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Journal of Chromatography A, 1057 (2004) 49–57
JOURNAL OF CHROMATOGRAPHY A
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A fast, convenient and rugged procedure for characterizing the selectivity of alkyl-silica columns

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Received 6 February 2004; received in revised form 17 September 2004; accepted 20 September 2004

Abstract

Previous work has shown that the selectivity of reversed-phase columns for HPLC can be described by means of five column parameters: **H** (hydrophobicity), **S*** (steric resistance), **A** (hydrogen-bond acidity), **B** (hydrogen-bond basicity) and **C** (cation-exchange capacity). Values of **H**, **S***, etc. can be determined by carrying out retention measurements for 18 test solutes under standardized conditions. The reproducibility of the latter procedure has been evaluated by comparison testing in four different laboratories and found acceptable. An alternative 10-solute test procedure which is more reproducible and convenient (but somewhat less accurate), requires only 2–3 h per column.

Keywords: Column selectivity; Selectivity parameters; Hydrophobicity; Steric resistance; Hydrogen-bond acidity; Hydrogen-bond basicity; Cation-exchange capacity

1. Introduction

Previous work has described an empirical equation for characterizing the selectivity of reversed-phase liquid chromatography (RP-LC) columns [1–8]:

$$\log\alpha = \log\left(\frac{k}{k_{EB}}\right) = \eta'\mathbf{H} - \sigma'\mathbf{S}^* + \beta'\mathbf{A} + \alpha'\mathbf{B} + \kappa'\mathbf{C} \quad (1)$$

A separation factor α, defined as the retention factor k for a given solute divided by k for the reference solute ethylbenzene (k_{EB}), is related to conditions-dependent properties of the solute (η', σ', etc.) and conditions-independent properties of the column (**H**, **S***, etc., except for **C**, which varies with mobile phase pH). The symbols in Eq. (1) are defined in Section 5. Values of **H**, **S***, etc. have been reported for several hundred columns [4–8], which allows the selectivity of any two of these columns to be compared in terms of these column-selectivity parameters [4]. Columns with sufficiently similar values of **H**, **S***, etc. can be used interchangeably with little change in a given separation. See the immediately following paper [9] for details, including several examples of this approach.

Given experimental values of α for appropriate test solutes and a given column under specified conditions, plus values of η', σ', etc. for these test solutes and conditions, the column selectivity parameters (**H**, **S***, etc.) can be calculated by multiple linear regression. Prior to the present study, all previously reported measurements of column selectivity via Eq. (1) have been carried out in a single laboratory [1–8]. As other laboratories undertake similar measurements for additional columns, it is important to investigate those

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doi:10.1016/j.chroma.2004.09.063

Table 1
Test solutes used in present study

Solute		η'	σ'	β'	α'	κ'
Mix #1	Thiourea[a,b,e]					
	Amitriptyline[b,e] (#1)	−1.094	0.163	−0.041	0.300	0.817
	n-Butylbenzoic acid[b,e] (#2)	−0.266	−0.223	0.013	0.838	0.045
Mix #1a	*N*,*N*-Diethylacetamide[b,f] (#3)	−1.390	0.214	0.369	−0.215	0.047
	5-Phenylpentanol (#4)	−0.495	0.136	0.030	0.610	0.013
	Ethylbenzene[b,f] (#5)	0	0	0	0	0
Mix #2	*N*,*N*-Dimethylacetamide (#6)	−1.903	0.001	0.994	−0.012	0.001
	5,5-Diphenylhydantoin (#7)	−0.940	0.026	0.003	0.568	0.007
	Toluene (#8)	−0.205	-0.095	0.011	−0.214	0.005
Mix #2a	Nortriptyline (#9)	−1.163	-0.018	−0.024	0.289	0.845
	Acetophenone[b,f] (#10)	−0.744	0.133	0.059	−0.152	−0.009
	Mefenamic acid (#11)	0.049	0.333	−0.049	1.123	−0.008
Mix #3	4-Nitrophenol (#12)	−0.968	0.040	0.009	0.098	−0.021
	Anisole[b,f] (#13)	−0.467	0.062	0.006	−0.156	−0.009
	4-*n*-Hexylaniline[c] (#14)					
Mix #3a	Benzonitrile[b,e] (#15)	−0.703	0.317	0.003	0.080	−0.030
	cis-Chalcone (#16)	−0.048	0.821	−0.030	0.466	−0.045
	trans-Chalcone[b,e] (#17)	0.029	0.918	−0.021	−0.292	−0.017
Mix #4	Berberine[b,d] (#18)					

Values of η', σ', etc. from [4].
[a] Used to calculate values of *k*.
[b] Used in short (8-solute) procedure.
[c] Not used to calculate values of **H**, **S***, etc., because of excessive variation of *k* with pH.
[d] Used with Eq. (2).
[e] Mix A used in alternate procedure.
[f] Mix B used in alternate procedure.

factors which might contribute to the inter-laboratory or day-to-day variability of resulting values of **H**, **S***, etc. Because the original procedure for measuring values of these column parameters [4,5] is somewhat tedious, requiring retention data for 18 test solutes (all solutes but #14 of Table 1), a simpler, more convenient test method would also be preferable.

