

Characterization of reversed phases by chemometric methods

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ABSTRACT

The characterization of stationary phases by empirically selected test solutes is compared with chemometric methods. Cluster, principal component, correspondence factor and discriminant analysis were applied to a data set consisting of capacity factors, selectivities and asymmetry factors. Commercially available RP-8 and RP-18 columns from German distributors with various specifications, and laboratory-prepared polymer-coated stationary phases were chosen. The different chemometric methods showed a uniform picture of the multi-dimensional data set. However, factor and correspondence factor analysis provide the most useful information. Factor analysis confirmed the pragmatic finding that the elution behaviour of aniline and phenol is well suited for the characterization of polar and toluene and ethylbenzene for that of hydrophobic phase properties. Correspondence factor analysis indicated similarity within some groups of the investigated stationary phases and emphasized the importance of proper selection of the test solutes. The deviation of some columns from the typical trend observed for RP-8, RP-18 or polymeric columns was detected by either of the chemometric methods and can be explained by their extreme physico-chemical properties.

INTRODUCTION

Reversed-phase (RP) chromatography is the main separation system applied in high-performance liquid chromatography (HPLC). The analyst can select columns from a large number of available stationary phases. There have been long and sometimes controversial discussions on the description of stationary phase properties. Their retention characteristics depend not only on the type of alkyl groups bonded to the surface but also on the silica base material and on the chemistry of the bonding procedure. Although the physico-chemical properties of the base silica gel and the chain length and type of the bonded alkylsilane are available from the manufacturers, it is difficult to correlate these data completely with the chromatographic behaviour.

Several test procedures [1–8] are used to characterize the chromatographic properties of packed columns and to transfer a separation system from one column to another. In a simple test [9] with nine benzene derivatives, valuable information on

hydrophobic, silanophilic and polar interactions of solutes and stationary phases was obtained. Standard chromatographic parameters such as capacity factors (k'), relative retentions (selectivities) and peak asymmetries are used to evaluate the chromatographic properties of packed columns. Major emphasis in this test procedure is placed on the selection of columns for the separation of basic, nitrogen-containing organic solutes. This pragmatic test procedure has been developed by chromatographic experts and is the result of knowledge and intuition. With this test, it is possible to select groups of columns showing similar properties for a given separation problem, especially for the separation of basic solutes.

Chemometric methods have been successfully applied in the characterization of gas-liquid chromatographic stationary phases [10] and also liquid chromatographic stationary phases [11]. In the latter study, non-polar solutes were used to characterize ten different stationary phases; basic solutes, however, were not included.

The objective of this paper is to evaluate pragmatically obtained data by chemometric methods [10-15], to confirm the suitability of the test procedure, to select groups of stationary phases showing similar properties and to eliminate redundant stationary phases. Moreover, chemometrics should reveal whether the test solutes describing the chromatographic properties have been properly selected and if it is possible to obtain the same information regarding stationary phase properties with fewer test solutes.

EXPERIMENTAL

Chromatographic data set

The test procedure and the chromatographic data described in detail recently [9] were used for chemometric evaluation. A methanol-water mixture (55:45, v/v) was used as the eluent, without addition of buffers or salts. All columns showed expected efficiencies ($H < 4 d_p$). The abbreviations of the test solutes and the pK_a values of the bases are as follows: EB = ethylbenzene; T = toluene; BE = ethyl benzoate; P = phenol; A = aniline, $pK_a = 4.58$; N = N,N-dimethylaniline, $pK_a = 5.06$; OT = *o*-toluidine, $pK_a = 4.39$; MT = *m*-toluidine, $pK_a = 4.69$; PT = *p*-toluidine, $pK_a = 5.12$.

The retention data of these solutes with 26 different stationary phases (seven RP-8, eleven RP-18 and eight polymer-coated) were used for chemometric evaluation. The stationary phases summarized in Table I were purchased from various German suppliers between 1983 and 1986. Seven of the eight polymer-coated stationary phases were prepared in our laboratory by using the same silica base material, different reaction conditions for the polymerization process being applied for each of the eight phases, resulting in various degrees of modification, e.g., carbon load. One polymer-encapsulated stationary phase (No. 25) was obtained from Bischoff. The column lengths (12.5 or 25 cm) varied depending on the particle diameter (5 or 10 μm) used. The retention data (k') of the test solutes with these columns are summarized in Table II.

