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Repeatability and reproducibility of retention data and band profiles on reversed-phase liquid chromatography columns III. Results obtained with Kromasil C_{18} columns

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Abstract

The reproducibility of the retention data and the band profiles was investigated with Kromasil C_{18} columns (silica-based monomeric type reversed-phase packing material). High precision data were obtained and statistically compared among five columns from the same batch (column-to-column reproducibility) and six columns, one from each of six different batches (batch-to-batch reproducibility). These data were acquired under five different sets of chromatographic conditions, for a group of 30 neutral, acidic and basic compounds selected as probes following an experimental protocol previously described. Data characterizing the retention time, the retention factor, the separation factor, the column efficiency and the peak asymmetry for the different probe compounds are reported. Factors describing the silica surface interaction with the selected probe compounds, such as the hydrophobic interaction selectivity, the steric selectivity, and the separation factors of basic compounds at different pH values were also determined. The influence of the underlying silica on these data and correlations between the chromatographic and physico–chemical properties of the different batches are discussed.

Keywords: Band profiles; Kromasil C_{18}; Stationary phases, LC; Retention data

1. Introduction

Chromatography is the most widely used separation method. It remains the only practical one for the analysis of most of the mixtures encountered in the pharmaceutical, fine chemicals, food and beverage industries. As such, it is attracting considerable attention from the agencies which over the world regulate these industries. The problems of analytical repeatability are ever present, are of foremost importance in regulatory analyses, and have not been investigated in a systematic fashion for a long time [1]. Accordingly, we set out to investigate the state of the art in this matter by determining the current limits to the precision of chromatographic analyses due to the contributions of the column-to-column repeatability and the batch-to-batch reproducibility within a given brand.

Another important motivation for this work is the current interest in the characterization of the station-

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ary phases in high-performance liquid chromatography (HPLC) and, more specifically, in reversed-phase liquid chromatography (RPLC). Chromatographers realized that the prediction of chromatographic behavior from the physico–chemical properties of a packing material is not yet a successful approach. Several authors developed systematic chromatographic tests to characterize the available brands of C_{18} bonded silica under either isocratic [2–14] or gradient elution conditions [15–17] and used the results of these tests for the classification of different brands [15,18–26]. Recently, Claessens et al. [27] studied four test methods using 18 different RPLC stationary phases and compared the results of the different tests in the column classification. However, most of the data reported in these publications was acquired with a single column for each brand. The precision of the parameters derived from these data and used to characterize the packing materials is unknown, so it is impossible to decide whether certain materials are significantly different from others. At this stage, we need information on the repeatability and reproducibility of chromatographic data to interpret charts such as plots of the hydrophobic selectivity vs. the silanophilic activity. Knowing the precision of the parameters measured (retention factors, column efficiencies and peak asymmetries for a series of probe compounds) and how this precision varies from compound to compound would improve our understanding of the retention mechanisms. This would also permit a comparison of the suitability of the most widely used test methods for the characterization of stationary phases.

Some authors [28] complained about the lack of a single standardized test method for the chromatographic characterization of RPLC stationary phases. We are of the opinion that it is fortunate that there is no such prescribed method since a fixed test method could generate misleading results when applied to some of the existing stationary phases (as will be shown later in this paper). Also, it would rapidly become obsolete with the constant introduction of new and improved stationary phases. We agree with Unger [7] who states that the characterization of stationary phases requires the combination of the results of several test methods, using different test compounds and experimental conditions. This conclusion was also reached more recently by McCalley and Brereton [21].

Although Dorsey et al. stated in a recent review [29] that ''many of the early problems of reproducibility of columns from lot-to-lot were solved 10 or more years ago'' few papers dealing with this topic were published during the last ten years. Kirkland and co-workers [30–32] and Neue and co-workers [12,33,34] published data proving the excellent batch-to-batch reproducibility of their packing materials based on the results of one chromatographic test but no systematic, independent data were published on this topic. In a previous publication [35], we described a comprehensive and rigorous protocol for the systematic investigation of the column-to-column and the batch-to-batch reproducibilities of the chromatographic data acquired from commercially available columns packed with silica-based stationary phases for reversed-phase chromatography. In this report as in the previous study [35], we used isocratic test conditions. Previously, we used this approach to characterize a brand of chromatographic columns, Symmetry C_{18} made by Waters (Milford, MA, USA) [36]. Here, we report on the results obtained with our protocol applied to another brand, Kromasil C_{18} (Eka, Bohus, Sweden), supplied to us through BTR Separations (Wilmington, DE, USA).

The parameters derived from the tests used in this study can be classified into two groups. The first group consists of those parameters which are affected both by the performance of the column bed and by the surface properties of the packing material, i.e., the retention time, the retention factor, the column efficiency and the peak asymmetry. The second group includes those parameters which characterize only the surface properties of the stationary phase, i.e., the hydrophobic selectivity, the steric selectivity, the separation factors of basic compounds in unbuffered and buffered (at pH 7.0 and 2.7) mobile phases and the concentration of metal impurities in the surface layer of the solid adsorbent. They will be discussed successively.

2. Experimental

The experimental conditions were described in

detail and discussed earlier in the previous report [35]. The following is a summary description of the essential points. It includes the changes required for the application of the protocol to a different column brand as well as their justification, the relevant information regarding the new packing material, the different batches studied, and the properties of the columns used in this work.

2.1. Instrument

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 (Pentium processor, 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. They are also uploaded to a computer for further evaluation.

The instrument tests corresponding to the operational qualification and performance verification procedures for the HP 1100 Series HPLC modules [37] were performed weekly and after each maintenance of the equipment.

2.2. Conditions

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 [35], confirm the stability of this parameter. The mobile phases (see Section 2.4) 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 1.39 ml/min for all the tests (see Section 2.5 why it was not 1.00 ml/min as in the protocol [35]). This procedure allows sparging the pure mobile phase components with helium for long periods of time without affecting the composition of the eluent. This permits the elimination of the possible influence of gases or vapors (e.g., carbon dioxide) present in the atmosphere of the laboratory. Test 1 was repeated at flow-rates of 1.0 and 0.5 ml/min after completion of all the other tests. The columns were equilibrated with the required mobile phase for 5 h before the first injection.

The injection volume was 18 μl. Each sample was injected in five replicates. The signals were detected with the UV detector at 220, 230, 254, 270 and 290 nm. The 254 nm signal was used for the data interpretation, except for amitryptiline (test 4) for which the 220 nm signal was used to improve the signal-to-noise ratio.

2.3. Stationary phase

The experimental results reported in this work were acquired with 10 columns (250×4.6 mm) packed with Kromasil C_{18}, an RPLC packing material from Eka (Bohus, Sweden). Five columns were packed with material from the same batch and six columns with materials from six different batches. The columns were packed by the manufacturer and used as received. Kromasil C_{18} is based on a spherical, porous silica prepared by the sol–gel technique. The silica surface is chemically bonded with monofunctional octadecylsilane then end-capped. The main characteristics of the bare silica and the packing material are summarized in Table 1. The values were measured and supplied by the manufacturer. The pH of the 5% aqueous slurry of these packing material batches were between 5.5 and 6.0 (value supplied by the manufacturer).

The six batches of packing material were based on four batches of silica. One silica batch was bonded at three different times. The batches were made in 1997 and stored dry until packed into columns in 1998. The six batches represent a one year period of stationary phase production. The physico–chemical properties (Table 1) of this silica and those of the previously tested Waters silica [36] are very close.

2.4. Samples and chemicals

The qualitative and quantitative compositions of the five test mixtures used are given below. For reasons explained in the next section, they are

Table 1
Physico–chemical properties of the six batches of stationary phase (Kromasil C_{18}) supplied by the manufacturer (Eka Chemicals)

Silica batch	Particle size (μm)	Particle size distribution (90:10, % ratio)	Pore size (Å)	Pore volume (ml/g)	Surface area (m^2/g)	Na, Al, Fe content (ppm)	Particle shape
AT124	6.03	1.38	112	0.90	322	15; <10; <10	Spherical
AT130	5.98	1.44	112	0.88	314	11; <10; <10	Spherical
AT132	6.24	1.48	107.5	0.90	333	23; <10; <10	Spherical
AT140	6.11	1.46	114	0.89	313	15; 13; 14	Spherical
Mean	6.09	1.44	111.38	0.8925	320.5	N/A	
RSD (%)	1.86	3.00	2.47	1.07	2.89	N/A	

Kromasil C_{18} batch[a]	Corresponding silica batch	Total carbon (%)	Surface coverage[b] (μmol/m^2)
DT200	AT130	19.65	3.51
DT201	AT130	19.85	3.55
DT202	AT130	20.00	3.59
DT204	AT124	20.00	3.50
DT208	AT132	20.60	3.52
DT220	AT140	19.80	3.55
Mean		19.98	3.54
RSD (%)		1.65	0.94

[a] All batches of Kromasil C_{18} were end-capped.
[b] Calculated according to Ref. [39].

slightly different from those described in the protocol [35].

Sample 1: Thiourea (15.4 mg/l), phenol (153.6 mg/l), 1-chloro-4-nitrobenzene (25.6 mg/l), toluene (668.2 mg/l), ethylbenzene (554.9 mg/l), butylbenzene (1321 mg/l), *o*-terphenyl (56.3 mg/l), amylbenzene (1326 mg/l), triphenylene (15.4 mg/l) in methanol–water (80:20).

Sample 2: Thiourea (15.4 mg/l), aniline (104.6 mg/l), phenol (153.6 mg/l), *o*-toluidine (102.1 mg/l), *p*-toluidine (25.6 mg/l), *m*-toluidine (75.9 mg/l), *N*,*N*-dimethylaniline (48.9 mg/l), ethylbenzoate (669.4 mg/l), toluene (1114 mg/l), ethylbenzene (1110 mg/l), in methanol–water (55:45).

Sample 3a: Thiourea (15.4 mg/l), theobromine (23 mg/l), theophylline (38.4 mg/l), caffeine (41 mg/l), phenol (205 mg/l), 2,3-dihydroxynaphthalene (256 mg/l) in methanol–water (30:70).

Sample 3b: Thiourea (15.4 mg/l), pyridine (125.8 mg/l), 2,2-dipyridyl (256 mg/l) in methanol–water (30:70).

Sample 4: Thiourea (15.4 mg/l), propranolol (512 mg/l), butylparaben (25.6 mg/l), dipropylphthalate (435.2 mg/l), naphthalene (76.8 mg/l), acenaphthene (256 mg/l), amitriptyline (128 mg/l), in methanol–water (65:35) buffer (20 m*M*) with potassium phosphate, monobasic/dibasic at pH 7.00.

Sample 5: Thiourea (15.4 mg/l), procainamide (15.4 mg/l), benzylamine (251.4 mg/l), benzylalcohol (801.8 mg/l), benzoic acid (256 mg/l) in methanol–water (30:70) buffer with phosphoric acid–potassium monophosphate buffer (20 m*M*) at pH 2.70.

The tests were carried out in the order listed. When a test was finished on all columns, the first column was connected again, equilibrated and retested. The comparison of the values measured initially and the ones found in the repeated experiments allowed the calculation of the long-term repeatability. The time elapsed was usually 10 days.

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 chemicals used in the work described here were recently acquired, and not leftovers from a previous study. They were used as received. In order to avoid any possible errors caused by fluctuations of

the buffer composition due to the lack of reproducibility of the buffer preparation, the same buffer solution was used for all the columns tested, for each test.

2.5. Changes made to the initial protocol

Because the column dimensions were different, the flow-rate, the sample concentration, and the sample load were scaled up compared to those described in the protocol [35] and used with the first brand studied (Symmetry C_{18}, Waters, Milford, MA, USA). In order to keep the mobile phase velocity constant, the flow-rate was scaled up based on the ratio of the square of the column diameters, according to the formula:

$$V_2 = V_1 \cdot \frac{A_2}{A_1} = V_1 \cdot \frac{d_{c,2}^2}{d_{c,1}^2} \quad (1)$$

The volume and the mass of the loaded amounts were increased to keep constant the volume and mass loading of the column. Accordingly, the injected volume was scaled up by the ratio of the product of the square of the column diameter and the square root of the column length

$$V_{inj,2} = V_{inj,1} \cdot \frac{d_{c,2}^2\sqrt{L_2}}{d_{c,1}^2\sqrt{L_1}} \quad (2)$$

The mass of the sample injected was scaled in proportion to the surface area of the packing material, the square of the column diameter, and the column length, in order to maintain a constant loading factor, according to the following relationship

$$m_{inj,2} = m_{inj,1} \cdot \frac{d_{c,2}^2 L_2 S_{a,2}}{d_{c,1}^2 L_1 S_{a,1}} \quad (3)$$

The concentrations of the test mixtures were adjusted according to these values (see Section 2.4).

Because of the accidental coelution of caffeine and pyridine under the experimental conditions of test 3 and because a possible tailing of 2,2-dipyridyl could affect the peak shape of the 2,3-dihydroxynaphthalene which is eluted immediately after, this test mixture was divided into two (see Section 2.4, tests 3a and 3b). The first sample was analyzed on all the columns first. When the five injections were finished the column was flushed and stored on the storage solvent (acetonitrile). The columns were equilibrated with the mobile phase again before the second part of the sample was injected. Because of the accidental coelution of phenol and benzylalcohol, phenol was removed from the test mixture 5.

2.6. 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 relative standard deviation (RSD) for 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 another brand were already published and discussed [35,36]. The values obtained in this study closely match those previously published. For example, the short term repeatability of the retention times were characterized by an RSD below 0.12% for all the compounds in all the tests, most of the compounds giving values below 0.05%; the short-term repeatability of the selectivity factors for pairs of neutral–neutral compounds were around 0.02% while those for basic–neutral and basic–basic compound pairs were below 0.15%. Short-term repeatability values of the efficiency presented in this paper are between 1 and 2.5%, except for 2,3-dihydroxynaphthalene.

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.

The long-term repeatability values were obtained by repeating the series of five consecutive analyses of the test mixture on the same column after the measurements had been completed on all the columns tested (a total of 10). This interval was typically 10 days.

3. Results and discussion

3.1. Absolute retention data

The column-to-column and the batch-to-batch reproducibilities as well as the long-term repeatability of the retention times are plotted in Figs. 1–5. The values of the RSDs measured are impressively small, as in the previous case [36].

The long-term repeatabilities of the retention times in the first three tests, which are carried out with unbuffered solutions, are characterized by RSDs lower than 0.1% for all compounds, except for *N*,*N*-dimethylaniline (0.6%) in test 2. It is hard to explain this high value. It is not related to the equipment performance since all the other compounds, including those eluted just before or after *N*,*N*-dimethylaniline, give excellent repeatability values. One might consider that this phenomenon could be related to a lack of chemical stability of the packing but it was shown [38] that *N*,*N*-dimethylaniline is the least sensitive basic test compound for silanol interactions and none of the other basic compounds in these test mixtures exhibit a similar effect. The long-term repeatability values in the buffered test mixtures are not so good as those in the unbuffered ones. In general, the RSDs are below 0.4%, except for two of the basic compounds (propranolol and amitriptyline, both high pK_a bases) in test 4. This could be explained by minor stability problems, although we have no further verifications of that explanation.