The present investigation is divided into two parts. First, we examined the transferability of the original test procedure [4,5] to other laboratories, by carrying out replicate testing of several different columns among four different laboratories. We also analyzed the effects of possible differences in equipment and separation conditions on resulting values of **H**, **S***, etc.

Second, we used results from the present and prior studies to evaluate the reliability of a simpler, more repeatable and more convenient test procedure which makes use of only eight test compounds (indicated by superscript "b" in Table 1) plus thiourea and berberine.

2. Experimental

Four laboratories participated in the reproducibility studies described in Section 3.1: BASi, Wyeth Research, 3M, and Eli Lilly. Replicate testing of identical columns from the same lot was carried out for 44 different column lots (see Table 2).

2.1. Equipment

The BASi laboratory used a Shimadzu HPLC system that is described in [1]. The remaining three laboratories each used Agilent 1100 HPLC systems.

2.2. Procedure

The procedure used is described in detail in [4]. The 15 cm × 0.46 cm column (with 5 μm diameter particles) was first flushed with pH 2.8 mobile phase (50%, v/v, acetonitrile/buffer; buffer is pH 2.80, 60 mM potassium phosphate), capped off (static equilibration) and stored at ambient conditions for 8–16 h. Following static equilibration, the column was connected to the system and mobile phase flow was begun. After 20 min of mobile phase flow, the seven samples of Table 1 (Mixes #1–4) were successively injected at 10-min intervals. A repeat injection of Mix #1 was made, and the column was stored in 50% (v/v) acetonitrile/water. At a later time, the column was reinstalled for testing with pH 7.0 mobile phase (50%, v/v, acetonitrile/buffer; buffer is pH 7.00, 60 mM potassium phosphate). After flow of mobile phase through the column for 20 to 40 min, Mix #4 was injected three times at 20-min intervals.

The column temperature was 35 ± 0.5 °C, the flowrate was 2.0 mL/min, and UV-detection was at 205 nm.

Table 2
Summary of results for all columns in the collaborative study

Column	Laboratories testing each column[a]				Average S.D. for each column (units of log α)[b]
	BASi	Wyeth	3 M	Lilly	
BetaBasic C18	X	X			0.006
BioBasic C8	X	X			0.006
BioBasic 18	X	X			0.006
Hypersil BetaBasic-8	X	X			0.007
Hypersil BetaMax Neutral	X	X			0.006
Inertsil C8-3	X	X		X	0.006
Inertsil ODS-3	X	X		X	0.010
Inertsil ODS-P	X	X		X	0.006
Symmetry C18	X	X	X	X	0.008
Xterra MS C18	X	X		X	0.006
Symmetry-300 C18	X	X		X	0.003
Delta Pak C18(300A)	X	X		X	0.007
Polarity C18	X	X		X	0.007
YMC-Pack Pro C18	X	X		X	0.007
Delta Pak C18100A	X	X		X	0.005
YMC-Pack Pro C8	X	X		X	0.003
Xterra MS C8	X	X		X	0.005
Symmetry C8	X	X		X	0.004
Discovery BIO Wide Pore C18[c]	X		X		0.041
Discovery C8	X		X		0.006
Discovery BIO Wide Pore C5	X		X		0.014
Discovery C18	X		X		0.007
Discovery BIO Wide Pore C8	X		X		0.009
Chromolith Performance RP18e-3 M	X		X		0.005
StableBond C18	X	X	X	X	0.004
Ace 5 C18	X	X			0.004
Ace 5 C8	X	X			0.005
Chromegabond WR C8	X	X			0.008
Chromegabond WR C18	X	X			0.006
ProntoSil 120-5-C18-SH	X	X			0.022
ProntoSil 120-5-C8-SH	X	X			0.008
Prontosil 120-5-C18-AQ	X	X			0.005
Prontosil 120-5-C18-H	X	X			0.005
Synergy Max RP-80	X		X		0.008
Luna C8	X		X		0.013
Prodigy ODS-3100A	X		X		0.011
Kromasil 100-5C18	X	X			0.003
Genesis EC C8120A	X	X			0.005
Genesis C8120A	X	X			0.006
Genesis AQ 120A	X	X			0.007
Genesis C4300A	X	X			0.008
Genesis C4 EC 120A	X	X			0.004
Genesis C18120A	X	X			0.003
Genesis C18300A	X	X			0.003
Average for all columns[d]					0.007

For sources of various columns, see [4].

[a] E.g., for the first column (BetaBasic C18), the BASi and Wyeth laboratories tested this column.

[b] The average S.D. of log α values was calculated for solutes (#1–13, 15–17), then averaged for all solutes for a given column.

[c] The very large S.D. for this column suggests a major error in either separation conditions or column identity; this value of S.D. is not included in the average value of 0.007 for all columns.

[d] Average of all S.D. values (except for the Discovery Bio Wide Pore C18 column).

On-line mixing of acetonitrile and buffer was employed, and the HPLC pumps were calibrated to deliver 50% (v/v) of each solvent (±0.05%, v/v). Pump calibration was carried out by comparison of retention times from on-line mixing versus the use of accurately pre-mixed mobile phase (based on the weights of buffer and acetonitrile).

