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Repeatability and reproducibility of retention data and band profiles on reversed-phase liquid chromatography columns V. Results obtained with Vydac 218TP C_{18} columns

Marianna Kele[a,b], Georges Guiochon[a,b,*]

[a]*Department of Chemistry, The University of Tennessee, 552 Buehler Hall, Knoxville, TN 37996-1600, USA*
[b]*Division of Chemical and Analytical Sciences, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6120, USA*

Abstract

The general goal of this work is to investigate the precision of chromatographic data and to determine which properties of chromatographic columns influence this factor. Chromatographic data were acquired under five different sets of experimental conditions for 30 neutral, acidic and basic test compounds on columns packed with Vydac 218TP C_{18}, a polymeric, wide-pore silica-based stationary phase. Five columns packed with samples from the same batch of this packing material were used to measure the column-to-column reproducibility and six columns packed with material from six different batches to measure the batch-to-batch reproducibility. The parameters studied were the retention time, the retention and separation factors, the hydrophobic and the steric selectivities, the column efficiency, and the tailing factor.

Keywords: Retention data; Band profiles; Stationary phases, LC; Vydac 218TP C_{18} columns

1. Introduction

The purpose of this work is to further our understanding of the relationship between the column-to-column and batch-to-batch reproducibilities of commercial columns packed with reversed-phase packing materials, the precision of chromatographic analyses, and the properties of these materials. In previous papers we published the experimental protocol used [1] and discussed results obtained on two monomeric type stationary phases, Waters Symmetry C_{18} [2] and Kromasil C_{18} [3], and on a stationary phase with proprietary surface bonding, Luna C_{18} (2) [4]. Based on our results we concluded that the batch-to-batch reproducibility achieved with these columns should satisfy the needs of most analysts. However, reproducibility problems were observed for the kinetic data (i.e., peak efficiency and symmetry) pertaining to some basic or chelate forming compounds. The compounds affected and the test conditions involved were different for the different brands. This result suggests that the type and the acidity of the residual surface silanols are different on the different brands of stationary phases. As in previous studies [1–4], this study was undertaken using columns from production batches considered as typical by the manufacturer.

The Vydac 218TP C_{18} stationary phase used in this study differs from those previously studied in two ways. First, it is based on a wide pore silica (270 Å instead of 91 [2], 111 [3], and 104 [4]Å). Second,

*Corresponding author. Department of Chemistry, The University of Tennessee, 552 Buehler Hall, Knoxville, TN 37996-1600, USA. Tel.: +1-865-9740-733; fax: +1-865-9742-667.
E-mail address: guiochon@utk.edu (G. Guiochon).

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the surface modification is carried out using polyfunctional octadecylsilanes in the presence of water, resulting in the formation of a polymeric C_{18} layer on the silica surface. Polymeric stationary phases are generally considered to be less reproducible or more difficult to reproduce than monomeric ones [5,6], in spite of the results of Sander and Wise [7] who showed that polymeric phases can be prepared reproducibly by using a single lot of silica and carefully controlling the water content in the reaction mixture. They reported the preparation of four different samples of a polymeric phase with a relative standard deviation (RSD) of only 0.96% for their surface coverage. Thus, the present study could illustrate the combined effect of a pore enlargement and of the polymerization of the reagent on the reproducibility of stationary phase batches.

Silica gels having an average pore size of 300 Å can be prepared by manipulating the average size of the sol used to prepare the silica particles or by enlarging the pores of narrow-pore silicas. This second approach involves a hydrothermal treatment either under pressure or with a stream of steam at atmospheric pressure [8,9]. The method is based on the dependence of silica solubility on the radius of curvature of the silica surface. Another method, introduced by Krebs and Heintz [10], is based on filling the pores of silica gel with an inorganic salt with a high melting point and calcinating the composite. This method gives wider pores than hydrothermal treatments and is used for preparation of silica with pore size 500 Å and higher. Tanaka et al. [11] studied the pore structure of wide pore silicas and of polymer gels using transmission electron microscopy of Vydac TP (''totally porous'' 300 Å) silica, among other samples. They concluded that this silica exhibits an irregular internal structure (and not a corpuscular structure) and contains a few huge pores.

In addition to the pore size enlargement step, the preparation of this stationary phase involves the polymerization of the silanization reagent for surface modification. Two major approaches can be used in the preparation of polymeric stationary phases. The reagent can be polymerized and covalently attached to the surface silanols or the surface can be covered with a thin layer of a polymer (encapsulation) which does not react with the surface silanols.

The first approach includes the solution and the surface polymerization processes. In the so-called solution polymerization (or vertical polymerization [7]) process water is added to a the reaction slurry. The polymerization of di- or trifunctional silanes takes place in solution, with subsequent linkage of the polymer to the silica surface. Surface polymerization (also referred to as horizontal polymerization [12–16]) takes place in anhydrous solvents if water is adsorbed on the silica surface prior to the introduction of the di- or trifunctional silanes. The density of the C_{18} chains is controlled by the addition of silanes having shorter alkyl chains (spacers).

Polymer encapsulated (capsule-type, coated) stationary phases [17–20] were designed on the assumption that the inner surface of silica particles can be covered with a thin layer of a polymer. This layer makes the particles more resistant to harsh pH conditions, shields the surface silanols, and binds polymers that can be further modified.

The Vydac stationary phase is prepared by reacting a wide pore silica with a trifunctional silane, in a solution polymerization process. It is recommended for peptide separations. The characterization of wide-pore reversed-phase packing materials is carried out with both small molecule standards and peptides and proteins [21–23]. In order to investigate whether the original test compounds [1] used in this study are able to predict and characterize the chromatographic behavior of a stationary phase used for peptide separations, we included an additional test suggested by Mant and Hodges [24,25]. According to these authors, the separation of the four synthetic undecapeptides of the test mixture demonstrates the presence and the extent of free silanols.

2. Experimental

The experimental conditions were described in detail and discussed earlier [1]. We merely summarize below the essential points of the protocol and discuss the minor changes required for its application to the new packing material.

2.1. Experimental conditions and columns

The experimental data were acquired using a Hewlett-Packard (Palo Alto, CA, USA) HP 1100

liquid chromatograph including a binary solvent delivery system, an autosampler, a diode array UV detector, a column thermostat, and a data station. All these units were controlled by a dedicated computer (equipped with a Pentium processor and operating under Windows 95). Automatic data acquisition and the determination of most parameters were performed using the standard features of this instrument (ChemStation Software, Rev. A. 05.03). The data are regularly ''burnt'' into a CD-ROM for archiving and authentication purposes.

The instrument tests corresponding to the operational qualification and performance verification procedures for the HP 1100 Series HPLC modules were performed weekly and after each maintenance of the equipment.

The column temperature was maintained at 25.0°C by the instrument controller. Systematic measurements of the temperature with an independent thermometer, as previously described [1], confirm the stability of this parameter within 0.1°C. The mobile phases (see composition later) were obtained by instructing the solvent delivery system to pump and mix the two required streams (pure water or buffer and pure methanol) in the proper ratio, using the binary pump. The total flow-rate was scaled up compared to the original protocol (see discussion in Ref. [3]) to adjust for the difference in column diameter. This flow-rate was 1.39 ml/min in all tests. The columns were equilibrated with the required mobile phase for 5 h before the first sample injection.

The injection volume was 18 μl. Each sample was injected in five replicates. The changes in eluent composition at column outlet were detected with the UV detector at 220, 230, 254, 270 and 290 nm. The 254 nm signal was used for the data interpretation.

The experimental results reported in this work were acquired with 11 columns (250×4.6 mm) packed with Vydac 218TP54 C_{18}, a reversed-phase liquid chromatography (RPLC) packing material from Vydac (Separation Group, Hesperia, CA, USA) based on wide-pore (300 Å pore diameter) silica. The particles are spherical, with an average size of 5 μm. The main characteristics of the bare silica are summarized in Table 1. The values listed there were measured and supplied by the manufacturer. The silica surface is covered with a polymeric C_{18} layer using polyfunctional octadecylchlorosilanes. All the batches studied had also been end-capped (C_1 and C_3).

We used five columns packed with samples from the same batch of packing material (batch number E970225-8-3) and six columns packed with samples from six different batches (batch numbers E960610-3-2, E960912-9-3, E970225-8-3, E970416-9-1, E970416-9-2 and E970401-4-2). The six reversed-phase batches were based on five batches of silica. The columns were packed by the manufacturer and used as received.

2.2. Samples and chemicals

The qualitative and quantitative compositions of the five test mixtures used are given below. Because the column dimensions and the average surface area were different from those of the first brand of packing material studied [1,2], the flow-rate, the

Table 1
Physico-chemical properties of the six batches of silica used for the stationary phase preparation (Vydac 218TP C_{18}) supplied by the manufacturer (Separation Group, Hesperia, CA, USA)

Silica batch No.	Particle size (μm), median	Particle size ±1 μm (%)	Particle size ±2 μm (%)	Pore size (Å)	Pore volume (ml/g)	Surface area (m^2/g)
E960610	4.7	94.6	99.7	256	0.41	67.2
E960912	5.0	92.4	99.5	279	0.47	75.0
E970401	4.8	90.7	99.1	273	0.40	65.5
E970416	5.3	86.8	99.1	279	0.47	72.6
E970225	4.8	90.7	99.8	253	0.45	72.2
Mean	4.98	91.25	99.38	269.83	0.445	70.85
RSD (%)	5.30	4.30	0.33	4.50	7.21	5.17

sample concentration, and the sample load were scaled up compared to those described in the protocol [1] and used with the first brand studied (Symmetry C_{18}; Waters, Milford, MA, USA) [2]. Detailed explanations of the scale-up calculations are provided elsewhere [3].

Sample 1: thiourea (3.2 mg/l), phenol (32.4 mg/l), 1-chloro-4-nitrobenzene (5.4 mg/l), toluene (140.9 mg/l), ethylbenzene (117.0 mg/l), butylbenzene (278.6 mg/l), *o*-terphenyl (11.9 mg/l), amylbenzene (279.7 mg/l), triphenylene (3.2 mg/l) in methanol–water (80:20).

Sample 2a: thiourea (3.2 mg/l), phenol (32.4 mg/l), aniline (22.1 mg/l), ethyl benzoate (141.2 mg/l), toluene (234.9 mg/l), ethylbenzene (234.1 mg/l) in methanol–water (55:45).

Sample 2b: thiourea (3.2 mg/l), phenol (32.4 mg/l), *N*,*N*-dimethylaniline (10.3 mg/l) in methanol–water (55:45).

Sample 2c: thiourea (3.2 mg/l), phenol (32.4 mg/l), *o*-toluidine (21.1 mg/l), *m*-toluidine (15.8 mg/l), *p*-toluidine (30.0 mg/l) in methanol–water (55:45).

Sample 3: thiourea (3.2 mg/l), theobromine (4.9 mg/l), theophylline (8.1 mg/l), caffeine (8.6 mg/l), phenol (43.2 mg/l), 2,3-dihydroxynaphthalene (54.0 mg/l) in methanol–water (30:70).

Sample 4: thiourea (3.24 mg/l), butylparaben (5.4 mg/l), dipropylphthalate (91.8 mg/l), propranolol (108.0 mg/l), acenaphthene (54 mg/l) in methanol–water buffer (20 m*M*) with potassium phosphate, monobasic/dibasic at pH 7.00 (65:35).

