see[5]). They can be used to classify both columns and substances and to furnish qualitative information about retention mechanism.

For this purpose, one of the most powerful multivariate statistical technique is *principal component analysis* (PCA).[6-8] Strongly related to factor analysis (FA), it is applied to uncover hidden or underlying nature of the database. To set up the starting database, chromatographic data are arranged in matrix format so that rows represent HPLC columns while columns of the

matrix represent test substances. The mathematical process aims to find the lowest number of fundamental factors required to account for the greatest possible variance. By combining the original variables into few artificial variable called principal component or factor, PCA sorts out the significantly different test substances and reduces the number of compounds needed for characterization. The projection of the subsequently calculated "factor scores", i.e. the share of each column from a certain abstract factor can provide a good visualisation of column differences.

Spectral mapping analysis (SMA) developed by Massart and co-workers[9] employs also PCA as redundancy extraction method. However, the input database is a matrix derived from double-centering of the original data. This scaling technique allows the user to project columns and substances on the same multidimensional space. One can attribute chemical meaning to compounds lying far from each other and thereby obtain characterization of columns.

Both in PCA and SMA, the weakest point of the data evaluation is to find out the certain chemical interaction or property a test substance is indicating.

It is also possible to classify columns on the basis of chromatographic data without embarking on interpreting the often ambiguous factor structures extracted. Cluster analysis (CA) offers a quick view of column similarity/dissimilarity.[17] On the icicle plot (or dendrogram) presentation the columns are arranged according to their similarity distances computed. Several distance measures and linkage rules can be selected. To obtain a set of clusters, the dendrogram is cut at a certain level of similarity resulting in grouping of columns into specific sets.

We have previously reported the characterization of nine prepacked wide-pore columns, using six different test mixtures and single parametric methods.[12] The results indicated that amino acids, peptides or proteins as test compounds can not reveal the physico-chemical differences of the stationary phases examined. Interestingly, they led to nearly uniform characterization, mainly in order of column hydrophobicity. To achieve sufficient discrimination we extended our small molecule test group and included several substances recommended earlier for NP column characterization.

For the interpretation of chromatographic data and for evaluating the nine WP, stationary phases spectral mapping analysis (SMA) was carried out. Under identical mobile phase conditions only the strength of the stationary phases could have been characterised. After equalising the conditions for different column strength, also, the selectivity differences could have been

revealed. From the retention values obtained under balanced conditions for some specific compounds selected by SMA, single characteristics were computed, which provided a flexible scheme for comparing and evaluating stationary phases of widely different type.

MATERIALS

The test components used for characterization - toluene (T), ethylbenzol (EB), methyl p-hydroxybenzoate (ME), nitrobenzol (NB), aniline (A), N,N-dimethyl-aniline (DA) and phenol (P) - were all of analytical-reagent grade and were obtained from different sources. For chromatography, a Merck-Hitachi (Merck, Darmstadt, Germany) fully automated system was used consisting of an L-4250-UV/VIS detector, L-6200 programmable pumps and a Rheodyne injector (Cotati, CA, USA) with a 10 μl loop. System control, data acquisition and evaluation were performed with HPLC Manager D-6000 software (Merck) running on an IBM386-compatible computer. All the columns investigated were prepacked with silica based, wide-pore (300Å) reversed phase stationary phases. Further characteristics are listed in Table 1.

METHODS

Measurements were carried out under isocratic conditions with acetonitrile-water mixtures. No buffer or salt was added, since it was shown that additives of this kind can mask the actual quality of the stationary phase, i.e. moderate the effect of surface silanols.[13] It was also demonstrated that the silanol effect can not be completely suppressed at pHs applicable on silica based RP phases. This effect can influence significantly the peak shape of basic compounds even in buffered eluents.[9] However, application of such additives under real conditions should be considered just on the basis of characterisation obtained for a column as it is. Consequently, no pH control and no masking additive was applied here.

According to our earlier practice[12] the composition of the eluent was always adapted to the test compounds, i.e.; it was varied so as to obtain 4-5 retention values in the range $-1 < \ln k < 3$ for all the components on all the columns investigated. This region is not only advisable for isocratic separations but it is optimal for stationary phase tests.[1] The hold-up time was measured with aqueous solution of sodium nitrite. All measurements were repeated at least twice and the average values were used for calculations.