Chemometric methods

Cluster analysis was performed with a variety of algorithms, including the commercially available program CLUE [16] (Elsevier, Amsterdam, The Netherlands)

TABLE I
CHROMATOGRAPHIC COLUMNS

No.	Column	Name
1	RP-8	Merck, LiChrosorb 60 RP-8, Select B
2	RP-18	Merck, LiChrosorb 100 RP-18
3	RP-8	Merck, LiChrospher 100 RP-8
4	RP-18	Merck, LiChrospher 100 RP-18
5	RP-8	Merck, LiChrospher 60 RP-8
6	RP-18	Waters Assoc., Radialpak Nova Pak C ₁₈
7	RP-18	Waters Assoc., Radialpak Resolve C ₁₈
8	RP-8	Merck, LiChrosorb 100 RP-8
9	RP-8	H-90-10, RP-8
10	RP-18	Shandon, Hypersil MOS
11	RP-18	Shandon, Hypersil ODS
12	RP-8	Phase Separation, Spherisorb C ₈
13	RP-18	Phase Separation, Spherisorb C ₁₈
14	RP-18	Whatman, Partisil, 5% C
15	RP-18	Whatman, Partisil, 10% C
16	RP-18	Whatman, Partisil, 15% C
17	RP-8	Macherey, Nagel & Co., RP-8
18	RP-18	Macherey, Nagel & Co., RP-18
19	PE	Laboratory prepared, 8.5% C
20	PE	Laboratory prepared, 11.1% C
21	PE	Laboratory prepared, 11.9% C
22	PE	Laboratory prepared, 12.7% C
23	PE	Laboratory prepared, 13.2% C
24	PE	Laboratory prepared, 14.2% C
25	PE	Bischoff, 14.7% C
26	PE	Laboratory prepared, 11.1% C

and with the method described in refs. 17 and 18. For discriminant analysis algorithms described in ref. 13 were used. In addition, correspondence factor analysis [14,19] and principal component analysis as described in ref. 20 were applied.

RESULTS AND DISCUSSION

Chromatographic evaluation

The hydrophobic properties of the stationary phases can be described by the retention of toluene (T) and ethylbenzene (EB). The retentions of both samples show a linear relationship with the carbon content of the stationary phase [9]. The relative retention increases up to a carbon content of *ca.* 10% and then remains nearly constant. Usually with RP-18, the relative retention (EB/T) is 1.8 or higher. Ethyl benzoate (BE) elutes before toluene with RP-18. However, it always elutes behind toluene with RP-8 and with polymer-coated phases, although in some instances coelution occurred.

To characterize the stationary phases with respect to their behaviour towards basic solutes, aromatic amines with different basicity were used. The isomeric toluidines (OT, MT, PT) are extremely sensitive indicators of silanophilic interactions. If their retention is based solely on hydrophobic interaction and no silanophilic

TABLE II
RETENTION DATA (k') FOR TEST SOLUTES

Eluent: methanol-water (55:45, v/v).

No.	EB	T	BE	P	A	N	OT	MT	PT
1	10.64	6.04	6.69	1.06	0.86	4.83	1.46	1.52	1.69
2	12.27	6.91	6.28	0.62	0.71	5.62	1.28	1.32	1.40
3	8.62	4.94	5.41	0.86	0.67	3.97	1.12	1.19	1.27
4	16.20	10.1	8.5	1.04	0.90	6.40	1.05	1.72	1.88
5	19.71	10.76	11.2	1.49	1.28	8.37	2.08	2.26	2.38
6	21.72	10.72	9.06	1.06	0.69	6.94	1.47	1.48	1.50
7	16.93	8.93	7.09	0.89	0.65	5.94	1.19	1.27	1.32
8	6.35	3.82	4.40	0.76	0.62	3.16	0.99	1.06	1.16
9	8.14	4.67	5.26	0.86	0.69	3.67	1.13	1.22	1.37
10	7.86	4.47	4.40	0.71	0.54	3.87	0.92	1.01	1.16
11	12.34	6.81	5.59	0.79	0.58	5.03	1.05	1.13	1.27
12	7.16	4.27	4.64	0.80	0.58	3.18	0.97	1.01	1.05
13	17.44	9.42	7.65	0.92	0.71	6.40	1.30	1.36	1.36
14	2.12	1.37	1.64	0.32	0.44	1.79	0.63	0.70	0.85
15	17.34	9.27	9.08	1.21	0.83	6.39	1.50	1.58	1.59
16	20.13	11.04	10.60	1.30	1.44	9.62	2.66	2.76	3.10
17	6.64	4.03	4.89	0.78	0.69	3.57	1.12	1.20	1.35
18	20.15	10.76	9.00	1.02	0.82	7.95	1.58	1.64	1.70
19	5.07	3.05	1.51	0.62	0.48	2.14	0.72	0.70	0.63
20	8.25	4.87	2.19	0.81	0.66	6.42	1.03	1.00	0.9
21	8.87	5.22	2.36	0.89	0.70	3.76	1.12	1.09	0.86
22	11.77	6.91	3.05	1.15	0.91	4.93	1.48	1.43	1.29
23	12.2	7.16	3.15	1.15	0.92	5.14	1.51	1.45	1.31
24	12.92	7.40	—	1.22	0.96	5.22	1.56	1.52	1.58
25	14.52	8.53	3.60	1.38	1.00	5.67	1.66	1.60	1.35
26	9.66	5.70	2.56	0.72	0.89	4.00	1.23	1.15	1.04

