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Chemometric categorization of octadecylsilyl bonded-phase silica columns using test mixtures and confirmation of results with pharmaceutical compound separations

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Abstract

The chromatographic properties of seventeen commercial octadecylsilyl phases were characterized in order to determine column similarities and differences that would aid in column selection for method development, choice of an alternate column and method ruggedness testing. Chromatographic test mixtures from the literature were used to probe the hydrophobicity, free silanol interactions, trace metal activity, and shape selectivity of these columns. Principal components and cluster analysis methods were used to categorize the columns into groups that tended to display similar chromatographic properties. The validity of the groups was tested with mixtures of pharmaceutical compounds under a variety of mobile phase conditions. In general, columns that were categorized together from the test mixture analysis showed similar behavior for the analysis of the pharmaceutical compound mixtures.

1. Introduction

Octadecylsilyl (ODS) bonded-phase silica is arguably the most widely used stationary phase for reversed-phase high-performance liquid chromatography [1]. This has led to a proliferation of ODS phases from many manufacturers. One survey listed 117 suppliers of reversed-phase columns, so even allowing for duplication, a large number of ODS phases is available [2]. It is also well-recognized that all ODS phases do not exhibit similar separation behavior. Factors such as particle size, surface area, pore size, trace metal activity, bonded-phase surface density, bonding chemistry, silica deactivation, and bonded-phase stability can all influence retention, selectivity, and peak shape properties of analytes. While some general rules can be applied, such as smaller particle sizes will provide higher efficiency, it is often difficult to predict the properties of a column based on stationary phase characteristics normally listed by manufacturers.

The large number of ODS phases available and the variability in their properties can make column choice for method development a difficult or somewhat arbitrary process. This choice is often made based on experience with given columns and compounds, advertising claims, or by trial and error. Choosing an ODS column could be more efficient if the choices were reduced by categorizing columns into groups that display similar behavior. If the initial column choice was not acceptable (and it is still deemed desirable to employ an ODS phase), a column

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from a different category would be more likely to show different behavior than one from the same category. Such a categorization would also be useful in selecting an alternative column that is likely to be similar to one specified in a literature or compendial method.

Investigation of method ruggedness toward changes in stationary phase characteristics can be valuable in method development to give an indication of whether the separation will be sensitive to these characteristics. Changes in chromatographic behavior from batch to batch or when a manufacturing change is implemented for a given stationary phase are often problematic for method performance over time. These difficulties may not be apparent in a manufacturer's quality control testing but can be manifested as selectivity, retention, or peak shape changes compared to previous columns in end-user applications. In testing method ruggedness with columns categorized into groups as mentioned above, similar results obtained with a column from a different group would suggest that the method will be quite rugged, while different behavior on a column from the same group would indicate the opposite. This approach may be more informative than performing the method with several columns of the same type to test ruggedness.

Classification of columns for improving the choice of alternate columns or evaluation of behavior for basic compounds have been goals addressed in the literature. Methods employing various test mixtures and data analysis techniques have been described. Several relatively simple test mixtures have been used to probe hydrophobic, silanophobic and polar interactions of solutes and stationary phases [3–11]. Other investigators have used more specialized compounds to characterize columns [12–15] or focused on specific interactions such as shape selectivity [16] or trace metal activity [17]. Data analysis techniques have included qualitative description of results [3,13,18], classification based on performance parameters or indices [4,5,7,9,10,14–17], and chemometric methods such as principal components and cluster analysis [6,8,12]. In earlier related work, Massart and co-workers [19–23] described cluster analysis/numerical taxonomy techniques for the determination of optimal sets of gas chromatographic phases or thin-layer chromatographic systems.

In this work, test mixtures based on those described by Schmitz et al. [6], Sander and Wise [16] and Verzele and Dewaele [17] were used to characterize seventeen commercially available ODS columns. Principal components and cluster analysis methods were used to categorize the columns according to the similarity of their chromatographic behavior with the test mixtures. Furthermore, investigation of several columns was conducted using "real" analysis conditions for pharmaceutical compounds to check the validity of the categories derived from the test mixtures. This also tested the usefulness of the category groupings for determination of method ruggedness as described above.

2. Experimental

2.1. Reagents

HPLC-grade acetonitrile and methanol were obtained from EM Science (Gibbstown, NJ, USA). Buffers were prepared using appropriate concentrations of potassium phosphate, mono- and dibasic (EM Science), sodium acetate (EM Science), and orthophosphoric acid (85%, Fisher Scientific, Fair Lawn, NJ, USA). Water for mobile phases and sample solutions was purified with a Milli-Q system from Millipore (Milford, MA, USA).

Aniline, phenol, *o*-toluidine, *m*-toluidine, *p*-toluidine, methylbenzoate, N,N-dimethylaniline, 2,4-pentanedione (acetylacetone), naphthalene, 1-nitronaphthalene, amitriptyline hydrochloride and 4-acetamidophenol were obtained from Aldrich (Milwaukee, WI, USA). Toluene was from Mallinckrodt (Paris, KY, USA). Standard Reference Material 869–Column Selectivity Test Mixture for LC (polycyclic aromatic hydrocarbons, PAHs) was from the National Institute of Standards and Technology (Gaithersburg, MD, USA) The mixture was diluted with acetonitrile to the following concentrations: benzo[*a*]pyrene

(BaP, 0.4 μg/ml), tetrabenzonaphthalene (TBN, 0.6 μg/ml) and phenanthro[3,4-*c*]phenanthrene (PhPh, 2.2 μg/ml). Nortriptyline hydrochloride, dirithromycin, cephalexin, loracarbef and cefaclor were from Lilly Research Labs.

