Fast HPLC with a Silica-Based Monolithic ODS Column

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Abstract

Fast high-performance liquid chromatography is becoming routine in laboratories that require high throughput or for combinatorial libraries. Reduced analysis time is commonly achieved by using shorter columns and higher flow rates. Shorter columns require smaller particles in order to maintain efficiency. However, smaller particles increase backpressure, which limits both column length and higher flow rates for typical LC pumps. This disadvantage has been addressed by the emergence of monolithic liquid chromatographic columns (1). Unlike particle-base columns, monolithic columns consist of a continuous rod-shaped porous network with a bimodal pore distribution. In this study, a commercially available 50- ¥ 4.6-mm silica-based octadecyl silane monolithic column (Chromolith SpeedROD RP18e, EM Science, Gibbstown, NJ) was used to separate a seven-component test mixture with a wide range of polarity. The primary goals of this investigation were: (*a*) to study the chemistry (selectivity) of the new silica-based monolithic columns and (*b*) to study their run-to-run and column-to-column performance (retention times and peak areas). The selectivity (α factor) is a ratio of partition coefficients and, if comparable for a variety of solutes, would mean that methods could be readily transferred between particulate and monolithic columns.

Introduction

Fast high-performance liquid chromatography (HPLC) for traditional, particulate columns usually includes faster flow rates, increased column temperature, small particle diameters, and shorter column lengths (2–4). To maintain efficiency with shorter columns, smaller particle size is necessary. Because particle size has an inverse squared dependence on column pressure, particle-based chromatographic columns are inherently limited by pressure (3).

The demand for higher throughput has resulted in the development of new column technology (5). Of these developments, monolithic columns show promise for fast analysis (5–10). Monolithic columns significantly reduce column backpressure compared with particulate columns by using a porous network of macropores (6). This pressure reduction permits flow rates that are unattainable with typical particulate columns and instrumentation. Flow rates as high as 9 mL/min have been reported with reasonable pressure drops (1). Monolithic columns may consist of an organic or inorganic polymer network. Organic monoliths, although showing promise for capillary electrochromatography (11–14), have not performed well for HPLC because of problems associated with poor mechanical strength, network swelling in the presence of organic solvents, and the existence of micropores within the network (1). Recently, silica-based monolithic HPLC columns became commercially available from Merck KGaA (Darmstadt, Germany) (1). These silica-based monolithic columns consist of a single porous silica rod with a bimodal pore distribution. Within the network, macropores (2-µm) allow higher flow rates than particulate columns and mesopores (130-Å) provide good analyte capacity (9). Initial evaluations of these monolithic columns have been promising (1,5,8,9).

The main focus of this work was to evaluate these monolithic columns using a test mixture with a wide polarity range while maintaining the shortest possible analysis time. A seven-component test mixture developed by Scynexis (Research Triangle Park, NC) was used throughout this study. It was chosen to represent a wide range of hydrophobicity that is characteristic of combinatorial libraries. Additionally, five commercially available particulate columns were obtained for a selectivity comparison with the Chromolith column.

Experimental

Chromatographic analyses were carried out using an HPLC system consisting of a Shimadzu (Columbia, MD) LC-10AT pump and SCL-10A photodiode array detector with manual injection using a Rheodyne 7725i injector (Cotati, CA). Acetonitrile was obtained from Burdick & Jackson (Muskegon, MI) and water was from EM Science (Gibbstown, NJ). HPLC-grade solvents were used throughout. The polarity test mix consisted of 70 ppm of each benzamide, *N*-methylbenzamide, biphenyl, acetophenone (ACROS, Morris Plains, NJ) benzyl alcohol, ethylparaben, and propylparaben (Sigma-Aldrich, St. Louis, MO) in 50:50 acetonitrile–water. Structures of each component along with c*log *P* values are listed in Table I. Six 50- × 4.6-mm Chromolith SpeedROD RP18e columns (Merck KgaA) were evaluated. Each column was manufactured from a separate batch.

Column properties supplied by the manufacturer were as follows: macropore size, 2 µm; mesopore size, 13 nm; pore volume,

1 mL/g; surface area, 300 m^2/g; surface modification, C-18, encapped; and surface coverage, 17%.

For the column comparison, five commercially available columns were obtained: Waters Symmetry and XTerra (Milford MA), Phenomenex Luna (Torrance, CA), Optimize Technologies Velocity (Oregon City, OR), and Varian Chrompack (Walnut Creek, CA). The column parameters are listed in Table II.