In spite of these minor uncertainties on the retention times of these three basic compounds, the measurement precision allows some meaningful comparisons between the data obtained with the five columns packed from the same batch of packing material and used with the unbuffered mobile phases (Figs. 1–3). The RSDs of the retention times measured on these five columns (column-to-column reproducibility) show a systematic trend. They increase with increasing retention times. The RSD of

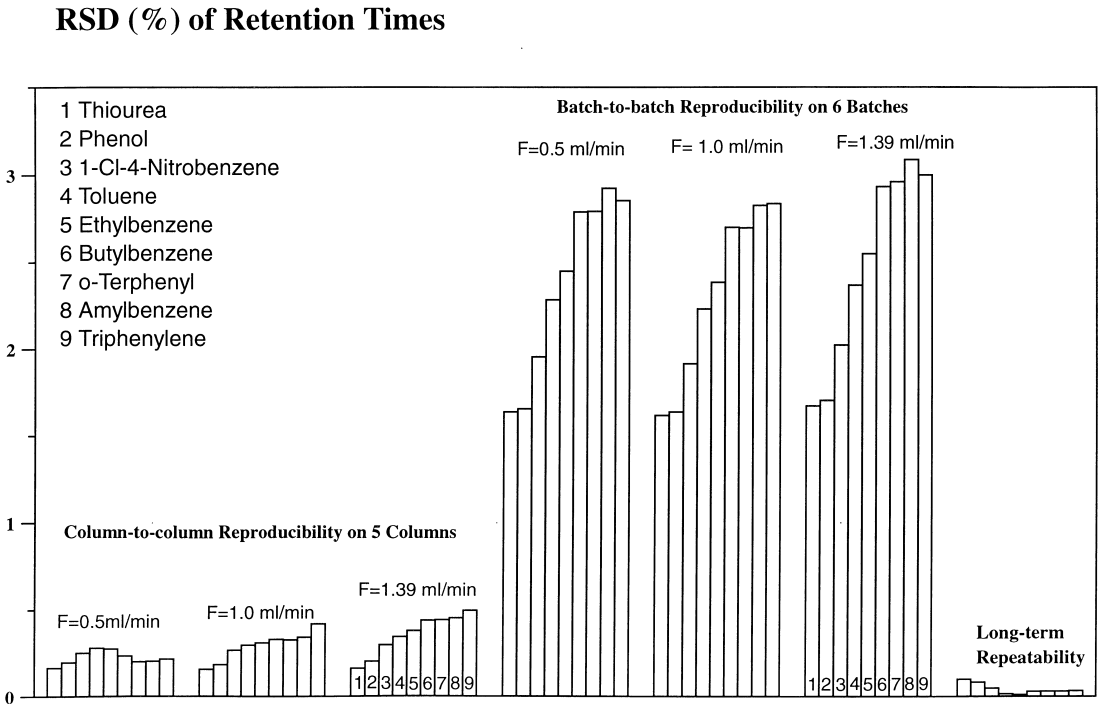


Fig. 1. Reproducibility of the retention time measured in the first test. 1=Thiourea, 2=phenol, 3=1-chloro-4-nitrobenzene; 4=toluene; 5=ethylbenzene; 6=butylbenzene; 7=*o*-terphenyl; 8=amylbenzene; 9=triphenylene. Mobile phase, methanol–water (80:20) at 0.5, 1.0 and 1.39 ml/min.

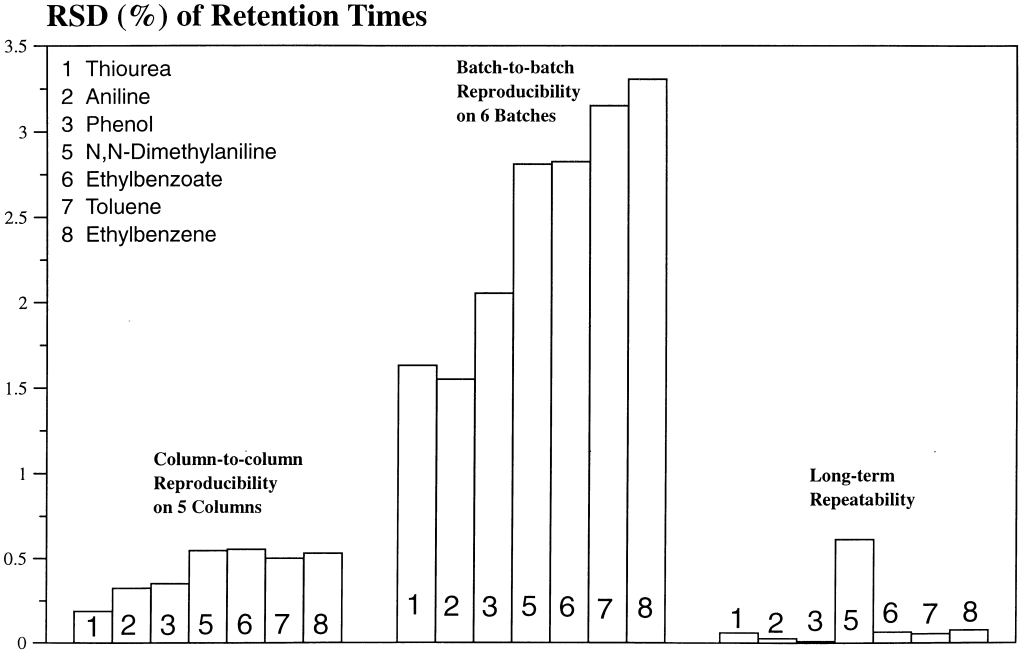


Fig. 2. Reproducibility of the retention time measured in the second test. 1=Thiourea; 2=aniline; 3=phenol; 5=*N*,*N*-dimethylaniline; 6=ethylbenzoate; 7=toluene; 8=ethylbenzene. Mobile phase, methanol–water (55:45) at 1.39 ml/min.

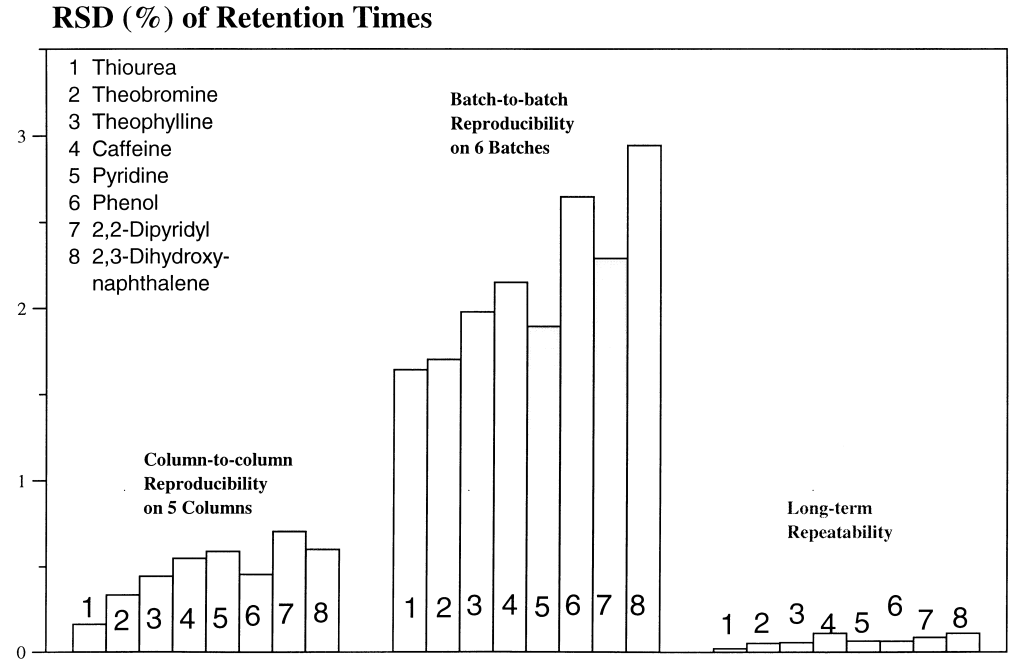


Fig. 3. Reproducibility of the retention time measured in the third test. 1=Thiourea; 2=theobromine; 3=theophylline; 4=caffeine; 5=pyridine; 6=phenol; 7=2,2-dipyridyl; 8=2,3-dihydroxynaphthalene. Mobile phase, methanol–water (30:70) at 1.39 ml/min.

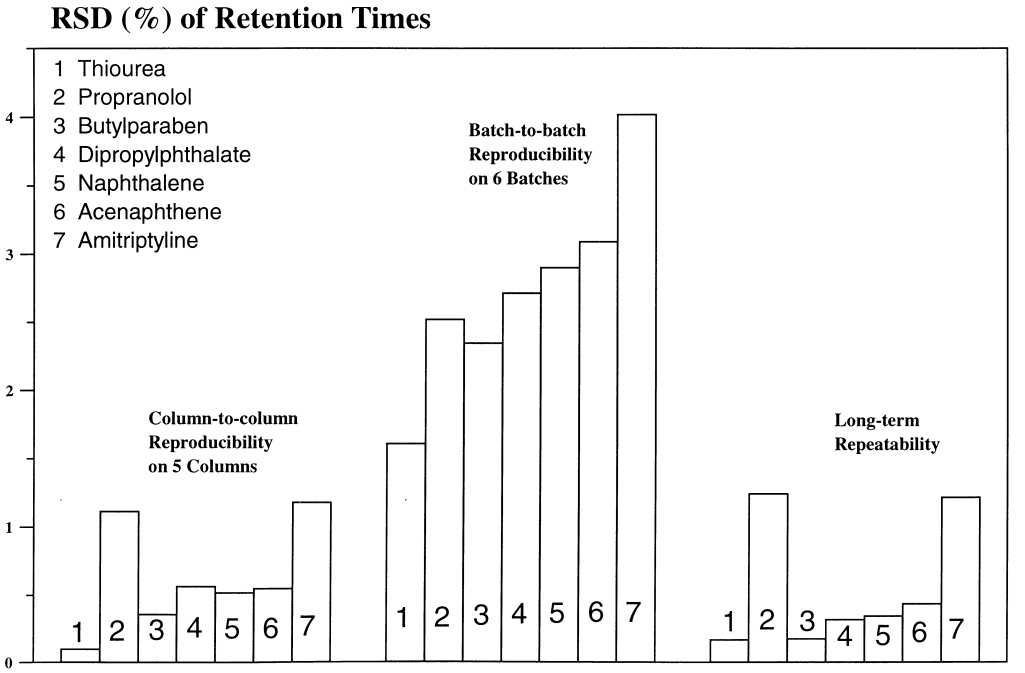


Fig. 4. Reproducibility of the retention time measured in the fourth test. 1=Thiourea; 2=propranolol; 3=butylparaben; 4=dipropylphthalate; 5=naphthalene; 6=acenaphthene; 7=amitriptyline. Mobile phase, methanol–water (65:35) buffer with potassium phosphate, monobasic/dibasic at pH 7.00 at 1.39 ml/min.

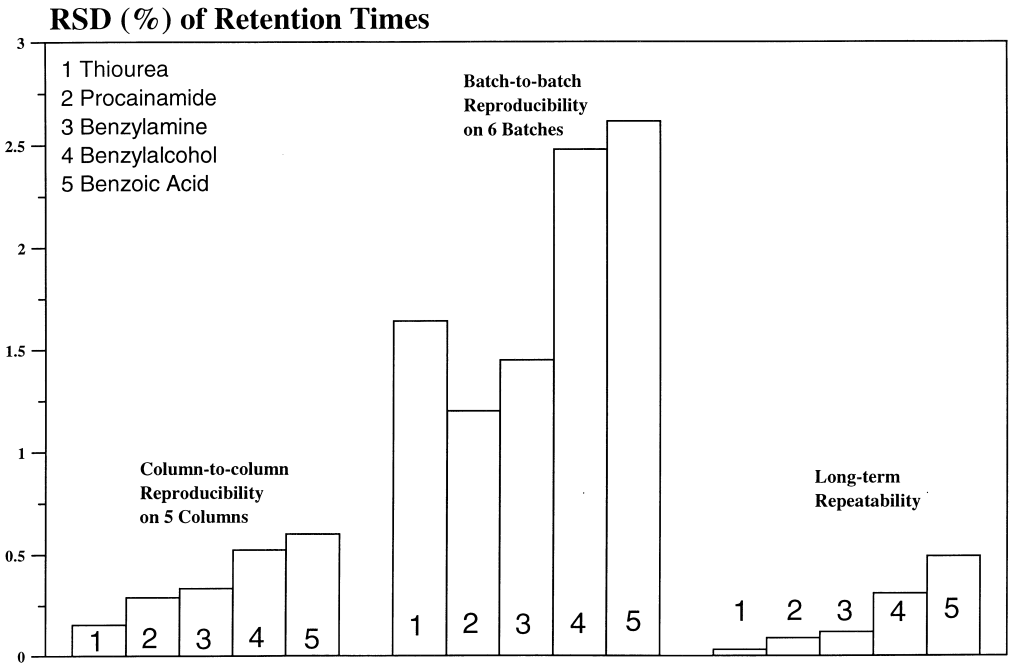


Fig. 5. Reproducibility of the retention time measured in the fifth test. 1=Thiourea; 2=procainamide; 3=benzylamine; 4=benzylalcohol; 5=benzoic acid. Mobile phase, methanol–water (30:70) buffer with phosphoric acid–potassium monophosphate buffer at pH 2.70 at 1.39 ml/min.

the hold-up time, taken as equal to the retention time of thiourea, which is unretained under these experimental conditions, is between 0.1 and 0.19%, depending on the series of results. In most cases, this is slightly higher than the long-term repeatability value. The RSDs of the retention times of the most retained compounds are ~0.6%. The column-to-column fluctuation of the retention times on columns packed with the same stationary phase is affected by the fluctuations of the size of the column tube, the flow-rate, the temperature and the packing density.

The fluctuation observed for the column volume is smaller than the value which can be derived from the tubing specification (±0.0075 cm for the inner diameter, and ±0.025 cm for the length, which gives a volume fluctuation of 3% of the tube volume).