Sample 5a: thiourea (3.2 mg/l), benzylamine (53.0 mg/l), benzyl alcohol (169.1 mg/l), benzoic acid (54.0 mg/l) in methanol–water buffer with phosphoric acid/potassium monophosphate buffer (20 m*M*) at pH 2.70 (30:70).

Sample 5b: procainamide (3.2 mg/l), phenol (43.2 mg/l), in methanol–water buffer with phosphoric acid/potassium monophosphate buffer (20 m*M*) at pH 2.70 (30:70).

Sample 6: 1. Ac–Gly–Gly–Gly–Leu–Gly–Gly–Ala–Gly–Gly–Leu–Lys–amide [p*I*=6.0], 2. Ac–Lys–Tyr–Gly–Leu–Gly–Gly–Ala–Gly–Gly–Leu–Lys–amide [p*I*=7.9], 3. Ac–Gly–Gly–Ala–Leu–Lys–Ala–Leu–Lys–Gly–Leu–Lys–amide [p*I*=9.75], 4. Ac–Lys–Tyr–Ala–Leu–Lys–Ala–Leu–Lys–Gly–Leu–Lys–amide [p*I*=9.95] in water. The separation was carried out with a linear gradient from 5% B to 65% B in 120 min, with eluent A: 0.02% (v/v) trifluoroacetic acid (TFA) in water and eluent B: 0.02% (v/v) TFA in acetonitrile. Sample injection was made at the dwell time of the equipment and detection at 210 nm.

The samples (test mixtures) 2 and 5 initially described [1] and used [3–5] could not be resolved completely on this phase. Reliable data regarding the peaks of their components were obtained by splitting them into several partial mixtures. Because pyridine and 2,2′-dipyridyl did not elute from this stationary phase under the experimental conditions of test 3, these compounds were removed from the test mixture. The last test was added because the phase studied is mostly used for the separation of oligopeptides. The tests were carried out in the order listed.

The chemicals were obtained from Fluka, a Sigma–Aldrich Company (Milwaukee, WI, USA), except *o*-toluidine, benzylamine, methanol, and water, which were from Fisher Scientific (Pittsburgh, PA, USA). The undecapeptide standard kit was purchased from Alberta Peptide Institute (University of Alberta, Edmonton, Canada). The chemicals used in the work described here were all recently acquired. They were used as received. In order to avoid possible errors caused by fluctuations of the buffer composition due to the lack of reproducibility of its preparation, the same buffer solution was used for all the columns tested, for each test. For the same reason, the flasks containing the two mobile phase components were constantly sparged with a helium stream in order to avoid the dissolution of carbon dioxide from the laboratory.

2.3. Presentation of the data

For the sake of clarity, the terms used in this paper are now defined and explained.

The **short-term repeatability** is the RSD of the results of five consecutive runs carried out with one column over a period of a few hours. Short-term repeatability data of retention times, retention factors and selectivity factors measured on columns of other brands were already published and discussed [1–4].

The values obtained in this study closely match those previously published. The **long-term repeatability** is the RSD obtained by repeating the series of five consecutive analyses of the test mixture

on the same column after the whole series of measurements involved in the study had been completed on all the columns tested (a total of 11). This interval was typically 10 days.

The **column-to-column reproducibility** is the RSD of the 25 injections (five consecutive injections on each column) made on the five columns packed with packing material coming from the same batch. The **batch-to-batch reproducibility** is the RSD of the 30 injections made on six columns packed with material from the six different batches of the reversed-phase packing material.

3. Results and discussion

3.1. Absolute retention data

The column-to-column reproducibility (five columns packed with identical packing material), the batch-to-batch reproducibility (six columns packed with packing material from six different batches) and the long-term repeatability of the retention times are plotted in Figs. 1–5. The RSDs of the retention times vary between 0.06 and 0.25% on the five-column set, with the exception of three basic compounds (aniline, *N*,*N*-dimethylaniline and propranolol). The large RSDs observed for these compounds (2.07, 1.66 and 0.74%, respectively) are related to the high long-term repeatability of these values that are comparable with the RSDs measured for the five columns.

In general, the column-to-column reproducibility is not significantly different from the long-term repeatability of the experiment. This is not due to high long-term repeatability values (those are practically the same as the values obtained previously for other brands of stationary phases) but to the very small differences between the five columns. We showed earlier [26] that the retention times differences on two columns packed with the same packing material are due to column-tubing volume and to total porosity differences, the former being the major factor. The values obtained with this set of columns indicate that the five columns were packed using a column tubing with very low size fluctuations. Since the specifications of the column tubing dimensions predict larger retention time differences on the five columns than the observed ones, we can assume that

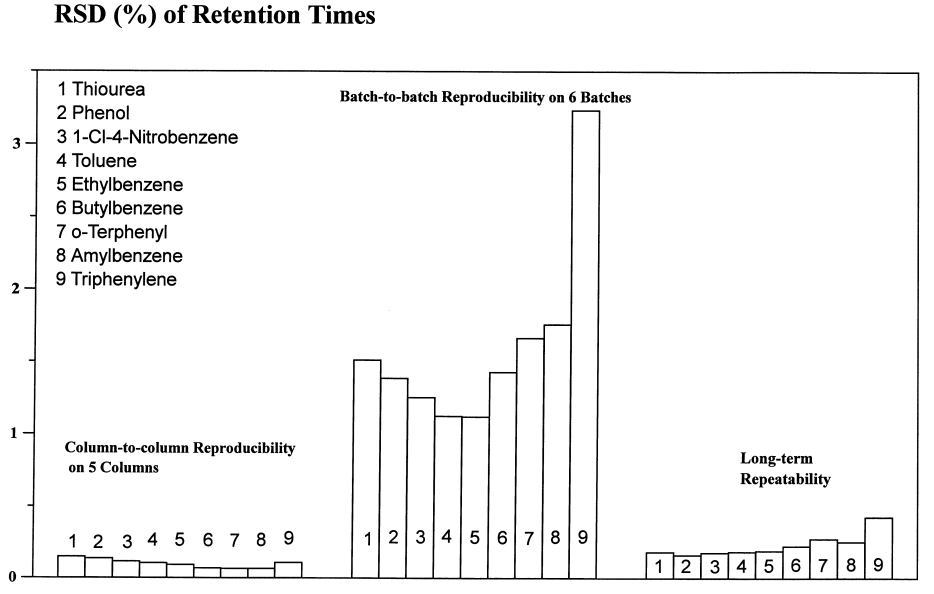


Fig. 1. Reproducibility of the retention time measured in the first test. 1=Thiourea, 2=phenol; 3=1-chloro-4-nitrobenzene; 4=toluene; 5=ethylbenzene; 6=butylbenzene; 7=*o*-terphenyl; 8=amylbenzene; 9=triphenylene. Mobile phase, methanol–water (80:20) at 1.39 ml/min.

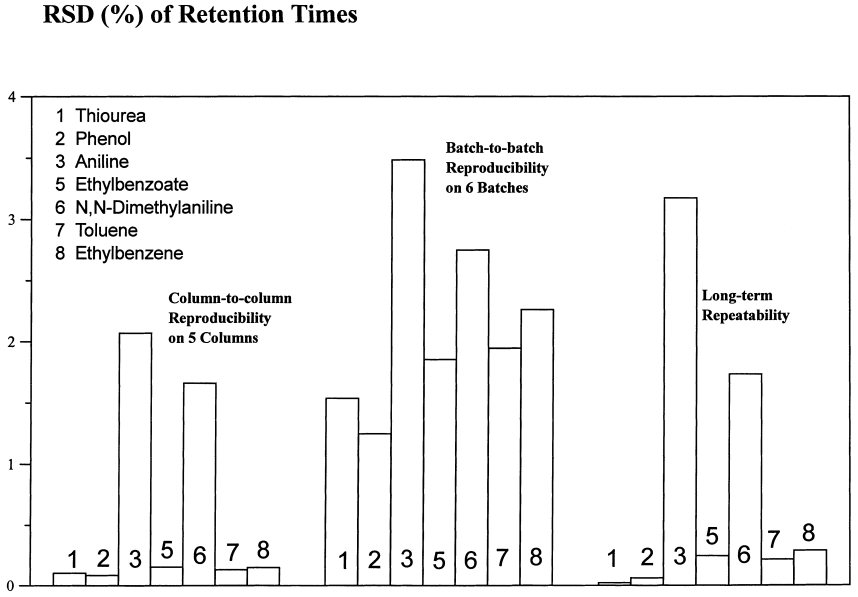


Fig. 2. Reproducibility of the retention time measured in the second test. 1=Thiourea; 2=phenol; 3=aniline; 5=ethyl benzoate; 6=*N*,*N*-dimethylaniline; 7=toluene; 8=ethylbenzene. Mobile phase, methanol–water (55:45) at 1.39 ml/min.

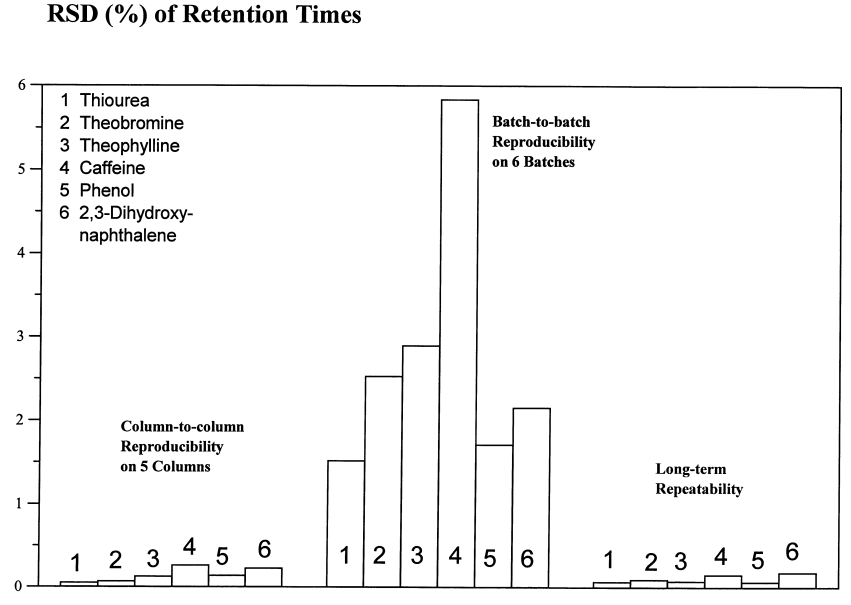


Fig. 3. Reproducibility of the retention time measured in the third test. 1=Thiourea; 2=theobromine; 3=theophylline; 4=caffeine; 5=phenol; 6=2,3-dihydroxynaphthalene. Mobile phase, methanol–water (30:70) at 1.39 ml/min.