2.2. Apparatus

The chromatographic system consisted of a Model 600 pump with column heater (Waters, Bedford, MA, USA), a Model 728 autoinjector (Alcott, Norcross, GA, USA) with a fixed-loop injection valve (Valco, Houston, TX, USA), and a Model 787 variable-wavelength UV detector (Applied Biosystems, Ramsey, NJ, USA). Chromatograms were recorded using an in-house data acquisition system. The columns investigated are listed in Table 1.

2.3. Software

The principal components and cluster analyses were performed using the statistical analysis software JMP (for the Macintosh), version 3.0.2 from the SAS Institute, Cary, NC, USA. Some cluster analysis was performed using the CLUSTER procedure in SAS software also from the SAS Institute.

3. Results and discussion

The test mixtures were chosen for their ability to show differences among columns based on various factors known to affect chromatographic performance. A mixture of basic and neutral compounds was used to probe hydrophobic and free silanol interactions [6]. Trace metal activity was tested using 2,4-pentanedione (acetylacetone), a strong metal chelator, with naphthalene and 1-nitronaphthalene to test silanol interactions [17]. Shape selectivity was determined using polynuclear aromatic hydrocarbons differing in degree of planarity [16]. Data obtained for these test mixtures on 17 different columns are given in Table 2. Data from three Zorbax Rx-C_{18} columns with duplicate measurements taken on different days from one of these columns were included. Observations from each test mixture are summarized below.

Table 1
Columns investigated using test mixtures

Column	Supplier
Kromasil C_{18}	Keystone Scientific, Bellefonte, PA, USA
Nucleosil C_{18}	Keystone Scientific
LiChrosorb RP18	Keystone Scientific
ODS Hypersil	Keystone Scientific
BDS Hypersil C_{18}	Keystone Scientific
Partisil 5 ODS-3	Whatman, Clifton, NJ, USA
Partisil 10 ODS-2 (10 μm)	Whatman
Zorbax Rx-C_{18}	MacMod Analytical, Chadds Ford, PA, USA
Zorbax ODS	MacMod Analytical
Spherisorb ODS	Regis, Morton Grove, IL, USA
Spherisorb ODS-II	Regis
Inertsil 5 ODS (2)	MetaChem, Redondo Beach, CA, USA
YMC-Pack ODS-A	YMC, Wilmington, NC, USA
Supelcosil LC18	Supelco, Bellefonte, PA, USA
Supelcosil LC18-DB	Supelco
Ultrasphere ODS	Beckman, San Ramon, CA, USA
μBondapak C_{18} (10 μm, 300 × 3.9 mm)	Waters, Milford, MA, USA

All columns were 250 × 4.6 mm with 5-μm particles unless otherwise indicated.

Table 2
Data from analysis of test mixtures

Column	Aniline		Phenol, k'	*o*-Toluidine, k'	*m*-Toluidine, k'	*p*-Toluidine, k'	N,N-Dimethylaniline		Methylbenzoate, k'
	k'	Peak width					k'	Peak width	
Kromasil C_{18}	0.85	26	0.91	1.25	1.34	1.59	4.60	34	2.68
Nucleosil C_{18}	0.82	16	0.72	1.16	1.30	1.80	3.70	48	2.25
Partisil 5 ODS-3	0.80	17	0.74	1.10	1.32	1.94	3.04	50	2.03
BDS Hypersil C_{18}	6.58	179	0.56	6.18	13.27*	22.37*	23.88*	687*	1.51
Partisil 10 ODS-2	1.76	40	0.90	2.36	3.07	5.22	7.30	131	3.04
Inertsil 5 ODS (2)	0.66	15	0.68	1.03	1.03	1.03	3.73	23	2.37
Zorbax Rx-C_{18}-1, day 1	0.60	14	0.59	0.88	0.96	1.24	3.48	22	1.98
Zorbax Rx-C_{18}-1, day 2	0.59	13	0.59	0.88	0.94	1.19	3.48	22	1.99
Zorbax Rx-C_{18}-2	0.49	13	0.51	0.76	0.76	0.90	3.05	21	1.74
Zorbax Rx-C_{18}-3	0.55	17	0.54	0.81	0.87	1.22	3.26	24	1.81
Spherisorb ODS-II	3.62	125	0.56	3.82	6.81	11.68*	13.61*	470*	1.55
YMC ODS	0.62	11	0.77	0.96	0.96	0.96	3.48	22	2.21
Supelcosil LC18-DB	9.98*	341*	0.61	9.57*	20.26*	34.24*	35.54*	1340*	1.76
Ultrasphere ODS	1.15	48	0.62	1.39	2.00	4.06	4.43	80	1.95
μBondapak C_{18}	0.56	13	0.56	0.77	0.84	1.09	2.17	24	1.51
Supelcosil LC18	10.38*	354	0.62	9.98*	21.07*	35.63*	36.93*	1390*	1.85
Zorbax ODS	1.64	89	0.79	1.94	2.80	5.79	8.32	366	2.87
Spherisorb ODS	2.99	141	0.34	3.14	5.72	9.94*	11.6*	534*	1.20
Hypersil ODS	4.06	206	0.57	4.14	8.64	13.5*	15.22*	796*	1.69
LiChrosorb RP18	0.61	13	0.63	0.96	0.96	1.10	3.36	46	2.00