The RSDs of the retention times measured on the six columns packed with six different batches vary between 1.25% (procainamide in test 5, Fig. 5) and 4% (amitriptyline in test 4, Fig. 4). The values measured on the peaks of the three toluidine isomers are not included in Fig. 2. We observed a partial separation of the three isomers on all six batches of packing material. Because the resolutions of these isomers vary slightly from column to column, however, a comparison of the RSDs of these retention times would be meaningless. Instead of this value, we show in Fig. 6 the chromatograms obtained for the toluidine peaks on the five columns of one batch and in Fig. 7 those obtained for the six columns of six different batches. Four out of six batches meet the requirements (symmetrical peaks with a separation factor or ratio of the extreme retention factors less than 1.3) which were defined by Engelhardt and

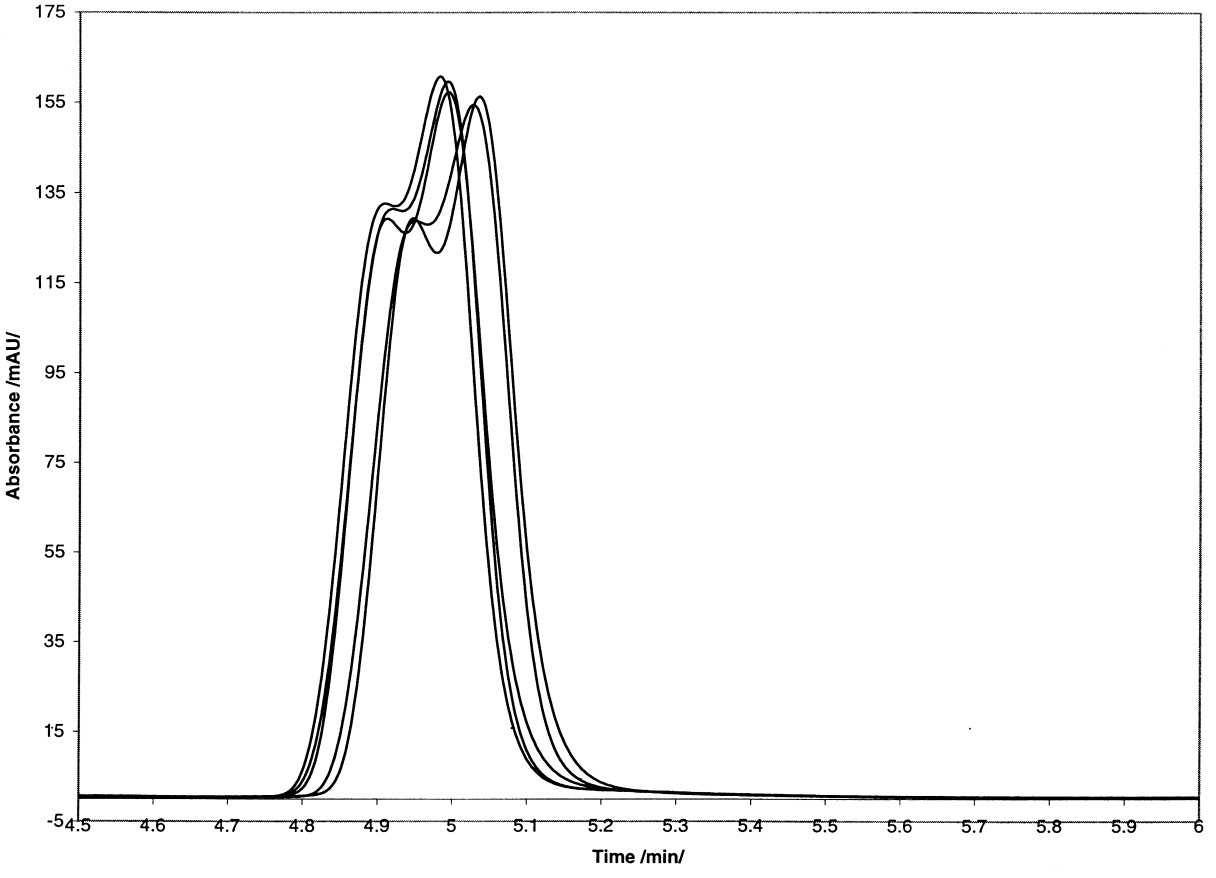


Fig. 6. Chromatograms of *o*-, *m*-, *p*-toluidine on the five columns of the first batch. Mobile phase, methanol–water (55:45); flow-rate 1.39 ml/min; detection: 254 nm UV.

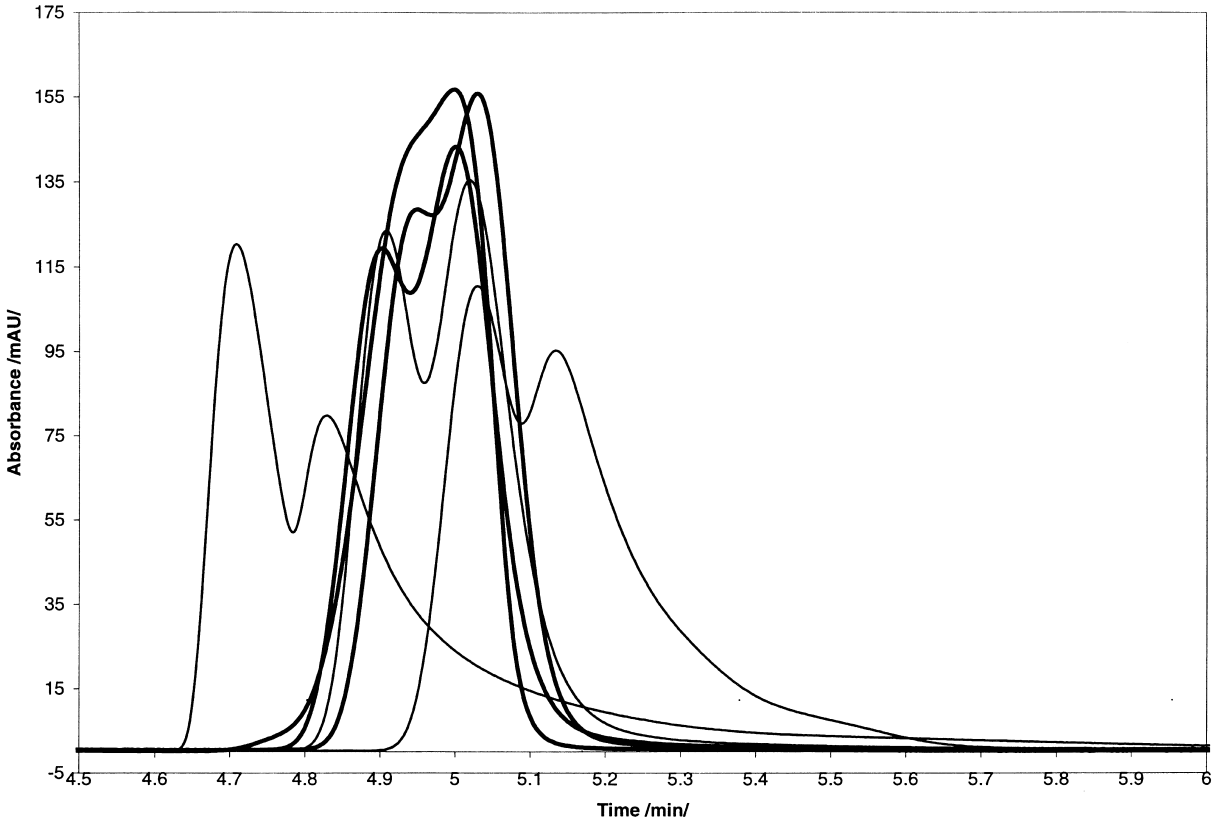


Fig. 7. Chromatograms of *o*-, *m*-, *p*-toluidine on the six columns of the different batches. Mobile phase, methanol–water (55:45); flow-rate 1.39 ml/min; detection: 254 nm UV.

Jungheim [8] as those that a column must fulfill to be suitable for the separation of basic compounds. On two batches, the toluidine peaks tailed strongly.

Three batches of the packing material were derived from the same batch of silica. In Fig. 8, the RSDs of the retention times of all compounds, in all test mixtures, on these three batches are compared with the RSDs of the retention times of all the compounds on the four batches of packing material based on four different batches of silica. On the three batches made with the same silica batch, the RSD is 0.89% for the total carbon content and 1.18% for the surface coverage (calculated according to Ref. [39] for monomeric stationary phases) while the surface area measured before the bonding is the same for the three batches. The RSDs for the total carbon content and the surface coverage are 2.08 and 0.61%, respectively, on the four batches of packing material based on four different batches of silica. The RSD of the surface area of the four initial silica batches is 2.89%. The RSDs of the retention times measured on the three batches made with the same silica batch are not significantly different from the RSDs of the same data measured on the five columns packed with the same batch of packing material (compare Figs. 1–5 and Fig. 8). The RSDs of the retention times measured on the four batches of packing material made from the four different silica batches are approximately four-times higher than those observed on the three batches based on the same silica batch (except for *N*,*N*-dimethylaniline in test 2).

This last observation suggests, first, that the influence of surface area fluctuations has a significant effect on the retention and, second, that the preparation of the silica is more difficult to control precisely and contributes more to the relatively minor fluctua-

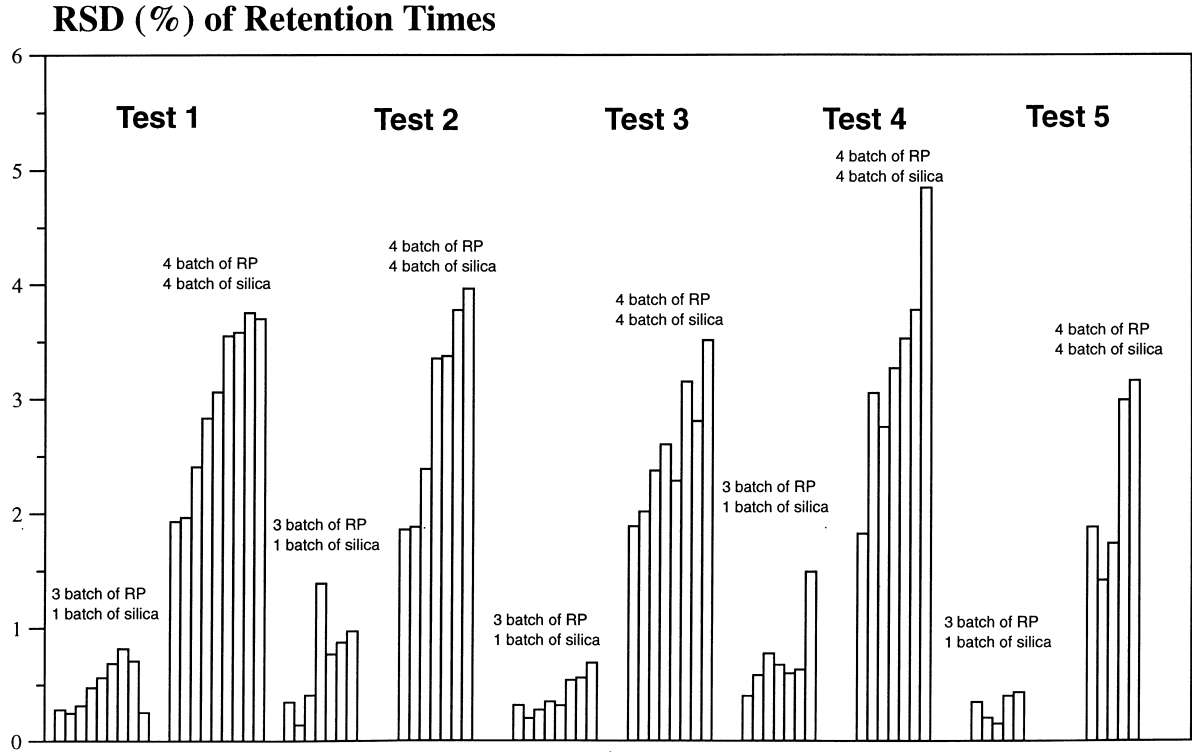


Fig. 8. RSDs on the retention times of the components of all the test mixtures.

tions of the retention time observed than the chemical reactions used in the surface modification process (e.g., C_{18} bonding).

3.2. Retention and separation factors

The RSDs of the retention factors derived from the measurements made on five columns of one batch and on six columns of different batches are listed in Tables 2–6 and illustrated in Figs. 9–13. The RSDs of the retention factors are practically constant and are all below 0.7% on the five columns packed from the same batch of packing material, except for propranolol and amitriptyline in test 4 and pro-

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

	RSD (%) of k					
	Column-to-column reproducibility on five columns			Batch-to-batch reproducibility on six batches		
	$F=0.5$ ml/min	$F=1.0$ ml/min	$F=1.39$ ml/min	$F=0.5$ ml/min	$F=1.0$ ml/min	$F=1.39$ ml/min
Phenol	0.360	0.413	0.471	2.349	2.273	2.401
1-Chloro-4-nitrobenzene	0.322	0.388	0.462	2.610	2.527	2.677
Toluene	0.320	0.37S	0.448	2.888	2.812	2.976
Ethylbenzene	0.293	0.352	0.450	2.934	2.848	3.040
Butylbenzene	0.226	0.298	0.453	3.063	2.948	3.204
o-Terphenyl	0.20S	0.281	0.447	3.072	2.951	3.236
Amylbenzene	0.201	0.283	0.448	3.133	3.005	3.287
Triphenylene	0.208	0.327	0.485	2.887	2.806	3.015

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	Batch-to-batch reproducibility on six batches
Aniline	0.543	2.179
Phenol	0.572	2.218
Toluidine	0.611	
N,*N*-Dimethylaniline	0.611	3.283
Ethylbenzoate	0.615	2.659
Toluene	0.541	3.050
Ethylbenzene	0.557	3.147

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	Batch-to-batch reproducibility on six batches
Theobromine	0.679	2.130
Theophylline	0.633	2.238
Caffeine	0.687	2.314
Pyridine	0.675	2.627
Phenol	0.468	2.476
2,2-Dipyridyl	0.691	2.470
2,3-Dihydroxynaphthalene	0.573	2.611

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	Batch-to-batch reproducibility on six batches
Propranolol	1.348	2.519
Butylparaben	0.321	2.516
Dipropylphthalate	0.525	2.804
Naphthalene	0.466	3.006
Acenaphthene	0.472	3.120
Amitriptyline	1.121	4.061

cainamide and benzylamine in test 5. The batch-to-batch reproducibilities of the retention factors are four- to seven-times larger than the column-to-column reproducibilities.

Table 7 reports the average relative retention data

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	Batch-to-batch reproducibility on six batches
Procainamide	2.412	6.272
Benzylamine	0.728	1.911
Benzylalcohol	0.515	2.381
Benzoic acid	0.580	2.372

(i.e., separation factors, α) for pairs of successively eluted peaks, for all the tests carried out, and their RSDs. In the first test, these RSDs are all below 0.05% on the five columns packed with the same packing material. On the six columns from different batches, they vary from 0.08% (ethylbenzene/toluene) to 0.8% (triphenylene/amylbenzene). In the other tests, the RSDs of the separation factors of the neutral compound pairs are comparable to those observed in the first test. They are at least one-order of magnitude higher for the pairs of neutral/basic or basic/basic compounds. The highest RSD value (5.2%) was obtained for the relative retention of the pair benzylamine/procainamide (test 5), carried out for the six column of different batches. Out of a total of 27 separation factors calculated in Table 7, 14 have batch-to-batch reproducibilities within 0.5%, 23 are better than 1.5%, and 26 better than 2%.

The separation factors discussed above are arbitrary since they result from the elution order of a set of arbitrarily chosen compounds. Thus, we calculated also the values of the separation factors suggested by various authors [6,8,11,40–42] for the characterization of surface properties of different brands. These values are now discussed.