Table 1

Characteristics of the Columns

Column Name	Manufacturer	Ligand Type	Dimensions (mmxmm i.d.)	Particle Size (μm)	Symbol
AQUAPORE OD-300	Applied Biosystems (San Jose, CA, US)	C18	100 x 4.6	7.0	A-C18
AQUAPORE BUTYL	Applied Biosystems (San Jose, CA, US)	C4	100 x 4.6	7.0	A-C4
SYNCHROPAK RP-PC18	Synchrom (Linden, IN, USA)	C18	100 x 4.6	6.5	S-C18
SYNCHROPAK RP-C4	Synchrom (Linden, IN, USA)	C4	250 x 4.1	6.5	S-C4L
SYNCHROPAK C4	Synchrom (Linden, IN, USA)	C4	100 x 4.6	6.5	S-C4
ZORBAX SB 300 C8	Rockland Technol. (Newport, DE, USA)	C8	150 x 4.6	5.0	Z-C8
ZORBAX SB 300 C3	Rockland Technol. (Newport, DE, USA)	C3	150 x 4.6	5.0	Z-C3
ZORBAX SB 300 CN	Rockland Technol. (Newport, DE, USA)	CN	150 x 4.6	5.0	Z-CN
ZORBAX SB 300 TRIF	Rockland Technol. (Newport, DE, USA)	trifluoro-acetamid	150 x 4.6	5.0	Z-TFA

The retention data obtained on different columns can be arranged in a data matrix. The rows of this matrix represent objects (here, the stationary phases), the columns are the variables (here, the test solutes) describing the objects.

Accordingly, a complete classification of the objects are given in a hyperspace having as many dimensions as many test components were applied. Clearly, graphical interpretation of such multivariate data tables is impossible, and thus, visual evaluation being more useful than any numerical representation is also infeasible.

For revealing the underlying structure of database principal component analysis (PCA) and related techniques have been proposed. These methods reduce the dimensions of the hyperspace by defining new variables called principal components (PC) along which the objects can be represented in a plane having significantly lower dimensions than the raw data while reserving all or most of the variation in the original data set..

PCs are calculated as weighted sum of the original variables. The corresponding weights are called the loadings of variables on the PCs. The objects are characterised by their values on these new variables. These values are called scores. For displaying the results of PCA both the loadings and scores are applied. Prior to extraction of PCs, the data are generally pre-treated. These mathematical operations transform the raw data to a different set, thus, PCA can reveal different aspects of the original data structure.[15]

Spectral mapping analysis (SMA) is a technique of PCA which was proposed for characterisation of stationary phases.[9] In SMA log double centering is applied as pre-treatment i.e. a logarithmic transformation followed by double centering. The graphical display of the SMA results (the so called biplot) is of central importance. It is a joint graphical representation of the objects and the variables by means of the scores and loadings on two selected factors; i.e. the biplot superimposes the scores plot for the objects and loadings plot for the variables. The position of objects and variables with respect to the origin of graph indicates contrast. Points located far away from (close to) the centre of plot have high (low) specificity or high (low) contrast.

On such a display the relationships or interactions between objects and/or variables can be easily identified. The closer two variables or objects on the plot, are the more similar behaviour these test compounds or columns exhibit. The proximity of a variable and an object being in the same direction means attraction, i.e. the high affinity of that test compounds for the corresponding column. This affinity can be related to specific feature(s) of the stationary phases and, thus, it can be used for characterisation. On the contrary, variables and objects located in the opposite direction repel each other, which are also typical for them.

The characteristic variables can be selected according to contrasts. Test compounds with high contrast located far from one another are the most dissimilar and are therefore the most specific. These variables can be considered poles and can be used for constructing bipolar axes. If the number of poles is less than that of the variables (which is generally held true), the original set of test compounds can be reduced[9] which makes it possible to use much simpler characterisation techniques, e.g. ternary diagrams.

RESULTS AND DISCUSSION

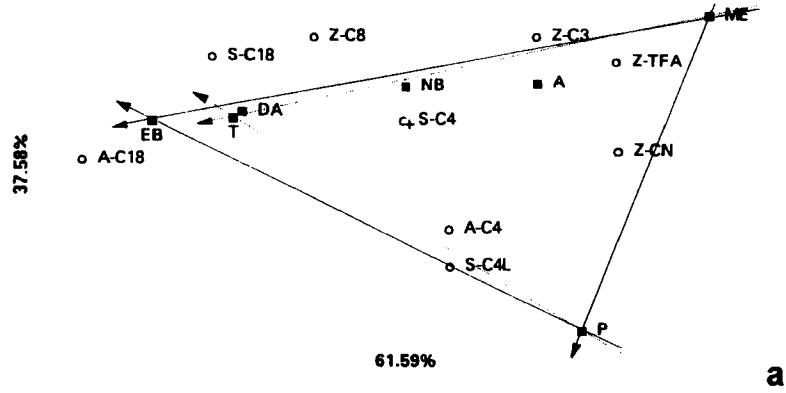
A rough estimate of retentive strength of the stationary phases could have been established immediately from the retention measurements. The order of columns obtained conformed to the hydrophobicity of ligands. It prompted that the characteristic playing dominant role on retention process is the hydrophobicty of columns determined mainly by the ligand type. It also involved that any characterisation with respect to hydrophobicity must reflect, at least approximately, that of the ligands.