Table 2 (*continued*)

Column	Toluene, k'	BaP, k'	PhPh, k'	TBN, k'	α TBN/BaP	Acetylacetone		Nitronaphthalene, k'	Naphthalene, k'	α naphthalene/nitronaphthalene
						k'	Peak width			
Kromasil C_{18}	6.54	8.47	7.88	13.44	1.59	0.53	20	3.89	7.00	1.80
Nucleosil C_{18}	4.24	4.60	4.73	8.21	1.78	0.51	48	3.37	4.44	1.32
Partisil 5 ODS-3	3.25	2.81	3.31	5.12	1.82	1.25	208	2.32	2.88	1.24
BDS Hypersil C_{18}	3.47	4.72	4.42	7.56	1.60	0.30	19	2.08	3.65	1.75
Partisil 10 ODS-2	5.61	8.27	6.93	11.86	1.43	5.22*	500*	4.39	6.00	1.37
Inertsil 5 ODS (2)	5.22	6.10	6.10	10.48	1.72	0.48	15	3.41	5.38	1.58
Zorbax Rx-C_{18}-1, day 1	4.85	6.40	5.47	9.47	1.48	0.39	15	2.80	5.20	1.86
Zorbax Rx-C_{18}-1, day 2	4.87	6.40	5.47	9.47	1.48	0.40	14	2.88	5.34	1.86
Zorbax Rx-C_{18}-2	4.44	6.40	5.47	9.47	1.48	0.36	21	2.46	4.74	1.93
Zorbax Rx-C_{18}-3	4.59	6.40	5.47	9.47	1.48	0.38	14	2.74	5.09	1.86
Spherisorb ODS-II	2.43	5.65	5.17	8.84	1.57	1.38	260	3.32	4.82	1.45
YMC ODS	4.90	5.50	6.10	10.58	1.92	0.44	13	3.15	5.05	1.61
Supelcosil LC18-DB	3.93	4.84	5.23	9.11	1.88	5.10*	500*	2.48	4.13	1.67
Ultrasphere ODS	4.74	5.44	5.72	10.05	1.85	0.43	97	2.99	5.33	1.78
μBondapak C_{18}	2.49	2.51	2.90	4.59	1.83	0.47	124	1.73	2.18	1.26
Supelcosil LC18	4.16	4.93	5.28	9.23	1.87	0.39	70	2.53	4.22	1.67
Zorbax ODS	6.89	7.65	7.60	13.43	1.76	5.79*	500*	4.11	7.05	1.72
Spherisorb ODS	1.95	2.37	1.84	3.05	1.29	0.59	118	1.58	1.99	1.26
Hypersil ODS	3.71	3.85	4.40	7.50	1.95	0.35	31	2.31	3.71	1.61
LiChrosorb RP18	3.83	4.94	4.94	8.65	1.75	0.67	202	2.99	4.06	1.36

Peak widths at 10% of peak height are given in s for selected compounds. Entries with asterisks were obtained by regression from other peak data or were assigned as indicated in text. Capacity factors, peak widths and separation factors were used as raw data for principal components and cluster analysis.

3.1. Base/neutral test mixture

This test mixture was perhaps the most revealing of those investigated. Chromatograms from four columns representing the range of behavior for basic compounds are shown in Fig. 1. Large differences in retention and peak shape were apparent. All columns gave sharp peaks for toluene and methylbenzoate, with the retention of these peaks giving an indication of column hydrophobicity. The YMC ODS column gave sharp peaks for all components and the toluidine isomers were not separated, indicating minimal residual silanol activity [5]. Although the Zorbax Rx-C_{18} column gave good peak shape for aniline and N,N-dimethylaniline (DMA), the toluidine isomers were partially separated. Complete separation of the toluidine isomers was obtained on some columns, such as the Nucleosil C_{18}. This column also exhibited tailing peaks for the basic compounds. Finally, for the Supelcosil, Hypersil and Spherisorb columns, the basic compounds were eluted very late as poorly shaped or almost indiscernable peaks or were not eluted at all during the run time of the experiments.

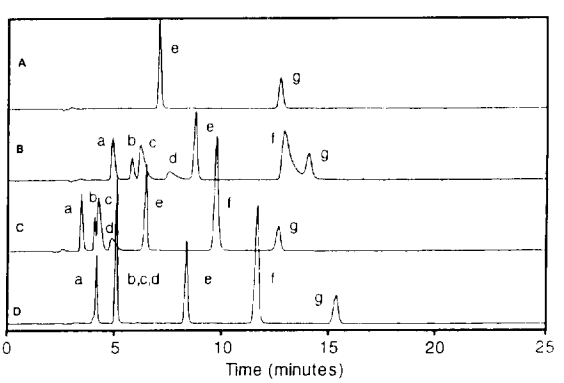

Fig. 1. Representative chromatograms for base/neutral test mixture. Columns: A = Supelcosil LC18; B = Nucleosil C_{18}; C = Zorbax Rx-C_{18}; D = YMC ODS. Peaks: a = aniline (10 μg/ml); b = *o*-toluidine (8.8 μg/ml); c = *m*-toluidine (16 μg/ml); d = *p*-toluidine (6 μg/ml); e = methylbenzoate (34 μg/ml); f = N,N-dimethylaniline (7.2 μg/ml); g = toluene (86 μg/ml). Phenol (18 μg/ml) was injected separately. Mobile phase: methanol–water (65:35). Flow-rate: 1.0 ml/min. Detection: absorbance at 254 nm. Injection volume: 20 μl.

