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HPLC Column Classification

Brian Bidlingmeyer,[*] *Agilent;* Chung Chow Chan,[†] *Eli Lilly and Company;* Patrick Fastino,[†] *FDA;* Richard Henry,[*] *ThermoHypersil Keystone;* Philip Koerner,[*] *Phenomenex;* Anne T. Maule,[†] *3M Pharmaceuticals;* Margareth R.C. Marques,[*] *U.S. Pharmacopeia;* Uwe Neue,[*] *Waters;* Linda Ng[*] *USP Pharmaceutical Analysis 2 Expert Committee;* Horacio Pappa,[‡] *U.S. Pharmacopeia;* Lane Sander,[*] *NIST;* Carmen Santasania,[*] *Supelco;* Lloyd Snyder,[†] *LC Resources;* Timothy Wozniak,[*] *USP Pharmaceutical Analysis 2 Expert Committee*

ABSTRACT This *Stimuli* article represents the conclusions and recommendations of the USP Working Group on HPLC Columns. The working group included the five largest manufacturers of HPLC columns in the United States, along with the National Institute of Standards and Technology (NIST) and USP. This work attempts to facilitate the selection of HPLC columns by the analyst when performing a USP test.

INTRODUCTION

The L nomenclature to designate high-performance liquid chromatography (HPLC) column type was introduced for the first time in the *Fourth Supplement* to *USP XIX* in 1978. The L1 designation is for columns with octadecilsilane as the bonded phase. When *USP XX* was published in 1980, only seven columns were classified and given a brief description. Since then the list has grown without pause to 56 descriptions, some of them very broad or with imprecise wording (*1, 2*). For years, this classification system has generated an increasing number of inquiries to USP regarding which column brand is appropriate for a particular compendial procedure. Today, column packings are developed for specific applications, resulting in columns with distinct characteristics even though they belong to the same original USP classification. For example, more than 220 columns currently available in the worldwide market can be classified as L1, but not all of them have the same applications (*3, 4*). This situation makes the process of selecting a column for a particular application very difficult. The problem is partially controlled by the *System Suitability* test in most of the USP chromatographic procedures, but in many cases these tests are not conclusive to ensure column interchangeability. Evidently the current classification nomenclature does not provide sufficient information to fill the needs of modern liquid chromatography.

THE USP APPROACH

In an attempt to facilitate the selection of possible columns by the analyst, the chair of the USP Expert Committee (EC) on Pharmaceutical Analysis 2, Dr. Timothy Wozniak, and the vice-chair of the same EC, Dr. Linda Ng, created a group to define a proposal about how to subclassify initially only L1 columns, and, perhaps in the future, extend the approach to other USP column designations. The USP Working Group on HPLC Columns was created, and its membership represented the National Institute of Standards and Technology (NIST) and the five largest manufacturers of HPLC columns in the United States. Dr. Ng chaired this group.

At the beginning the group considered several existing approaches. After a series of meetings, they decided to use the NIST Standard Reference Material (SRM) 870 to carry out the evaluation of C18 columns according to the procedure described in the certificate of analysis for this SRM (*5, 6*).

This procedure uses a mixture of five organic compounds (uracil, toluene, ethylbenzene, quinizarin, and amitriptyline) in methanol to characterize column performance. This test mixture is intended primarily for the characterization of C18 columns used in reversed-phase liquid chromatography. Selection of the components in SRM 870 was based on published testing protocols (*7, 8*) and commercial column literature (*9*) to provide a broad characterization of column performance in a single, simple test.

On the basis of the results obtained and the problems faced during the evaluation of the C18 columns using NIST SRM 870, the group identified four parameters to be used in the characterization of the columns: hydrophobicity (capacity factor of ethylbenzene); chelation (tailing factor of quinizarin); activity toward bases (silanol activity, capacity factor, and tailing factor of amitriptyline); and shape selectivity (bonding density). The term *shape selectivity* is commonly used to denote a chromatographic quality exhibited by certain stationary phases for which enhanced separations of geometric isomers may result based on their molecular structure rather than other physical or chemical properties of the solute (*10*). Although SRM 870 does not characterize shape selectivity, the property can be assessed by use of other chromatographic tests, such as SRM 869a, *Column Selectivity Test Mixture for Liquid Chromatography,* or by measuring the bonding density of the stationary phase.