Two procedures are described in this paper. The above procedure based on 18 test solutes (#1–13 and 15–17 of Table 1) will be referred to as the "18-solute procedure". A second, abbreviated procedure is described in Section 3.3 that is based on the use of only ten test solutes ("10-solute procedure"). (For each procedure, retention times for thiourea and berberine were also determined.)

2.3. Materials

Solvents and other chemicals were of HPLC grade. The solutes of Table 1 were obtained from Aldrich, with the exception of compound #16 (*cis*-chalcone). The latter compound was prepared by UV-light radiation of a 50 mg/mL solution in acetonitrile of the *trans*-isomer (#17), giving a mixture of both isomers. In separations by RP-LC, the *trans*-isomer always elutes after the *cis*-isomer [4,10]. The compounds of Table 1 are present in each mixture at a concentration of 50 μg/mL; the injection volume is 10 μL.

2.4. Calculations

Retention factors k were determined for solutes #1–18 of Table 1 and (for berberine only) the two mobile phases described in Section 2.2 (i.e., pH 2.8 and 7.0): $k = (t_R - t_0)/t_0$, where t_0 is the retention time of thiourea at pH 2.8 (assumed the same at pH 7.0). Values of α were calculated for solutes #1–17, equal to k for the solute divided by k for ethylbenzene (#5). Given values of α at pH 2.8 for 16 solutes (#1–13 and 15–17) and a given column, values of **H**, **S***, **A**, **B** and **C**(2.8) were calculated by multiple linear regression of Eq. (1), using the solute parameter values (η', σ, etc.) of Table 1 (see [4] for details). **C**(2.8) refers to the value of **C** at pH 2.8. Values of **C** at pH 7.0 were determined [2] from:

$$\mathbf{C}(7.0) = \mathbf{C}(2.8) + \log\left(\frac{k_{7.0}}{k_{2.8}}\right), \quad (2)$$

where $k_{7.0}$ and $k_{2.8}$ refer to values of k for berberine (a quaternary ammonium salt) at pH 7.00 and 2.80, respectively. Note that the value of **C**(2.8) is obtained from values of k for the solutes of Table 1 *apart* from berberine.

If the equilibration of the column prior to collection of values of k is incomplete, this will be manifested by varying values of k for amitriptyline at pH 2.8 and berberine at pH 7.0.

Replicate values of the latter measurements should agree within ±2%, and the average values of these replicates are used in the above calculation. If a larger change in k is noted, the measurement should be repeated until successive values agree within ±1%. In the present study, replicate measurements always agreed well within the latter limits.

3. Results and discussion

3.1. Comparative studies in four different laboratories

A summary of the present study is given in Table 2. For each of the 44 columns tested, replicate measurements of $\log\alpha$ were carried out on presumably identical columns from the same production batch by two to four of the collaborating laboratories. For each solute and column, the average standard deviation (S.D.) of $\log\alpha$ was determined (except for berberine as solute, where $\log k$ was measured at both pH 2.8 and pH 7.0). Table 2 (last column) lists the average of these S.D. values for all solutes (except 4-*n*-hexylaniline and berberine, which are not used in the determination of values of **H**, **S***, etc. at pH 2.8) and a given column. The average S.D. for all columns is ±0.007 log units, equivalent to ±1.6% in α (1 S.D.). As discussed previously [4], an accuracy of ±3% in α is needed for a reliable comparison of column equivalency. The overall results of Table 2 therefore suggest that values of the column selectivity parameters **H**, **S***, etc. obtained in any one of the four collaborating laboratories are adequately reproducible. Values of **H**, **S***, etc. were also determined for each column in each laboratory, and average S.D. values were calculated for each column parameter: **H**, ±0.003; **S***, ±0.001; **A**, ±0.022; **B**, ±0.001; **C**(2.8), ±0.010; **C**(7.0), ±0.019. Values of **H**, **S***, etc. for the columns of Table 2, as well as for an additional 48 type-B alkyl-silica columns, are reported in [4].

The above variability in values of **H**, **S***, etc. would predict a similar variability in values of $\log\alpha$ calculated from Eq. (1) (with these values of **H**, **S***, etc.) as was observed experimentally (±1.6%, 1 S.D.). However, the sample of Table 1 (excluding 4-*n*-hexylaniline and berberine) is atypical, in that half of these solutes have extreme values of one of the solute parameters (η', σ', etc.). The effect of variability in values of **H**, **S***, etc. for a much larger and presumably more representative sample has been reported (Table 5 of [4]), and for such a sample the average S.D. of values of $\log\alpha$ calculated from Eq. (1) is only ±1.0%; i.e., a somewhat smaller error than for the sample of Table 1. For most samples, therefore, experimental error in the measurement of values of α for the test solutes of Table 1 and a given column translate into less important errors in values of **H**, **S***, etc. for that column.