The chemometric techniques used for analysis require that a specific data value be present for all potential clustering units. Otherwise, either the clustering unit —in this case, the column— or the measured variable must be eliminated from the analysis. In this study, some of the peaks had censored retention times; that is, the retention times were known to exceed a certain value, but the exact time could not be determined. To retain all clustering units and all variables in the analysis, the censored data were replaced by estimates obtained using regression analysis. Because strong correlations existed between variables with some censored data and variables with existing data, it was possible to obtain predictions for the censored observations. For example, DMA is highly correlated with aniline. The Spherisorb and Hypersil columns have observed data for aniline but censored data for DMA. A linear model based on complete data from other columns was used to predict the DMA value from the existing value for aniline. Other censored data were handled in a similar fashion.

The lack of a starting point (no observed retention time for aniline) for the Supelcosil columns was not a major issue. Because of the strong correlations between the Supelcosils for other variables, it is assumed the aniline retention times would be very close. Although the assignment of aniline retention times for the Supelcosils at greater than 25 min (the experimental run time) was somewhat arbitrary, this is not expected to influence the results of the subsequent analyses.

3.2. Chelator test mixture

The main difference among columns observed with this text mixture was the behavior of acetylacetone. As shown in Fig. 2, the elution behavior ranged from a sharp peak near the void volume of the column to a very broad or even completely retained peak. When an acetylacetone peak was not observed, the retention time was chosen to be the maximum run time of the experiment (15 min) and the peak width was assigned as about twice the maximum

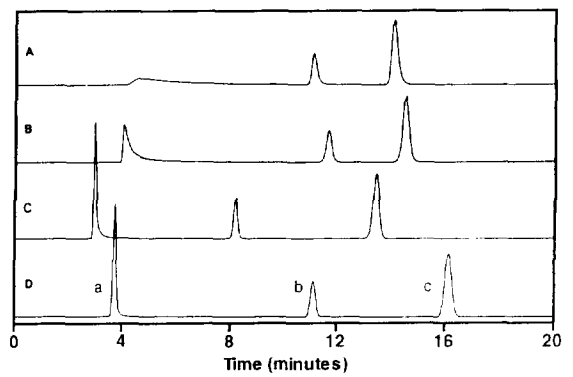

Fig. 2. Representative chromatograms for chelator test mixture. Columns: A = LiChrosorb RP18; B = Nucleosil C_{18}; C = Zorbax Rx-C_{18}; D = Inertsil C_{18}. Peaks: a = 2,4-pentanedione (0.2 μl/ml); b = 1-nitronaphthalene (54 μg/ml); c = naphthalene (300 μg/ml). Mobile phase: methanol–0.5% aqueous sodium acetate (70:30). Flow-rate: 1.0 ml/min. Detection: absorbance at 254 nm. Injection volume: 10 μl.

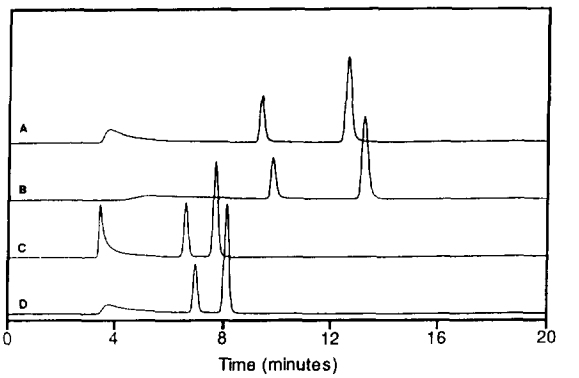

Fig. 3. Effect of column exposure to phosphate buffers (50 m*M*, pH 2.5 and 7.5) on response of chelator test mixture. A = Spherisorb ODS-II, used; B = Spherisorb ODS-II, new; C = μBondapak C_{18}, used; D = μBondapak C_{18}, new. Conditions as in Fig. 2.

observed value on other columns (500 s). The separation factor for naphthalene to 1-nitronaphthalene was included in Table 1 as an indication of silanophobic interactions [17].

Verzele and Dewaele [17] indicated that trace metal activity may change depending on mobile phase conditions to which the column has been exposed. In particular, mobile phases containing phosphoric acid, were reported to reduce trace metal activity. To check the effect of column history on acetylacetone elution, several columns were examined by running the chelator test mixture on a new column and on the same column after exposure to phosphate mobile phases. As shown in Fig. 3 for Spherisorb ODS-II and μBondapak C_{18} columns, some changes were evident after exposure to phosphate mobile phases (see Figs. 6–8 for conditions), but in agreement with the observation by Verzele and Dewaele [17], the trace metal activity was not completely suppressed. The peak width of the acetylacetone peak decreased somewhat, but the values obtained before exposure to phosphate were used in Table 1. Over an extended period of time this effect could contribute to changes observed in column behavior attributed to column "aging" [24,25].