In the preceding part of this paper[12] the stationary phases were characterised on the basis of the relative hydrophobicity (x^*) of the test components. (x^* is the composition of that eluent in which the retention factor equals 1, i.e. the component is distributed equally between the stationary and the mobile phase.). Earlier we found that x^* is a sensitive indicator of the nature of the phase system (the stationary and mobile phase), therefore, it is applicable for characterisation of the stationary phases.[16] The relative order concerning the strength of columns could have been established very well with x^* but some differences in selectivity was also indicated which could not have been identified unanimously.[12]

"Traditionally", columns are characterised in isocratic mode on the basis of retention factors of some test compounds measured under identical eluent conditions, i.e. in the same mobile phase. When conditions are properly selected all or most of the test components are significantly retained on all columns. Under such conditions, the variation of retention values obtained can be related to the differences between the stationary phases. When nominally identical columns are to be compared the reference conditions can be easily selected and even varied, some deviations from the methods recommended do not alter significantly the characterisation.

However, the strength of stationary phases investigated here vary in wider range which limited seriously the selection of such reference eluent composition. When all columns were taken into consideration a very narrow range (approx. 30 - 40 % ACN) was only applicable. This range corresponds to the requirements outlined above within which three compositions (30%, 35%, 40%) were selected for the characterisation of the stationary phases.

The retention factors obtained at the specified compositions were subjected to SMA subsequently. The biplots obtained are shown on Figure 1a-c. In each case, the first two principal components account for more than 98% of the variance in the data table.



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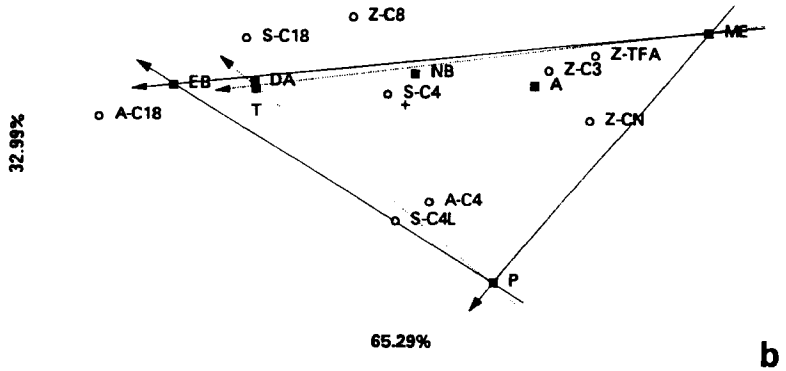

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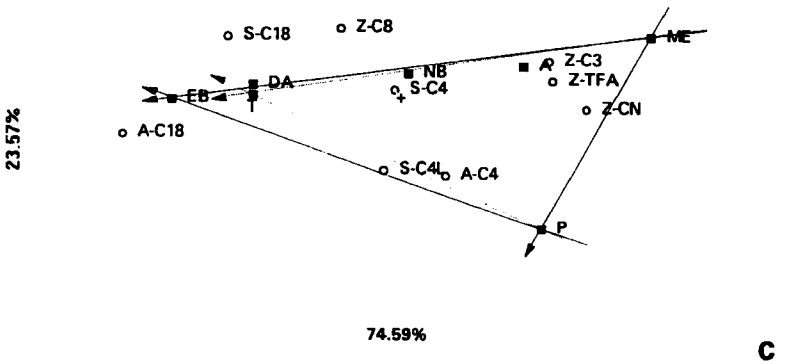

c

It means, the objects and the variables can be described by these two PCs without meaningful loss of information. As seen, the most specific (dissimilar) variables are EB, P and ME, thus they can be considered poles and were selected to construct bipolar axes.

The EB-ME axis seems to represent a kind of polar-apolar axis. Clearly, the spread of variables (test compounds) between the two extrema reflects their polarity. It is seen that DA is very similar to T, i.e. the basic character of amino group is fairly hindered by the methyl substitutions. This kind of behaviour of DA is well known.[1,13] It seems, under conditions applied here T and DA are equivalent with respect to characterisation.

The order of stationary phases obtained by orthogonal projection of the respective points on the EB-ME axis corresponds well to that expected from the hydrophobicity of ligands, as denoted above. The extreme position of A-C18 indicates its profound strength with respect to the other columns. The S-C18 is somewhat weaker, however, the two octadecyl columns are located near to the most hydrophobic test compounds which indicates their large hydrophobic strength. The other columns are bunched to two groups. Indeed, within each group the average retention of test compounds were approximately the same, only selectivity differences could have been experienced. The first group consists of the butyl phases and Z-C8. Note, the octyl column exhibits lower hydrophobicity than would be expected from the ligand type. The second group comprises the three weakest columns. Note, here the "weakness" of stationary phases is resulted from different effects. The strength of columns decreases as the hydrophobicity of ligand decreases (Z-C3) or as its polarity increases (Z-CN, Z-TFA). The order obtained along this axis reflects correctly the strength of stationary phases experienced.

When poles are properly selected, the corresponding ratio of retention factors can be reproduced by orthogonal projection of objects (stationary phases) upon a bipolar axis formed by two test compounds. In this meaning the EB-P axis is incorrect, since it shows the retention of P relative to EB was significantly higher on Z-CN than on the butyl columns (A-C4 and S-C4L). However, the experimental values obtained for these columns were in the same range at all concentration investigated.

Figure 1 (left). SMA biplots obtained from k retention factors measured in 30% (a), 35% (b) and 40% (c) acetonitrile. (For details and explanation see text.)