[*] USP Group

[†] PQRI Group

[‡] Correpondence should be addressed to: Horacio Pappa, 12601 Twinbrook Pkwy., Rockville, MD, 20852-1790; e-mail: hp@usp.org.

To compare columns on the same basis, the user should evaluate column performance by separating the mixture isocratically using a mobile phase constituted by 80% methanol and 20% buffer phosphate at pH 7.0.

The certificate of analysis for NIST SRM 870 contains a series of chromatograms representative of possible types of retention behavior. In most instances, peak identification can be made on the basis of elution order (uracil, toluene, ethylbenzene) and detector response (quinizarin, amitriptyline). Relative peak areas depend on the detection wavelength. Quinizarin has significant absorbance at 480 nm, and separations of SRM 870 carried out at this wavelength are selective for this single component. Conversely, quinizarin exhibits reduced absorbance at 210 nm, permitting measurement of amitriptyline in the presence of quinizarin.

The retention behavior of reversed-phase liquid chromatography (LC) columns often differs in a variety of ways. The components in this test mixture were selected as indicators of several types of chromatographic properties. The determination of peak width (efficiency; theoretical plates), peak asymmetry (A_S), absolute retention (k'), and selectivity factor (α, i.e., relative retention, k'_1/k'_2) for these components may provide useful measures of these properties.

Uracil

This component is commonly used as an indicator of the void volume (unretained volume) in an LC column, which is required to calculate the retention factor (k').

Toluene/Ethylbenzene

The retention of these compounds can be considered to result primarily from solvophobic interactions. The selectivity factor $\alpha_{E/T}$ is the k' ratio of ethylbenzene and toluene, and this value has been used to characterize differences among C18 and C8 columns. Absolute retention of a nonpolar component such as ethylbenzene provides a measure of column retentiveness (column strength). Toluene and/or ethylbenzene are also useful markers for calculation of column efficiency (theoretical plates, N).

Quinizarin

Quinizarin (1,4-dihydroxyanthraquinone) is a metal-chelating reagent. The retention behavior of this component is expected to be indicative of the presence or absence of metals in the chromatographic system. Columns demonstrate one of two types of retention behavior. Low activity toward chelating reagents is indicated by symmetric peak shape, and high activity toward chelating reagents is indicated by tailing, asymmetric peak shape. Quinizarin typically elutes after ethylbenzene and before amitriptyline. It is interesting to note that for columns known to contain certain embedded polar functional groups, quinizarin elutes last, with good peak symmetry. Peak asymmetry is not strongly correlated with retention for quinizarin.

Amitriptyline

Amitriptyline is a basic compound (pKa = 9.4) commonly used by column manufacturers for column characterization. Elution of organic bases with severe peak tailing is often associated with high silanol activity; however, the elution of such compounds with symmetrical peak shape is considered indicative of column deactivation. Because peak tailing is the most objectionable property associated with silanol activity, A_S is an appropriate measure of this property. Peak asymmetry is not strongly correlated with retention for amitriptyline.

The influence of chromatographic conditions on test results was examined for several different parameters. Because retention, efficiency, and peak shape are influenced by testing conditions, column evaluation should be carried out under standardized conditions to facilitate column comparisons. The largest changes in retention behavior occur with changes in mobile phase composition. The absolute retention of the polar and nonpolar components increases with the percentage of buffer in the mobile phase (at pH 7.0 and constant ionic strength in the mixed solution). A composition of 80% methanol and 20% buffer was selected to provide appropriate retention for a broad range of column types.

Changes in column temperature influence the absolute retention of the components in SRM 870; however, relatively small effects are observed in the peak shape of quinizarin or amitriptyline. It is recommended that column temperature be controlled to 23 °C ±1 °C.