3.3. Hydrophobic selectivity

As in a previous paper [36], we derived the hydrophobic selectivity of different batches using the retention data measured in three different tests. First, we calculated α(CH2) as the ratio of the retention factors of the three following pairs of compounds, amylbenzene/butylbenzene (test 1, Fig. 14a), butylbenzene/ethylbenzene (test 1, Fig. 14b), and ethylbenzene/toluene (tests 1 and 2, Fig. 14c and d,

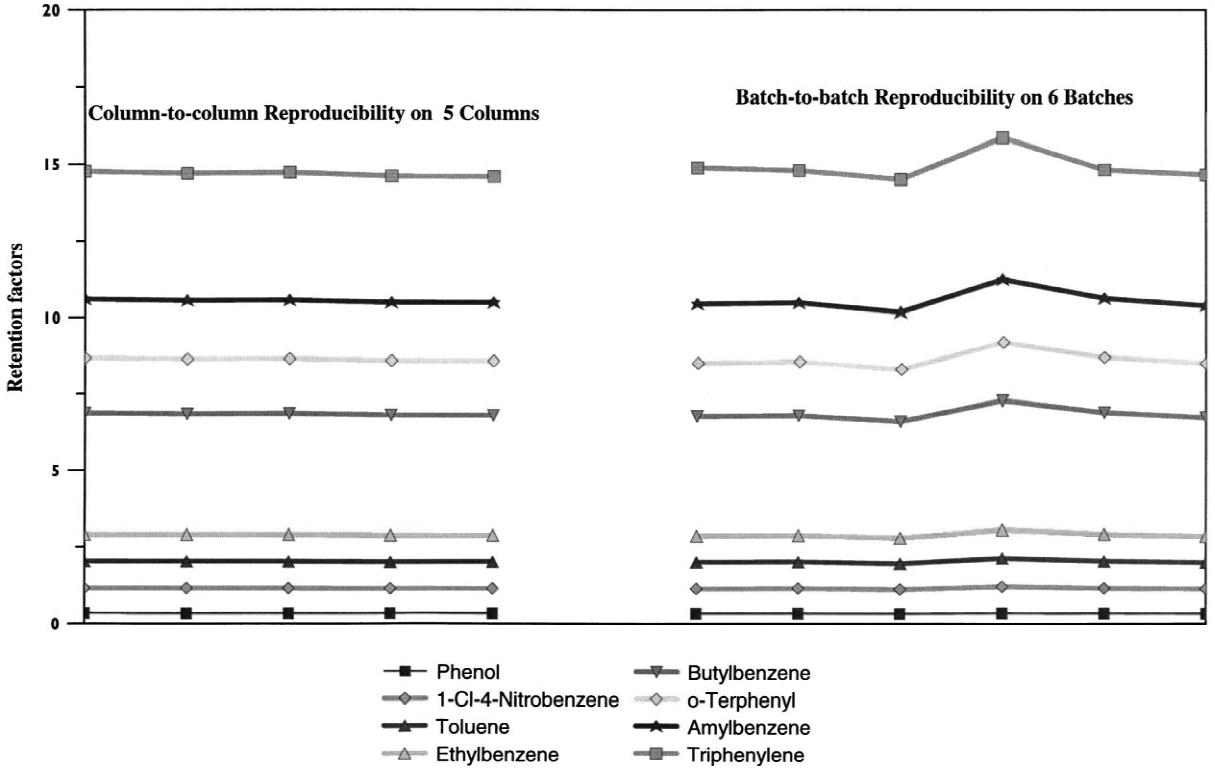


Fig. 9. Retention factors of the components of the first test mixture. Each data point represents the average of five consecutive injections carried out on a column.

respectively). Second, from the data measured in the fourth test, we derived the separation factor of the pair acenaphthene/naphthalene (Fig. 14e). The figures report both the values obtained and their RSDs (denoted as six batches of RP). The long-term repeatability values were of the same order as the column-to-column reproducibility while the batch-to-batch reproducibility was three- to five-times higher (Fig. 14a–e). The correlations between the values derived under different test conditions are satisfactory.

The first, the second and the sixth data points measured on the six different batches correspond to the three batches of packing material which were prepared from the same batch of silica. The RSD values of the selectivity factors on the three batches of packing based on the same batch of silica and the four batches based on different batches of silicas are indicated on the Fig. 14a–e (denoted as three batches of RP and four batches of RP, respectively). The RSDs of the selectivity values derived from the retention factors of the pairs amylbenzene/butylbenzene and butylbenzene/ethylbenzene are five-times higher on the four batches of RP based on different batches of silica than on the three batches based on the same batch of silica. The differences are less pronounced for the ethylbenzene/toluene and the acenaphthene/naphthalene pairs. The correlation between the total carbon content of the phase and the hydrophobic selectivity are weak ($R^2 = 0.53$) while the surface coverage and the selectivity values show no correlation. These results seem to indicate that even minor differences between the chemistry of the silica surface of different batches of silica play an important role in determining the fluctuation of hydrophobic selectivity, a role which seems more important than that of small variations of the surface coverage by alkyl groups or of the total carbon content. Obviously, it cannot be ruled out (although this seems unlikely) that the measurements of the

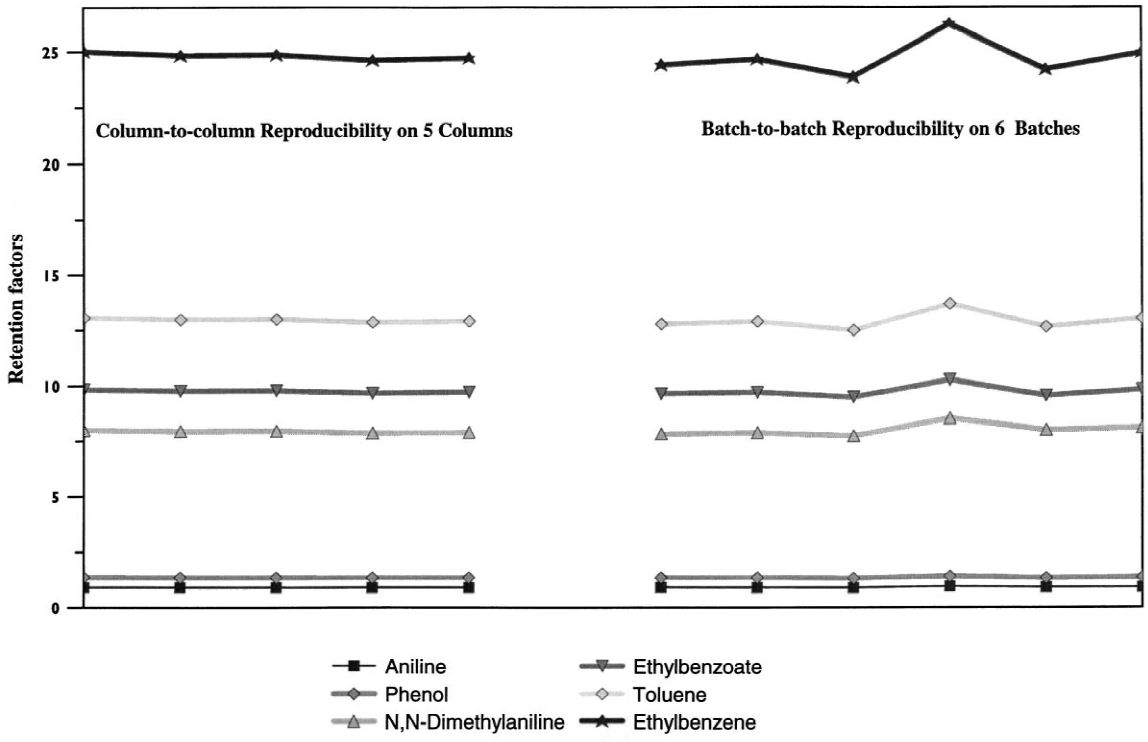


Fig. 10. Retention factors of the components of the second test mixture. Same data presentation as in Fig. 9.

carbon content and the specific surface area and, as a consequence, the determinations of the surface coverage are so much less accurate than those of the chromatographic parameters reported here that no correlation should be made between their fluctuations and those of the parameters characterizing the size of the bonded layer.

This result is consistent with previous findings [8,43–46] that the carbon content of a monomeric-type RPLC packing material does not correlate well with its hydrophobic selectivity when the density of surface coverage of the alkyl groups or the carbon content are high. According to Engelhardt and Jungheim [8], the hydrophobic selectivity, expressed as the selectivity of ethylbenzene and toluene, is a quasi-linear function of the carbon content up to 12% carbon, above which value the dependence is less pronounced.

Finally, Tan and Carr [46] concluded that adsorption of the analytes does not have a major influence on the separation factors of neutral compounds for C_{18} packing materials with a high carbon content but that partition governs the separation process. According to one of the proposed models, the surface partition model, the ''chemical potential of the solute is influenced by the water and organic solvent associated with the surface bonded ligands or with the solid surface per se or its silanol groups''. Based on this theory, the differences observed here could be explained by similar differences of the analyte constants of distribution between the mobile phase and the layer of mobile phase components adsorbed on the surface of the stationary phase. The excess adsorption isotherms would vary from batch-to-batch and the composition of the mobile phase adsorbed on the stationary phase surface would change slightly.

3.4. Steric selectivity

In this study, the steric selectivity is characterized by the separation factor of triphenylene and *o*-terphenyl which have a similar polarity but different shapes [6]. All the values of the steric selectivity

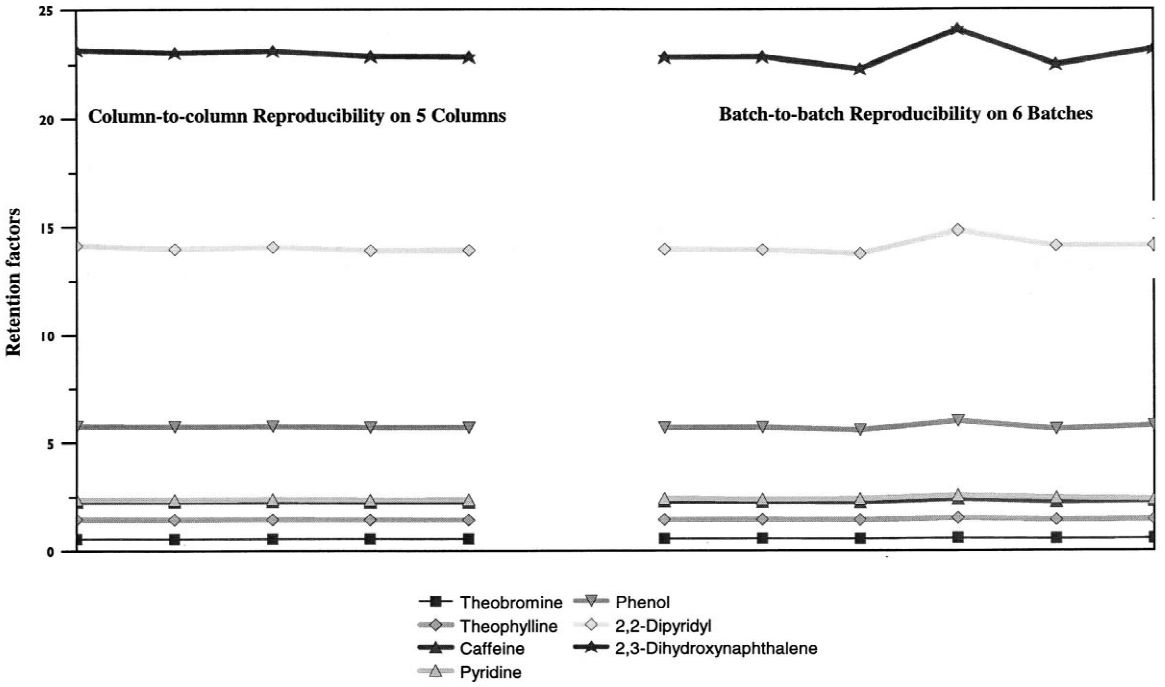


Fig. 11. Retention factors of the components of the third test mixture. Same data presentation as in Fig. 9.

measured are plotted in Fig. 15. Each set of five successive data points correspond to one column. The steric selectivities of the five columns packed with the packing material from the same batch are highly reproducible (RSD = 0.06%). The RSD for the columns of the six different batches is much higher, 0.89%. The differences observed between the different batches cannot be correlated with their carbon content but there is a correlation with the surface coverage ($R^2 = 0.85$). The values obtained on the three batches of packing made from the same batch of silica (the first, second and sixth sets of data points) show higher fluctuations (1.15%) than the values measured on the packing material batches made from different silica batches (0.65%).

The precision of these measurements is high, both for the short- (0.016% RSD) and the long-term (0.033% RSD) repeatability, so the differences observed between the batches are significant. Finally, we note that the RSD of the relative retention of the pair triphenylene/amylbenzene was 0.80%, barely different from the value found for the polyaromatic hydrocarbon pair.

3.5. Separation factors of the basic compounds

In a previous paper [36], we discussed the complexity of the retention of basic compounds and listed the factors which may affect their separation. These factors originate from the properties of the stationary, the mobile phase and the solute properties [47–51].

The large number of effects and the paucity of the available data considerably complicate the interpretation of most experimental results. For example the concentrations of the different silanol groups are measured by most manufacturers. Unfortunately, they keep these data confidential. The effect of the organic modifier on the pK_a (or on the point of zero charge) of the silanol groups and, in cases in which ion-exchange is the dominant form of interaction between solute and stationary phase, its effect on the ion-exchange equilibrium constant should also be taken into account. They are difficult to estimate.

The large number of factors influencing the retention of basic compounds makes it impossible to use only a few test solutes in an attempt to character-

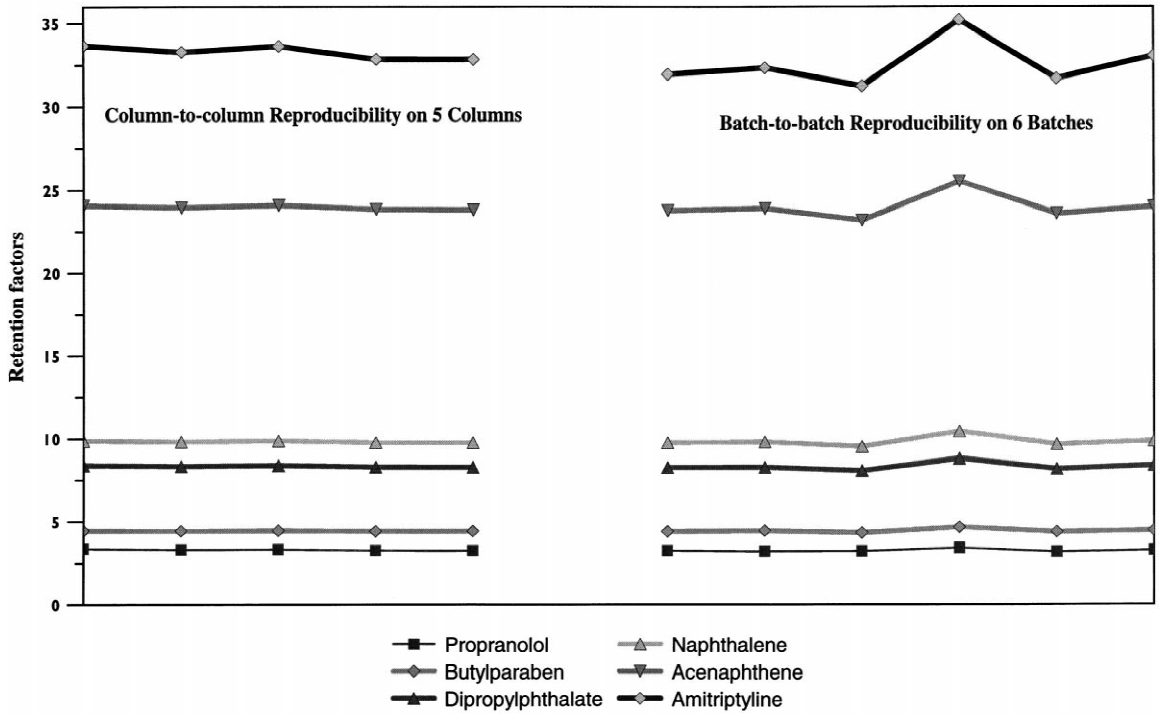


Fig. 12. Retention factors of the components of the fourth test mixture. Same data presentation as in Fig. 9.

ize properly the ability of any stationary phase to separate basic compounds. This is why, in our investigations, we used nine different basic compounds, under four test conditions. Still, we agree that the characterization achieved with this small array is merely qualitative and insufficiently detailed.