Table I. Name, Structure, and c*log *P* Values for the Seven-Component Mixture

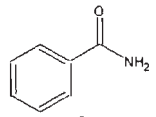
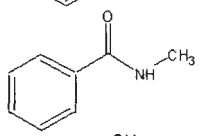
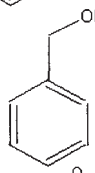
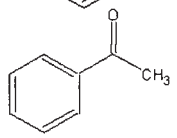
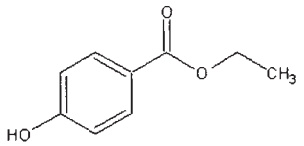
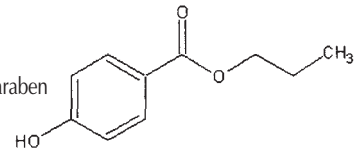
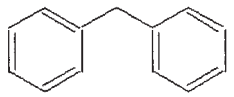
Name	Structure	CAS #	clog*P*
Benzamide		55-21-0	0.64
N-methylbenzamide		613-93-4	0.86
Benzyl alcohol		100-51-6	1.1
Acetophenone		98-86-2	2.13
Ethylparaben		120-47-8	2.51
Propylparaben		94-13-3	3.04
Biphenyl		92-52-4	4.09

Table II. Columns Used for Selectivity Comparison with the Chromolith SpeedROD

Column	Dimensions (mm)	Particle size (µm)	Pore size (Å)	Carbon load (%)
Phenomenex Luna	50 × 4.6	5	100	18
Waters Symmetry	50 × 4.6	3.5	100	19
Waters XTerra	50 × 3.0	3	125	15
Optimize Velocity	50 × 4.6	3	100	n/a*
Varian Chrompack	50 × 4.6	3	100	n/a

* n/a, not available.

Results and Discussion

Pressure measurements

The backpressure was measured with and without a Chromolith column using flow rates up to 9.00 mL/min with a 50:50 mixture of acetonitrile–water. Figure 1 shows both sets of data to be linear through the entire range of flow rates studied. Column backpressure for the Chromolith column and the HPLC system at 9 mL/min was less than 1700 psi.

Column comparison

The seven-component test mixture (Table I) was used to assess the selectivity of five particulate columns (Table II) and the Chromolith SpeedROD. Each column was tested under identical gradient conditions with analyses in triplicate for each column. Figure 2 shows chromatograms from the Chromolith column and the Waters XTerra and lists the chromatographic conditions. The average retention time (t_R) of each component was determined and the selectivity of adjacent peak pairs was calculated for each column.

Selectivity (a) was determined by first calculating the retention factor (k) of each of seven components under gradient conditions

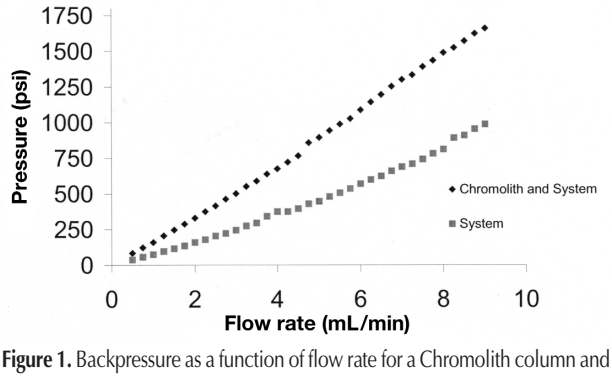


Figure 1. Backpressure as a function of flow rate for a Chromolith column and HPLC system and HPLC system alone.