Forty-one commercial C18 columns were used in the development of SRM 870. Columns were selected to represent a broad sampling of chromatographic retention properties and included alkyl phases prepared with embedded polar functional groups. No two columns exhibit identical retention behavior; however, similarities do exist among several columns. Among columns utilized, values of k' for ethylbenzene ranged from 0.2 to 2.8. In contrast, only slight differences were observed for methylene selectivity ($\alpha_{E/T}$; range, 1.26 to 1.45). The retention of quinizarin ranged from $k' = 1$ to $k' = 23.6$. In two instances, no elution of this compound was detected. Peak asymmetry values ranged from $A_S = 1.1$ to $A_S = 5.7$ (in two instances, peaks were not defined well enough to permit determination of A_S). Finally, the retention of amitriptyline ranged from $k' = 1.4$ to $k' = 72.9$ ($A_S = 1.0$ to $A_S = 11$).

Besides the members of the USP Working Group, several other column manufacturers tested their columns using this protocol. The results from these tests are presented in *Table 1*.

Pharmacopeial Forum
Vol. 31(2) [Mar.–Apr. 2005]

Table 1. Characterization of C18 columns using NIST SRM 870

Column Number	Hydrophobicity Capacity Factor (k') Ethylbenzene	Chelating Tailing Factor Quinizarin	Silanol Activity		Shape Selectivity Bonding Density (μmols/m^2)
			Capacity Factor (k') Amitriptyline	Tailing Factor Amitriptyline	
1	2.8	No peak	No peak	No peak	3.4
2	2.1	1.4	8.2	6.7	3.5
3	2.0	1.1	7.3	2.3	2.0
4	2.4	1.0	6.1	1.8	4.0
5	2.4	1.1	5.9	3.4	3.8
6	1.0	6.0	7.5	4.0	1.1
7	1.5	7.5	4.6	3.0	2.7
8	2.2	1.7	5.1	1.7	3.2
9	1.6	1.5	3.1	1.2	3.3
10	0.7	No peak	23	3.0	1.7
11	2.0	No peak	11.5	7.0	2.6
12	1.0	1.2	1.7	1.1	2.3
13	1.5	1.1	3.3	1.3	2.2
14	2.0	No peak	35	8.0	2.7
15	1.7	1.1	5.1	2.4	1.6
16	2.0	2.0	6.3	1.9	3.5
17	1.5	1.9	23	2.8	2.2
18	1.6	6.6	4.1	2.7	3.2
19	4.2	1.6	11	3.9	3.6
20	3.2	1.6	7.6	2.0	3.6
21	0.9	1.3	2.2	2.1	4.2
22	0.4	2.5	1.0	4.9	Not available
23	1.5	1.5	3.5	2.0	3.1
24	1.5	3.4	4.3	3.6	3.2
25	1.5	2.0	5.6	4.1	2.4
26	1.2	2.2	12	2.6	4.6
27	1.3	1.4	3.5	2.1	3.3
28	2.2	1.2	5.3	1.1	3.4
29	0.7	No peak	2.1	1.4	2.3
30	2.6	1.2	—	3.3	4.0
31	2.2	1.0	—	3.6	Not available
32	2.5	1.6	—	1.2	3.3
33	2.0	1.2	—	1.0	5.5
34	1.0	1.4	3.0	2.6	3.0
35	1.3	No peak	3.8	3.9	3.1
36	1.3	2.0	4.5	13	3.1
37	1.8	1.5	13.6	2.8	2.6
38	1.9	1.5	5.0	2.4	2.6
39	1.9	1.5	5.1	2.4	2.7
40	1.9	1.5	6.0	2.9	2.2
41	3.3	1.3	8.8	2.9	3.2
42	1.6	1.4	5.0	2.7	1.4
43	0.9	1.4	3.0	2.8	0.9
44	1.9	1.3	5.0	1.5	2.5
45	1.5	1.3	4.4	1.9	1.9
46	3.3	1.2	7.5	1.3	3.0
47	2.0	1.0	6.7	2.6	2.1
48	1.0	2.2	3.1	2.4	2.1
49	2.2	1.4	14.2	3.5	3.2
50	2.2	1.8	10.2	2.2	3.0
51	3.9	1.7	12.5	4.0	2.9
52	2.3	1.0	6.1	1.8	2.9