The behavior toward basic compounds of the columns studied was characterized by the relative retention of the test basic compounds with respect to that of neutral, nonpolar compounds (although phenol was used in the last case). Fig. 16a–f illustrate the results obtained, showing the separation factors of the following pairs: aniline and toluene (Fig. 16a), *N*,*N*-dimethylaniline and toluene (Fig. 16b) – both from test 2 – amitryptiline and acenaphthene (Fig. 16c) and propranolol and acenaphthene (Fig. 16d) – both from test 4 – benzylamine and benzylalcohol (Fig. 16e) – from test 5 – and pyridine and phenol (Fig. 16f) – from test 3. There are two methods to interpret these data, using either a pH scale defined in water or an apparent pH scale defined for the methanol–water (buffer) mixture. Unfortunately none of these two methods can take into account the possible effect of the stationary phase on the pH of the solution inside the column.

In test 2, the mobile phase was unbuffered and the pH of the water used was close to 7. The pK_a of the solutes used in pure water is 4.63 for aniline and 5.15 for *N*,*N*-dimethylaniline. Under these conditions most of the silanol groups (at least the most acidic sites which are believed to be the strongest interaction sites) are dissociated and the aromatic amines are both unprotonated, so only rather weak (i.e., nonelectrostatic) interactions can take place between them and the adsorbent. In methanol–water mixtures the pK_a of the amines decreases with increasing methanol content (in methanol–water, 1:1, the pK_a of aniline is 4.23 [50]). The effect of the organic modifier on the dissociation constant of the silanol groups is not as clear, although data published [50] on the variation of the dissociation constants of weak acids with methanol concentration in methanol–water mixtures predicts an increase of approximately 1 pK_a unit from pure water to a methanol–water

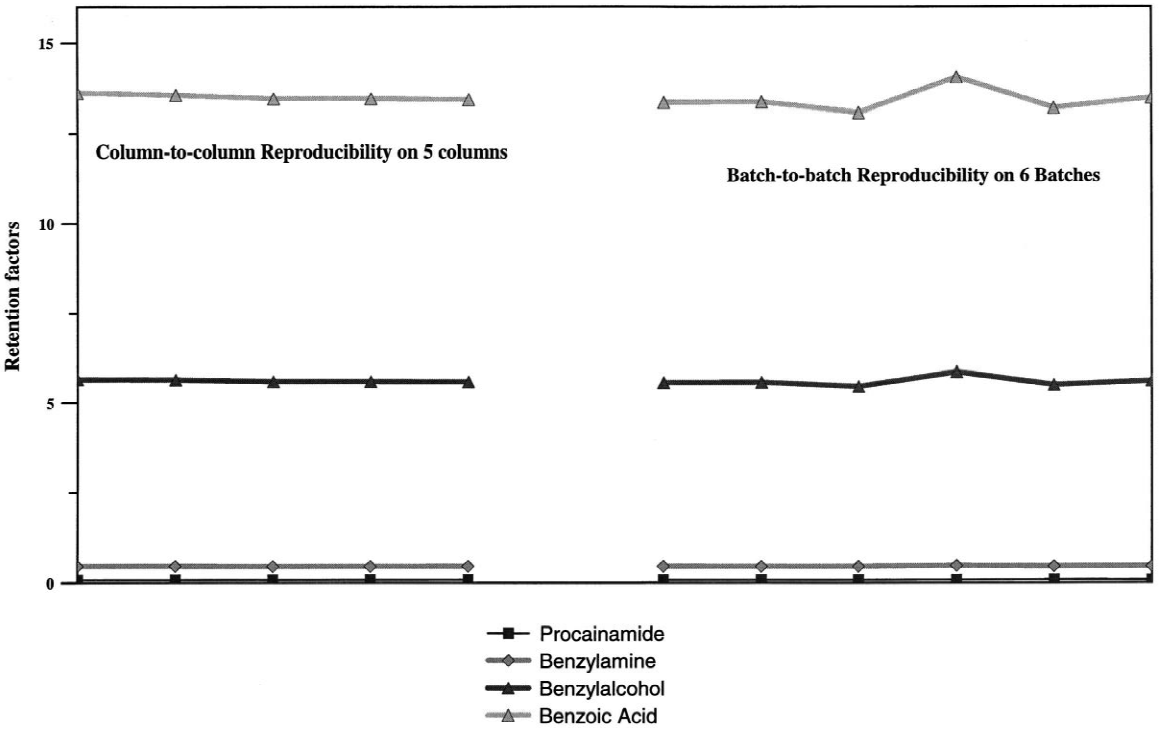


Fig. 13. Retention factors of the components of the fifth test mixture. Same data presentation as in Fig. 9.

(1:1) mixture. The pH of the mobile phase is close to 7, so it is clear that it is the neutral forms of the bases that are chromatographed. Under these conditions, we can assume that the separation of the toluidine isomers is strongly influenced by steric effects.

The RSDs of the measurements of the relative retentions of the two pairs aniline/toluene and *N*,*N*-dimethylaniline/toluene on the five columns packed with the packing material coming from the same batch were low, 0.14 and 0.08%, respectively. The RSDs of these separation factors were approximately 10-times higher for the measurements made on the six columns packed with different batches of packing material. The RSD of the measurements of the relative retention of two neutral compounds (ethylbenzene/toluene) was 0.08 on the same series of columns, under the same test conditions. Surprisingly, the RSDs obtained on the batches of packing materials made from the same batch of silica (marked with stars in Fig. 16a and b, 0.88%) are only twice lower than those of the measurements made on the batches prepared from different batches of silica (1.6%). These results suggest that the chemistry of the silica surface is not seriously involved in the (minor) effects observed here and/or that the reproducibility of the end-capping procedure has an observable effect. The fact that the trends observed on the six batches are different for the two pairs, aniline/toluene and *N*,*N*-dimethylaniline/toluene, indicates that the interactions on and/or the accessibility of the silica surface by the two amines are most probably different (steric effects might play an important role in the separation).

In test 4, the mobile phase is a methanol–water (65:35) mixture, made of a pH 7.0 buffer (pH measured before addition of the organic solvent). The pK_a values of the basic compounds, amitriptyline and propranolol, in water are 9.4 and 9.5, respectively. The pH and pK_a values measured in water suggest that the silanol groups are dissociated while both amines are still completely protonated. Thus, strong ion-exchange interactions are expected to take place between these amines and the silica

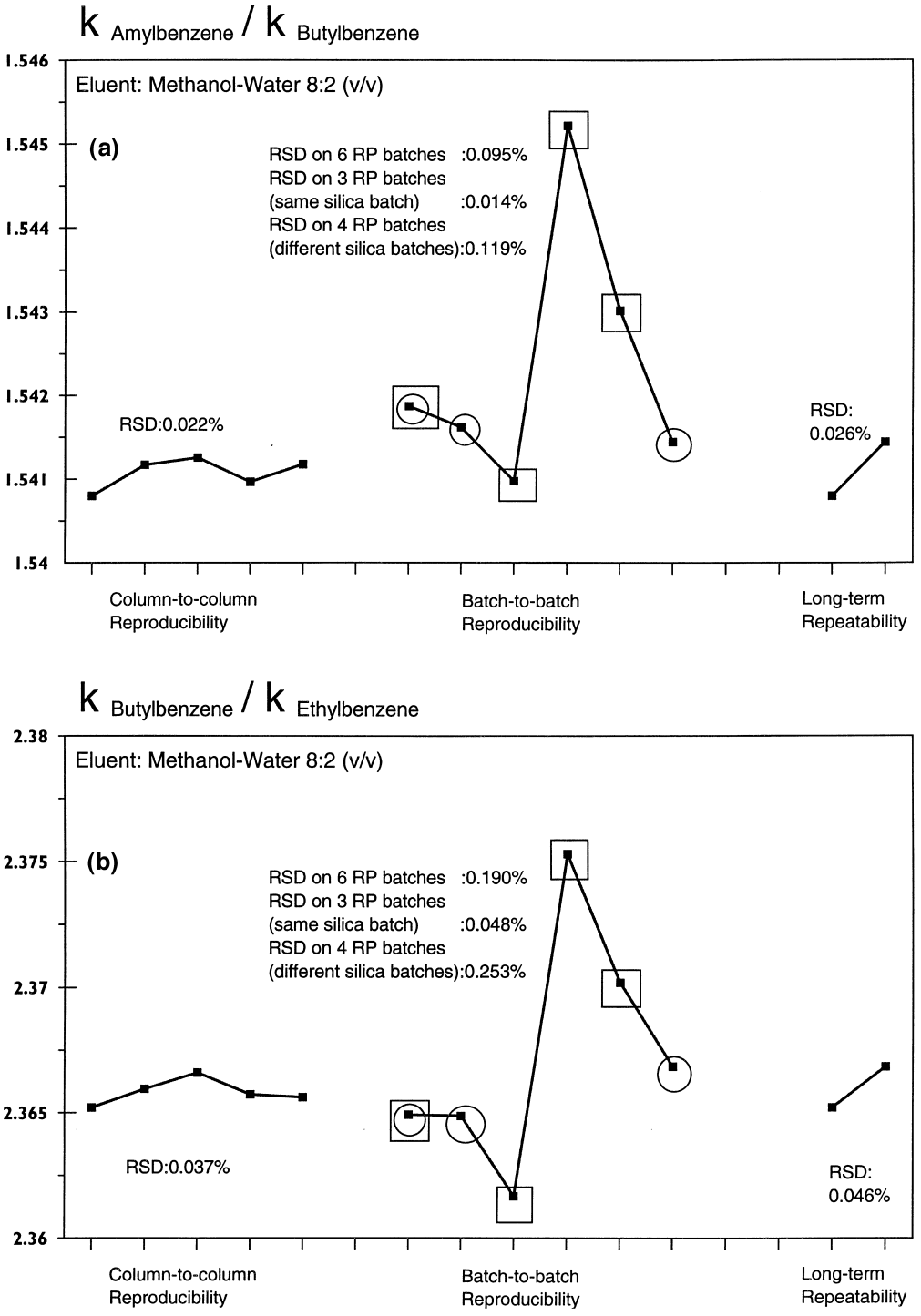
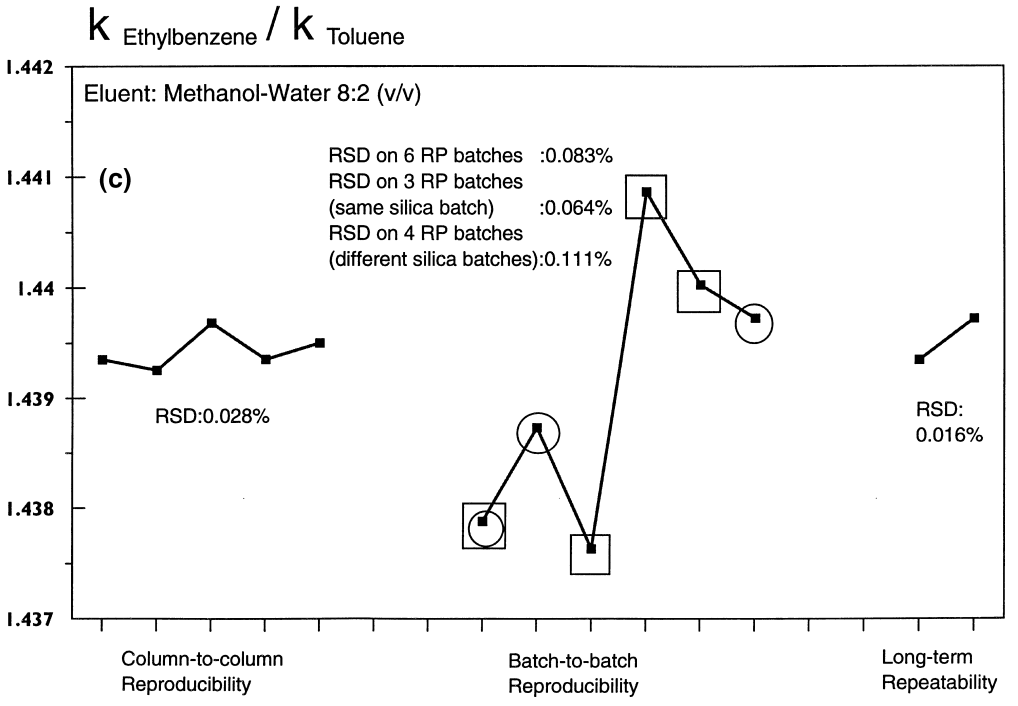


Fig. 14. Reproducibility of the hydrophobic selectivity. (a) Amylbenzene/butylbenzene (test 1). (b) Butylbenzene/ethylbenzene (test 1). (c) Ethylbenzene/toluene (test 1). (d) Ethylbenzene/toluene (test 2). (e) Acenaphthene/naphthalene (test 4).