The correlation between the calculated and the experimental values can be improved significantly by selecting T as pole instead of EB (dashed axes on the biplots). Note, this alteration does not affect the characteristics concerning the polar-apolar nature of stationary phases outlined above, since the hydrophobicity axis (T-ME) remains practically the same as was before (EB-ME).

The property indicated by the third pole selected (P) must be different from that represented by the former ones. It is very likely the polar-ionic (acid-base) character of the stationary phases. The relatively high affinity of A-C4, S-C4L and Z-CN towards P can be attributed to this kind of feature. This interpretation is supported by the behaviour of benzoic acid also used for characterisation. Retention appropriate for evaluation could have been obtained only on A-C18, A-C4, S-C4L and Z-CN for this compound, which corresponds to the postulation given. On other columns no retention was achieved, consequently, benzoic acid was excluded from the evaluation. Since the first step of SMA is a logarithmic transformation, zero retention factors can not be included. So as to keep this compound for evaluation, a "small value" of retention should have been used instead of zero. Arbitrarily selected "small values" may, however, distort significantly the result of analysis just because of the logarithmic transformation.

The relatively high affinity of P towards Z-CN can be attributed to the strong dipole interaction induced by the cyano groups. But this interpretation can not be true for the butyl phases. In these instances another kind of interaction (e.g. acid-base) induced by the uncovered part of silica support must be presumed. The position of stationary phases on the biplot clearly shows this difference. The butyl columns are located relatively far from the polar-neutral pole (ME) but they are close to the polar-ionic pole (P). Z-CN is rather closer to ME but the closeness to P seems to be also significant. It is very likely that on this column both interactions contribute to the whole retention process.

The retention behaviour of benzoic acid on these columns can be explained similarly. However, the position of A-C18 on the biplots does not indicate any kind of polar or ionic activity, therefore, it can not explain the retention behaviour of this compound. In this instance it can be attributed, rather, to the large hydrophobicity of this stationary phase. As seen, quite different column characteristics can result in the same retention phenomena which can not be revealed by single parametric evaluation, i.e. by examining the absolute or relative retention values obtained. For the decomposition and identification of the underlying effects multivariate techniques are needed, e.g. PCA or related techniques such as SMA.

Besides the diversity indicated above a common feature of these four columns can be found, namely, they all have negative scores on the second PC, i.e. they all are located under the origo (marked with + on biplots). The retention of acidic compounds seems to be proportional rather to the distance from the EB-ME axis and not simply to the affinity towards some selected poles.

The bigger is the angular distance between an object and a variable, the more they "repel" each other. In this meaning Z-C8 and, to a lesser extent, S-C18 and S-C4 exhibit lack of affinity towards P. However, this repulsion is not associated with attraction to any of the basic compounds, thus this characteristic (P-affinity) can be considered "one dimensional". In this meaning, the EB-ME axis represents a "two dimensional" characteristic. It seems as if the deficiency of one of the features (e.g. hydrophobicity) was associated with the excess of the other (polarity).

However, this interpretation seems to be somehow oversimplified. The position of Z-C3, Z-CN and Z-TFA indicates the low strength of these columns, but the effects from it's results is not revealed clearly by the biplots. The polarity of silica-based RP stationary phases can be the consequence of the ligand polarity and/or the accessibility of the polar support. The latter effect can be pronounced even for apolar ligands, especially for shorter ones, or, irrespective of size, at lower ligand density. In addition, depending on the manufacturing and ligand bonding chemistry the silica surface can induce polar-neutral and/or polar ionic interactions.[3] Under practical conditions the result of these factors is the same, namely, an overall decrease of hydrophobicity, but from a viewpoint of characterisation these effects should be exactly identified.

A further problem of the characterisation scheme given is that the composition of the mobile phase affects the results. The spread of points on the biplots decreases as the organic content increases which can be attributed to the overall decrease of retention. The biplots are equally scaled for better comparison. It means, at higher organic content only larger differences can be revealed, but this problem can be overcome by the proper selection of the mobile phase strength. The bigger problem is that the relative position of the stationary phases also changes with the acetonitrile concentration. At 30% ACN S-C4L exhibits higher affinity towards P than does A-C4 which indicates larger polarity for S-C4L. But this order is highly dependent on the mobile phase conditions, and, as it is seen, it is reverted at 40% (cf. Figure 1a and Figure 1c). It is the consequence of the non-uniform behaviour of this compound on the different columns.

The retention of P decreases much steeper on S-C4L than on A-C4 as the organic content of eluent increases. The relative characteristics of the weakest columns are also different from plot to plot. The position of these columns changes not only on the P-ME axis but also on the T-ME axis as the concentration of acetonitrile varies.

The eluent composition must be varied even within one test if the strength of stationary phases to be compared differ too much or if the different kind of characteristics to be evaluated necessitate it. In these instances the strength of mobile phase applied depends on the average retention of test compounds[9] or it follows different recommendations given in the literature.[17] However, the above results indicate that the selection of mobile phase conditions for characterisation of stationary phases of widely different type is not a trivial task. Most of the test methods published in the literature are designed and optimised for stronger, alkyl type stationary phases (C18 and C8). These schemes can not be applied on columns of different type without some modifications, but, as was shown, different selection of mobile phase conditions could result in different classifications.