A further analysis of the data summarized in Table 2 is of interest. Fig. 1a is a plot of average S.D. values (units of $\log\alpha$) for all laboratories and each solute (and all 44 columns) versus the average value of $\log k$ for each solute and all columns. With the exception of two data points marked by circles (4-*n*-hexylaniline [#14] at pH 2.8 and berberine [#18] at pH 7.0 of Table 1), values of S.D. are seen to increase as solute retention k decreases. This inverse correlation of values of S.D. and k appears to be a consequence of small, random variations in retention time (or the *measurement* of retention time), as indicated by the solid curve labeled "t_R error" = 0.008 min). Thus, the latter curve corresponds to the calculated increase in $\log\alpha$ (for different values of k) as a result of an increase in t_R by 0.008 min. Because four measurements of t_R are involved in a calculated value of α, the implied uncertainty in t_R is about $0.008/4^{1/2} = 0.004$ min. Regardless of the reason for the increase in S.D. with decrease in k, it is apparent that values of α are less reliable when based on small values of k. This is a particular concern for *N*,*N*-dimethylacetamide [#6], as well as (for other reasons) berberine [#18] at pH 7 (the dashed line marked "error limit" represents an [acceptable] accuracy of ±3% in α); see also Section 3.3 below.

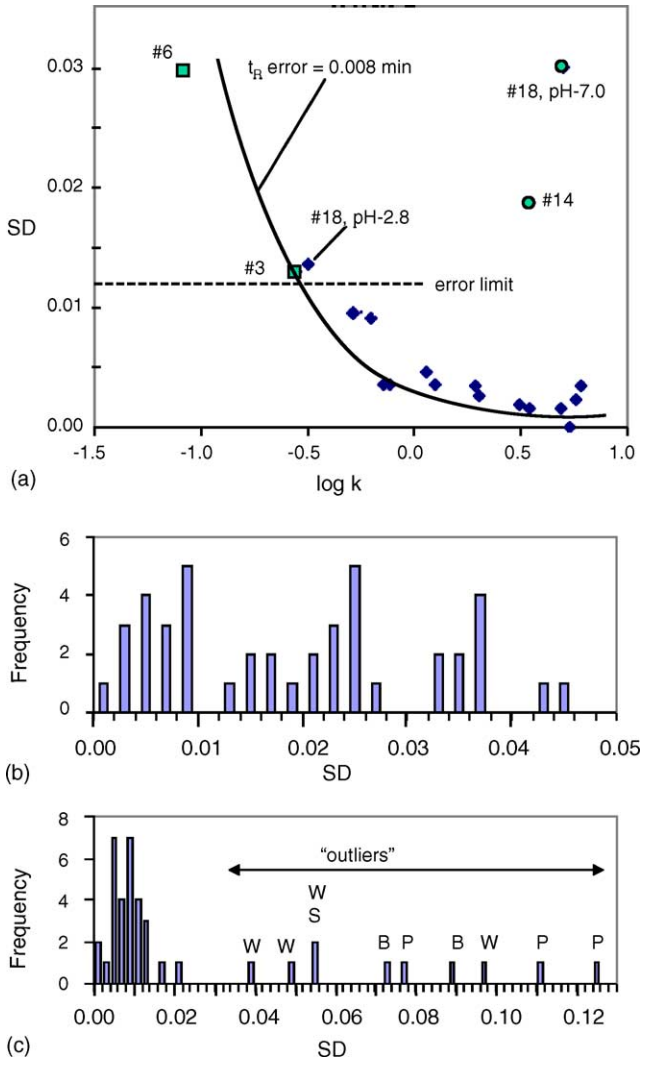

Fig. 1. Repeatability of retention measurements by the four laboratories; all values are for pH 2.8 unless otherwise specified. (a) Plot of average standard deviation S.D. for individual solute α values vs. average value of log k for each solute (for solute numbering, see Table 1); (b) frequency-distribution of S.D. values for log α values of solute #14 (4-*n*-hexylaniline); increments of 0.002 S.D. units; (c) plot as in (b) for solute #18 (berberine) at pH 7.0. Outlier S.D. values in (c) are identified by the initial letter of the manufacturer for that column: B, Bischoff; P, Phenomenex; S, Supelco; W, Waters. See text for details.

3.1.1. *Experimental uncertainty in values of k and α for partially-ionized solutes*

4-*n*-Hexylaniline [#14] is a weak base that is about 70% ionized in the mobile phase (50% acetonitrile, pH 2.8) [2]. It is the only solute of Table 1 whose retention varies significantly at pH 2.8 with small changes in pH [2]; an 0.2 unit change in pH results in a change in log k by 0.13 units (±35% in α). The larger value of S.D. = 0.019 for 4-*n*-hexylaniline, versus that of other solutes in Fig. 1a, can reasonably be attributed to small errors in adjusting mobile phase pH among the four collaborating laboratories. Thus, the random variation in pH can be estimated equal to (0.019/0.13) × 0.2 = ±0.03 units (1 S.D.). This pH-variability seems reasonable for typical, well-performing laboratories. However, this example also emphasizes a need for test solutes that are either completely ionized (#1, 9 and 18) or largely non-ionized (#2–13 and 15–17) under the conditions of column testing. For this reason, 4-*n*-hexylaniline is neither included in the column-test procedure of Section 2, nor was this solute used in previously reported measurements of **H**, **S***, etc. for different columns [4–8]. As seen in Fig. 1b, the frequency-distribution of S.D. values for 4-*n*-hexylaniline and different columns roughly approximates a Gaussian distribution; i.e., no clearly observable outliers.