Table 1. Characterization of C18 columns using NIST SRM 870 *(Continued)*

Column Number	Hydrophobicity Capacity Factor (k') Ethylbenzene	Chelating Tailing Factor Quinizarin	Silanol Activity		Shape Selectivity Bonding Density (μmols/m^2)
			Capacity Factor (k') Amitriptyline	Tailing Factor Amitriptyline	
53	1.1	1.7	2.9	2.2	2.9
54	0.7	1.6	2.2	2.7	2.9
55	2.0	1.2	3.8	1.6	2.8
56	1.1	1.2	2.2	1.2	3.2
57	0.7	1.3	1.4	1.8	3.2
58	2.6	1.5	1.7	—	—
59	0.6	1.5	1.7	—	—
60	2.0	1.1	4.0	1.1	3.2
61	3.4	1.1	13.5	5.4	2.8
62	2.0	1.0	23.0	4.5	2.6
63	0.4	1.4	14.5	3.5	1.2
64	1.4	1.3	4.3	5.3	5.2
65	2.6	1.4	7.8	1.9	3.3
66	2.1	1.2	5.7	1.5	3.3
67	0.8	1.5	3.1	2.9	3.3

Figure 1 illustrates typical elution patterns for SRM 870. To improve chromatographic performance toward bases, five of the columns utilized are known to contain embedded polar functional groups within the stationary phase. The separation of SRM 870 was similar for these columns. In each case, quinizarin eluted last, and both amitriptyline and quinizarin exhibited symmetrical peak shape (e.g., *Figure 1A*).

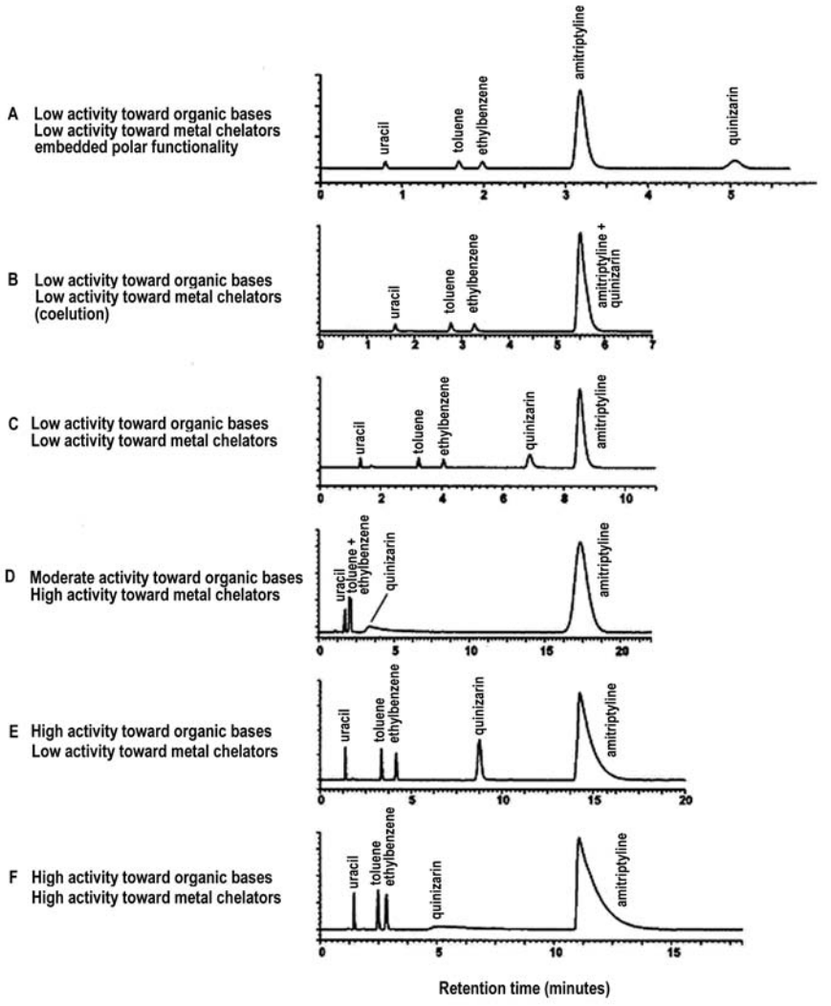

Fig. 1. Examples of separations of SRM 870 on commercial C18 column (reproduced from SRM-870 Certificate of Analysis, NIST)