Here we postulate that the uncertainty of the above characterisation is resulted from the confounding of strength and selectivity of the stationary phases. A practically relevant characterisation should concentrate also on the selectivity of stationary phases and not only on their strength, as do most of the test methods. A C18 phase is trivially stronger than a C8 or C4, no evaluation is really needed. An overall decrease of retention could be expected on the latter ones. Theoretically, the differences in strength of stationary phases can always be compensated by adjusting the strength of the eluent, i.e. by varying its organic content. However, selectivity differences can only be counteracted by changing the type of the eluent, i.e. replacing the organic constituent with another or adding further modifier(s), e.g. using ternary eluents. The quite frustrating result of these modifications is that the complete method development process (optimisation and validation) must be repeated. Consequently, it is very useful and advisable to reveal these characteristics in advance.

So, as to test selectivity differences, the eluent conditions must be selected according to the strength of stationary phases, i.e. these kind of differences must be balanced by the mobile phase. Under such conditions the variation of retention behaviour of test compounds reflect only the selectivity differences amongst the columns.

For a precise selection of the eluent conditions it is better to test the behaviour of only one appropriate test component than using the average retention of a complete test mixture. Accordingly, the characterisation of stationary phases is to be performed in mobile phases providing definite retention for this compound. For reference value we selected unit retention (k = 1). Note, it means the use of x^* for defining test conditions (cf. definition of x* given above.). Under this conditions the strength of the mobile and stationary phase is equal with respect to the indicator, and the differences of column strength are also compensated for this compound.

By comparing our present and earlier results[12,14,16] toluene (T) seems to be a good choice. The retention behaviour of this compound is governed mainly by hydrophobic interaction with the stationary phase, the effect of polar or ionic interaction can be considered negligible. Identical retention conditions can be maintained for T even on most stationary phases designed for hydrophobic interaction chromatography[14] which means widespread applicability indeed.

The biplot obtained under mobile phase conditions equalised for unit retention of T is shown on Figure 2a. The most dissimilar compounds are DA, ME and P, which prompts that they should be selected as poles. Since the first two PCs account for only 94% of the variance in the data table, the third PC must be also taken into consideration. Figure 2b shows the result of projection onto the plane defined by PC1 and PC3.

As it is seen, the position of test components and also that of the stationary phases changed significantly compared to Figure 1a-c. Since the characteristics indicated by the poles selected are practically the same as were given for Figure 1a-c, the attraction and repulsion of objects (stationary phases) can be interpreted similarly, but the features represented by the poles relate here to the selectivity of columns, i.e. to the differences which can not be compensated by adjusting the strength of mobile phase.

Taking into account the similarity of DA and T indicated by the biplots above, DA can be considered an indicator of hydrophobicity. Accordingly, the DA-ME axis is a kind of polar-apolar axis. The order of columns obtained by orthogonal projection on it corresponds well to that of the ligands, however, it is not exactly the same as on Figure 1a-c. It indicates that higher value of some characteristics (e.g. hydrophobicity) does not imply inevitably higher selectivity. For example, the hydrophobic selectivity of butyl phases is higher than that of Z-C8 as indicated by the attraction towards the presumed hydrophobic pole (DA), while the hydrophobic strength of these phases is nearly equal (see Figure 1a-c).