3.3. Shape selectivity test mixture

This test mixture was chromatographed using conditions given by Sander and Wise [16]. The separation factor for TBN and BaP has been shown to indicate the polymeric or monomeric nature of C_{18} bonded phases, with factors less than or equal to 0.9 indicating a phase prepared with a polymeric surface modification process [16]. None of the columns used in this study were listed as polymeric and all gave shape selectivity factors greater than 1.0. The factors obtained were consistent with those reported previously and showed some columns to be monomeric in nature ($1.7 \leq \alpha \leq 2.2$) and the rest to have intermediate shape selectivity properties ($1.0 < \alpha < 1.7$) [16]. Data obtained for one of the Zorbax Rx-C_{18} columns were used for the others since this test mixture was run on only one column.

3.4. Column categorization

Principal components analysis (PCA) was performed on the correlation matrix for all variables of the combined test mixture data set to reduce the dimensionality and better understand the variability structure of the data [26]. The correlation matrix was used to counter the influence the

variables with larger variances would have on a PCA of the covariance matrix. The first four PCs from this analysis had eigenvalues greater than 1 and account for greater than 95% of the variability (Table 3). In addition to the raw data, the standardized PC scores for the first four PCs were used for categorizing the columns with cluster analysis.

PCA of the retention and peak width data showed that results for the basic compounds were highly correlated. As expected, toluene and methylbenzoate results also had a high correlation. For additional analysis, the raw data set was reduced to include only aniline and toluene k' values along with aniline peak width. Ratios of aniline to phenol and *p*-toluidine to *o*-toluidine k' values were also included as indicators of column performance, particularly regarding activity of residual silanols [5]. The TBN/BaP ratio from the shape selectivity test mixture and the acetylacetone peak width and naphthalene/nitronaphthalene ratio from the chelator test mixture were also included in this reduced data set.

Cluster analysis is another useful technique to give insight into patterns or structure that may be present in a data set. Results obtained from cluster analysis are dependent on which of several procedures is used. As recommended by Massart et al. [27], multiple clustering approaches were applied. Two similarity measures were examined; Euclidean distance and the Pearson product–moment correlation coefficient [28]. Columns that are proportionally or additively different in their retention properties, and that could be equalized by an adjustment of mobile phase strength, would be found to be different when using Euclidean distance as a measure of similarity. The correlation coefficient measure ignores proportional or additive differences which may not be critical in determining column similarity. Both measures were used to

Table 3
First four principal components of test mixture data including eigenvalues, eigenvectors, percent of variability accounted for by each PC and the cumulative percent variability accounted for by the PCs

	PC1	PC2	PC3	PC4
Eigenvalue	8.6478	6.1480	2.0829	1.2050
Percent	45.5150	32.3577	10.9626	6.3419
Cumulative percent	45.5150	77.8727	88.8352	95.1771
Eigenvectors				
Aniline, k'	−0.25816	0.25745	0.05321	0.01135
Aniline, peak width	−0.26205	0.24913	0.04267	0.00992
Phenol, k'	0.22365	0.18507	−0.23233	0.32766
o-Toluidine, k'	−0.25335	0.26442	0.03967	0.00571
m-Toluidine, k'	−0.26016	0.25448	0.06015	0.02210
p-Toluidine, k'	−0.25852	0.25796	0.05200	0.00441
DMA, k'	−0.25144	0.26666	0.06072	-0.00277
DMA, peak width	−0.26258	0.24886	0.02607	0.00497
Mebenzoate, k'	0.26909	0.18706	−0.19633	0.11437
Toluene, k'	0.26428	0.21157	0.11339	0.09650
BaP, k'	0.25685	0.21712	0.18031	−0.20624
PhPh, k'	0.24603	0.26175	0.13266	0.05642
TBN, k'	0.24038	0.26606	0.14529	0.04688
α TBN/BaP	−0.06875	0.09316	−0.15254	0.78525
Acetylacetone, peak width	0.00138	0.21824	−0.50038	−0.31263
α naphthalene/nitronaphthalene	0.08608	0.13937	0.57102	−0.08893
Acetylacetone, k'	0.04693	0.26484	−0.40809	−0.31274
Nitronaphthalene, k'	0.26774	0.20266	−0.12720	−0.00130
Naphthalene, k'	0.26688	0.22363	1.15506	−0.03809

cluster the standardized original data and scores from the first four PCs. Using the PCs for cluster analysis serves as a check on the categorization procedure using the raw data and can also determine whether PCA is an appropriate method for reducing the dimensionality of the data. Checking results using the reduced data set can add additional confidence in the final groupings.

Two commonly used clustering algorithms, average linkage and Ward's minimum variance method, were used to categorize the columns. Average linkage defines the similarity between clusters to be the average of the similarities among the objects in one cluster and the objects in the other. Ward's method combines, at each step, the two clusters that result in the smallest increase in the sums of squares within clusters. Ward's method in itself is sensitive to additive and proportional differences so the use of the correlation coefficient as the measure of similarity was not necessary.