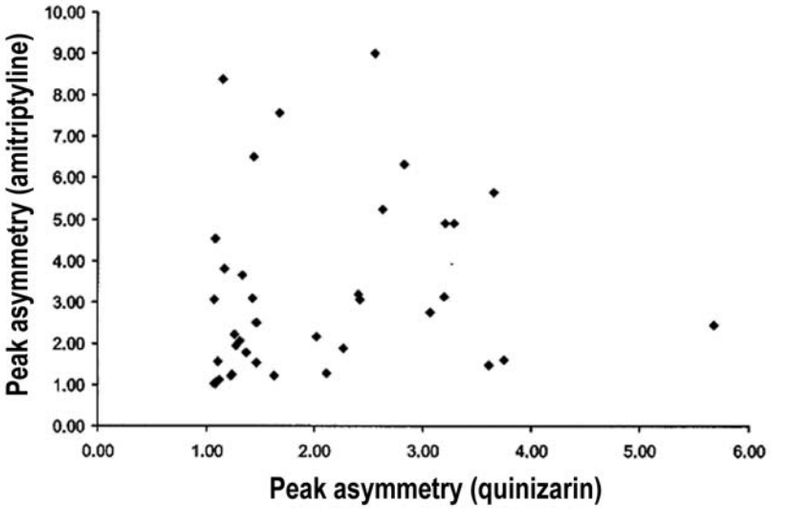

Fig. 2. Plot of peak asymmetry for amitriptyline vs. peak asymmetry for quinazirin for various C18 columns (reproduced from SRM-870 Certificate of Analysis, NIST)

Peak asymmetry data for quinizarin and amitriptyline are plotted in *Figure 2*. The scatter in the data indicates independence of the two terms. Thus, it is possible for a column to exhibit high activity toward chelating agents and low activity toward bases, or other combinations (e.g., *Figures 1C* through *1F*).

THE PQRI INITIATIVE

The Impurities Working Group of the PQRI Drug Substance Technical Committee had an objective to investigate the impact of technology on the characterization of impurities. This project is being carried out in collaboration with Dr. Lloyd Snyder, who has initiated work to create a database and software that will help to define conditions that will lead to equivalent separations for different columns. An application resulting from the project will be a way to obtain an equivalent separation for a monograph method that stipulates an L1 or L7 column.

The Snyder/Dolan column test procedure has been described in a series of publications (*11–18* and a review in *19*). Based on retention data for a series of standard mixtures (see *Table 2*) and the same separation conditions (50% acetonitrile/buffer; pH 2.8 and 7.0; 35 °C), every reversed-phase column can be characterized by six column-selectivity parameters (*15*): relative retention (k_{EB}), hydrophobicity (*H*), steric interaction (*S**), hydrogen-bond acidity (*A*) and basicity (*B*), and relative silanol ionization or cation-exchange capacity (*C*).

Table 2. Test mixtures for Snyder/Dolan procedure

Mixture 1 thiourea amitriptyline 4-butylbenzoic acid	**Mixture 2a** nortriptyline acetophenone mefenamic acid
Mixture 1a *N,N*-diethylacetamide 5-phenyl-1-pentanol ethylbenzene	**Mixture 3** *p*-nitrophenol anisole 4-hexylaniline
Mixture 2 *N,N*-dimethylacetamide 5,5-diphenylhydantoin toluene	**Mixture 3a** *cis/trans* chalcone benzonitrile
	Mixture 4 berberine

Column *hydrophobicity* increases with an increase in total carbon. *H* is somewhat larger for small-pore packings because of the compression of the ends of the alkyl chains. Because end-capping adds only a few tenths of a percent carbon to the column, end-capping has little effect on *H*. As noted before, *H* has only a minor effect on column selectivity.

Column *steric interaction* increases as the bonded phase becomes more crowded. That means an increase in *S** for increased chain length or concentration of the bonded phase. *S** also increases for narrow-pore packings because of the compression of the ends of the alkyl groups. *S** has a significant effect on column selectivity, especially for molecules of different shape.