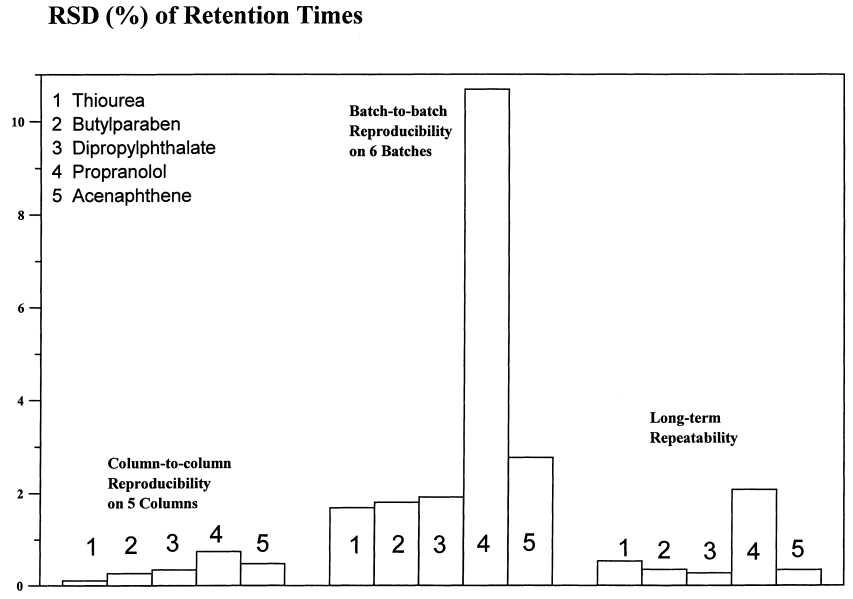


Fig. 4. Reproducibility of the retention time measured in the fourth test. 1=Thiourea; 2=butylparaben 3=dipropylphthalate; 4=propranolol; 5=acenaphthene; Mobile phase, methanol–water buffer with potassium phosphate, monobasic/dibasic at pH 7.00 (65:35) at 1.39 ml/min.

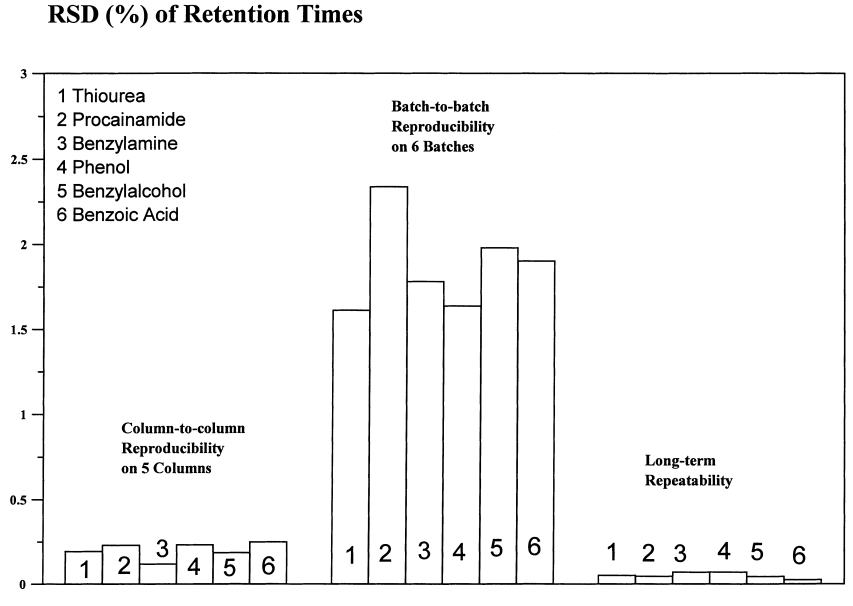


Fig. 5. Reproducibility of the retention time measured in the fifth test. 1=Thiourea; 2=procainamide; 3=benzylamine 4=phenol; 5=benzyl alcohol; 6=benzoic acid. Mobile phase, methanol–water buffer with phosphoric acid/potassium monophosphate buffer at pH 2.70 (30:70) at 1.39 ml/min.

the columns were packed using sections from the same batch of tubing, exhibiting fluctuations in a range narrower than the specifications.

The RSDs of the retention times measured on the six columns packed with six different batches vary between 1.1 and 11%. The values obtained for the basic compounds separated with a nonbuffered mobile phase (aniline and *N*,*N*-dimethylaniline in test 2) are not significantly different from those measured on the five-column set or from the long-term repeatability of the experiment. The high RSD (5.8%) of caffeine in test 3 cannot be explained by simple experimental errors since the long-term repeatability of the experiment is excellent (RSD=0.15%). It was stated by Kimata et al. [27] that the retention of caffeine is a good measure of the amount of residual silanols. On the packing materials previously studied [2–4], we observed no differences between the reproducibility of the retention of caffeine and that of the other test compounds in test 3. It is possible that the high RSD of caffeine retention on this brand of stationary phase can be attributed to the higher concentration (or ion-exchange ability) of the residual silanols on the surface. The high RSD of propranolol (a strong base with pK_a=9.6) and the behavior of the toluidine isomers seem to support this idea. The retention time reproducibilities of the toluidine isomers (test 2) are not included in Fig. 2. A typical chromatogram is given in Fig. 6 with indications of the RSDs of these retention times on the six batches. The three toluidine isomers are completely separated on all six batches. The separation of the toluidine isomers was explained by their interaction with residual silanol groups [28], since the hydrophobic properties of the three isomers are the same but not their pK_a values. Steric effects also

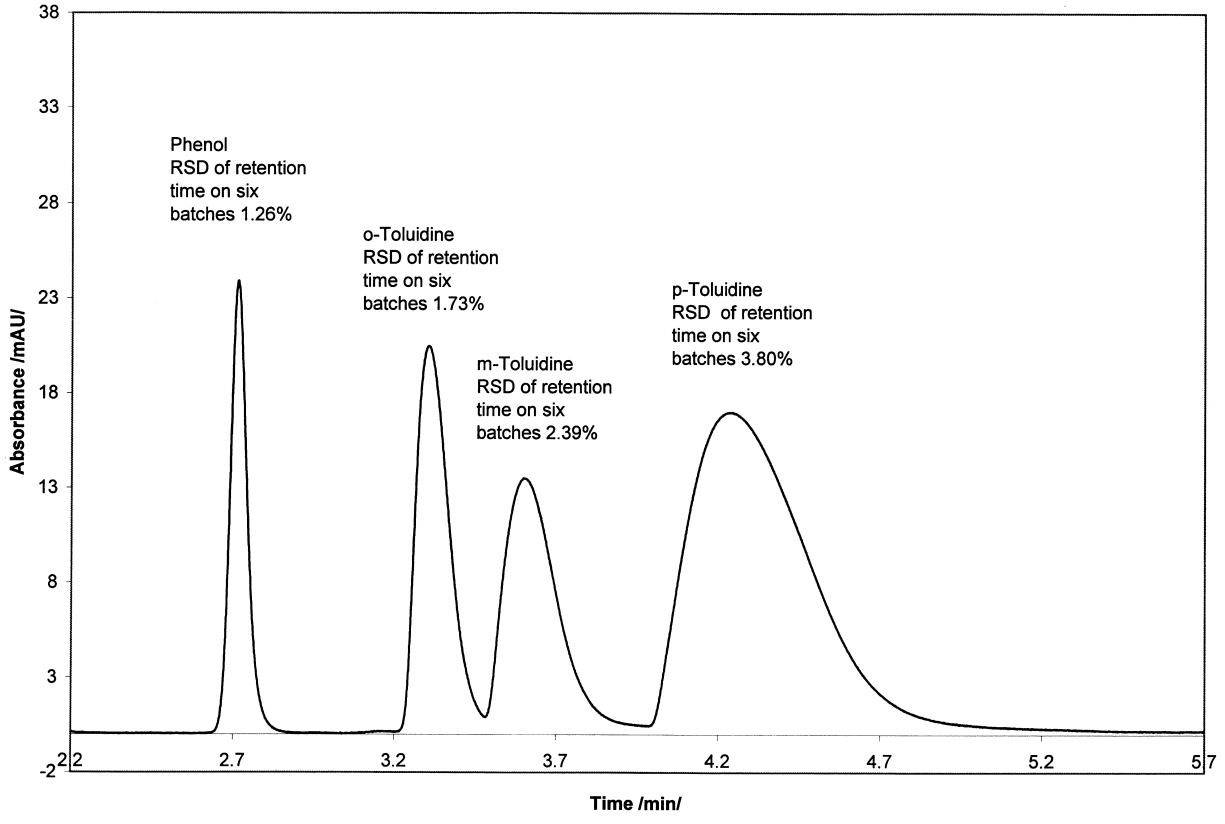


Fig. 6. Typical chromatogram of toluidine isomers; reproducibility of retention time of toluidine isomers on six batches. Mobile phase, methanol–water (55:45) at 1.39 ml/min.

Table 2
Reproducibility of the retention factors of the components of the first test mixture

	RSD (%) of k five columns from the same batch	RSD (%) of k six batches
Phenol	0.197	3.625
1-Chloro-4-nitrobenzene	0.166	3.539
Toluene	0.164	3.764
Ethylbenzene	0.168	3.772
Butylbenzene	0.157	3.877
o-Terphenyl	0.154	4.033
Amylbenzene	0.145	3.972
Triphenylene	0.131	4.533

might influence the separation of the three isomers [29]. The fact that aniline elutes after phenol is also an indication of stronger silanophilic interactions.

3.2. Retention factors

Tables 2–6 list the RSDs of the retention factors

Table 3
Reproducibility of the retention factors of the components of the second test mixture

	RSD (%) of k column-to-column reproducibility on five columns	RSD (%) of k batch-to-batch reproducibility on six batches
Phenol	0.176	3.140
Aniline	5.785	12.055
Ethyl benzoate	0.229	3.235
N,*N*-Dimethylaniline	2.202	3.905
Toluene	0.179	3.804
Ethylbenzene	0.182	3.891

Table 4
Reproducibility of the retention factors of the components of the third test mixture

	RSD (%) of k column-to-column reproducibility on five columns	RSD (%) of k batch-to-batch reproducibility on six batches
Theobromine	0.438	8.912
Theophylline	1.763	6.250
Caffeine	0.514	9.775
Phenol	0.203	3.343
2,3-Dihydroxynaphthalene	1.166	3.249

Table 5
Reproducibility of the retention factors of the components of the fourth test mixture

	RSD (%) of k column-to-column reproducibility on five columns	RSD (%) of k batch-to-batch reproducibility on six batches
Butylparaben	0.518	2.829
Dipropylphthalate	0.524	3.025
Propranolol	1.042	14.173
Acenaphthene	0.563	3.771

Table 6
Reproducibility of the retention factors of the components of the fifth test mixture

	RSD (%) of k column-to-column reproducibility on five columns	RSD (%) of k batch-to-batch reproducibility on six batches
Procainamide	12.849	42.777
Benzylamine	1.727	6.458
Phenol	0.200	3.412
Benzyl alcohol	0.374	3.536
Benzoic acid	0.316	3.138

of the different test compounds on the five columns of one batch and on the six columns of different batches. The averages of these retention factors for five successive injections are plotted in Figs. 7–11 versus the column rank. The column-to-column reproducibility varies between 0.2 and 0.5% for neutral and acidic compounds. For the basic compounds and for the neutral chelate-forming compound 2,3-dihydroxynaphthalene it varies between 1 and 13%. The batch-to-batch reproducibility varies between 2.8 and 4.5% for neutral and acidic compounds, between 3.9 and 43% for the basic compounds.