Results from the various clustering methods showed some differences but general trends in grouping were apparent. Four examples are shown in Fig. 4. As expected, the Zorbax Rx-C_{18} columns always clustered together. Pairs of columns based on the same silica (Hypersil, Supelcosil, Spherisorb), treated and untreated for basic compounds, usually grouped together. Also, those that gave broad peaks (Hypersil, Supelcosil, Spherisorb) and those giving narrow peaks (YMC, Inertsil, Kromasil, Zorbax Rx) for basic compounds tended to cluster together. Intermediate columns (LiChrosorb, Nucleosil, μBondapak, Partisil 5 ODS-3) tended to move around, sometimes clustering with each other

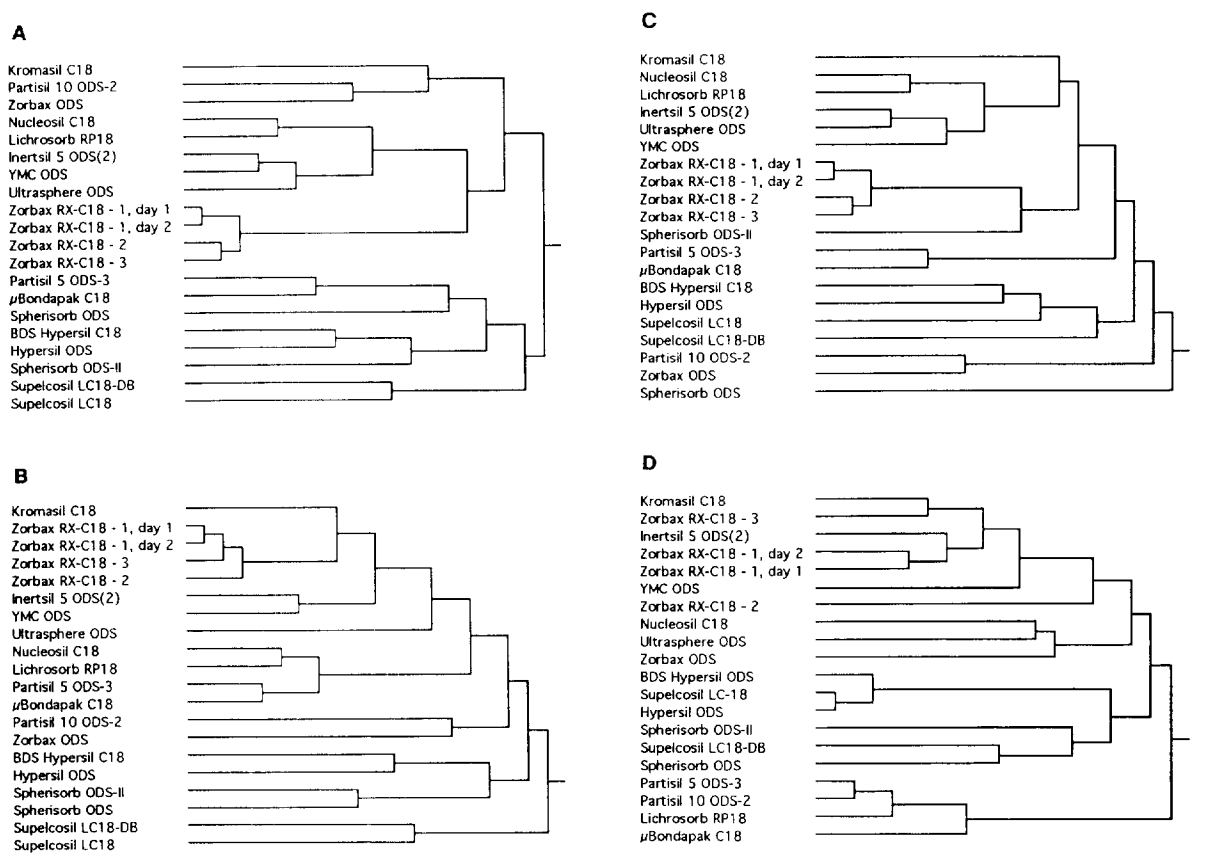

Fig. 4. Column clustering using different data sets and methods. (A) Raw data, Ward's method; (B) reduced data, average linkage; (C) first four principal components, average linkage; (D) raw data correlation matrix, average linkage.

and sometimes with other groups. In a few cases, unexpected or perhaps anomalous clustering results were obtained. For example, in Fig. 4A, Kromasil is grouped with Zorbax ODS and Partisil 10 ODS-2. This was not observed using other combinations of data set and clustering algorithm and underscores the need to use multiple methods for this type of analysis.

Relatively good consistency in clustering results was observed using the raw data, the PCs, and the reduced data. This provides an indication that the PCs and reduced data set each represent the raw data fairly well.

An important conclusion from this analysis is that a very rigid categorization of columns is not warranted. Some of the clusters were quite "loose" with relatively large distances between members of the group. However, it is possible to put the columns in small groups with quite similar behavior and in somewhat broader categories based on trends observed in the cluster analysis. Such a grouping is shown in Fig. 5. Columns within the same group would be expected to have a greater probability of giving similar results compared to columns in other groups. The consistency noted above among the data sets and clustering methods that were used adds confidence to these groupings. These categories are also generally consistent with visual comparison of chromatograms.