Column *hydrogen-bond acidity* due to non-ionized silanols increases with column acidity. Therefore, values of *A* are greater for more acidic type-A columns. When the column is end-capped, the number of accessible and unreacted silanols decreases, as do values of *A*. The column parameter *A* has a significant effect on column selectivity for non-ionized basic molecules such as amines and amides, especially aliphatic derivatives.

Column *hydrogen-bond basicity* arises from various functional groups within the bonded phase. For all type-B (high-purity silica) and some type-A (older, less pure silica) columns, it appears that water from the mobile phase partly dissolves in the bonded phase, and this water can preferentially interact with and bind to non-ionized carboxylic acids. So, columns with larger values of *B* preferentially retain acidic compounds.

In the case of columns with embedded polar groups, the basic polar group (urea, amide, carbamate) can strongly bind both phenols and carboxylic acids. Some type-A columns have larger values of *B,* believed to be the result of metal impurities in the silica.

Silanol ionization results in a negative charge on the column, and this charge attracts ionized (positively charged) bases and repels ionized (negatively charged) acids. For samples that contain ionized acids or (especially) bases, the column parameter *C* is a very important contributor to column selectivity. For samples that do not contain acids or bases, *C* is unimportant. Column ionization and values of *C* increase as mobile-phase pH is increased. End-capping results in decreased access to ionized silanols and a large decrease in *C*.

The ability to characterize column selectivity is of potential value for two different situations. First, routine HPLC procedures require the replacement of the column from time to time due to deterioration of the column during use. Also, when an HPLC method is transferred, it is necessary to obtain a suitable column for that procedure. In either situation, there exists the possibility that an equivalent column from the original supplier may no longer be available. For this reason, two or more equivalent columns with different part numbers can be specified as part of method development. ''Equivalent'' columns will have similar (ideally, ''identical'') values of the six column-selectivity parameters discussed above. This phase of the project was done and completed in collaboration with PQRI.

A second use of the six column-selectivity parameters outlined above is for the selection of columns of very different selectivity. Columns of different selectivity are often required during HPLC method development (for a deliberate change in selectivity) or for the development of orthogonal procedures that can be used to ensure that no new sample impurity is present in some samples.

The Snyder/Dolan procedure was originally developed for application to type-B C18 columns (*11–13*). It since has been extended to type-B alkyl–silica columns with C_1–C_{30} ligands (*14*), type-A C8 and C18 columns (*15*), columns with polar groups such as urea, carbamate, or amide that are either embedded into the ligand or used to end-cap the column (*16*), cyano columns (*17*), and phenyl and fluoro columns (*18*).

Columns with identical values of *H, S*, A, B*, and *C* are expected to give essentially identical selectivity (spacing of bands) for a given HPLC procedure (same mobile phase, temperature, and flow rate). Small differences in values of k_{EB} can be corrected by changes in flow rate. Although it is rare to find two reversed-phase columns that have identical values of *H, S*,* etc., small differences in these column parameters are still acceptable for any sample, and larger differences are allowable for some samples. A column comparison function F_S can be defined for two columns 1 and 2 as follows:

$$F_S = \{[12.5\ (H_2 - H_1)]^2 + [100\ (S^*_2 - S^*_1)]^2 + [30\ (A_2 - A_1)]^2 + [143\ (B_2 - B_1)]^2 + [83\ (C_2 - C_1)]^2\}^{1/2} \qquad [1]$$

Here, H_1 and H_2 refer to values of *H* for columns 1 and 2, S^*_1 and S^*_2 are values of *S** for columns 1 and 2, and so on for the remaining column parameters *A, B,* and *C*. If $F_S<3$ for any two columns 1 and 2, the two columns should provide equivalent selectivity and band spacing for any sample or set of conditions. Equivalent separation may still be achieved for $F_S>3$, but this is less certain. However, if it is known that the sample does not contain ionized compounds (e.g., no acids or [especially] bases), the term $C_2 - C_1$ of Equation 1 can be ignored, which usually means a much smaller value of F_S for two columns 1 and 2. Similarly, if carboxylic acids (ionized or not) are absent from the sample, the term $B_2 - B_1$ can also be ignored, again reducing the value of F_S.