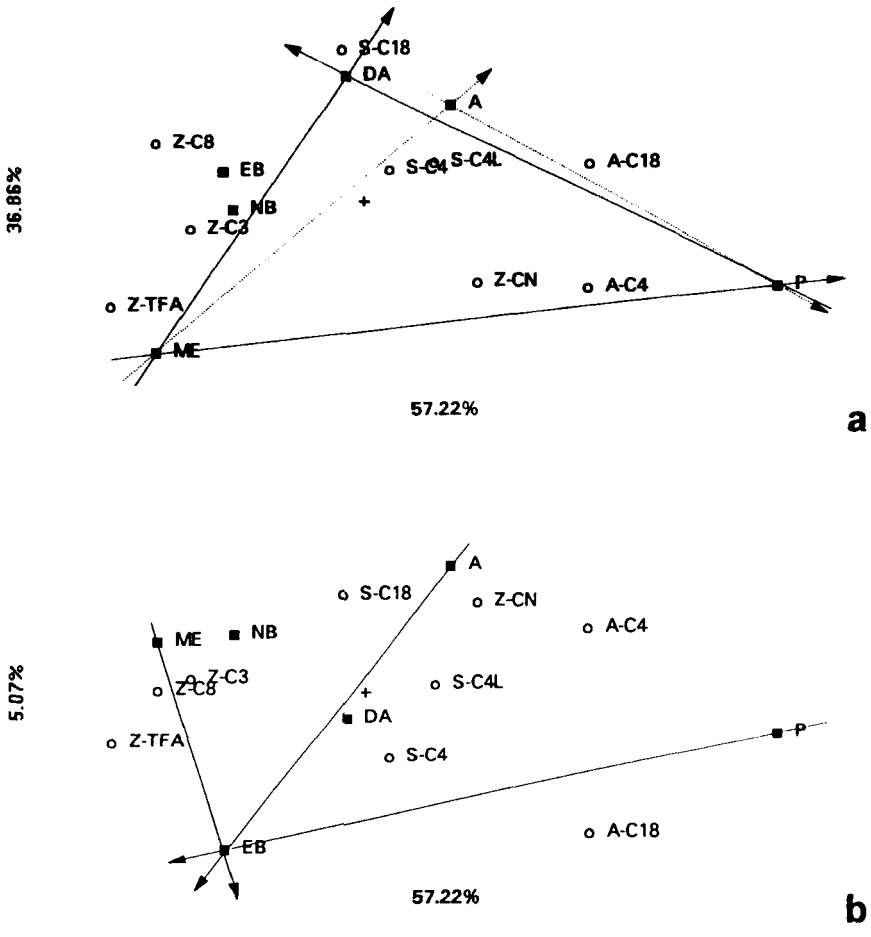

Figure 2. SMA biplots obtained under mobile phase compositions where k retention factor of toluene equals 1.(a) PC1 vs. PC2; (b) PC1 vs. PC3. (For details and explanation see text.)

The examination of the spread of stationary phases immediately reveals, that it is governed rather by their origin than by the ligand type. Columns from different sources are located at different regions on the biplot. The Zorbax columns are between the most hydrophobic (EB) and the polar-neutral (ME) test compounds, the Synchropak columns show the highest affinity towards the basic ones (A, DA) and the Aquopore columns are the nearest to the acidic pole (P). The only exception is Z-CN, which is shifted towards P, but this attraction can be attributed to the specific nature of ligand, as was indicated above.

This arrangement has nothing in common with the ligand type, rather it can be attributed to the different manufacturing and/or ligand binding chemistry of the columns.

The dissimilarity of nominally identical stationary phases from different sources is frequently experienced, and it is explained mainly by the unlike production process. Since this process is generally quite complex, many factors affect the final quality of stationary phases simultaneously. Consequently, similar selectivity differences can be expected for identical types of columns even from the same source, as it is shown by S-C4 and S-C4L. As seen, these differences can be revealed and identified under properly selected mobile phase conditions.

Note, these selectivity differences are less pronounced or less characteristic when conditions are not balanced for strength of the stationary phases. For example, Figure 1 indicates that the increased retention of acidic compounds on A-C18 results only from the large hydrophobicity of this column, but Figure 2 shows that some kind of polar interaction(s) also contribute. It is also seen that the differentiation between weaker columns is more relevant if the characterisation scheme is not dominated by the strength of columns.

Since the first two PCs explain only 94% of total variance, the spread of points along the third PC, i.e. the distance of objects and variables from the plane defined by the PC1 and PC2, must be also taken into consideration,. As it is seen on Figure 2b, EB and A are farest from this plane, the former is below and the latter is above. It prompts that these compounds should be also selected for poles. The closeness of the points in Figure 2a indicates that DA could be replaced with A (dashed axes on Figure 2a), but EB is a new pole. Correspondingly, three new axes must be formed, as shown on Figure 2b. It means, the complete representation of objects can be given in a tetrahedron instead of a triangle.

In most publications data are explained according to only the first two PCs. Further PCs are usually not used for interpretation or are completely neglected even if they have significant contributions. Here the third PC must be significant, since without it, i.e. only on Figure 2a, the position of EB can not be interpreted. The rather small contribution of this PC indicates that the feature represented by EB (hydrophobic selectivity) is less important with respect to present characterisation, the selectivity differences of stationary phases can be attributed mainly to polar interactions.

The inclusion of EB as indicator of hydrophobic selectivity involves the correction of interpretation given for DA, since this compound was used for the same purpose above. The intermediate position of DA between EB and A indicates that its retention is affected not only by hydrophobic interaction but by polar interaction too, originating from its basic character (see Figure 2b). Under non-balanced conditions this polarity is of secondary importance, but when the condition is equalised for different column strength, this feature becomes dominant. It means A and/or DA indicates the affinity of stationary phases towards basic compounds.

It is also seen that better selection of the basic indicator(s) is needed. The biplots show that the retention of A and DA does not result from a single effect, but their basic nature is suppressed, or at least confounded, by other stronger interaction(s). Since basic substances have primary importance in the pharmaceutical industry, the affinity or neutrality of stationary phases towards basic compounds should be clearly identified. This kind of affinity is generally attributed to the silanol activity of columns and it is often regarded as "polarity". However, the above results indicate that this classification is oversimplified, more exact distinction of possible interactions is needed.