interaction contributes to their retention, then they should coelute ($\alpha < 1.05$). Aniline (A) and phenol (P) usually elute close together in this sequence with small k' values. Where asymmetry caused by instrumental deficiencies affects both peaks identically, silanophilic interaction causes an additional contribution to peak asymmetry for the aniline peak. Hence the ratio of peak asymmetries of the aniline peak and the phenol peak is a good indication of silanophilic interactions. Stationary phases with an asymmetry ratio < 1.3 are well suited for the separation of basic solutes. Other relationships, such as the relative retentions of T and PT or the elution sequence of BE and T, have additionally been derived empirically for stationary phase characterization [9]. These parameters are summarized in Table III for the stationary phases used in the following chemometric studies (the empirically derived criteria for pragmatic phase characterization are also given).

Chemometric methods

Univariate and bivariate methods. A first treatment of the data set by means of univariate and bivariate methods (mean values, standard deviations and correlation factors) shows that the k' values with the RP-8 columns were the most widely scattered.

TABLE III
EMPIRICAL CONDITIONS FOR PHASE CHARACTERIZATION

No.	Type	Selectivity					Asymmetry, A/P
		EB/T	T/PT	PT/OT	MT/OT	PT/MT	
		Expert requirements					
		> 1.8 for RP-18	> 4	< 1.05	< 1.05	< 1.05	< 1.3
1	RP-8	1.76	3.57	1.16	1.04	1.11	1.00
2	RP-18	1.78	5.00	1.09	1.03	1.06	1.10
3	RP-8	1.75	3.85	1.13	1.06	1.07	1.40
4	RP-18	1.60	5.26	1.79	1.64	1.09	1.60
5	RP-8	1.83	4.55	1.14	1.09	1.05	1.70
6	RP-18	2.03	7.14	1.02	1.01	1.01	1.20
7	RP-18	1.90	6.67	1.11	1.07	1.04	1.46
8	RP-8	1.66	3.33	1.17	1.07	1.09	1.20
9	RP-8	1.74	3.45	1.21	1.08	1.12	1.20
10	RP-18	1.76	3.85	1.26	1.10	1.15	2.30
11	RP-18	1.81	5.26	1.21	1.08	1.12	1.28
12	RP-8	1.68	4.00	1.08	1.04	1.04	1.00
13	RP-18	1.85	7.14	1.05	1.05	1.00	1.00
14	RP-18	1.55	1.61	1.35	1.11	1.21	1.10
15	RP-18	1.87	5.88	1.06	1.05	1.01	1.20
16	RP-18	1.82	3.57	1.17	1.04	1.12	1.00
17	RP-8	1.65	2.94	1.21	1.07	1.13	1.16
18	RP-18	1.87	6.25	1.08	1.04	1.04	1.25
19	PE	1.66	4.84	0.88	0.97	0.90	1.00
20	PE	1.69	5.41	0.87	0.97	0.90	1.05
21	PE	1.70	5.44	0.86	0.97	0.88	1.05
22	PE	1.70	5.34	0.87	0.97	0.50	1.05
23	PE	1.70	5.47	0.87	0.96	0.90	1.00
24	PE	1.75	4.68	1.01	0.97	1.04	1.05
25	PE	1.70	6.32	0.81	0.96	0.84	1.10
26	PE	1.69	5.48	0.85	0.93	0.90	1.11