Toluene and ethylbenzene were used in two tests (tests 1 and 2). Under the different test conditions we obtained consistent reproducibilities for these compounds, both on the five-column set and on the six columns packed with samples of the six different batches.

The hydrophobicity or hydrophobic strength of a stationary phase relates to the overall strength of the dispersion forces between the solute and the station-

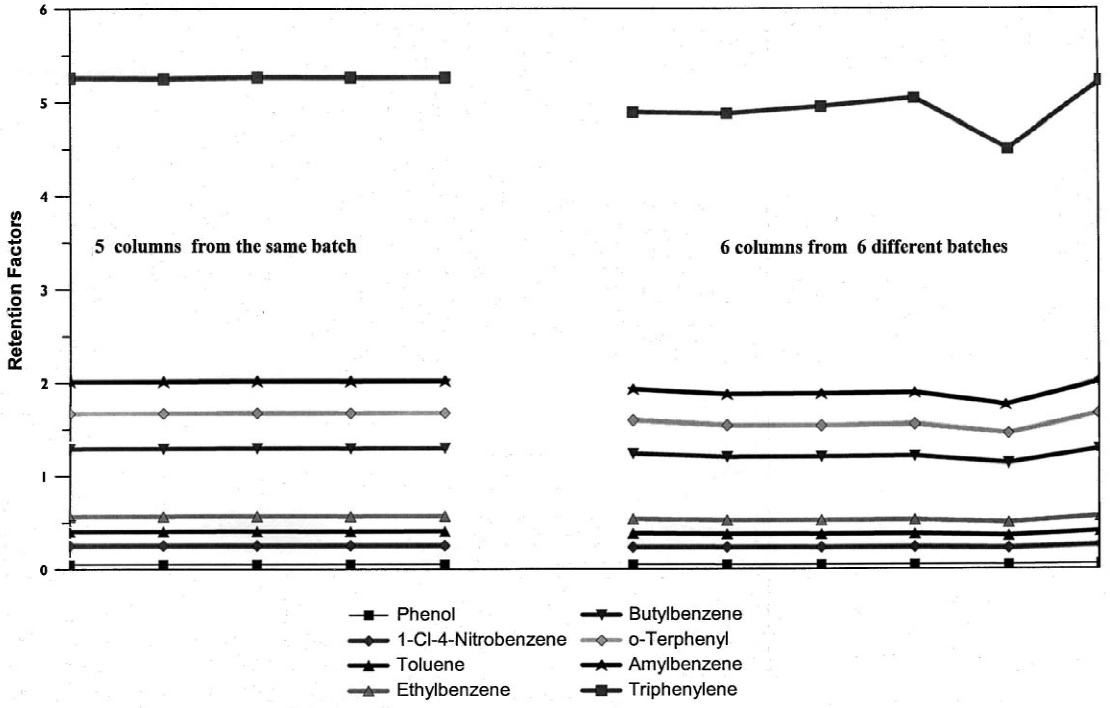


Fig. 7. Retention factors of the components of the first test mixture. Each data point represents the average of five consecutive injections carried out on a column.

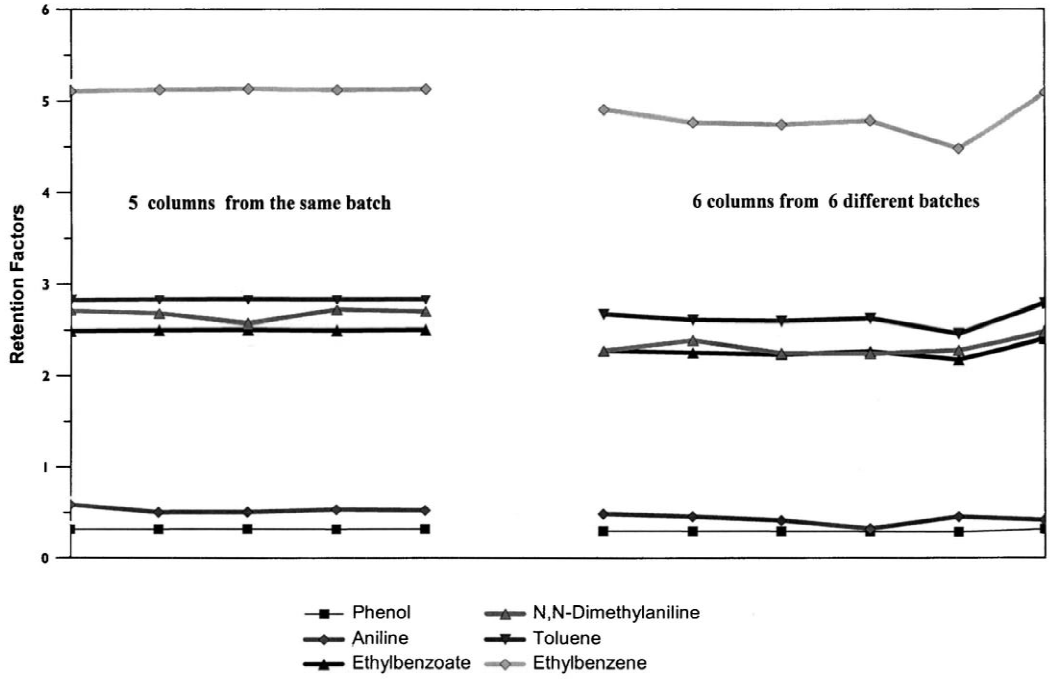


Fig. 8. Retention factors of the components of the second test mixture. Same data presentation as in Fig. 7.

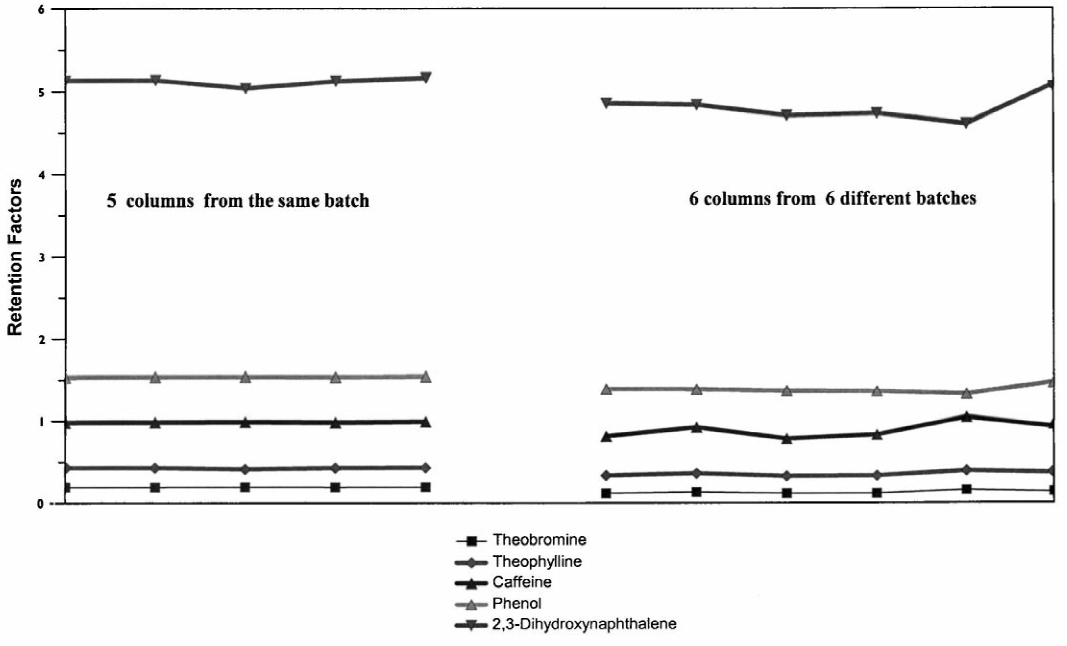


Fig. 9. Retention factors of the components of the third test mixture. Same data presentation as in Fig. 7.

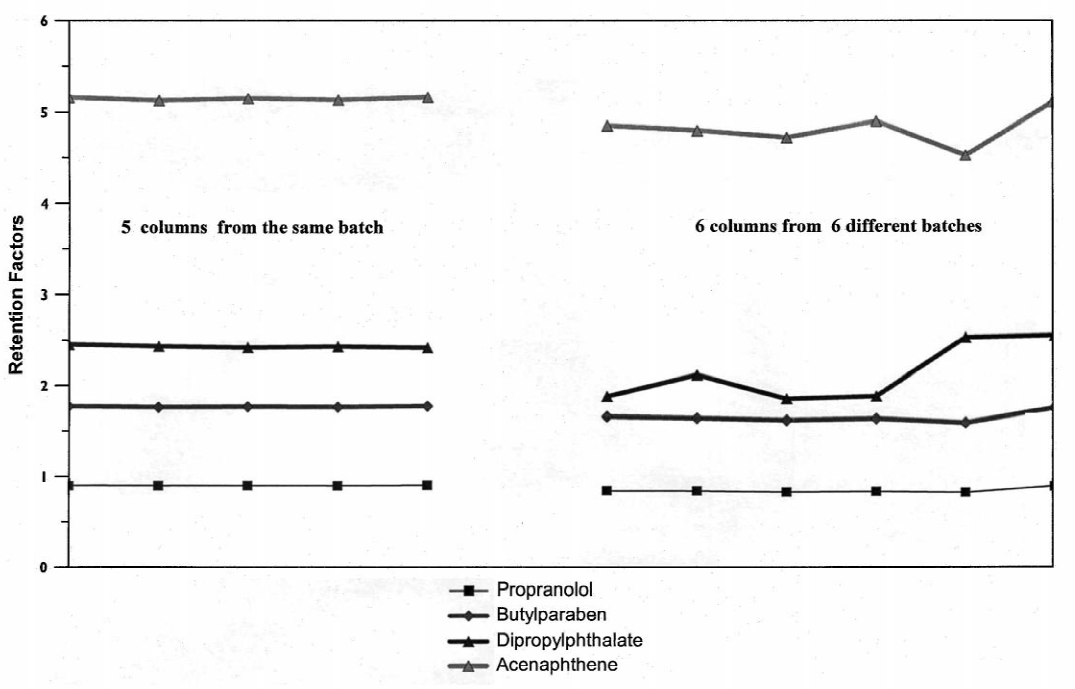


Fig. 10. Retention factors of the components of the fourth test mixture. Same data presentation as in Fig. 7.