In the event that columns of very different selectivity are desired, two columns 1 and 2 with a very large value of F_S would be preferred. *Figure 3* provides an example of the use of values of F_S to select columns of either similar (*a–c*) or different (*d*) selectivity.

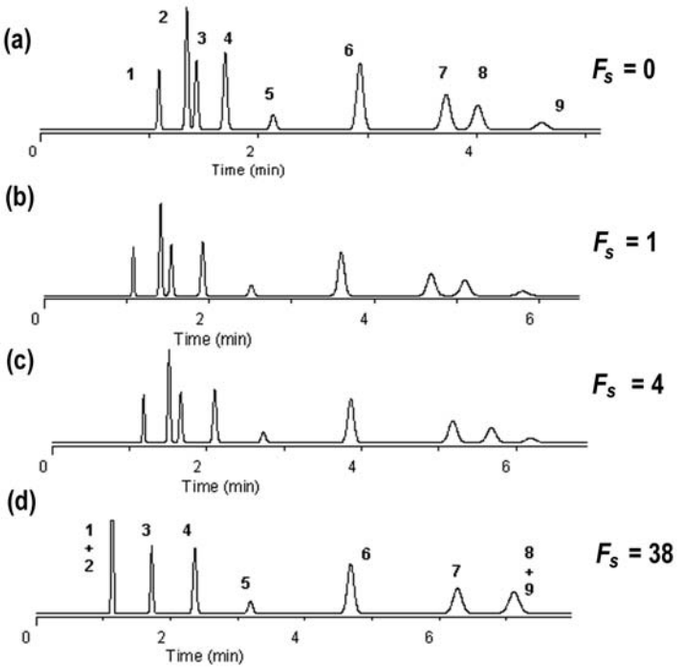

Fig. 3. Example of use of F_S values to select columns of either similar (*a–c*) or different (*d*) selectivity

The measurement of values of *H, S*,* etc. has been studied by four separate laboratories under PQRI sponsorship. On the basis of replicate testing of 42 different C8 and C18 columns, it was established that the measurement of values of *H, S*,* etc. is suitably repeatable (*20*). Further studies by these and other laboratories have since confirmed the reliability of Equation 1 as a basis for comparing column selectivity (*21*). These results have substantiated the ability of values of F_S to select columns of equivalent selectivity. The study of Snyder et al. (*20*) has also shown that the measurement of values of *H, S*,* etc. can be simplified and shortened, requiring only 2–3 hours per column. Five separate laboratories are currently evaluating the utility of the Snyder/Dolan approach for the development of orthogonal separations.

CONCLUSIONS

Both of these approaches have merit, and it is too soon to favor one over the other. The USP approach provides column performance characterization (theoretical plate count, good peak symmetry, etc.) and produces five data points to describe the column. The PQRI approach provides selectivity characterization (relative retention times), and the parameters are included in a searchable database that produces a list of suitable columns ordered by the F_S factor. The USP approach also could be provided in database form to permit ordering of columns based on a single factor derived from the measured parameters (analogous to the Snyder/Dolan approach). A third option might be to provide data from both characterization approaches. Algorithms could be developed to permit column assessment based on the combined data. Ultimately, the approach(es) utilized must balance ease of use with effectiveness.

In the USP approach, performance proprieties include tailing determination due to the presence of trace metals in the column packing and "active" silanols (which may also be the result of metal contamination), but the PQRI (Snyder/Dolan procedure) does not. However, it must be pointed out that poor column performance is associated mainly with older columns that utilize type-A silica.

As a result of the evaluation presented here, the USP Working Group on column classification resolved to publish the data obtained by these two approaches and encourage users to submit their comments in order to improve these tools. In the near future, the results obtained by the NIST SRM 870 and the searchable database will become public via the USP Web page (www.usp.org). This will allow users to evaluate the results obtained with both approaches. Both databases will be updated as new results are obtained. In the interim, USP will continue to update and publish *Chromat-*

ographic Reagents Used in USP–NF and Pharmacopeial Forum that lists the original column brand used during method development for compendial procedures.

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