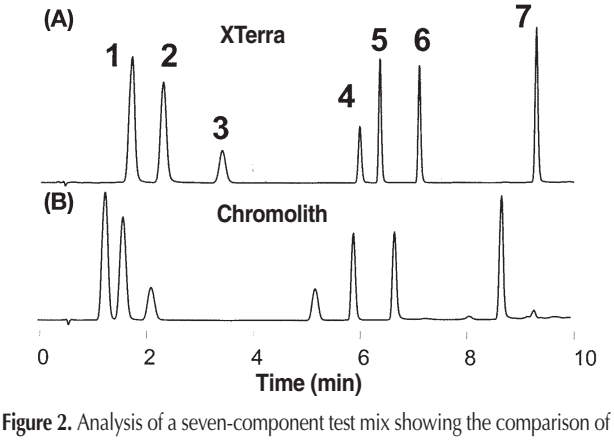


Figure 2. Analysis of a seven-component test mix showing the comparison of (A) Waters XTerra and (B) Chromolith SpeedROD. The peaks represent benzamide (1), *N*-methylbenzamide (2), benzyl alcohol (3), acetophenone (4), ethyl paraben (5), propyl paraben (6), and biphenyl (7), each at approximately 70 ppm. Chromatographic conditions: flow rate, 1.5 mL; manual injection, 10 µL; UV detection at 220 nm; ambient; acetonitrile–water gradient; initial 10:90, held for 1 min; and linearly to 85:15 in 7 min, held for 2 min.

using equation 1:

$$k = \frac{(t_R - t_o)}{t_o} \quad \text{Eq. 1}$$

where t_o is the dead time as measured by the elution of uracil, an unretained compound in a reverse-phase system. The selectivity (α) was then calculated using equation 2:

$$\alpha = \frac{k_2}{k_1} \quad \text{Eq. 2}$$

where k_2 is the later eluting peak and k_1 is the earlier eluting peak. Table III lists the selectivity for adjacent peak pairs, averages, and standard deviations (SDs) calculated for the analysis of the seven-component test mixture. Figure 3 shows a graphical interpretation of the same data.

The Chromolith column fell within the SD for each adjacent peak pair except for pairs 4,3 (acetophenone and benzyl alcohol) and 6,5 (propyl and ethyl paraben). For peak pair 4,3 the average selectivity of all four particulate columns was 2.08 ± 0.60, whereas the selectivity for the same pair on the Chromolith column was 2.97. This value, however, was less than the selectivity for pair 4,3 for the Waters XTerra column (α = 3.09, a difference of 0.91). For peak pair 6,5, the average particulate column selectivity was 1.12 ± 0.01, whereas the selectivity for the same pair on the Chromolith was 1.14, which indicated good agreement.

Fast LC method development

Figure 4 shows the fast analysis of the seven-component test mixture at 8.0 mL/min using a combination of linear and step solvent gradients and UV detection at 220 nm. The analysis run time was 1.25 min. Note that the solvent gradient returned to initial conditions before peak 7 eluted. This reduced re-equilibration time and therefore total analysis time. The resolution was calculated using equation 3:

$$R = \frac{2*(t_{R2} - t_{R1})}{(w_{b1} + w_{b2})} \quad \text{Eq. 3}$$

where w_b is the width at peak base. Table IV contains the calculated resolution of each adjacent peak pair. The peak resolution ranged from 1.63 (benzamide and *N*-methylbenzamide) to 6.81 (benzyl alcohol and acetophenone).

Re-equilibration time

Re-equilibration time is necessary in gradient HPLC in order ensure the column environment has returned to initial conditions. In this study, the difference in the initial and final organic composition was large (85%). A reasonable volume of initial mobile phase must be used to "reset" the column environment to ensure reproducible analyses. The total analysis time is the amount of time from injection to injection and includes both the run time and this re-equilibration time.

In order to determine the lowest re-equilibration time acceptable, eight re-equilibration times were studied: 3.00, 2.50, 2.00, 1.75, 1.50, 1.00, 0.75, and 0.50 min. Table V lists the re-equilibrium times studied, their corresponding volumes, and the number of column volumes. Column volume was measured with uracil to be 1.23 mL.

A re-equilibration time of 0.50 min was not sufficient. Figure V shows a chromatogram of an analysis with a 0.50-min equilibrium time. The first three peaks could not be evaluated; all three peaks were eluted in the void volume. Table VI lists the percent relative standard deviations (RSDs) for t_R with varying re-equilibration times. Precision decreased with re-equilibration times below 1.00 min for the early eluting peaks 1, 2, and 3. Figure VI shows the t_R as a function of re-equilibration time. The t_R increased then leveled off as the re-equilibration time was increased. A re-equilibration time of 1.00 min was sufficient to ensure column equilibration under fast LC conditions.

Run-to-run and column-to-column precision

Each column was subjected to 15 injections with a re-equilibration time of 1.50 min (~ 10 column volumes) between each run using the method developed previously (see Figure 1) and the seven-component test mixture. To evaluate column-to-column precision, the averages of the t_R and peak areas were compared for six Chromolith columns from different batches. Tables VII and VIII list the percent RSD values for t_R and peak areas, respectively, for each monolith column tested along with the averages and overall percent RSD for all six columns. The overall percent RSD ranged from 0.25 to 4.56 for t_R and from 1.58 to 4.07 for peak areas.