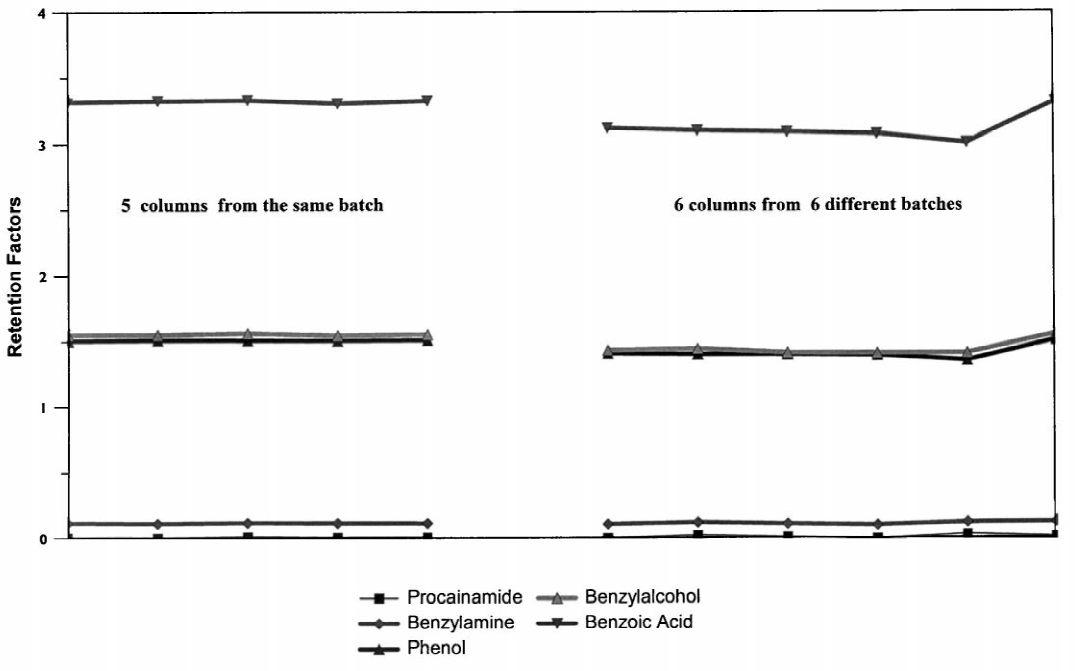


Fig. 11. Retention factors of the components of the fifth test mixture. Same data presentation as in Fig. 7.

ary phase. This parameter can be expressed as the retention factor of a nonpolar, nonionized compound (like an alkylbenzene). The hydrophobicity defined in this way depends on the eluent composition, i.e., on the organic content of the mobile phase. Our results indicate that the reproducibility of the hydrophobicity, expressed as the reproducibility of the retention factors of toluene and ethylbenzene is not a function of the eluent composition, at least in the range between 55 and 80% of methanol.

3.3. Relative retention

Relative retentions can be used for characterizing the differences in surface properties of stationary phases. This thermodynamic parameter is the ratio of the distribution constants of the selected compounds between the mobile and the stationary phase. At constant mobile phase composition and temperature, differences in relative retention or selectivity measured on different columns express the differences in the surface chemistry of the stationary phases. They may originate from differences in surface ligands (in the present case, differences in the degree of polymerization), from different surface coverages, and from differences in the types and ratios of the residual silanols.

The average relative retention data for the pairs of successively eluted peaks and their RSDs are listed in Table 7 for five columns from the same batch and for six columns representing the six different batches. The values reported differ from those previously obtained on monomeric stationary phases [2–4] for the pairs triphenylene/amylbenzene, aniline/phenol, caffeine/theophylline, and benzyl alcohol/phenol. In some cases, a reversal of the elution order was observed. The RSDs of the relative retentions vary between 0.015 and 10% on the five columns and between 0.16 and 42% on the set of six columns from different batches.

3.3.1. Methylene selectivity

In principle, the hydrophobic selectivity can be defined as the ratio of the retention factors of any two nonpolar, nonionic compounds which interact with the stationary phase surface only due to dispersion forces. The methylene selectivity is often used in stationary phase characterization and is defined as the relative retention of two successive members of an homologous series, differing by one methylene group.

The methylene selectivities are plotted in Fig. 12a–d. Individual data points are given in three groups, for the five-column sets, for the six-batch sets, and for the long-term repeatability. The corresponding RSDs are also included. The column-to-column reproducibility of the methylene selectivity corresponding to the different possible pairs varies between 0.01 and 0.028%. As expected, these values are not significantly different from the long-term repeatability of the measurements. The RSDs for the six batches vary between 0.16 and 0.43%. Although these values are at least twice as high as the corresponding RSDs measured on monomeric stationary phases [2,3], the difference between the different batches is not important. The relative retention for amylbenzene/butylbenzene correlates well with that for butylbenzene/ethylbenzene, except for one data point, while the ethylbenzene/toluene data for the two different mobile phases show an excellent correlation on the different batches.

3.3.2. Steric selectivity

The steric selectivity (or shape selectivity) is expressed as the relative retention of triphenylene/*o*-terphenyl in Fig. 13. This parameter was introduced by Kimata et al. [27]. All the data measured are plotted for the five-column and the six-column sets. The average steric selectivity of the six batches is 3.13 while it was 1.770 (Symmetry C_{18}) and 1.727 (Kromasil C_{18}) on the monomeric-type stationary phases [2,3]. The steric selectivity expressed as the relative retention of tetrabenzonaphthalene/benzo[*a*]pyrene [30,31] measured in acetonitrile–water (85:15) was 0.661 on the Vydac polymeric type phase versus 1.560 (Symmetry C_{18}) and 1.669 (Kromasil C_{18}) on the monomeric type phases. These two tests give consistent results regarding the selectivity difference between monomeric and polymeric type stationary phases.

Fig. 13 also reports the reproducibility of the steric selectivity calculated for the five-column and the six-column sets, and the long term repeatability of the experiment. As expected, the column-to-column reproducibility and the long-term repeatability of the experiment are comparable (0.09 and 0.15%, respec-

Table 7
Reproducibility of the relative retention data of the components of the five test mixtures

	One batch, five columns; average value of relative retentions	One batch, five columns; RSD (%) of relative retentions	Six batches; average value of relative retentions	Six batches; RSD (%) of relative retentions
Test 1 (MeOH–water, 8:2)				
Chloronitrobenzene/phenol	3.464	0.076	3.461	0.751
Toluene/chloronitrobenzene	1.540	0.017	1.543	0.739
Ethylbenzene/toluene	1.384	0.010	1.386	0.161
Butylbenzene/ethylbenzene	2.224	0.028	2.229	0.431
o-Terphenyl/butylbenzene	1.287	0.018	1.282	0.306
Amylbenzene/*o*-terphenyl	1.204	0.015	1.212	0.556
Triphenylene/amylbenzene	2.593	0.083	2.582	1.729
Test 2 (MeOH–water, 55:45)				
Aniline/phenol	1.626	5.900	1.400	12.510
Ethyl benzoate/aniline	4.406	5.628	5.194	14.448
Dimethylaniline/ethyl benzoate	1.073	2.332	1.023	2.655
Toluene/ethyl benzoate	1.080	2.367	1.133	3.341
Ethylbenzene/toluene	1.816	0.015	1.819	0.295
Test 3 (MeOH–water, 3:7)				
Theophylline/theobromine	2.398	1.791	2.400	2.883
Caffeine/theophylline	2.356	1.925	2.353	3.731
Phenol/caffeine	1.570	0.324	1.552	9.770
Dihydroxynaphthalene/phenol	3.406	1.149	3.416	0.571
Test 4 (MeOH–pH 7.0 buffer, 65:35)				
Dipropylphthalate/butylparaben	1.932	0.055	1.928	0.580
Propranolol/dipropylphthalate	1.373	1.442	1.291	13.630
Acenaphthene/propranolol	2.108	1.478	2.285	13.728
Test 5 (MeOH–pH 2.7 buffer, 3:7)				
Benzylamine/procainamide	5.656	10.403	5.925	42.396
Phenol/benzylamine	11.315	1.767	11.088	6.618
Benzyl alcohol/phenol	1.029	0.341	1.023	1.153
Benzoic acid/benzyl alcohol	2.130	0.233	2.150	0.989

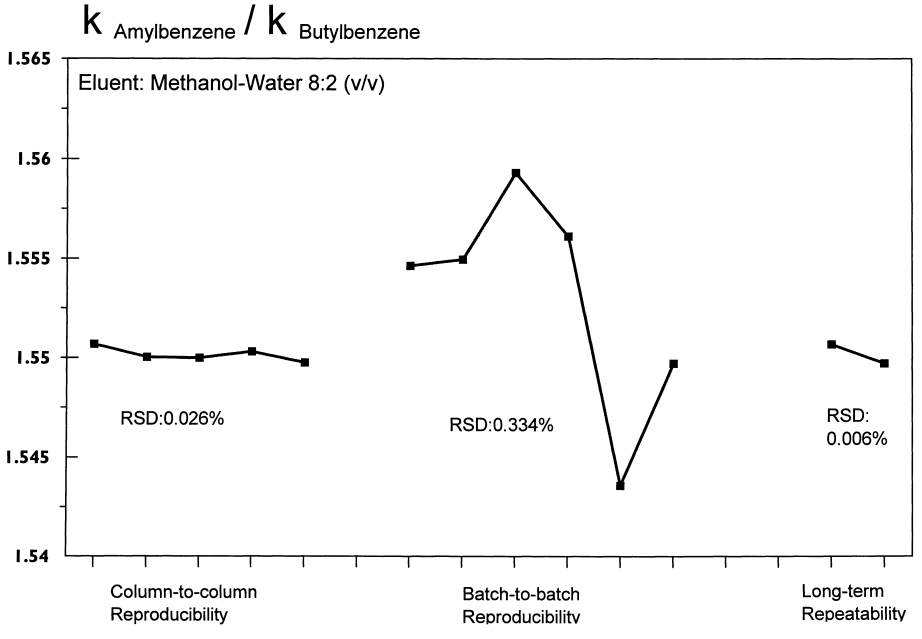
tively). The batch-to-batch reproducibility was 15 times higher than the repeatability of the experiment, indicating that the significant steric selectivity differences between the batches are real and do not originate from experimental errors.

3.3.3. Relative retention of basic compounds

Fig. 14a–d show the relative retention of the basic compounds relative to neutral ones measured on the five-column and the six-column sets, and the long-term repeatability of the experiment (indicated by stars). The relative retentions were measured in an unbuffered mobile phase for aniline/toluene and *N*,*N*-dimethylaniline/toluene, in a buffered mobile phase at pH 7.0 for propranolol/acenaphthene and at pH 2.70 for benzylamine/benzyl alcohol.

The RSD of the relative retention of the aniline/toluene pair is high on the six batches (13%) but the value obtained on the five-column set is also high. The retention time, the retention factor, and the relative retention values indicate that the experimental error contribution to these parameters is not negligible.