3.1.2. *Experimental uncertainty in values of* **C** *at pH 7.0.*

Values of k for solute #18 (berberine) are used to determine the value of **C** at pH 7.0 (**C**(7.0) from Eq. (2)). Since the S.D. for berberine at pH 7.0 (0.030 log units, or ±7% in α) is an unexpectedly large value (see Fig. 1a), values of **C**(7.0) are similarly uncertain. While berberine is a quaternary ammonium compound whose ionization does not change with mobile phase pH, the ionization of the silica stationary phase can change rapidly with pH near pH 7 [2,11], and this directly affects the retention of berberine and derived values of **C**. We have determined that a change in pH from 7.0 to 7.2 for a Symmetry C18 column results in an increase in log k for berberine of 0.15 units. This implies a variation in mobile phase pH among the four laboratories of Table 2 equal to (0.03/0.15) × 0.2 = ±0.04 pH units, which is not unexpected (see above related discussion for 4-*n*-hexylaniline).

The frequency-distribution for values of S.D. for berberine at pH 7 is shown in Fig. 1c, which is seen to differ from that for 4-*n*-hexylaniline at pH 2.8 (Fig. 1b). Most of the S.D. values cluster in the range 0.000–0.015, and for these data the average S.D. (0.009 log units, or ±2.1% in α) is acceptable. The occurrence of higher S.D. values (S.D. values >0.022) for berberine at pH 7 (designated "outliers" in Fig. 1c) was similar for all four collaborating laboratories, but columns from certain manufacturers tended to give a higher proportion of high S.D. values (see identification of manufacturers of each column by letter in caption of Fig. 1c). Three out of a total of 11 manufacturers represented in Table 1 account for nine out of 10 total outliers. The data of Fig. 1c suggest that the characterization of columns from some manufacturers may require special care in the adjustment of mobile phase pH. It was also determined for the same system (Symmetry C18 column, pH 7.0 mobile phase) that an increase in temperature of 1 °C results in a decrease in k for berberine of 6%, suggesting that close temperature control is also important in the measurement of values of **C**(7.0). However, this appears *not* to be necessary, for the following reason.

As discussed in the following paper [9], the effect of **C** at higher pH on values of α can be reduced significantly, because of the partial de-protonation of ionized basic compounds. For a mobile phase pH > 6, we estimate that the effect of **C** on column selectivity can be reduced by as much as 10-fold. It can therefore be concluded that measurement errors as large

Table 3
Effect of errors in separation conditions on measured values of the column parameters **H**, **S***, etc. obtained with a pH 2.8 mobile phase

Column parameter	Effect on column parameters of a change in conditions		
	+1 °C	+1% B	+0.2 pH units
H	0.000	0.008	0.000
S	0.000	−0.047	0.000
A	0.024	0.067	−0.001
B	0.000	−0.019	−0.001
C(2.8)	0.007	0.031	0.005
C(7.0)	0.024	–[c]	0.15
Cum %[a]	0.9	6.4	0.4
Allowed[b]	1.1	0.2	0.5

[a] Cumulative average effect of change in condition on values of α (1 S.D.) at pH 2.8; see [4].
[b] Allowed change in conditions for ±1% average change in α (excluding berberine at pH 7.0, see text).
[c] Not determined.

as ±7% for berberine retention at pH 7 are unlikely to significantly affect calculated values of **C**(7.0). Consequently, special care in the control of mobile phase pH or separation temperature when measuring berberine retention at pH 7.0 appears unnecessary.

3.2. Robustness of the present test procedure in terms of equipment and/or conditions

Variations in equipment can lead to differences in measured values of retention time t_R, as well as derived values of k, α, and **H**, **S***, etc. In addition, experimental error in the formulation of the mobile phase can also affect final results. Critical aspects of the equipment include extra-column hold-up volume, and the control by the equipment of mobile phase composition (assumes on-line mixing of acetonitrile and buffer) and temperature. On the basis of previous data for each solute (Tables 1 and 8 of [2]), it is possible to estimate the impact on column testing of both equipment differences and the external control of mobile phase composition.

Differences in equipment hold-up volume lead to differences in t_R and derived values of k, but these differences are cancelled for final values of α [1]. Consequently, differences in hold-up volume do not influence the present column-test procedure. If on-line mixing of acetonitrile and buffer is used, errors in mobile phase composition of a few tenths of a percent or more are possible for some HPLC systems (unpublished observation). Similarly [12], we have observed that the temperature controller of many HPLC systems can be in error by as much as 2 °C when set at the temperature of our column-test procedure (35 °C). Finally, the discussion of Section 3.1 and related observations by others suggests that errors in mobile phase pH of ±0.05 units are not unlikely.

Table 3 summarizes our estimates of the required limits on each separation condition, for a maximum error in derived values of α at pH 2.8 that is no larger than 1%. Starting with previously measured changes in log k for each of the test solute solutes of Table 1, we can determine the effect of these changes on each column parameter – as shown in Table 3. The cumulative effect of these changes for each condition on the average value of log α is shown in the next-to-last row of values in Table 3, and if we accept no more than ±1% error in values of log α, the required limits on separation condition errors are given in the final row of Table 3: not greater than ±1.1 °C, ±0.2% B, and ±0.5 pH units. These are fairly generous limits on the possible variability of these conditions, except possibly for % B. In the present study, each laboratory calibrated their HPLC systems for any bias in the on-line mixing of 50% acetonitrile with 50% buffer (by comparisons of sample retention for Mix 1a of Table 1, using: (a) on-line mixing versus (b) mobile phase prepared gravimetrically). The instrument setting was then adjusted to deliver the required 50% B (±0.05%). A similar procedure is recommended for future measurements of this kind.