as indicated by the standard deviations. Table IV summarizes the mean values and standard deviations for the different classes of stationary phases and solutes. The average relative retention ratio of the pair EB/T is 1.84 for RP-18 and 1.74 for RP-8 and 1.70 for polymer-coated phases (silica coated with butyl acrylate). The possible differentiation between RP-8 and RP-18 by the elution order of BE and T could be verified. The selectivity of BE/T is always greater than 1.0 for RP-8. Further, the elution sequence of the three isomeric toluidines strictly follows their pK_a values with classical RP-8 and RP-18. However, this is not the case with the investigated polymer-coated stationary phases. The F -value, the ratio of variances between the different classes of stationary phases and the pooled within-class variances has also been included in Table IV. From these F -values, it can be expected that the test solutes BE, T, EB and N should be the most suitable compounds for differentiating between the

In the first approach, the 26 stationary phases and the nine k' values of the test solutes were considered. In a further attempt, the pragmatic phase evaluation criteria (Table III) were used. Ward's strategy (described in refs. 17 and 18) and the average linkage method provide the most consistent clusters of stationary phases and show improved performance compared with the competitive algorithms, such as single and complete linkage, medium, centroid or a divisive method [16]. The clusters obtained by using the empirically derived criteria are shown in Fig. 1. Distinct clusters are formed for the polymer-coated phases (columns 19–26) with only one column (24) assigned to a different cluster. Most of the RP-18 (6, 7, 13, 15, 18) and RP-8 (3, 8, 9, 17) columns also give a consistent picture. It is worth noting that columns 2, 4, 5, 10, 14 and 16 are always badly classified. Some explanation for this can be derived from the retention behaviour of the test solutes with these columns by comparing Tables II and III. Columns 14 and 16 are only partially silanized RP-18 columns with a high concentration of unreacted silanols [9]. Column 14, which is different from all other RP-18 columns, contains only 5% of bonded carbon, shows the highest PT/MT selectivity and thus forms a defined single cluster. Additionally, a higher retention for ethyl benzoate than for toluene was obtained, suggesting that this phase is behaving more like an RP-8 column. According to our hypothesis, the polar part of the ethyl benzoate molecule has a higher accessibility to the polar surface and will be retained longer owing to polar interactions.

Columns 4 and 10 form a single cluster separate from all other columns. These two phases show the worst retention behaviour with the basic solutes (high asymmetry relationship of A and P, and highest selectivities for PT/OT and MT/OT).

Principal component analysis (PCA). The most subtle technique for the recognition of similar acting variables within a multi-dimensional data set is factor analysis (FA). It provides a deeper insight into the possible relationships of the variables and objects, and it is possible to extract meaningful and interpretable features

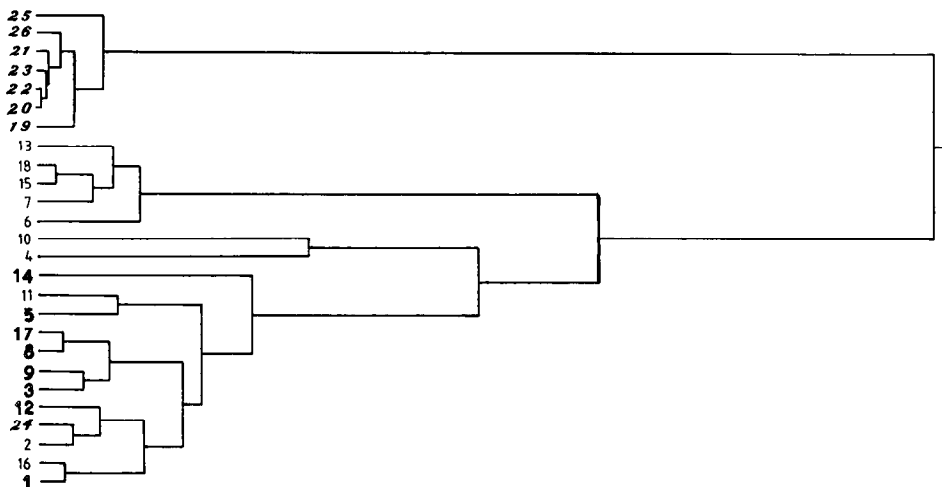


Fig. 1. Clustering of the 26 RP columns according to the empirical parameters given in Table III. Open-face numbers, RP-8; normal numbers, RP-18; italic numbers, polymer-coated phases.