Summarising the results obtained from the biplots, it seems, at least four different types of test compounds - hydrophobic, polar-neutral, acidic and basic- should be used for complete evaluation of the stationary phases. If the number of test compounds can be significantly reduced, more simple characterisation techniques providing the same information (e.g. ternary diagrams) can be used instead of SMA involving rather complex mathematical operations.[1,9] Although the calculations of these schemes are much simpler, they have the same shortage. Adding or removing stationary phases and/or test compounds is not a trivial task when multivariate techniques are used for characterisation. Modifications of this kind can significantly alter the results, consequently, after each changes the complete data set must be recalculated and re-evaluated . It is also true for testing column ageing in regular use.

After selecting the appropriate test components from the biplots obtained by SMA, the stationary phases are characterised according to their relative positions along the bipolar axes. However, these values can be derived directly from the retention data by calculating the corresponding relative retention factors, which provides a much simpler characterisation scheme. The strength (hydrophobicity) of columns can be tested by the relative hydrophobicity of T (x^*_T), i.e. by the mobile phase composition in which T is eluted with unit retention. This composition is calculated from the retention profile of T.[12,14,16]

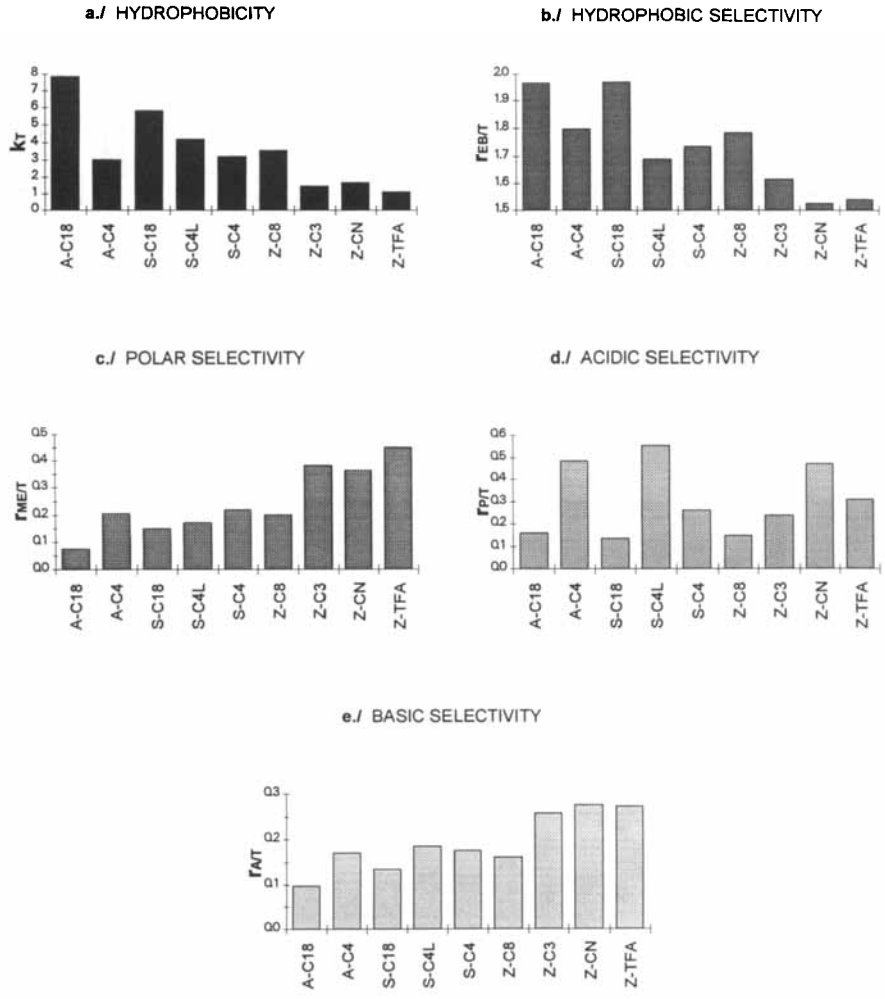

Figure 3. Single characteristics calculated from retention data obtained in 30% acetonitrile. (a) Retention factors of toluene; (b) Relative retentions of EB/T; (c) Relative retentions of ME/T; (d) Relative retentions of P/T; (e) Relative retentions of A/T.

Selectivity differences can be evaluated on the basis of retention of other test compounds. Confounding can be avoided, or at least minimised, if the measurements are carried out in mobile phases corresponding to x^*_T instead of using identical eluent on all columns.