Note that the data of Table 3 do *not* pertain to values of k for berberine at pH 7.0. The discussion of Section 3.1 suggests that no special care is required for the measurement of k values for berberine.

3.3. Development and evaluation of a simplified column-test procedure

The column-test procedure used in the study of Table 2 involves the preparation and injection of seven test mixtures for injection at pH 2.8, with one additional injection at pH 7.0.

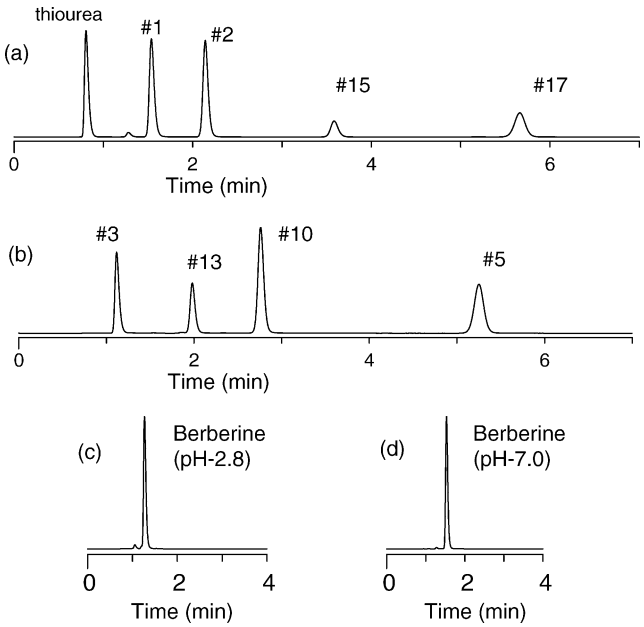

Fig. 2. Chromatograms from the application of the 10-solute procedure to an Altima C18 column. (a) Mix-A, pH 2.8; (b) Mix-B, pH 2.8; (c) berberine, pH 2.8; (d) berberine, pH 7.0. Experimental conditions are given in Section 2; numbering of peaks is given in Table 1.

Aside from the time and effort involved in sample preparation and injections, a significant amount of procedural complexity is involved in data interpretation. Furthermore, we have noted (Section 3.1) that measurements of k for *N*,*N*-dimethylacetamide (#6) are necessarily less precise. Also, *cis*-chalcone (#16) must be prepared by the user, since to our knowledge this compound is not commercially available. For these and other reasons, it is desirable to simplify the present 18-solute test procedure, while eliminating the latter two test solutes (#6 and 16).

On the basis of our previous experience and the results of the present study, it is possible to reduce the number of test solutes from 18 to 10 compounds, while eliminating troublesome solutes #6 and 16. Furthermore, on the basis of our