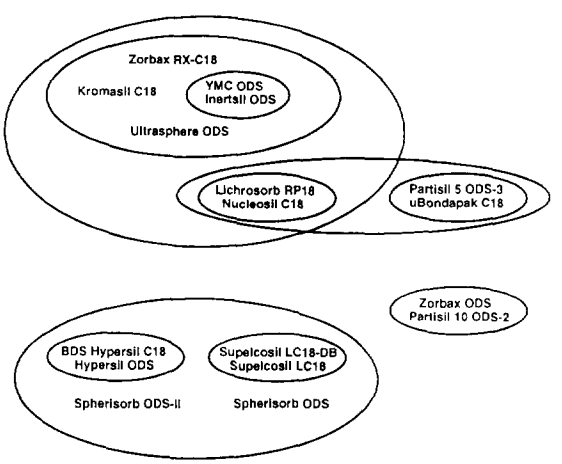

Fig. 5. Qualitative column groupings based on cluster analysis results.

3.5. Validation of column categories with pharmaceutical test mixtures

Test mixtures and mobile phase conditions used for column evaluation and categorization were chosen to accentuate differences between stationary phases. This is in contrast to methods for real applications in which mobile phases are usually developed to mask column differences. This is often accomplished by manipulation of pH, ionic strength, and the addition of silanol-masking reagents such as triethylamine. Additional confidence in the column categorization developed above can be obtained by comparison of results from different columns for analysis of mixtures representing real applications and much different mobile phase conditions than used for the original test mixture evaluation.

Pharmaceutical separations provide a good way of checking the column categorization. Three different applications were run on columns from similar and different clusters. If the clusters are valid, it would be expected that the separation behavior of columns within the same cluster will be very similar. Different behavior observed for columns from different clusters would also support the validity of the groupings. Similar behavior for different clusters could indicate that the clusters are not valid, but more likely would suggest that the method is not sensitive to column characteristics, i.e., the method is rugged.

Tricyclic antidepressants

The chromatographic behavior of the tricyclic antidepressants, amitriptyline and nortriptyline, using the *US Pharmacopeia* mobile phase conditions for amitriptyline hydrochloride injection [29] was examined. These compounds are amines and can be expected to give tailing peaks with some columns and mobile phases. Chromatograms obtained from several columns are shown in Fig. 6. The retention and peak shapes for amitriptyline and nortriptyline were similar for columns that would be expected to exhibit similar behavior based on the test mixture analysis. The YMC, Inertsil and Kromasil columns were grouped with each other and gave relatively

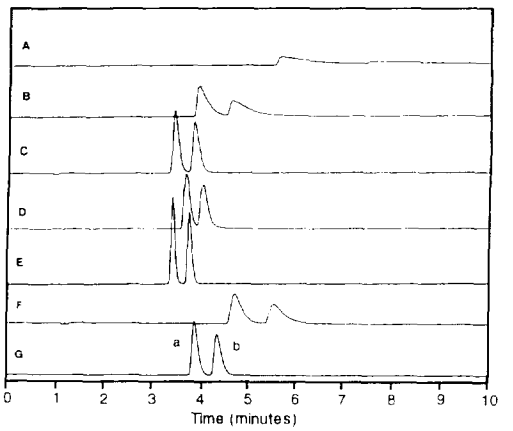

Fig. 6. Tricyclic antidepressant separation. Columns: A = Supelcosil LC18-DB; B = BDS Hypersil ODS; C = Kromasil C_{18}; D = Inertsil ODS; E = YMC ODS; F = Partisil 5 ODS (3); G = μBondapak C_{18}. Peaks: a = nortriptyline · HCl (0.2 mg/ml); b = amitriptyline · HCl (0.2 mg/ml). Mobile phase: acetonitrile–0.09 *M* aqueous sodium phosphate (pH adjusted to 2.5 with phosphoric acid) (58:42). Flow-rate: 2.0 ml/min. Detection: absorbance at 254 nm. Injection volume: 10 μl.

sharp peaks that were eluted within about 4 min. Supelcosil LC18-DB and BDS Hypersil ODS columns were also grouped together and each gave longer retention with greater tailing compared to the other group of columns. The μBondapak C_{18} and Partisil 5-ODS (3) columns were predicted to behave similarly. In this case, the Partisil 5-ODS (3) column was closer to the BDS Hypersil ODS column and the μBondapak C_{18} was closer to the YMC group, especially in terms of peak shape. The μBondapak C_{18} phase is recommended for this application [30]. It is apparent that the YMC group could provide equivalent separations that would probably not be obtained from columns in other groups. The column groupings appear to be fairly consistent for this application and the results show which groups of columns are likely to be acceptable for the method and which are not.

Dirithromycin

Dirithromycin is a macrolide antibiotic known to exhibit poor chromatography on several columns [31]. A mixture of dirithromycin, *epi*-dirithromycin and erythromycylamine is easily generated by allowing a sample solution to degrade partially. This solution was analyzed using several columns with the results shown in Fig. 7. The Supelcosil LC18-DB and Hypersil ODS (column recommended for this method) columns which grouped together with test mixtures gave similar chromatography for this sample. Also grouped with these columns was Supelcosil LC18 which gave similar retention but significantly greater tailing. Columns from other groups provided separations that were quite similar (YMC ODS, Zorbax Rx-C_{18}) or drastically different (Spherisorb ODS-II, μBondapak C_{18}, Nucleosil C_{18}). In this case, the separation appears to be rugged over two different column groups, but not so for the columns outside these groups. Also, the secondary grouping of Hypersil columns with Supelcosil and Spherisorb was valid for Supelcosil, but not for Spherisorb. This