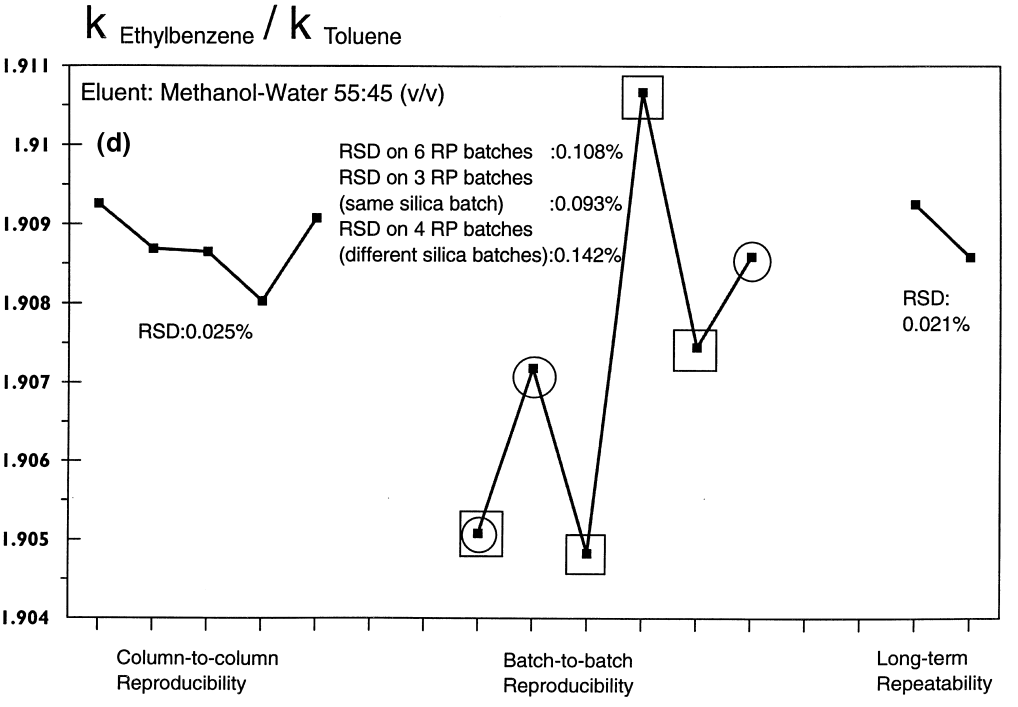


Fig. 14. (*continued*)

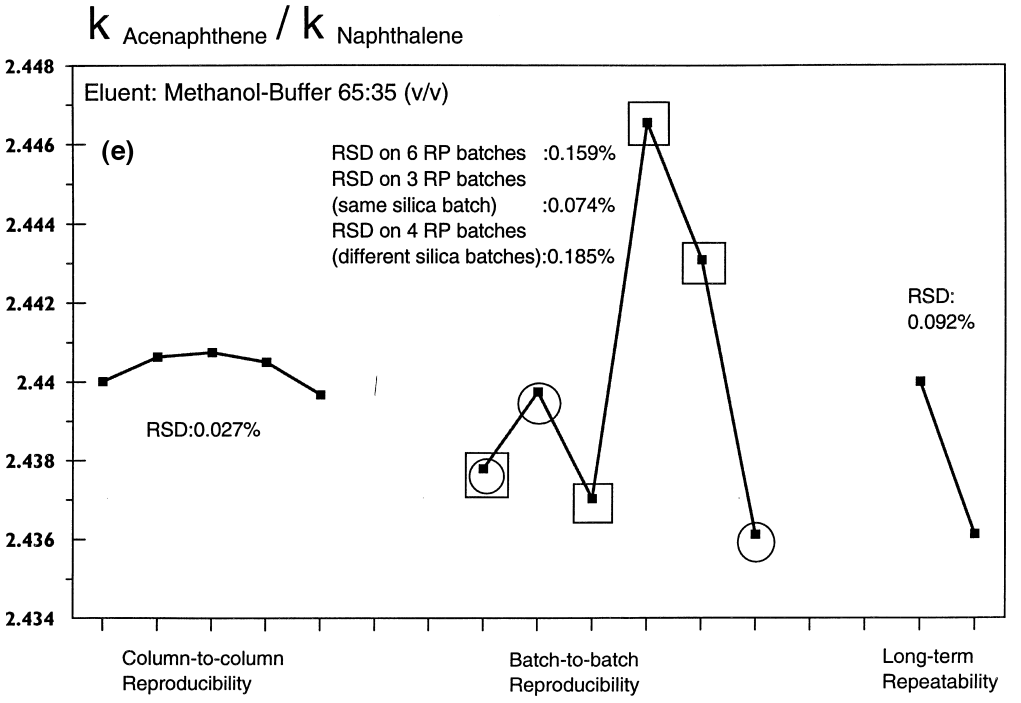


Fig. 14. (*continued*)

surface. One would expect to see more pronounced differences between the column batches than was observed for the bases in test 2 but this is not the case. The RSDs of the measurements of the separation factors of these two basic compounds relative to neutral acenaphthene are approximately the same as those found in the second test, 1.30% for propranolol/acenaphthene and 1.13% for amitriptyline/acenaphthene on the six batches (Fig. 16b and c). This phenomenon is probably explained by the influence of the methanol content of the mobile phase on the dissociation constants. The pK_a of the phosphate buffer is increased while those of the amines are decreased [50,51]. In the case in point, equal amounts of the two salts are used and the pH of the mobile phase is approximately 8.3 [50] while the pK_a of the amines in the mobile phase are decreased by approximately 0.5 unit [51]. The two effects combine and the amines are close to their half-dissociation state.

The protonated amine ions are still able to undergo strong ion-exchange interactions with the surface silanols but, because of the simultaneous presence of the two forms, worse peak shapes are expected than at lower pH values while column loading is supposed to have less influence on the peak shape. Note that the shifts of the dissociation constants of the phosphate buffer and the acidic silanols compensate each other, so the dissociation of the silanol groups is nearly the same in water or in buffer–methanol solutions.

In test 5, the methanol–water (30:70) mobile phase is buffered at pH 2.7 (pH of the aqueous buffer measured before addition of the organic modifier). This pH is believed to be low enough for most of the silanol groups to be protonated. The pK_a of benzylamine is 9.3 in water, certainly making the amine fully protonated at this pH. Under these conditions ion-exchange interactions can take place. This conclusion holds true no matter which pH scale is used in the discussion. The RSD of the measurements of the relative retention of the pair benzylamine/benzylalcohol on the six batches of packing material was 1.19%. We observed a correlation between the results obtained on the six batches of packing material with the pairs aniline/toluene

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

	One batch, five columns		Six batches	
	Average value of relative retentions	RSD (%) of relative retentions	Average value of relative retentions	RSD (%) of relative retentions
Test 1 (MeOH–water, 8:2)				
Chloro-nitrobenzene/phenol	3.5696	0.0427	3.5795	0.3386
Toluene/chloro-nitrobenzene	1.7489	0.0312	1.7455	0.3118
Ethylbenzene/toluene	1.4394	0.0281	1.4391	0.0831
Butylbenzene/ethylbenzene	2.3658	0.0367	2.3673	0.1900
o-Terphenyl/butylbenzene	1.2619	0.0157	1.2618	0.1597
Amylbenzene/*o*-terphenyl	1.2212	0.0245	1.2224	0.1503
Triphenylene/amylbenzene	1.3927	0.0475	1.4127	0.8036
Test 2 (MeOH–water, 55:45)				
Phenol/aniline	1.5038	0.1301	1.4913	1.1152
Dimethylanhline/phenol	5.9661	0.0496	6.0263	1.5425
Ethylbenzoate/dimethylaniline	1.2286	0.0455	1.2163	1.1923
Toluene/ethylbenzoate	1.3324	0.0758	1.3309	0.4136
Ethylbenzene/toluene	1.9087	0.0245	1.9073	0.1079
Test 3 (MeON–water, 3:7)				
Theophylline/theobromine	2.5142	0.0653	2.5108	0.1760
Caffeine/theophylline	1.5923	0.0648	1.5949	0.1332
Pyridine/caffeine	1.0427	0.2408	1.0632	1.3665
Phenol/pyridine	2.4093	0.3251	2.3473	1.7926
2,2-Dipyridyl/phenol	2.4429	0.2610	2.4667	1.0374
Dihydroxynaphthelene/dipyridyl	1.6408	0.2255	1.6248	1.0335
Test 4 (MeOH–pH 7.0 buffer, 65:35)				
Butylparaben/propranolol	1.3397	1.1531	1.3617	1.1200
Dipropylphthalate/butylparaben	1.8824	0.2655	1.8738	0.3944
Naphthalene/dipropylphthalate	1.1761	0.0612	1.1806	0.3271
Acenaphthene/naphthalene	2.4403	0.0270	2.4401	0.1592
Amitriptyline/acenaphthene	1.3880	0.6599	1.3571	1.1316
Test 5 (MeOH–pH 2.7 buffer, 3:7)				
Benzylamine/procainamide	5.7406	1.7609	5.6936	5.2525
Benzylalcohol/benzylamine	12.1037	0.7313	12.0311	1.1804
Benzoic acid/benzylalcohol	2.3981	0.1199	2.3925	0.0500

and benzylamine/benzylalcohol (cf. Fig. 16a and e). This suggests that the same kind of interactions take place with both amines. Finally, a similar correlation was observed previously [36] with the Waters Symmetry columns.

The RSD of the measurements of the relative retention of the pair pyridine/phenol was 1.79% in test 3 which uses an unbuffered mobile phase with 30% methanol content. Under these conditions, the silanol groups are mainly dissociated and pyridine is not protonated. Except for one batch we did observe on the six column batches the same trend with pyridine as we did with aniline/toluene and benzylamine/benzylalcohol.

3.6. Column efficiency

The ChemStation supplies five values of the efficiency for each recorded peak, derived from the measured signal using five different algorithms. In this study we report only the RSDs on the measurements of the peak efficiency derived from the peak width measured at half-height. The values obtained

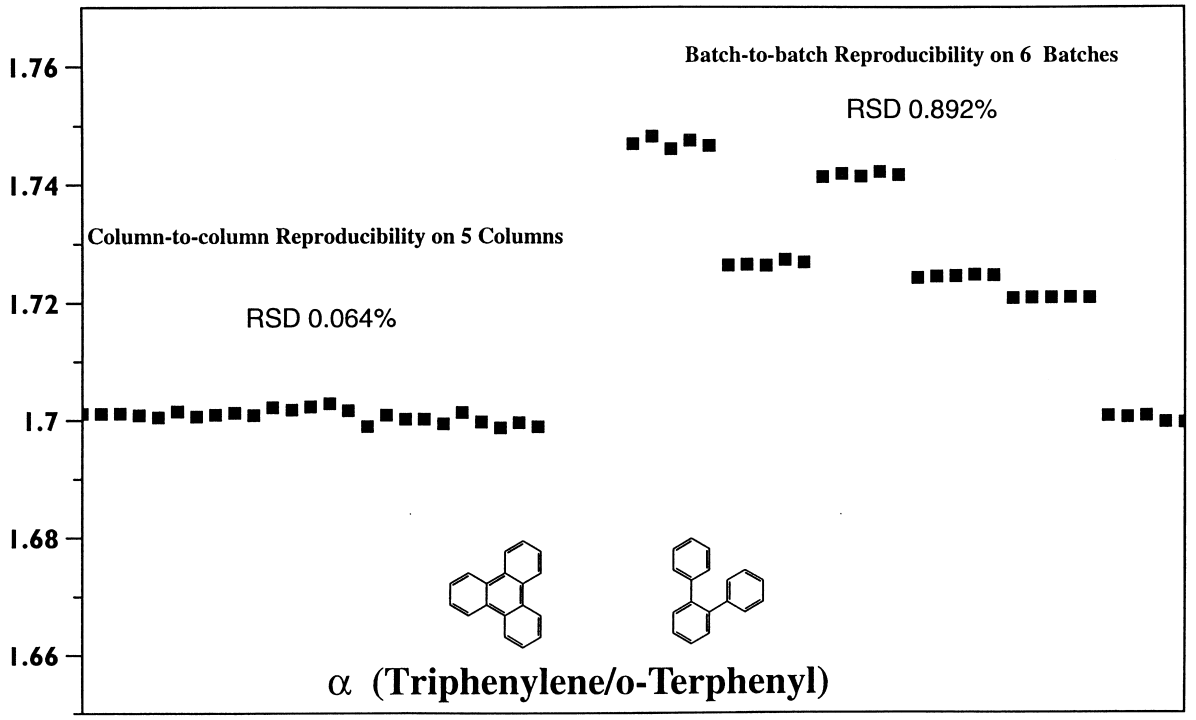


Fig. 15. Reproducibility of the steric selectivity.

are presented in Figs. 17–21. Similar trends were observed with the other values.

The short- and long-term repeatabilities closely match those found in a previous study [35]. The RSDs are all below 2%, except those for the long-term repeatability of the compounds of test 4 and for the short-term repeatability of 2,3-dihydroxynaphthalene in test 3. However, these RSDs are almost always more than one-order of magnitude larger than those observed for the other parameters determined in this study. The RSDs characterizing the column-to-column reproducibility differ slightly from those characterizing the short- and long-term repeatabilities, except for those found for 2,2-dipyridyl and 2,3-dihydroxynaphthalene in test 3 and for amitriptyline in test 4. We ensured that the shape of the peak of 2,3-dihydroxynaphthalene was not affected by the tailing of 2,2-dipyridyl, by injecting these two components in two separate samples. The software (HP ChemStation) used for the data processing offers different methods for the peak delimitation, hence, for the peak width determination. For a small peak with a high retention time and a strong tailing, like the amitriptyline peak, the integration method might affect the results. To check this possible effect, we tried all the features allowed by the software and found that the different measures of the peak efficiency had RSDs differing from each other by less than 0.1% for the efficiency of the amitriptyline peak and by less than 0.5% for the efficiency of the 2,3-dihydroxynaphthalene peak.