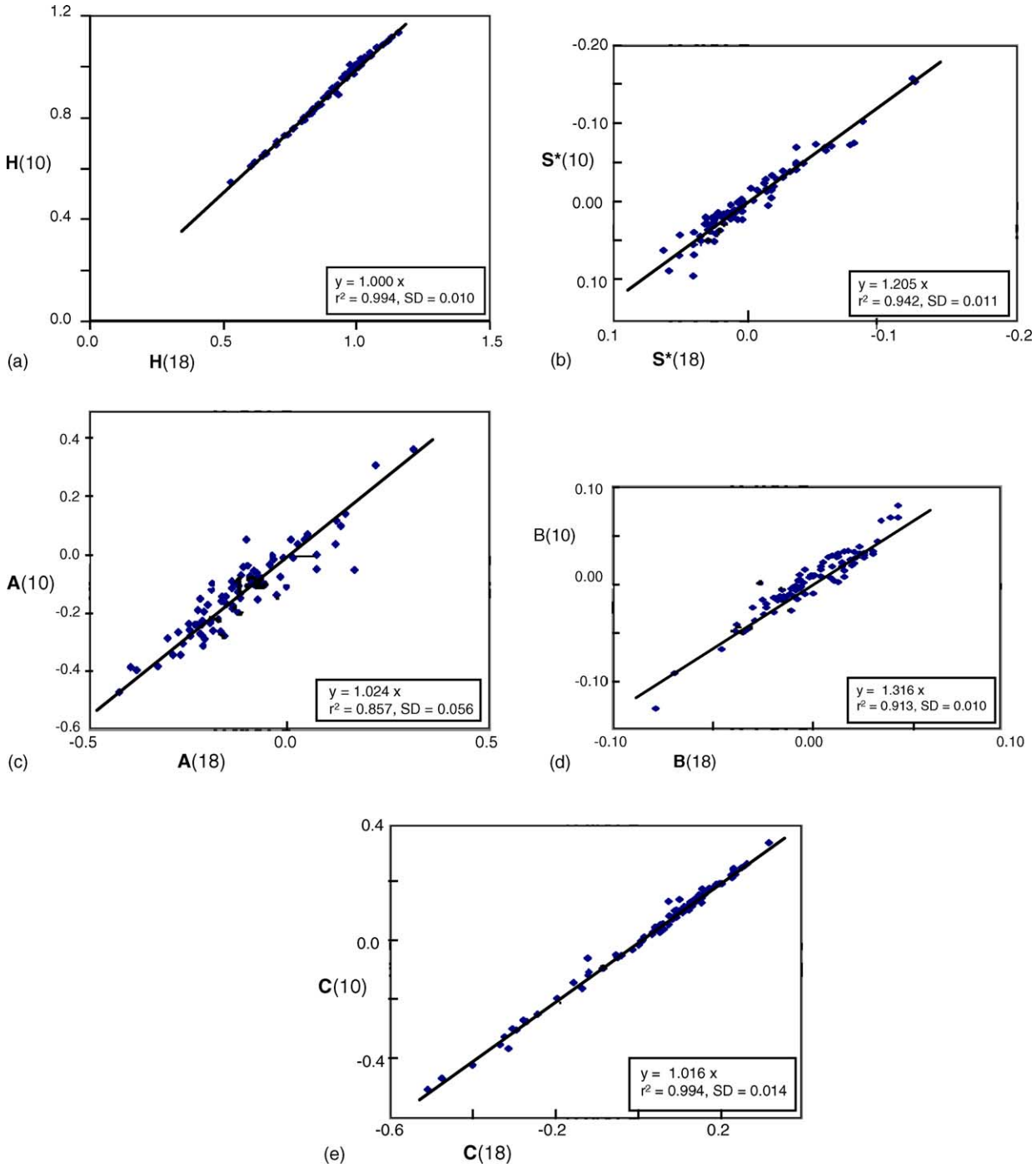

Fig. 3. Comparison of column selectivity parameter values obtained by the 10-solute procedure (**H**(10), **S***(10), etc.) with values from the 18-solute procedure (**H**(18), **S***(18), etc.). (a–e) Plots for each column parameter. Data for 87 columns described in [4].

experience with the use of the 18-solute procedure for 87 type-B alkyl-silica columns [4], it is possible to formulate these 10 compounds into just three mixtures: Mix A, thiourea (used to calculate values of *k*) plus #1, 2, 15, 17; Mix B, #3, 5, 10, 13; Mix C, #18 (for the measurement of **C** at pH 7; Eq. (2)). The possibility of retention reversals, which can complicate the interpretation of individual chromatograms, should not be a problem for mixtures A and B; thus, for 87 type-B alkyl-silica columns that were previously studied [4] there were no retention reversals, and the smallest separation between adjacent peaks within mixtures A and B corresponded to $\alpha = 1.2$ (i.e., baseline resolution). However, older ("type-A") columns [5] made from less-pure silica may show significantly greater changes in the relative retention of amitriptyline (solute #1), requiring its separate injection as a means of peak identification in Mix A. Fig. 2 shows representative separations for the 10-solute test procedure.

Values of $\log \alpha$ for the test solutes of Mix A and B can be used with the solute parameters of Table 1 to derive values of **H**, **S***, etc. (multiple linear regression via Eq. (1)) at pH 2.8. Values of the column parameters obtained in this way agree fairly well with values based on the 18 test solutes of the original procedure. This is illustrated in Fig. 3 for 87 type-B alkyl-silica columns described in [4], where values of **H**, **S***, etc. based on 10 test solutes (**H**(10), **S***(10), etc.) are plotted versus values using 18 solutes (**H**(18), **S***(18), etc.).

The observed values of S.D. for the plots of Fig. 3 imply some reduction in the accuracy of values of **H**(10), **S***(10), etc. versus values of **H**(18), **S***(18), etc. from the 18-solute procedure. The magnitude of this decrease in accuracy can be inferred by comparing the S.D. values of Fig. 3 (0.010–0.056) with the repeatability of values of **H**(18), **S***(18), etc. cited in Section 3.1 (S.D. values of 0.001–0.022); i.e., a significant decrease in accuracy for the 10-solute test procedure. For typical samples, however, the latter errors in values of **H**(18), **S***(18), etc. correspond to an average error in calculated values of α of only ±2.8% (1 S.D.), which falls within our target of ±3%.

The repeatability of the 10-solute procedure can be obtained by averaging S.D. values (units of $\log \alpha$) for the eight test solutes used in this test (#1, 2, 3, 5, 10, 13, 15, 17). The resulting average S.D. equals 0.004 (±1.0% in α), which is somewhat better than the reproducibility of the 18-solute procedure (S.D. = 0.006, or ±1.6%). The reproducibility of values of [**C**(7.0)–**C**(2.8)] is the same for both procedures, since there is no change in the measurement of this quantity (Eq. (2)).

Limited comparisons of the 10- versus 18-solute column test (similar to those above for type-B columns) were also carried out for several type-A alkyl-silica columns and columns with embedded or end-capping polar groups (data of [5,6]). It appears that the 10-solute procedure is unreliable for columns of the latter type, and possible band reversals in separations such as Fig. 2 are more likely. This suggests that the 18-solute procedure is preferable for columns other than type-B alkyl-silica.