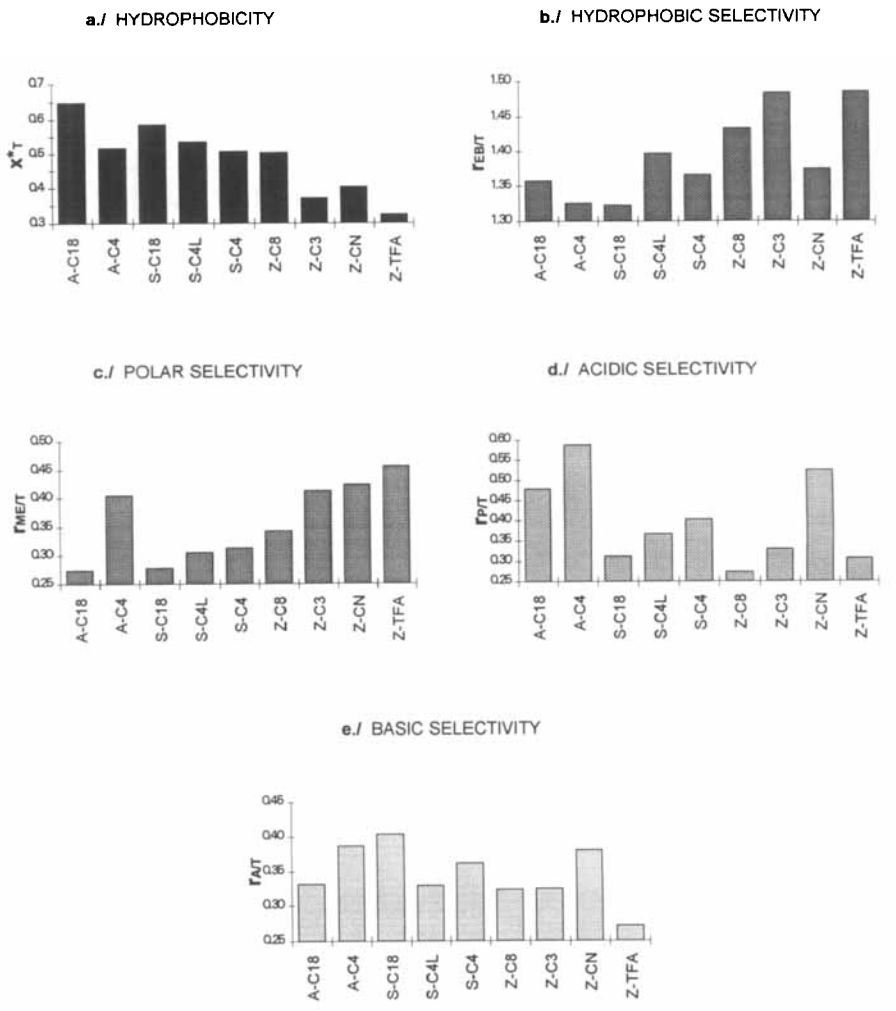

Figure 4. Single characteristics calculated from mobile phase compositions where k retention factor of toluene equals 1. (a) Retention factors of toluene; (b) Relative retentions of EB/T; (c) Relative retentions of ME/T; (d) Relative retentions of P/T; (e) Relative retentions of A/T.

The two approaches should give the same results for identical or very similar RP phases. In these instances, the retention behaviour of T does not change significantly from column to column; thus, hydrophobicity can be related directly to its retention and selectivity differences can be evaluated by

the retentions of other types of test compounds relative to T. In our case, the two methods result in dissimilar characterisations. Figures 3a-e show the characteristics calculated from retention values measured in 30% ACN. These characteristics were computed for all compounds found to be relevant with respect to characterisation.

This means some of the bipolar axes were neglected (e.g. P-ME), but some new ones, considered meaningful, were constructed. The retention of P relative to T ($r_{P/T}$) indicates the affinity of stationary phases towards polar-acidic compounds, $r_{A/T}$ relates to affinity towards polar-basic compounds, $r_{EB/T}$ measures the hydrophobic selectivity.

The same characteristics obtained under mobile phase conditions equalised for column strength are shown on Figure 4a-e. Here the hydrophobicity of stationary phases are described by the relative hydrophobicity of T (x^*_T). Under such conditions all the relative retention values used for characterisation equal to the absolute retention factors of test compounds ($k_T = 1$), however, the same notation was used on the figures for better comparison.

Note, Figure 3a-e and Figure 4a-e do not contain more information than Figure 1a and Figure 2a-b, they are only one-dimensional representations of the corresponding bipolar axes. However, the construction and interpretation of these scales are more straightforward than that of SMA biplots. In addition, this procedure is very convenient to use. Inserting a new column, or deleting an old one, does not affect the characterisation of others, similarities and differences remain relevant and characteristic. It can be easily improved further by applying new test compounds. In addition, the method can be used even for a single column, e.g., when the stability of a column is tested during regular use.

The results should be evaluated according to the task to be solved. During method development the separation may be further improved by changing to a stationary phase having different strength and/or selectivity. However, when a column in regular use must be exchanged, the most similar stationary phase is needed. The characteristics to be taken into account depend always on the features of the sample components. For example, the acidic or basic selectivity has no relevance if the mixture to be separated contains no compound of this type. On the other hand, for complex mixtures all characteristics should be taken into consideration. In this instances the use of biplots instead of single scales are more advantageous for selecting similar or different columns, since on biplots all the attractions and rejections towards different kind of test compounds are shown on the same plot.

ACKNOWLEDGEMENTS

We gratefully acknowledge the financial support given by the Hungarian Academy of Sciences under grants OTKA No. F7634/1993 and OTKA No. 014977/1995.

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Received May 23, 1996
Accepted September 10, 1996
Manuscript 4186