The values obtained in the buffered mobile phase at pH 7.0 indicate that under this test conditions the propranolol/acenaphthene pair is very sensitive to



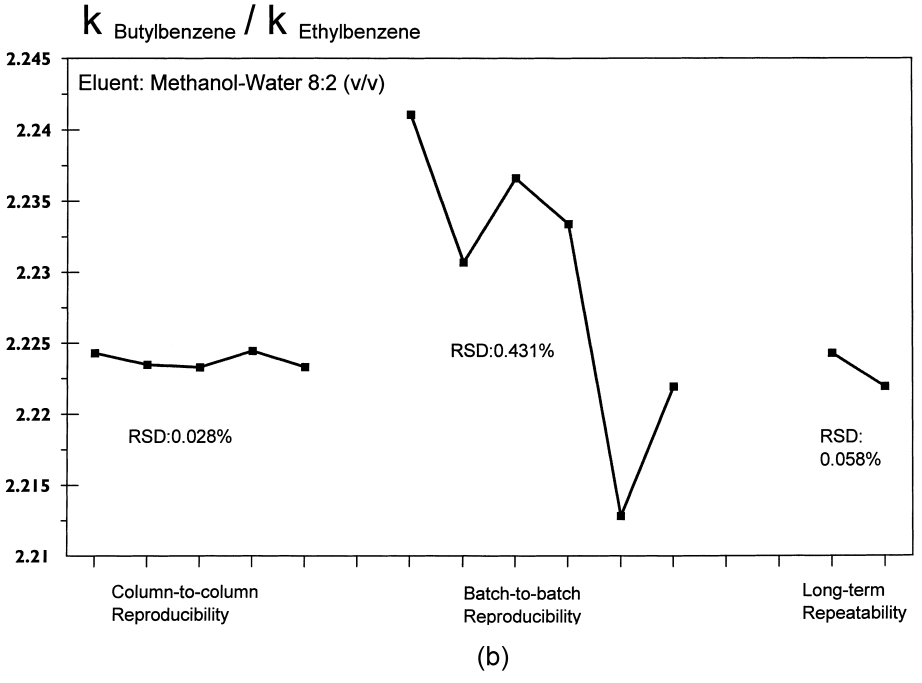
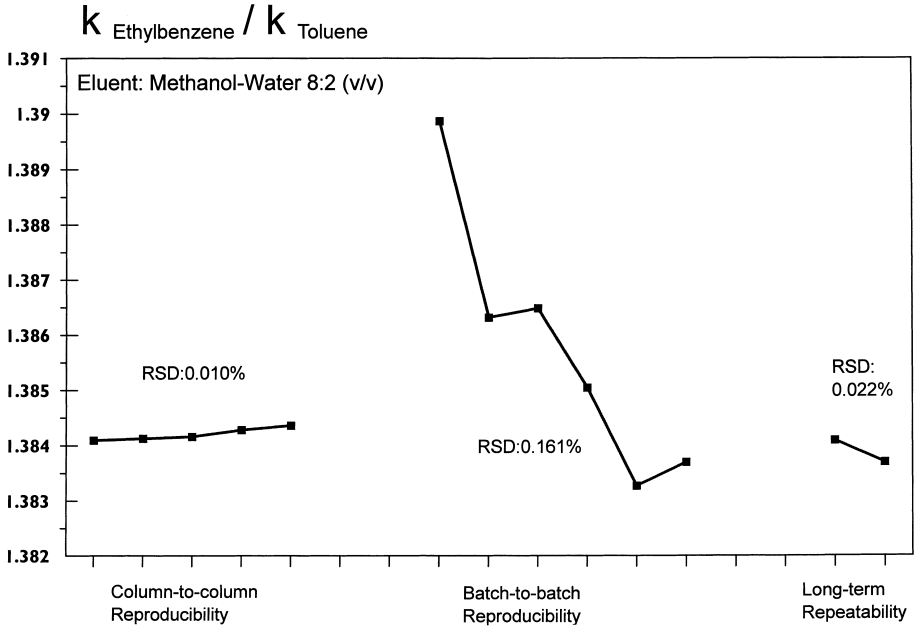
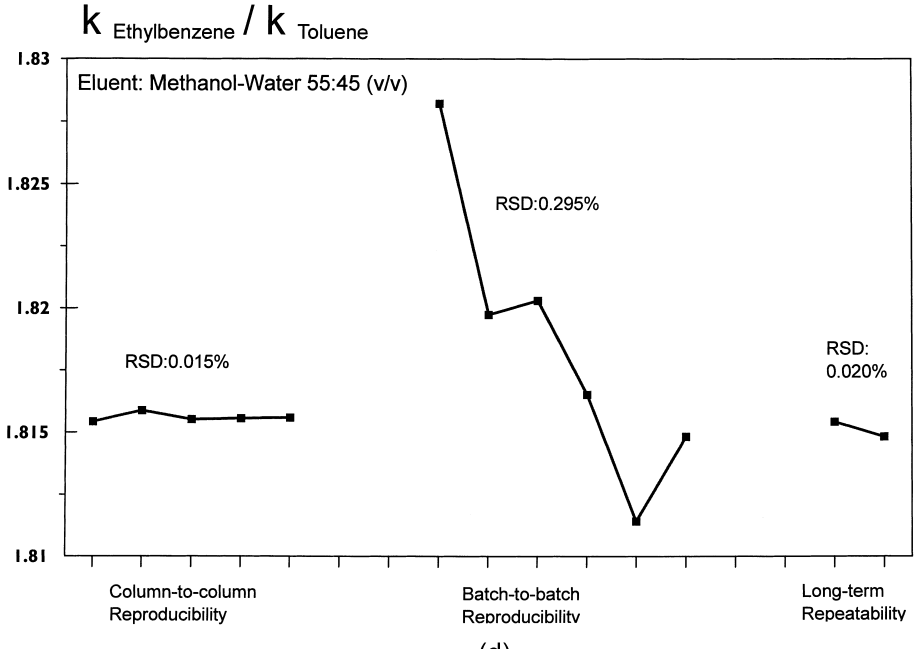


Fig. 12. Reproducibility of the hydrophobic selectivity. Each datapoint represents the average relative retention value calculated from the results of five consecutive injections carried out on a column. (a) Amylbenzene/butylbenzene (test 1). (b) Butylbenzene/ethylbenzene (test 1). (c) Ethylbenzene/toluene (test 1). (d) Ethylbenzene/toluene (test 2).



(c)



(d)

Fig. 12. (*continued*)

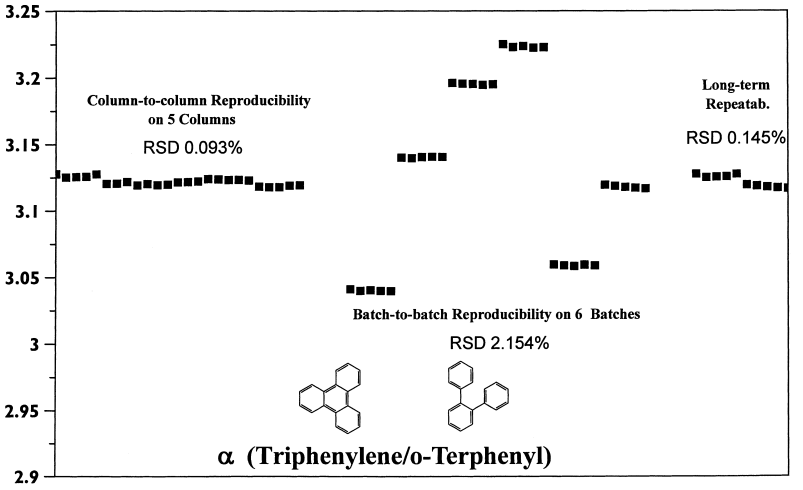


Fig. 13. Reproducibility of the steric selectivity.

batch differences. The RSD of this relative retention is 1.5% for the five-column set and 15%, 10 times higher, for the six-column set. The RSD of the relative retention of benzylamine/benzyl alcohol at pH 2.70 is four times higher on the six-column set than on the five-column set (5.6 and 1.5%, respectively).

3.4. Column efficiency

In this study, the reproducibilities of the band profiles are characterized by the RSDs of the column efficiencies and the tailing factors. The efficiencies are derived from the peak width measured at half-height. These RSDs are plotted in Figs. 15–19. For the first test mixture, which contains neutral nonpolar and polar test compounds and the weakly acidic phenol, the column-to-column reproducibility and the short-term repeatability closely match (Fig. 15). Although there might be slight differences between the five columns, due to differences in the heterogeneity of their bed, those are hidden by the ''experimental noise''. The long-term repeatability of the experiment is higher than the differences observed between the five columns for almost all compounds in the first test. The batch-to-batch reproducibility of the efficiency is typically twice as high as the long-term repeatability of the experiment. The RSDs vary between 3.2 and 5.6% for the compounds in test 1.

The RSDs of the efficiency of neutral compounds in test 2, which uses nonbuffered methanol–water (55:45), closely match the values obtained for the first test. The RSDs for the basic compounds are high for the five-column and the six-column sets but, because of the high value of the long-term repeatability of the experiment, no conclusions can be drawn. The RSDs of 2,3-dihydroxynaphthalene, the chelate-forming compound in the third test are 12 and 14% for the short-term and long-term repeatability of the experiment, respectively. The RSD on the five-column set is ca. twice that on the six-column set, i.e., four times higher (26 and 48%, respectively) than the repeatabilities of the experiment. This proves that the differences observed between the five column and the six-column sets do not originate from experimental errors but from the different degree of metal contamination of the silica surface. Two types of metal contaminations may affect the peak shape of a chelate-forming compound. Metal ions embedded in the silica matrix at the surface of the particles increase the acidity of the adjacent silanol groups and are able to form chelate complexes. Metal ions which are not embedded in the silica matrix but are attached to the silica surface by electrostatic forces affect the peak shape of complexing solutes but have no or negligible effect on the silanol acidity. Engelhardt and Lobert [32] concluded from their results that the main source of metal ions in a chromatographic column is the column hardware, especially the metal sieves and