4. Conclusions

A procedure for characterizing column selectivity at low and high pH in terms of five parameters (**H**, **S***, **A**, **B**, **C**) was previously reported [4]. In the present study, the reproducibility of this procedure was investigated for 44 different type-B (low metals content) alkyl-silica columns for RP-LC. Two to four columns of each kind from the same production lot were repetitively tested by four different laboratories, resulting in values of *k* and α for 18 test solutes plus berberine (each column). An average, overall repeatability of values of $\alpha = \pm 1.6\%$ (1 S.D.) was found for these 18 solutes and 44 columns among the four laboratories. Inasmuch as a repeatability of ±3% in α is required for the purpose of selecting columns that can provide equivalent separation, we conclude that the repeatability of this procedure should be adequate for its intended purpose. A somewhat greater variability in values of *k* and **C** at pH 7.0 was found, corresponding to ±7% in α. However, the importance of values of **C** at higher pH values is considerably reduced for most samples [9], and we conclude that the latter experimental variability is of little general consequence.

A more convenient test procedure for type-B alkyl-silica columns is proposed for future use, based on about half as many (10) test solutes,. A comparison of the 18-solute and 10-solute procedures suggests that the two test procedures are equivalent within the required accuracy of values of α (±3%), but *only* for type-B alkyl-silica columns. The 10-solute procedure is also more repeatable (±1.0% in α), mainly because it avoids the use of a test-solute (*N*,*N*-dimethylacetamide) which elutes very early ($k \approx 0.1$).

Finally, it should be noted that *average* values of the standard deviation (S.D.) equal to 1–2% do not preclude errors in $\alpha > 3\%$ for certain solutes. On the other hand, it will be seen in the following paper [9] that the application of values of **H**, **S***, etc. for the purpose of comparing column selectivity is subject to other errors of comparable or greater magnitude.

5. Nomenclature

Definitions of symbols used in present and following paper [9] are given below. Equations (e.g., II-1) refer to present paper (I) or following paper [9] (II).

A	"type-A" column based on metal-containing silica
A	relative column hydrogen-bond acidity, related to number and accessibility of silanol groups in the stationary phase
A(10)	value of **A** obtained using the 10-solute procedure of Section 3.3
A(18)	value of **A** obtained using the 18-solute procedure of Section 3.1
B	"type-B" column based on pure silica
B	relative column hydrogen-bond basicity

B(10) value of **B** obtained using the 10-solute procedure of Section 3.3
B(18) value of **B** obtained using the 18-solute procedure of Section 3.1
C relative column cation-exchange activity, related to number and accessibility of ionized silanols in stationary phase
C(10) value of **C** obtained using the 10-solute procedure of Section 3.3
C(18) value of **C** obtained using the 18-solute procedure of Section 3.1
C(2.8) value of **C** for pH 2.8
C(7.0) value of **C** for pH 7.0 (Eq. I-2)
F_S column matching function (Eq. II-1)
F_S^* value of F corrected for absence of acids or bases (Eq. II-3)
H relative column hydrophobicity
H(10) value of **H** obtained using the 10-solute procedure of Section 3.3
H(18) value of **H** obtained using the 18-solute procedure of Section 3.1
k retention factor, equal to $(t_R - t_0)/t_0$
k_{EB} value of k for ethylbenzene
k_1, k_2 value of k for column-1 or -2
$k_{2.8}$, $k_{7.0}$ values of k for berberine at pH 2.8 and 7.0, respectively (Eq. I-2)
Q maximum allowable value of F_S^* for two "equivalent" columns, taking the critical resolution into account (Eq. II-4)
r correlation coefficient
RP-LC reversed-phase liquid chromatography
$\mathbf{S}^*$ relative steric resistance to insertion of bulky solute molecules into the stationary phase; as $\mathbf{S}^*$ increases, bulky solute molecules experience greater difficulty in penetrating the stationary phase and being retained; **S** as defined previously is equal to $-\mathbf{S}^*$
$\mathbf{S}^*$(10) value of $\mathbf{S}^*$ obtained using the 10-solute procedure of Section 3.3
$\mathbf{S}^*$(18) value of $\mathbf{S}^*$ obtained using the 18-solute procedure of Section 3.1
S.D. standard deviation
t_0 column dead time (min)
t_R retention time (min)
x_B, x_C correction factors (Eq. II-3)
α separation factor for two solutes (isocratic elution)
α^* average value of α in gradient elution
α' relative solute hydrogen-bond acidity
β' relative solute hydrogen-bond basicity
η' relative solute hydrophobicity
κ' relative charge on solute molecule (positive for cations, negative for anions)
σ' relative steric resistance of solute molecule to penetration into stationary phase (σ' is larger for more bulky molecules)

Acknowledgement

The present study was carried out with the support of the Impurities Work Group (IWG), Technical Committee and Steering Committee of the Product Quality Research Institute (PQRI). The help of the following members of PQRI is gratefully acknowledged: E. Asafu-Adjaye, Moji Adeyeye, Todd Cecil (Technical Committee Liaison), Henry Drew, P. Faustino, Matthew Gosnell, Chris Rutkowski, Ken Sigvardson, Pat Tway (Technical Committee Chair), Fred Wolff and Yafei Zheng. The generous gift of the various columns by the manufacturers (see [4] for participating companies) is also appreciated.

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