To evaluate run-to-run precision, the percent RSDs of both t_R and peak area for each of the seven components were compared. Table IX lists the percent RSDs for t_R and peak area for each monolithic column tested. The average percent RSD for t_R ranged from 0.89 to 5.09 and the peak areas ranged from 0.89 to 7.52.

Table III. Numerical Values of a for Seven-Component Test Mixture Under Identical Chromatographic Conditions*

Peak pair	Chromolith	Chrompack	XTerra	Symmetry	Luna	Velocity	Average (particulate)	Chromolith deviation from average
2,1	1.47	1.44	1.58	1.45	1.47	1.47	1.48 ± 0.06	0.01
3,2	1.51	1.54	1.23	1.58	1.53	1.57	1.49 ± 0.15	0.04
4,3	2.97	2.11	3.09	1.88	1.64	1.67	2.08 ± 0.60	0.89
5,4	1.15	1.08	1.31	1.07	1.06	1.05	1.11 ± 0.11	0.04
6,5	1.14	1.13	1.13	1.12	1.11	1.12	1.12 ± 0.01	0.02
7,6	1.33	1.33	1.23	1.33	1.31	1.33	1.31 ± 0.04	0.02

* See Figure 2.

Conclusion

A selectivity comparison was completed between the Chromolith SpeedROD monolith column and five commercially available particulate columns using a wide polarity, seven-component test mixture. Good agreement with the

particulate column average selectivity values was achieved on four of six adjacent peak pairs, with fair agreement being achieved for the peak pair propyl and ethyl paraben (particulate average $\alpha = 1.12 \pm 0.01$ and Chromolith $\alpha = 1.14$) and poor agreement for the peak pair benzyl alcohol and acetophenone (particulate average $\alpha = 2.08 \pm 0.60$ and Chromolith $\alpha = 2.97$). For this pair, the Chromolith column performed similarly to the Waters XTerra column ($\alpha = 3.09$). This suggests that these silica-based monolithic columns are good candidates for method transfer for neutral-polar and nonpolar analytes and that the monolithic columns performed similarly to a hybrid column for analytes in the mid-polarity range.

A fast LC method was developed for the same test mixture with a resolution exceeding 1.6 for each adjacent peak pair. Re-equilibration time between runs was investigated by monitoring t_R and its precision. The total analysis time (injection-to-injection) was determined to be 2.25 min.

Repeatability (run-to-run) and reproducibility (column-to-column) were evaluated for six Chromolith columns under fast

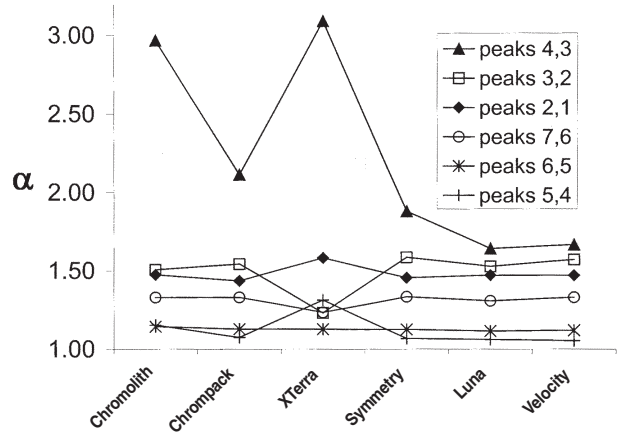


Figure 3. Selectivity comparison of Chromolith with five commercially available particulate columns. The chromatographic conditions are the same as those listed in Figure 2.