The RSDs corresponding to the batch-to-batch reproducibility for the neutral compounds were below 6% in the first test, below 7% in the second test, below 9% in the fourth test and, although low values were obtained for the long-term repeatability of this test, between 10 and 13% in the fifth test. These RSDs are definitely higher (nearly three times) for propranolol (6.2%) and amitriptyline (5.8%) than the RSDs for the five columns of the same batch. For several basic compounds (pyridine, propranolol, procainamide and benzylamine), these RSDs are close to those observed for the neutral compounds. In spite of the low RSDs observed for the long-term

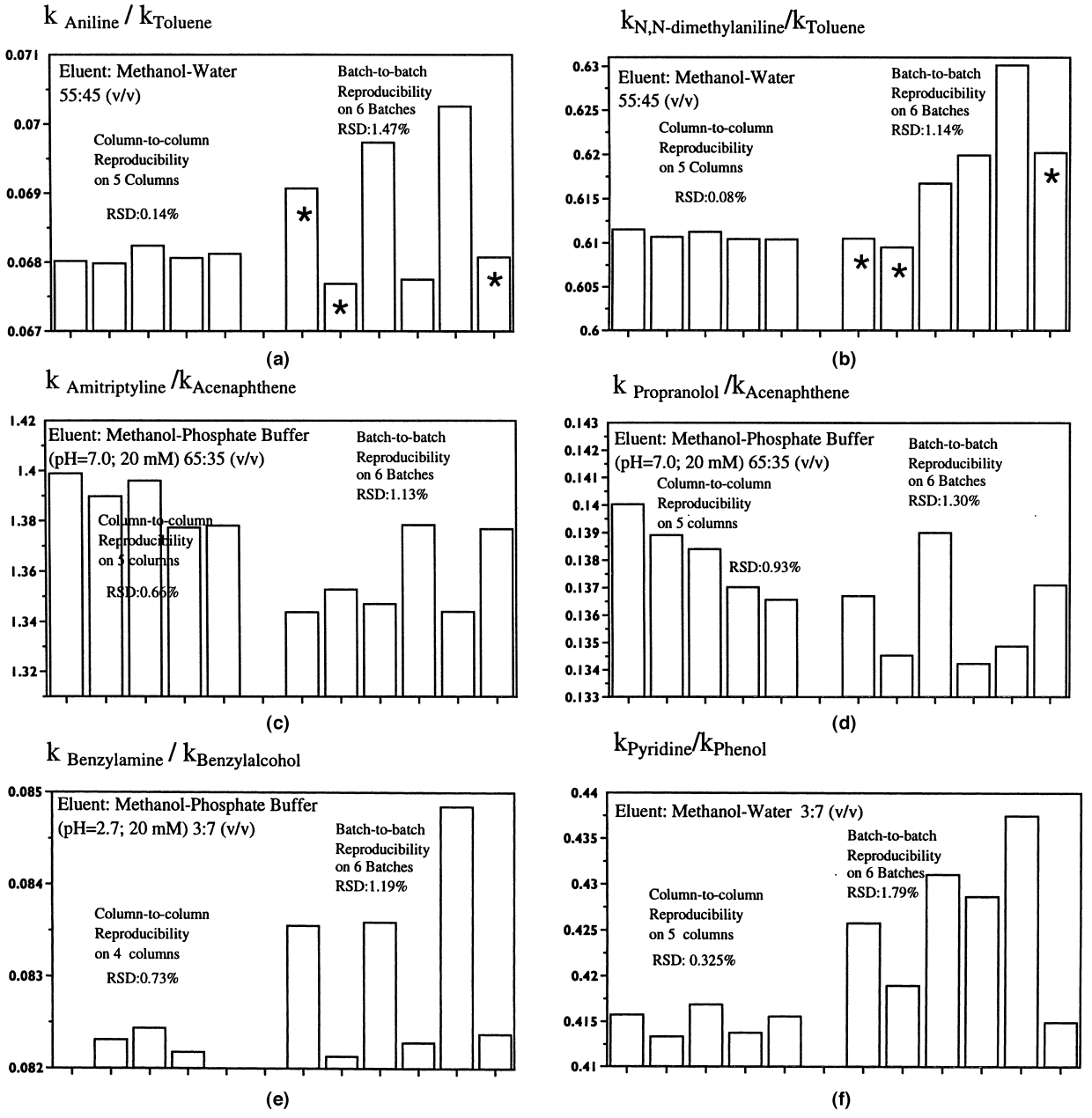


Fig. 16. Reproducibility of the separation factors of basic compounds. The batches labeled with a star were obtained by bonding the same batch of silica (cf. Table 1). (a) Aniline/toluene (test 2). (b) *N,N*-Dimethylaniline/toluene (test 2). (c) Amitryptiline/acenaphthene (test 4). (d) Propranolol/acenaphthene (test 4). (e) Benzylamine/benzylalcohol (test 5). (f) Pyridine/phenol (test 3).

repeatability in the second test (in which an unbuffered mobile phase was used), the RSDs for aniline and *N,N*-dimethylaniline on the six batches are high (16.4 and 13.4%, respectively). The same holds true for 2,2-dipyridyl (40.9%) and 2,3-dihydroxynaphthalene (25.8%) in test 3 and for amitriptyline (22.6%) in the buffered fourth test solution. Although the variations observed in the peak

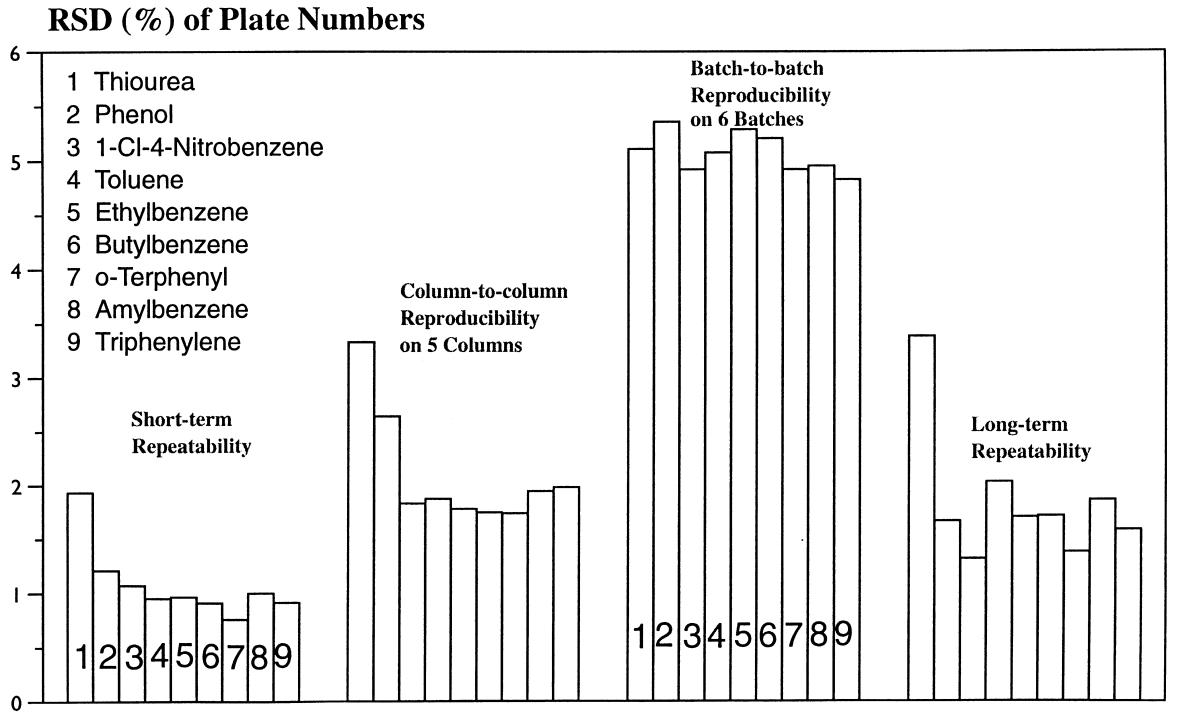


Fig. 17. RSD of the number of theoretical plates for the components of the first test mixture.

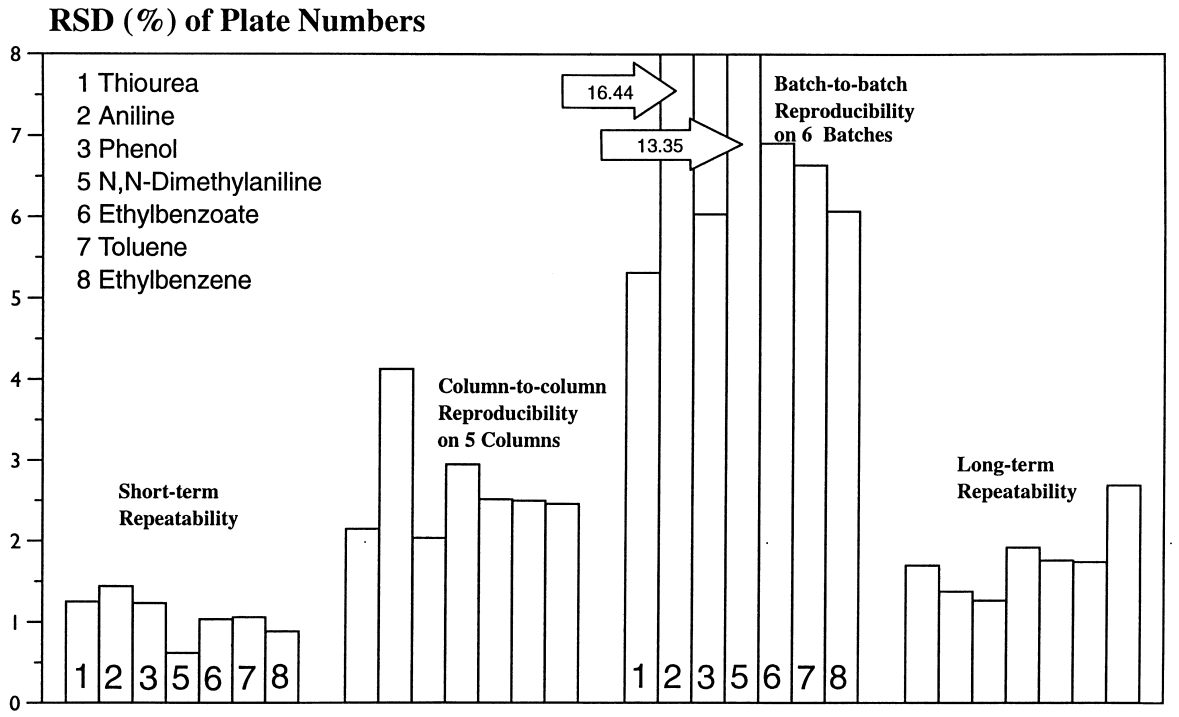


Fig. 18. RSD of the number of theoretical plates for the components of the second test mixture.

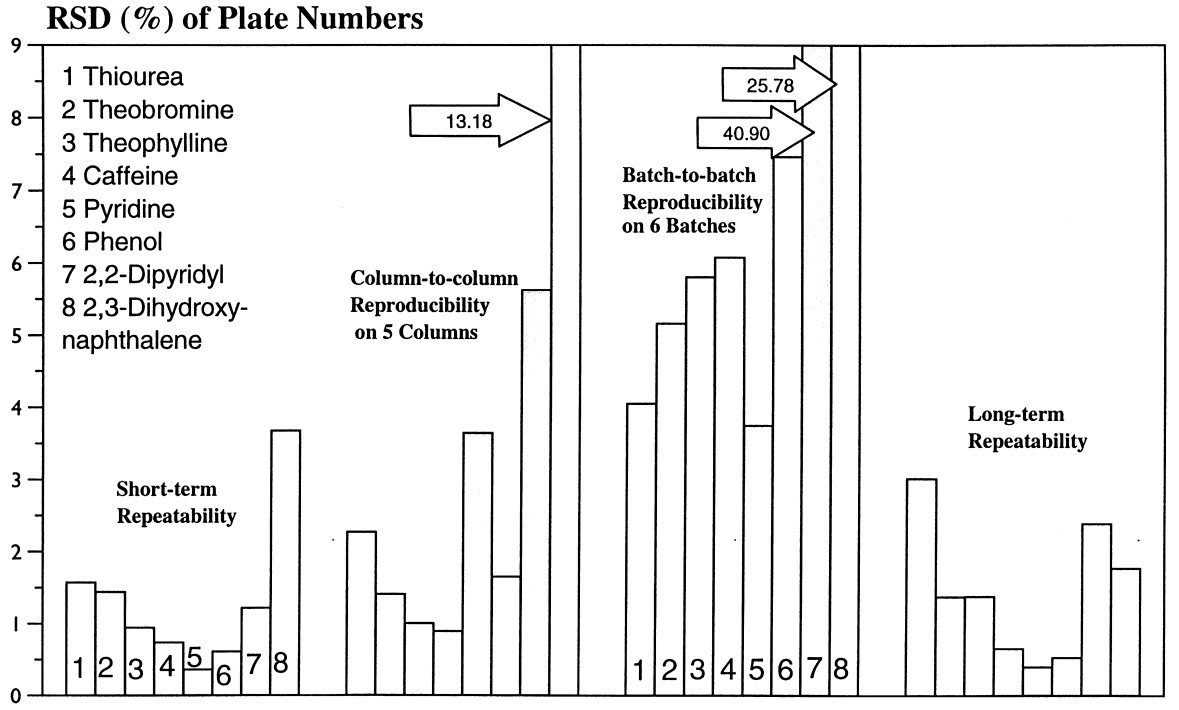


Fig. 19. RSD of the number of theoretical plates for the components of the third test mixture.

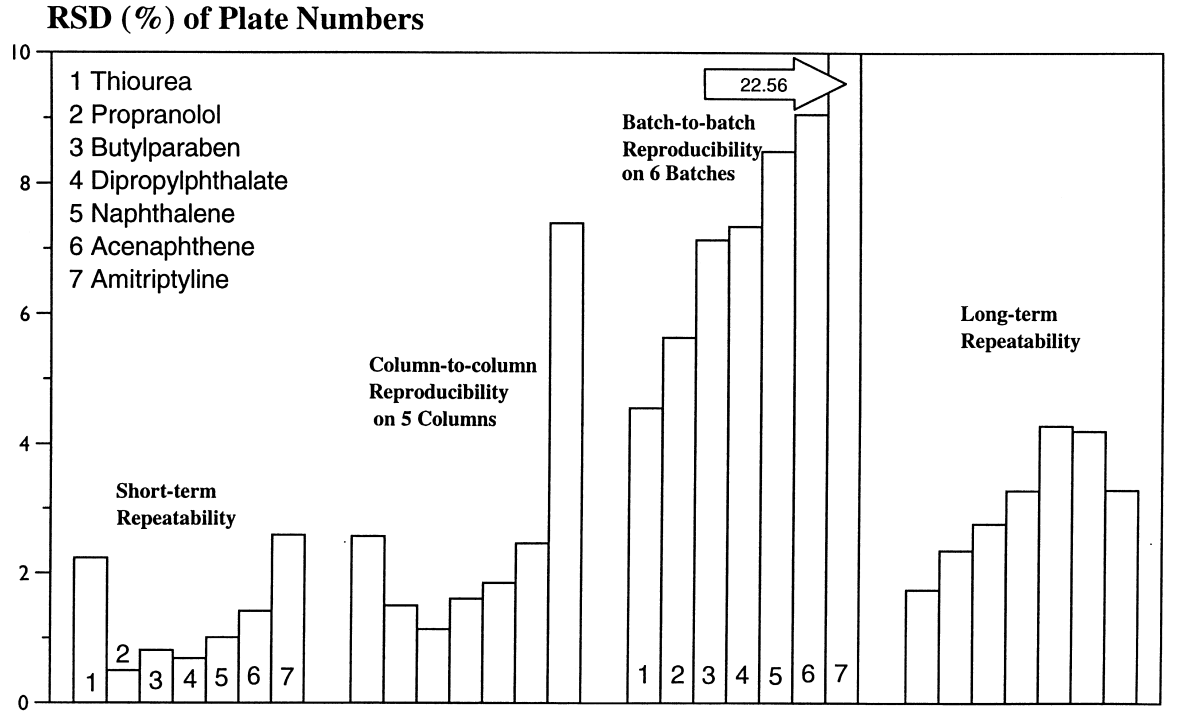


Fig. 20. RSD of the number of theoretical plates for the components of the fourth test mixture.