from the hidden structure of the experimental data. Because reliable specific variances of the originally measured values are not available, preference is given to a principal component analysis based on the correlation matrix given in Table V. It is evident that 96% of variance in this data set could be explained with three extracted orthogonal principal factors. After a varimax rotation of the three factors the loadings (Table VI) were calculated. The communalities given in this table show that these three factors are able to describe more than 90% of all original features. The high first factor loadings for aniline and phenol indicate that the retention data can be explained primarily by polar interactions, which is also confirmed by the high loadings of OT and MT. The second factor is highly correlated with the hydrophobic test solutes EB, T and N. The third factor is less significant, indicated by its small eigenvalue. It includes the strongly basic PT and the neutral but polar BE. The exact meaning of the last factor is not yet clear, but should not be overestimated in view of its small eigenvalue. However, the results of factor analysis reflect that in RP chromatography, predominantly hydrophobic and polar forces effect the separation process.

Correspondence factor analysis (CFA) [14,19,21]. This technique is closely related to factor analysis and permits a simultaneous projection of the objects (stationary phases) and the variables (test solutes) in a single plot. The advantage of this method is that relevant variables are plotted close to the objects. In Fig. 2, the correspondence of stationary phases and solutes is demonstrated (column 24 was not considered in this calculation owing to the missing k' value for BE). It can be seen that the different classes of test solutes are widely separated from each other, reflecting the differences in their chemical nature. The hydrophobic test solutes (EB, T and N) are in the same window, as are the most hydrophobic RP-18 columns. The test solutes are arranged according to increasing hydrophobicity (N, T, EB). BE, which can be used to differentiate between RP-8 and RP-18, is placed at an extreme position, close to column 5, which also showed extreme behaviour in cluster analysis. The other stationary phases which were also badly classified in cluster analysis (RP-18 columns 4,

TABLE VI

RESULTS FROM THE FACTOR ANALYSIS OF THE CHROMATOGRAPHIC DATA SET

Values in italics are the most significant factors.

Variable	Factor loadings			Communality (%)
	Factor 1	Factor 2	Factor 3	
EB	0.35	-0.89	0.26	98.8
T	0.41	-0.87	0.26	98.4
BE	0.18	-0.70	0.66	95.5
P	0.82	-0.49	0.06	92.0
A	0.88	-0.24	0.38	97.2
N	0.46	-0.74	0.39	91.1
OT	0.79	-0.37	0.42	94.6
MT	0.70	-0.43	0.56	99.5
PT	0.55	-0.36	0.74	99.3
Eigenvalue	7.41	0.82	0.44	
Cumulated variance	82.3%	91.4%	96.3%	

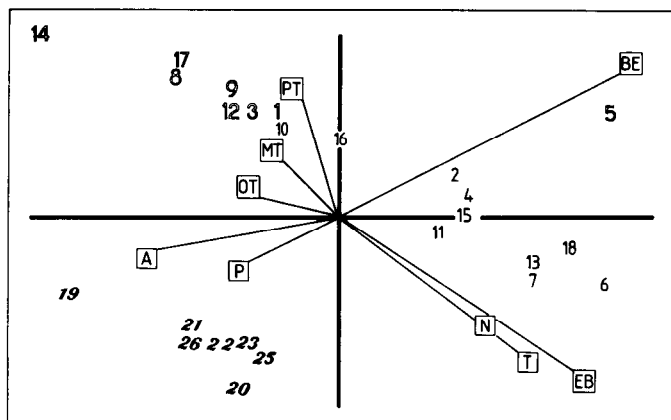


Fig. 2. Correspondence factor analysis plot for the 26 RP columns characterized by nine test solutes. Style for numbers as in Fig. 1.

10 and 16) are not projected in the window where the typical RP-18 phases are located. The polar solutes A and P with a relatively high dipole moment are in one segment together with the polymer-coated phases. As these stationary phases contain an ester group, it is appropriate to assume that this affinity is caused by polar interaction. The three isomeric toluidines are also in order of increasing basicity and are located in the same window as the RP-8 columns. The polymer-coated stationary phases form the narrowest group, as has been observed in cluster analysis. However, this is not surprising because these phases were prepared by the same polymerization process, although different temperatures, catalysts and reaction conditions were applied. This simultaneous projection of stationary phases and test solutes demonstrates the importance of chemical interactions in separation science.