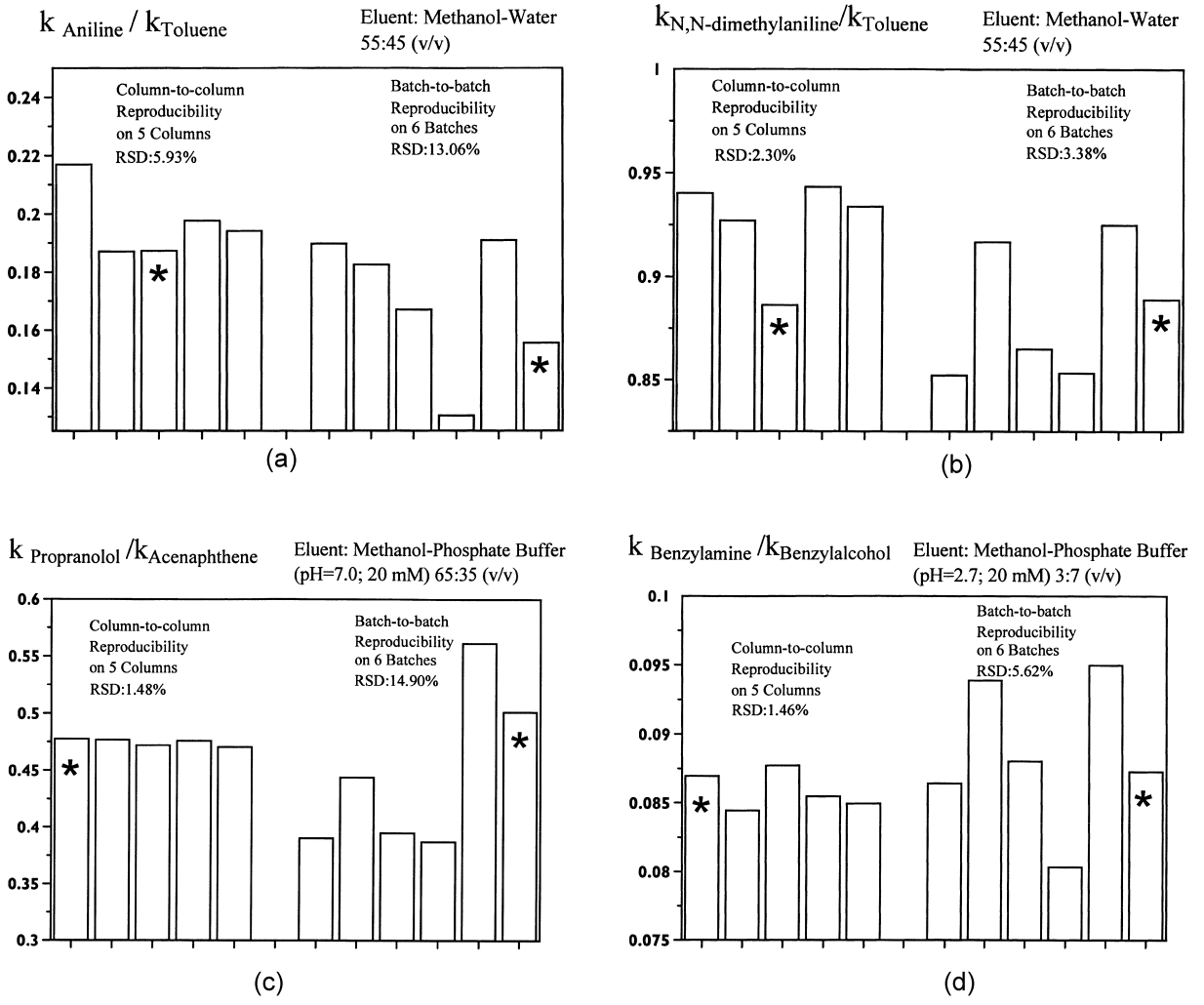


Fig. 14. Reproducibility of the separation factors of basic compounds. Each data point represents the average relative retention value calculated from the results of five consecutive injections carried out on a column. The stars indicate the long-term repeatability of the experiment. (a) Aniline/toluene (test 2). (b) *N,N*-Dimethylaniline/toluene (test 2). (c) Propranolol/acenaphthene (test 4). (d) Benzylamine/benzyl alcohol (test 5).

frits and also the HPLC-grade solvents. Unfortunately for this brand of stationary phase the metal contamination of the initial silica was not measured by the manufacturer, so we cannot define what type of metal contamination is responsible for the observed efficiency differences between the different batches. The relatively high value measured for the five-column set, however, indicates that metal contamination is introduced during the packing process and/or that the column hardware contribution varies from column-to-column (the five columns were packed with identical packing material and treated identically in our laboratory).

In test 4, the batch-to-batch reproducibility has practically the same RSD as the long-term repeatability, so we cannot make conclusions based on these results. However, it is interesting that the RSD of the efficiency of propranolol is always much lower than that of the other compounds of this test mixture. In test 5, we observed the same RSDs on

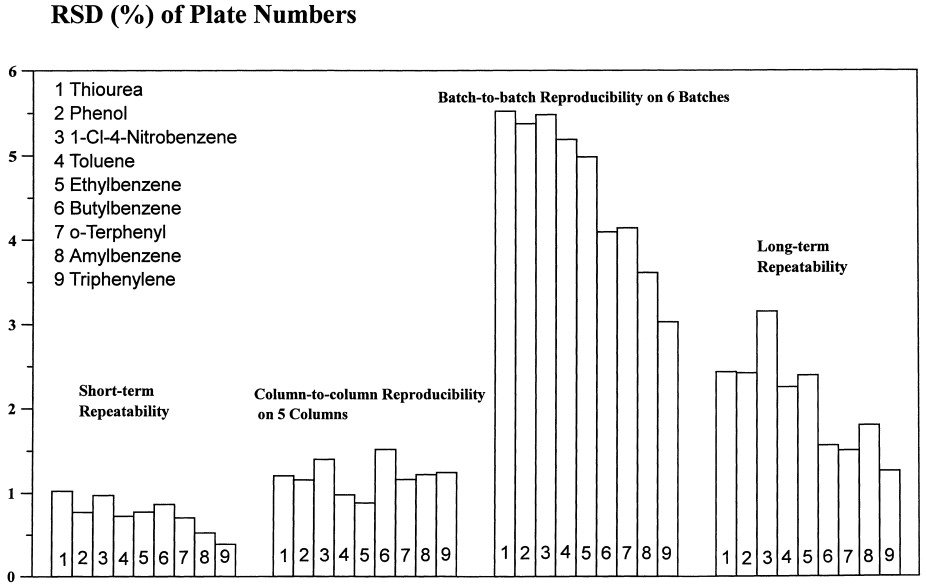


Fig. 15. Relative standard deviation of the number of theoretical plates for the components of the first test mixture.

the five-column and on the six-column sets, in spite of the low short-term repeatability of the experiment.

3.5. Peak asymmetry

In Table 8, we list the United States Pharmacopeia tailing factors determined from the peak width at 5% of the peak height and the RSDs of this parameter on the five-column and the six-column sets. The tailing factor is an empirical parameter without physical meaning but it is widely accepted to characterize peak asymmetry because of the ease of its derivation and the reasonable precision of its measurement. The tailing factors reported in Table 8 are not directly

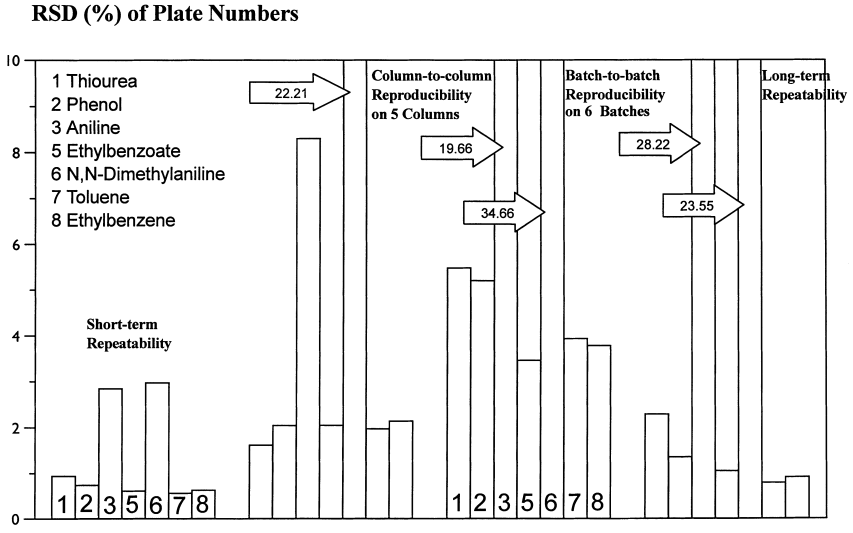


Fig. 16. Relative standard deviation of the number of theoretical plates for the components of the second test mixture.

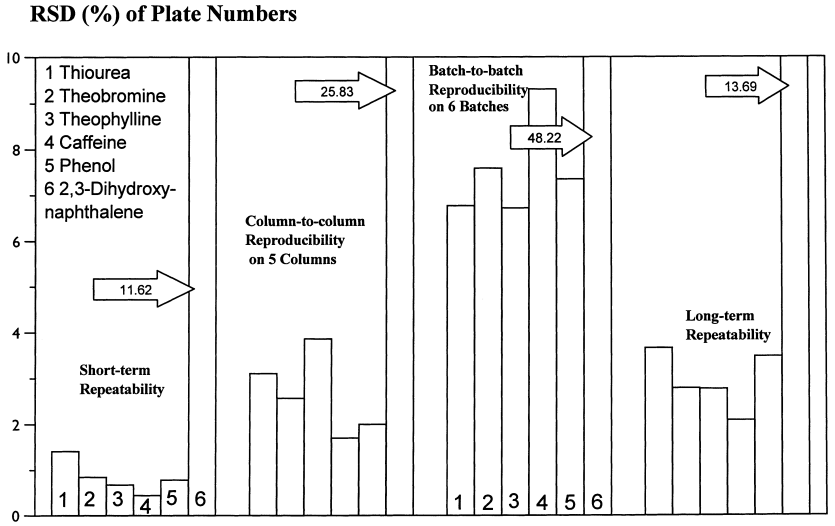


Fig. 17. Relative standard deviation of the number of theoretical plates for the components of the third test mixture.

comparable with those obtained on the previously studied brands [2–4] because the column sizes and the peak efficiencies are different.

The tailing factors of the first eluted peaks are affected by the contribution of the extracolumn volumes to band dispersion. Tailing factors of 1.1 were measured for neutral compounds with retention factors larger than one, which gives peaks whose shape is less affected by extracolumn effects. All basic compounds elute with tailing peaks (in contrast with the stationary phases previously studied for which *N*,*N*-dimethylaniline consistently gave leading peaks). The highest factor was observed for propranolol (3.8 on the five-column set, 3.4 on the six-column set) in a buffered mobile phase at pH 7.0, the second highest for *N*,*N*-dimethylaniline (2.7 on the

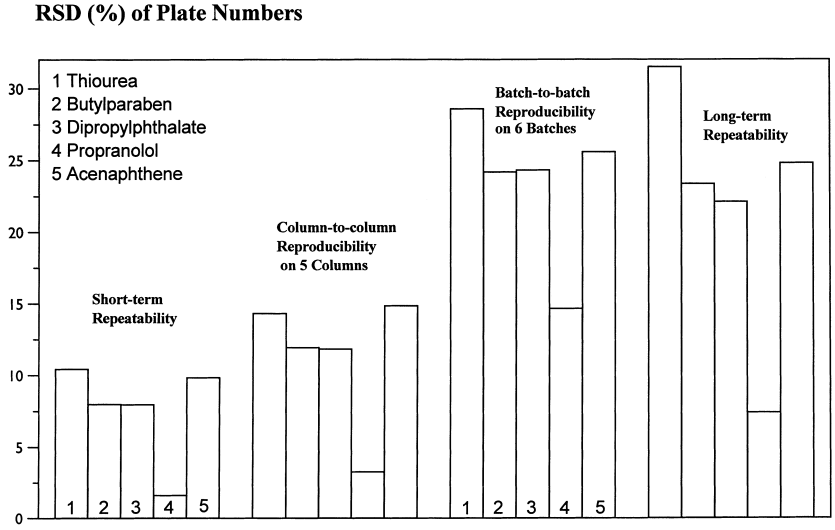


Fig. 18. Relative standard deviation of the number of theoretical plates for the components of the fourth test mixture.

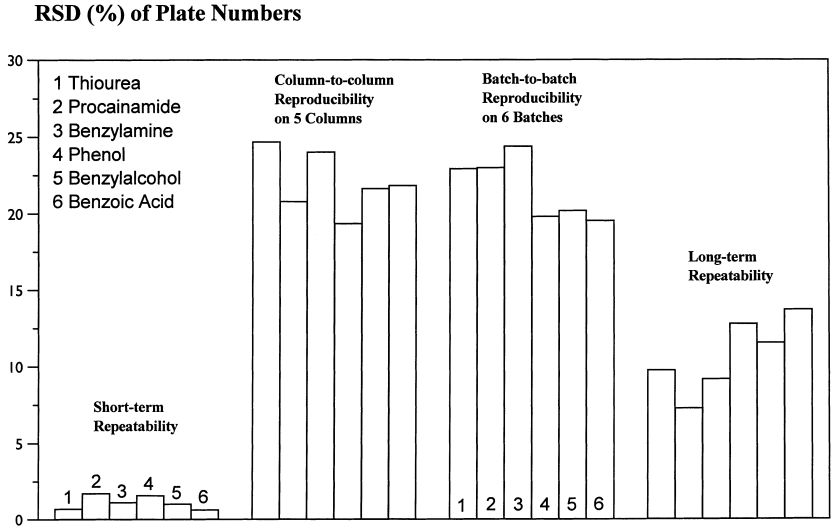


Fig. 19. Relative standard deviation of the number of theoretical plates for the components of the fifth test mixture.

five-column and the six-column sets). Benzylamine in a buffered mobile phase at pH 2.70 gives a factor (1.5) that is slightly higher than the one measured for phenol or benzyl alcohol under the same test conditions. The chelating compound, 2,3-dihydroxynaphthalene gives strongly tailing peaks with an average tailing factor 2.8 on the five-column set and 3.2 on the six-column set.