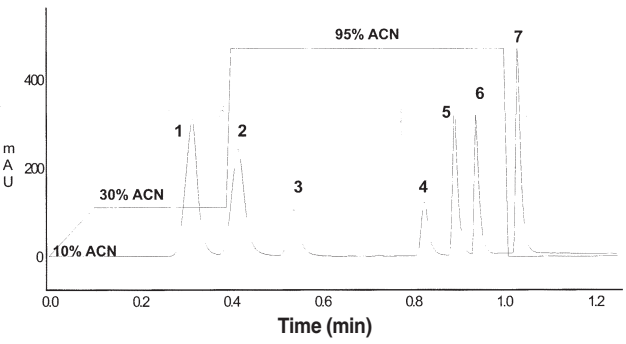


Figure 4. Chromatogram of a seven-component mixture analyzed under fast gradient conditions. The peaks are benzamide (1), *N*-methylbenzamide (2), benzyl alcohol (3), acetophenone (4), ehtyl paraben (5), propyl paraben (6), and biphenyl (7), each at approximately 70 ppm. Chromatographic conditions: flow, 8.0 mL/min; 10 µL injection (manual); UV detection at 220 nm; and ambient temperature. Gradient conditions: 10:90 acetonitrile–water to 30:70 linearly in 10 min, held at 30:70 for 0.30 min. Then increased to 95:5 acetonitrile–water at 0.40 min, held at 0.60 min, then increased to 10:90 (initial conditions) at 1.01 min.

Table IV. Resolution for Adjacent Peak Pairs for Seven-Component Analysis Under Fast LC Conditions*

Peak number	Resolution
1,2	1.63
2,3	2.21
3,4	6.81
4,5	1.95
5,6	1.55
6,7	2.47

* See Figure 4 for peak identification and chromatographic conditions.

Table V. Equilibration Times and Volumes for an 8-mL/min Flow Rate

Equilibration time (min)	Volume (mL)	Column volumes
0.50	4.0	3.3
1.00	8.0	6.5
1.50	12.0	9.8
1.75	14.0	11.4
2.00	16.0	13.0
3.00	24.0	19.5

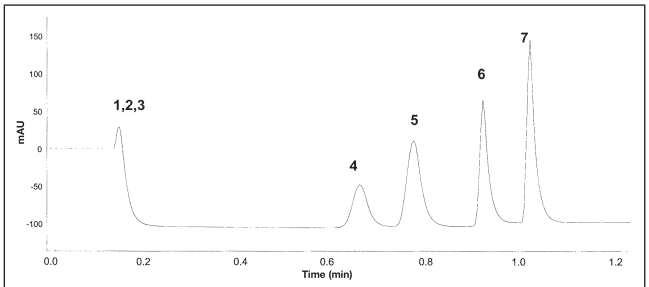


Figure 5. Chromatogram of a seven-component mixture analyzed under fast gradient conditions with a 0.50-min equilibration time. Chromatographic conditions and peaks are the same as listed in Figure 4. The first three peaks are eluted in the void volume.

Table VI. Percent RSD* for Different Re-equilibration Times for a 50- ¥ 4.6-mm Chromolith Column

	%RSD t_R						
Time (min)	Peak 1	2	3	4	5	6	7
0.50	n/a†	n/a	n/a	3.50	2.89	1.25	0.89
0.75	10.40	10.37	7.21	0.93	0.49	0.80	0.84
1.00	2.57	2.91	2.34	0.80	0.56	0.63	0.71
1.50	1.20	1.39	1.06	0.73	0.67	0.81	0.76
1.75	3.29	3.40	3.07	1.08	0.80	0.98	0.95
2.00	2.48	3.03	2.32	0.91	0.59	0.51	0.52
2.50	2.15	2.27	1.70	0.72	0.75	0.90	0.87
3.00	2.80	3.16	2.72	0.73	0.83	1.13	1.11

* $n = 5$.
† n/a, not available.

LC conditions for both t_R and peak area using the same mixture. Fifteen replicates per column were performed. In the column-to-column study, the RSDs for t_R ranged from 0.25% to 5.07% and for peak area from 1.08% to 6.77%. RSDs for the repeatability (run-to-run) study ranged for t_R from 0.25% to 4.56% and for peak area from 1.58% to 4.07%. It should be noted that the percent RSDs for t_R seem large for the early eluters because of their short t_R. For example, the range of retention times for peak one over the six columns is 0.012 min (0.72 s), which resulted in a percent RSD of 4.36. Overall, the percent RSDs show that the monolithic columns studied have excellent run-to-run and column-to-column precision for both t_R and peak area for fast HPLC analyses.

One disadvantage of using high flow rates is that they are only possible with lower viscosity solvent systems (i.e., acetonitrile–water). Higher viscosity systems (such as methanol–water) limit flow rates as much as 50%. Although the consumption of solvent and the production of solvent waste may seem to be a disadvantage, the solvent consumption per analysis was comparable with the fast LC method and the method developed for the particulate columns. A 10-min analysis at 1.5 mL/min (as shown in Figure 2) produces 15 mL of waste solvent, but the 1-min run at 8 mL/min (as shown in Figure 4) only produces 8 mL.