Discriminant analysis (DA). If for practical reasons the objects (RP columns) can be classified in different classes (RP-8, RP-18 and polymeric), discriminant analysis is the method of choice for data projection and is able to demonstrate the statistical differences of the classes in contrast to their scattering. The advantage of this method is that one obtains the whole content of information within a set of a limited number of optimum variables. Hence it is no longer necessary to measure the retentions of all test solutes in order to classify an unknown stationary phase into one of the three classes and the experimental effort can be reduced.

By reducing the number of variables necessary to describe the properties of a stationary phase, the three test solutes BE, PT and A proved to be sufficient to classify the investigated columns ($P = 95\%$). It is interesting that this statistically proven method selects from the three main factors (Table VI) the most sensitive feature, *i.e.*, the most useful test solute. The compound with the highest F -value (BE) is included. However, the two other solutes which are significant for this characterization showed less significant F -values (Table IV).

For graphical presentation, a linear combination of the k' values of the three selected solutes is formed giving the discriminant functions

$$D_1 = 0.64k'_{BE} - 20.32k'_A + 8.16k'_{PT}$$

$$D_2 = 0.62k'_{BE} - 20.82k'_A + 8.53k'_{PT}$$

The two discriminant values are the two coordinates for each RP column in Fig. 3 (RP-8 = 1, RP-18 = 2, polymer = 3). The scattering of the different RP columns can be clearly seen. The group of polymer-coated phases is significantly separated from the two other types of stationary phase. On the other hand, the similarity of RP-8 and RP-18 columns can be seen by the partial overlap of the projections. An unambiguous classification here is not possible, but the dashed line separates the two regions of classes. Only one RP-18 column (phase No. 2) would be classified as RP-8.

The axes in Fig. 3 have been arbitrarily set. The two discriminant functions were used solely for illustration. Only the discriminant function D_1 is statistically significant. Consequently, any stationary phase can be projected onto one axis with the following class mean values and confidence intervals ($P = 95\%$); RP-8, 0.054 ± 2.173 ; RP-18, 2.211 ± 2.149 ; and polymeric, -5.743 ± 2.190 . For group discrimination, the borderlines are at 2.89 for polymer-coated phases and RP-8 and at 1.14 for discrimination between RP-8 and RP-18.

CONCLUSIONS

Chemometric evaluation of chromatographic data obtained with nine test solutes and 26 stationary phases purchased from various German distributors revealed

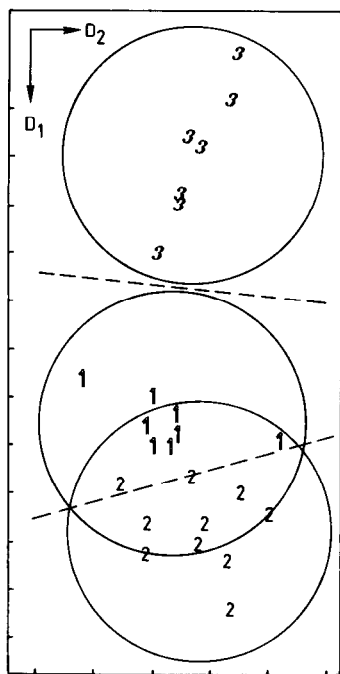


Fig. 3. Three classes of RP columns presented by two discriminant functions. Style for numbers as in Fig. 1.

that it is possible to identify groups of columns with similar behaviour. The study of the different chemometric methods (univariate and bivariate methods, cluster, principal component, correspondence factor and discriminant analysis) showed a uniform picture. The stationary phases were always grouped in the same classes of similar properties and the outliers were found to be the same. Additionally, reasonable explanations derived from physico-chemical properties of the stationary phases are provided to justify the existence of outliers within the group of the investigated stationary phases. Surprisingly, the strong base N,N-dimethylaniline included in the pragmatic test mixture to evaluate silanophilic properties shows similar characteristics to the hydrophobic solutes toluene and ethylbenzene.

Factor analysis showed that the test solutes aniline and phenol have the highest first factor loadings. According to the judgement of chromatographic experts, the elution behaviour of these two test solutes is most sensitively influenced by the presence or absence of surface silanol groups. Comparably high loadings for the second factors were achieved for the hydrophobic test solutes. Their retention depends strongly on the carbon load of the stationary phase. The plot obtained from correspondence factor analysis demonstrates impressively the affinity between the stationary phases and the test solutes.

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