The reproducibility of the tailing factors of the neutral compounds on the five-column set varies between 1.3% and 4%, except for benzyl alcohol in test 5, for which RSD=11%. The reproducibility of the tailing factor of the neutral compounds is 1.5 to three times higher on the six-column than on the five-column set, except for benzyl alcohol for which the RSD is lower on the six-column than on the five-column set. For the basic compounds, the tailing factor RSD varies between 2.8 and 7.9% on the five-column set and between 8.3 and 13% on the six-column set. Finally, the RSD of the tailing factor of the chelating 2,3-dihydroxynaphthalene is practically the same on the five-column and on the six-column sets (24 and 25%, respectively).

3.6. Peptide separation

The chromatograms obtained for the mixture of the four undecapeptides on the six columns packed with the materials from six different batches are plotted in Fig. 20. The insert indicates the long-term repeatability of the experiment. The composition of the four peptides is given in the Experimental section. All peptides are C-terminal amides and N-terminal N-acetylated. These standard peptides were designed to approximate the average size of cleavage fragments from tryptic digest of proteins. The four peptides contain one to four lysine residues. Their p*I* values are 6.0, 7.9, 9.75 and 9.95, respectively. In order to measure the batch-to-batch reproducibility, the typical conditions of reversed-phase analysis of tryptic digest samples were used (linear gradient of water–acetonitrile with the addition of 0.02% TFA, see Experimental). Without the addition of TFA the last two peptide standards, which contain three and four lysine units, did not elute at this gradient composition.

The RSDs of the retention times of the four peptides were 2.5, 3.6, 3.7 and 4%, respectively. These values are slightly higher than the RSDs of the retention times of nonionized compounds in the isocratic tests but lower than the values obtained for the basic compounds under such test conditions that they are partially ionized. This result was not unexpected for several reasons. First, small molecules are less sensitive to possible differences in the silica pore structures of the different batches. Unlike small

Table 8
Tailing factor of the different compounds studied[a]

	One batch, five columns; average value of tailing factors	One batch, five columns; RSD (%) of tailing factors	Six batches; average value of tailing factors	Six batches; RSD (%) of tailing factors
Test 1 (MeOH–water, 8:2)				
Thiourea	1.288	1.266	1.301	3.802
Phenol	1.248	1.284	1.262	3.958
1-Chloro-4-nitrobenzene	1.242	1.249	1.255	4.184
Toluene	1.208	1.585	1.216	4.349
Ethylbenzene	1.148	1.595	1.155	3.708
Butylbenzene	1.116	1.619	1.134	3.167
o-Terphenyl	1.124	1.321	1.112	3.240
Amylbenzene	1.130	1.618	1.150	3.461
Triphenylene	1.118	1.280	1.133	2.634
Test 2 (MeOH–water, 55:45)				
Thiourea	1.283	1.288	1.294	3.859
Phenol	1.231	1.471	1.241	5.807
Aniline	1.677	3.978	1.904	13.480
Ethyl benzoate	1.194	2.252	1.204	3.794
N,*N*-Dimethylaniline	2.735	2.900	2.753	13.231
Toluene	1.136	2.486	1.148	3.527
Ethylbenzene	1.115	2.555	1.143	3.583
Test 3 (MeOH–water, 3:7)				
Thiourea	1.300	1.403	1.264	5.349
Theobromine	1.747	6.035	1.710	8.444
Theophylline	1.567	2.762	1.631	8.950
Caffeine	2.276	1.338	2.255	2.953
Phenol	1.261	1.626	1.171	5.173
2,3-Dihydroxynaphthalene	2.786	23.845	3.193	24.650
Test 4 (MeOH–pH 7.0 buffer, 65:35)				
Thiourea	1.317	3.254	1.346	5.188
Butylparaben	1.209	2.897	1.227	5.304
Dipropylphthalate	1.221	2.839	1.218	6.043
Propranolol	3.812	6.399	3.410	11.331
Acenaphthene	1.156	4.153	1.247	6.709
Test 5 (MeOH–pH 2.7 buffer, 3:7)				
Thiourea	1.482	9.583	1.368	7.177
Procainamide	1.597	8.221	1.492	4.701
Benzylamine	1.526	7.854	1.503	8.278
Phenol	1.485	8.110	1.288	10.860
Benzyl alcohol	1.491	10.769	1.365	8.326
Benzoic acid	1.457	9.554	1.323	10.046

[a] Average values and their reproducibility.

molecules, peptides and proteins having a bigger size and several functional groups are able to interact with more than one functional group at a time. Second, TFA was used as a component of the mobile phase. This fully dissociated acid is often used in peptide separations as a weak hydrophobic ion-pairing reagent because it is completely volatile and noncorrosive. Under our test conditions (pH~2.0),

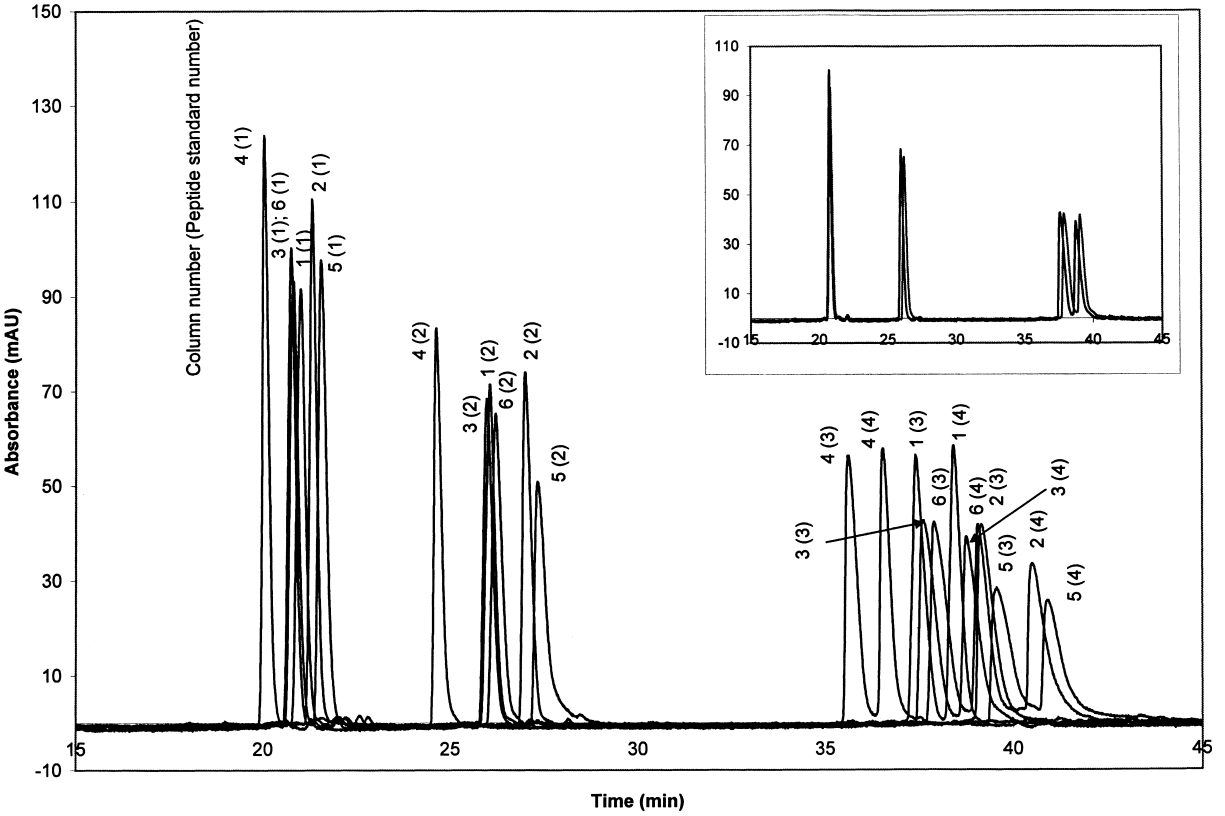


Fig. 20. Separation of peptide standards on six batches and the long-term repeatability of the experiment (insert) Eluent A: 0.02% (v/v) TFA in water. Eluent B: 0.02% (v/v) TFA in acetonitrile. Gradient: linear gradient from 5% B to 65% B in 120 min. Detection: UV at 210 nm; sample solvent: water. The peaks are identified by the column and peptide standard numbers. The column numbers correspond to the next reversed-phase batch numbers: 1-046; 2-156; 3-002; 4-541; 5-059; 6-449. The columns were tested in the order listed.

the basic lysine residues of the peptides are fully ionized. These positively charged ions are able to form ion pairs with the negatively charged trifluoroacetate ions, so the reproducibility of the retention times depends also on the fluctuations of the TFA concentration.

4. Conclusion

Although Vydac 218TP C_{18} is a polymeric type material, the batch-to-batch reproducibility of the chromatographic data obtained for nonionic compounds is almost as good as that observed on the monomeric or proprietary stationary phases previously studied [2–4]. The RSDs of the chromatographic data are only slightly higher. For example, the RSDs for the batch-to-batch reproducibility of the hydrophobicity (retention factors of alkylbenzenes) are around 4% while for the monomeric brands previously studied it varied between 1.1 and 3.5%. The same RSD for the steric selectivity (relative retention of triphenylene/*o*-terphenyl) is 2.1% on Vydac instead of between 0.8 and 1.1% on the monomeric brands. The RSDs of the hydrophobic selectivities are also comparable with those obtained on the monomeric phases and these results should satisfy most users.

The behavior of the basic compounds is markedly different on this packing material and on those previously studied. Aniline elutes after phenol, the three toluidine isomers are well resolved, pyridine and 2,2′-dipyridyl do not elute under the test conditions used in this study. Even in a mobile phase

buffered at pH 7.0, the strongly basic propranolol elutes after dipropylphthalate. All these observations indicate stronger (or, at least, quite different) interactions between the residual silanol groups and the basic test compounds on Vydac 218TP C_{18} silica and on the packing materials previously studied. The batch reproducibility (estimated as the RSDs of the chromatographic parameters) for the basic compounds is generally higher than that measured on the packing materials previously studied but, in most cases, the experimental error contribution (i.e., the column reproducibility) is also higher. At this stage, we cannot determine whether this difference originates from the properties of the initial silica, arising from the pore enlargement step or from the use of a trifunctional silane for the surface modification, or whether, during the bonding process, new silanol groups were generated.

Acknowledgements

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