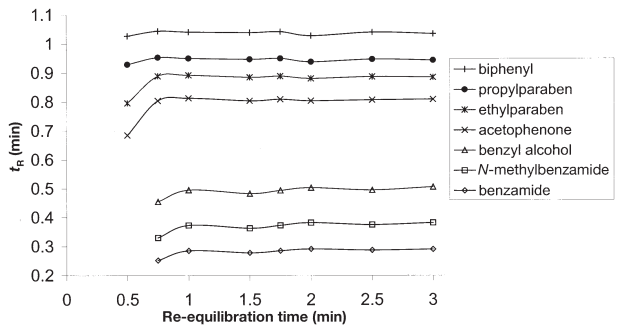


Figure 6. Retention times as a function of re-equilibration time for the seven-component test mix (Table I) using the Chromolith SpeedROD and chromatographic conditions listed in Figure 4.

Acknowledgements

EM Science, Phenomenex, Varian, Optimize Technologies, and Waters graciously donated the columns used in this study. Scynexis (Research Triangle Park, NC) developed the seven-component test mix and provided funding for this research. Portions of this paper were presented at the 52nd Pittsburgh Conference (New Orleans, March 2002) and the 50th International Symposia on Capillary Chromatography and Related Techniques (Riva del Garda, Italy, May 2002).

Table VII. Column-to-Column Precision. Comparison of the Average t_R for each of the Six Chromolith Columns*

	Average t_R (min)						
Column	Peak 1	2	3	4	5	6	7
1	0.267	0.347	0.455	0.778	0.868	0.939	1.031
2	0.264	0.345	0.462	0.782	0.867	0.935	1.027
3	0.283	0.371	0.490	0.797	0.877	0.941	1.034
4	0.296	0.391	0.513	0.805	0.885	0.943	1.035
5	0.291	0.384	0.502	0.800	0.880	0.941	1.033
6	0.280	0.367	0.486	0.793	0.876	0.940	1.032
Average	0.280	0.367	0.484	0.792	0.876	0.940	1.032
%RSD	4.56	5.07	4.67	1.36	0.79	0.26	0.25

* Fifteen replicates for each column.

Table VIII. Column-to-Column Precision. Comparison of the Average Peak Area for Each Component for Each of the Six Chromolith Columns*

	Average peak area						
Column	Peak 1	2	3	4	5	6	7
1	621771	537190	189518	157446	286998	281559	406450
2	596047	516236	185178	140665	272025	246185	362019
3	604251	521204	187680	143635	267027	247057	381607
4	611081	520134	187442	145332	277571	232630	376937
5	617698	517869	190944	147387	277820	241557	371786
6	603537	511233	186948	142864	277262	244196	366525
Average	609120	520711	187982	146216	276410	249066	377544
%RSD	1.58	1.70	1.08	4.07	2.42	6.77	4.18

* Fifteen replicates for each column.

Table IX. Run-to-Run Precision. Percent RSD of t_R and Peak Area for a Seven-Component Mixture Under Fast Gradient Conditions for Six Chromolith Columns

	%RSD t_R							%RSD peak area						
Column	Peak 1	2	3	4	5	6	7	1	2	3	4	5	6	7
1	4.89	5.47	4.47	1.64	1.12	0.72	0.66	5.16	5.66	5.30	4.90	5.56	4.60	6.96
2	5.58	6.51	5.82	1.94	1.54	1.31	1.27	5.68	5.92	4.56	6.03	6.25	8.95	8.86
3	4.77	5.32	4.50	1.68	1.37	1.04	1.06	6.53	6.77	6.75	8.06	11.65	11.67	7.17
4	4.77	5.52	4.18	1.62	0.99	0.79	0.80	3.94	3.31	3.45	4.97	4.47	10.80	6.84
5	5.39	5.60	3.36	0.95	0.73	0.80	0.73	4.44	4.45	4.33	5.55	6.46	6.36	8.15
6	1.87	2.14	1.69	0.52	0.55	0.82	0.82	3.39	3.15	3.55	4.49	2.66	2.46	7.14
Average	4.55	5.09	4.00	1.39	1.05	0.91	0.89	4.86	4.88	4.65	5.67	6.18	7.47	7.52

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