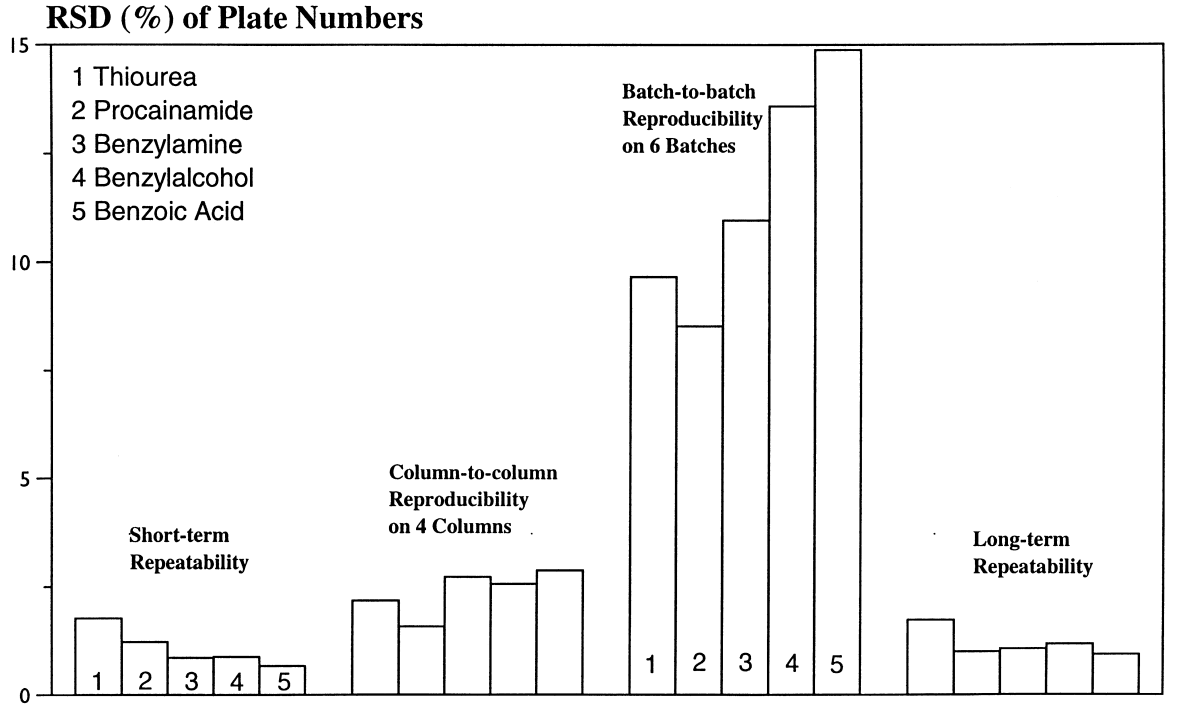


Fig. 21. RSD of the number of theoretical plates for the components of the fifth test mixture.

efficiencies on the different batches may have different origins for different compounds under different test conditions, the RSDs observed were very close.

These results are not easy to explain because they probably arise from the combined influence of several factors. However, they are real. The fact that the long-term repeatability and the column-to-column reproducibility give low RSDs proves that the differences observed between the different batches do not originate from the lack of reproducibility of the test methods. Similarly, test involving bases eluted by an unbuffered mobile phase are often criticized. Still, their results (whatever they mean) are nearly as reproducible than those obtained for bases in a buffered mobile phase. An equilibration time of 5 h under a steady stream of mobile phase was allowed (because an independent investigation showed that this was necessary and sufficient). The series of five measurements on a column took between two and five more hours. Nonequilibrium between the two phases would cause a drift of the results, which was not observed. The RSD would be high and it would be comparable for the columns of the same batch and those of different batches. This was not observed either.

We must consider the possibility that the columns are overloaded with the sample sizes injected, at least for some of basic compounds. There is a general belief [49,52–56] that the surface of RPLC packing materials contains a few strongly acidic ion-exchange sites and that, being few, these sites can be easily overloaded. When they are overloaded, the retention factor and the efficiency decrease and the peak asymmetry increases. The values published in the literature [52–56] indicate that the onset of column overloading takes place at concentrations that vary from compound to compound, depend on the specific stationary phase studied, as well as on the pH and the composition of the mobile phase. This is obvious from solution thermodynamics since the column loadability depends on the initial curvature of the isotherm [57]. However, the determination of the batch-to-batch reproducibility of the loadability of the packing materials was not part of the protocol and was not one of our goals. Instead, we decided always to inject the same amount of each

compound, except for the minor fluctuations of the amount injected in successive analyses (the repeatability of the automatic injector was not part of this study) and for the adjustments required by differences in the properties of the columns (their size) and of the packing material (its specific surface area), as described in Experimental.

The sample sizes are constant. So, if the random fluctuations of the peak efficiency observed were to originate from differences in the degree of overloading, this would mean that the different batches have different saturation capacities [57], hence that they have different specific surface areas or that their surface chemistries are different, e.g., that they have a different density of strongly interacting silanol sites. The possibility that packing density fluctuations would be the major factor should be ruled out because the RSDs of the column-to-column and the batch-to-batch reproducibilities are so different. Note that similar fluctuations of the peak efficiencies were observed on the batch-to-batch repeatability test made on the Waters Symmetry columns.

The high values of the RSDs observed for the batch-to-batch reproducibilities cannot be explained simply by surface area or packing density fluctuations. What we observed is more probably the cumulative result of effects arising from small fluctuations of the experimental conditions, of the packing density of the columns, of the specific surface area of the packing material, and, mainly, of the chemical properties of their surface.

3.7. Peak asymmetry

The parameter measured in this study is the United States Pharmacopeia tailing factor. It is determined from the peak width at 5% of the peak height and is defined as

$$T = \frac{a + b}{2a} \tag{4}$$

where a and b are the distances between the position of the peak maximum and the ascending and descending fronts, also called the ascending and descending half peak width. This is an empirical parameter which has no physical meaning and is not related directly to any of the characteristics of the elution peak. Its only advantage is that it is easy to derive and that the measurements are reasonably precise.

The neutral compounds have a tailing factor which is practically equal to 1, both on the five columns packed with the same batch of packing and on the six columns which represent six different batches of packing materials (Table 8). The RSDs of the asymmetry factors of these compounds were below 2% on both sets of columns.

The basic compounds give tailing peaks, except for *N*,*N*-dimethylaniline which gives a leading one. Although some unusual velocity distributions can give rise to leading peaks [58], the most common source of this effect is the overloading with an analyte having an antilangmuirian isotherm [57], the most probable cause in this case. Thiourea, which is not retained, gives a tailing factor of 1.33 on the five columns. This tailing may originate only from the extra-column effects and from the bed heterogeneity. Most other tailing factors are between 1.0 and 1.4, with RSDs for column-to-column reproducibility and for batch-to-batch reproducibility around 1%. We comment mainly on the few exceptions, the basic and the chelate forming compounds, aniline, pyridine, 2,2-dipyridyl and 2,3-dihydroxynaphthalene.

Aniline, which is slightly retained, gives the same average tailing factor as thiourea, probably for the same reason. However, the RSD value for this factor is high (5.9%), much higher than the column-to-column reproducibilities of the other compounds. This could suggest that even columns which were so far tested only with neutral compounds before the first injection of the test 2 sample suffer to a different degree of some surface contamination, possibly because of a different treatment during the packing process. The long-term repeatability of the aniline peak tailing factor had an RSD of 0.67% which illustrates the precision of the measurement process itself. Surprisingly, the average tailing factor measured on the six columns packed with the six different batches was high, at a value of 1.83. Neither this last result nor the exceptionally large RSD associated with it (55.7%) can be explained by extra-column effects. Results obtained with alternate methods to characterize peak asymmetry were compared in an attempt to reduce the error contribution due to the operation of peak delimitation, to no avail.

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

	One batch, five columns		Six batches	
	Average value of tailing factors	RSD (%) of tailing factors	Average value of tailing factors	RSD (%) of tailing factors
Test 1 (MeOH–water, 8:2)				
Thiourea	1.388	1.732	1.371	1.988
Phenol	1.264	1.346	1.246	1.218
1-Chloro-4-nitrobenzene	1.142	1.416	1.145	0.781
Toluene	1.099	1.259	1.101	0.838
Ethylbenzene	1.076	1.338	1.081	0.906
Butylbenzene	1.026	1.406	1.028	1.210
o-Terphenyl	1.064	1.388	1.064	1.177
Amylbenzene	1.015	1.268	1.018	1.073
Triphenylene	1.071	1.333	1.073	0.882
Test 2 (MeOH–water, 55:45)				
Thiourea	1.353	0.838	1.339	1.504
Aniline	1.327	5.868	1.833	55.722
Phenol	1.186	0.901	1.183	1.483
N,*N*-Dimethylaniline	0.769	0.584	0.952	25.144
Ethylbenzoate	1.109	1.172	1.122	1.910
Toluene	1.014	1.173	1.027	1.819
Ethylbenzene	0.976	1.033	0.994	1.596
Test 3 (MeOH–water, 3:7)				
Thiourea	1.320	1.705	1.333	2.983
Theobromine	1.202	1.056	1.211	1.776
Theophylline	1.163	0.627	1.174	1.705
Caffeine	1.194	0.785	1.200	1.969
Pyridine	2.039	2.856	2.671	48.980
Phenol	1.192	0.740	1.199	1.256
2,2-Dipyridyl	2.898	18.435	4.970	65.839
2,3-Dihydroxynaphthalene	0.804	5.774	0.966	23.779
Test 4 (MeOH–pH 7.0 buffer, 65:35)				
Thiourea	1.211	1.399	1.391	6.218
Propranolol	1.215	2.230	1.308	11.316
Butylparaben	1.187	3.046	1.263	7.550
Dipropylphthalate	1.149	3.108	1.263	8.685
Naphthalene	1.117	3.386	1.238	9.475
Acenaphthene	1.073	3.389	1.189	9.641
Amitriptyline	3.540	7.054	3.181	12.397
Test 5 (MeOH–pH 2.7 buffer, 3:7)				
Thiourea	1.322	1.189	1.371	3.895
Procainamide	1.097	5.783	1.169	8.233
Benzylamine	2.376	1.602	2.257	8.874
Benzylalcohol	1.273	1.949	1.333	5.781
Benzoic acid	1.316	2.452	1.362	6.745

[a] Average values and their reproducibility.

For example, the RSD of the second moment of the aniline peak was 65.2%. The RSD of the tailing factor on the three columns which were packed with RPLC packing material based on the same batch of silica was 16.9%. The RSD of the tailing factor on the four columns packed with packing materials based on different batches of silica was 48.2%. This confirms an earlier suggestion, that the properties of the underlying silica have a strong influence on the chromatographic performance of a packing material.

Pyridine gives a large average tailing factor of 2.0 on the five columns and of 2.7 on the columns representing the six batches in a methanol–water (3:7) mobile phase. The RSD on the five columns (2.8%) is half the RSD found for aniline (5.87%), while the RSD on the six columns representing the six batches (49%) is nearly as high at that for aniline. The columns packed from the three batches of packing based on the same batch of silica give an RSD of 1.82% (which value is just slightly higher than the long-term repeatability of 1.56%) while the value obtained on the four columns from four batches of packing based on four different batches of silica was 47.5%. This is another case in which the influence of the chemical properties of the underlying silica is clear.

2,2-Dipyridyl and 2,3-dihydroxynaphthalene form chelates with metal cations. 2,2-Dipyridyl gives the highest average tailing factor in the unbuffered solutions, 2.9 on the five columns and 4.97 on the six columns representing the six batches. The high value obtained for the six batches originates essentially from the high value (12) observed on one of these batches (which consistently exhibits lower performance than the others with basic compounds). As for aniline, there is a contrast between the RSD of the measurements made on the five columns of the same batch (18.4%) and the long-term repeatability of the experiment (RSD, 3.85%). The RSD on the six columns from the different batches was 65.8%. After excluding the batch with the extreme value of the tailing factor from the calculation, the RSD is reduced to 26.9%, still a high figure. The RSD of the tailing factors on the three columns packed with packing materials based on the same batch of silica was 13.9% while the value for the four columns for the four different batches of packing material based on different batches of silica was 65.4%. Although Engelhardt and Lobert recently [59] proved, that the stationary phases collect metal ions from the HPLC grade solvents even using metal free equipment and columns, the fluctuation we observe here is still an indication of the differences between the batches as the columns were flushed with the mobile phase for equal times in our laboratory.

2,3-Dihydroxynaphthalene gives peaks exhibiting a moderate degree of leading, except one batch (on the same batch with which 2,2-dipyridyl gives a peak with tailing factor of 12), for which a tailing factor of 1.44 was measured. This compound is the only one for which the tailing factor increases systematically from injection to injection on all the columns, except one, that gave a constant tailing factor. The RSD of 5.77% on the five columns originates from this injection to injection drift. The RSD on the six batches was 23.78%. After excluding the value measured on the column which exhibits a peak with a tailing factor of 1.44, the average value of the RSD drops to 6.4%, which is slightly higher than the RSD on the five columns.

4. Conclusion

For all practical purposes, the batch-to-batch reproducibility of the chromatographic properties of Kromasil C_{18} should satisfy most analysts. It is difficult to demand better reproducibility of the column performance when the RSDs of most retention factors are within a 0.5% range for the column-to-column reproducibility and within a 2 to 3% range for the batch-to-batch reproducibility and when the reproducibilities of the efficiencies and the tailing factors of most peaks are both within a few percent range. The replacement of one Kromasil column by another one should not result into any perceptible change in the analytical performance, at least in most cases. The same result was previously reported for another brand [36].

It is worth noting that the reproducibility of the thermodynamic data is always much better than that of the kinetic data and that the RSD figures for the neutral and acidic compounds are much lower than for the basic ones. The refinements brought to the precision of chromatographic measurements have reached such a level that the fluctuations of the tube

diameter and length are easily measured and contribute significantly to the RSD of the hold-up volume of columns, hence on that of the retention times measured at constant volume flow-rate of the mobile phase. Except for basic compounds, significant improvements in the precision of chromatographic analyses (if and when needed) could come only from a combination of better controlled instruments and packing materials.

Different column brands are prepared using different processes for the synthesis of porous silica and for the bonding of the paraffinic layer used in RPLC. It is not surprising that they have different physico–chemical properties. The aim of our current study was not to compare the position of the different brands studied on charts comparing their retention properties (i.e., plots of hydrophobic selectivity versus silanophilic activity), as was done by several groups [3–6]. It was to estimate the error range of the points on such charts. Knowing the precision of these correlation plots will markedly improve their usefulness for the selection of the column needed to perform a separation. Still, it is worth noting that, although Kromasil C_{18} and Symmetry C_{18} give similarly precise results overall and experience serious reproducibility problems only for a few compounds, these compounds are not the same for the two brands.

Our detailed investigations of the dependence of the precision of the measurements on the experimental conditions and of the fluctuations of the characteristics of the packing material showed some interesting features. In many cases, it was noted that fluctuations of the properties of the underlying silica have an important influence on the chromatographic properties of the packing material. A high reproducibility of the specific surface area and the surface chemistry of the porous silica seems to be more difficult to achieve than proper reproducibility of the bonded layer. Yet, fluctuations of the surface area affect more the retention of all solutes than fluctuations of the surface coverage or of the carbon content. Fluctuations of these last parameters also affect peak tailing much less than those of the local chemical properties of the underlying silica. However, this cannot explain entirely the fluctuations of the column efficiency. This parameter seems to be influenced also by the parameters that control the extent of radial heterogeneity of the column bed (radial heterogeneity of the packed bed).

High values of the RSD of some parameters for isolated compounds and the fact that these compounds are not the same for the different brand tested is a valuable information regarding the retention mechanisms involved. These results are still too few to allow their correct interpretation but this observation offers a new approach for the investigation of RPLC columns.

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