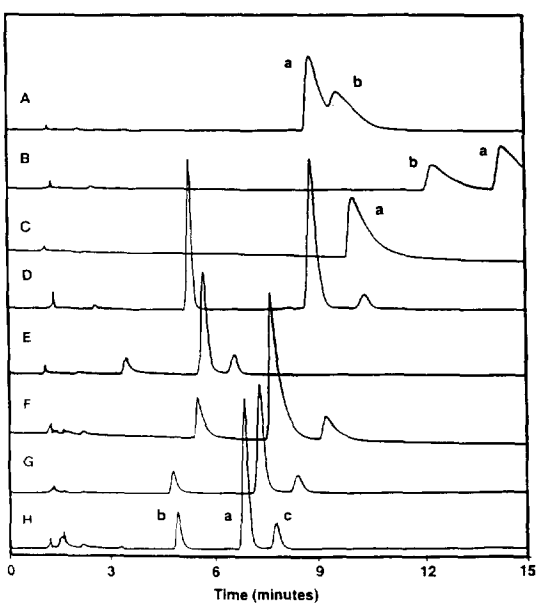

Fig. 7. Dirithromycin macrolide separation. Columns: A = μBondapak C_{18}; B = Nucleosil C_{18}; C = Spherisorb ODS-II; D = YMC ODS; E = Zorbax Rx-C_{18}; F = Supelcosil LC18; G = Supelcosil LC18-DB; H = Hypersil ODS. Peaks: a = dirithromycin, 2.5 mg/ml, degraded in mobile phase for at least 24 h to form b = erythromycylamine and c = epidirithromycin, additional erythromycylamine was added to some sample solutions. Mobile phase: acetonitrile–methanol–50 m*M* aqueous potassium phosphate, pH 7.5 (44:19:37). Flow-rate: 2.0 ml/min. Detection: absorbance at 205 nm. Injection volume: 10 μl. Column temperature: 40°C.

demonstrates that the groupings are only approximate and will not be valid for every separation.

β-Lactam antibiotics

Three β-lactam antibiotics, cephalexin, cefaclor and loracarbef, combined with acetaminophen as an internal standard, were investigated as another pharmaceutical compound separation. Each of these antibiotics is amphoteric, possessing carboxylic acid and primary amine groups. Results for different columns are shown in Fig. 8. The primary differences observed for columns from different groups are retention time and efficiency. Even the Zorbax ODS column, which did not group well with any columns other than the Partisil 10 ODS (2), gave a separation similar to the other columns. In this example, a simple adjustment of mobile phase strength could be used to make all the columns appear nearly identical. Therefore, the mobile phase conditions must be masking secondary retention mechanisms and stationary phase interactions that have led to differences among columns in the other examples. It is likely that this separation could be performed adequately on almost any ODS column.

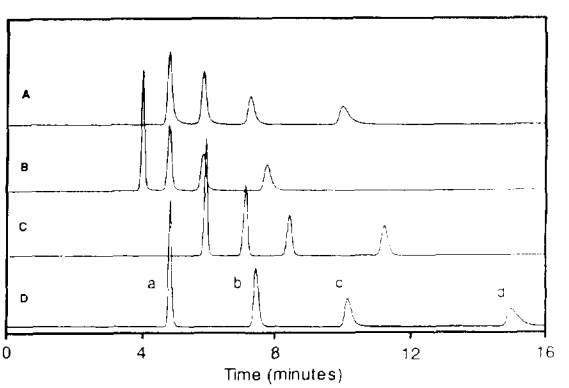

Fig. 8. Antibiotics separation. Columns: A = Zorbax ODS; B = Zorbax Rx-C_{18}; C = YMC ODS; D = Hypersil ODS. Peaks: a = 4-acetamidophenol (internal standard, 0.09 mg/ml); b = cefaclor (0.24 mg/ml); c = loracarbef (0.14 mg/ml); d = cephalexin (0.16 mg/ml). Mobile phase: acetonitrile–50 m*M* aqueous sodium phosphate, pH 2.5 (14:86). Flow-rate: 1.0 ml/min. Detection: absorbance at 254 nm. Injection volume: 10 μl.

4. Conclusions

Test mixtures containing compounds chosen to accentuate the differences among ODS bonded-phase columns are useful in categorizing columns according to the similarity of chromatographic behavior. The various cluster analysis methods used to categorize the raw data set or reduced sets derived from it showed some differences in groupings, but general column categories could be discerned. The general similarity of cluster analysis results also indicated that the principal components and reduced data sets were adequate representations of the raw data.

The categories determined from the analysis were tested for validity using separations of several pharmaceutical compounds. In general, columns in the same category behaved similarly in these examples and would be likely choices as alternates for each other. Columns from different categories were more likely to exhibit differences in behavior and could be explored during method development to achieve better retention, selectivity or peak shape. Similar behavior observed for columns from different categories can be an indication of method ruggedness with respect to column characteristics for the chosen separation conditions.

The work described here has been focused on small organic molecules. The test mixtures used may not be appropriate for prediction of column similarity with regard to behavior toward polypeptides and proteins where other interactions may become important.

The utility of the categories described here would be greatly extended if additional columns could be readily classified. This could be done qualitatively by visual examination of test mixture chromatograms or by using linear discriminant analysis to categorize a column not included in the original set.

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