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Repeatability and reproducibility of retention data and band profiles on six batches of monolithic columns

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Abstract

Chromatographic data were acquired for eight different mixtures, under five different sets of experimental conditions, for a total of 30 neutral, acidic and basic test compounds, on a series of six Chromolith Performance columns from Merck. These columns are made of a C_{18} chemically bonded silica monolith. Each column belonged to a different production batch, so the data reported here characterize their batch-to-batch reproducibility. The parameters studied in this work were the retention times, the retention and separation factors, the hydrophobic and the steric selectivities, the column efficiencies, and the tailing factors for all 30 compounds.

Keywords: Band profiles; Monolithic columns; Retention data

1. Introduction

The considerable potential advantages of monolithic columns were recognized by Knox [1] more than 30 years ago. He suggested the preparation of a rigid foam inside a column, the communicating bubbles providing the network of pores necessary for the flow of mobile phase. This column would provide nearly the same efficiency as the conventional column packed with particles having the size of the bubbles but a hydraulic resistance orders of magnitude lower. Unfortunately, in spite of numerous attempts, the concept of a monolithic column was reduced to practice only recently.

Based on the nature of the material they are made of, monolithic columns prepared so far can be classified as organic polymer or silica based columns. Based on their form and on their preparation process, we can differentiate between foams, monoliths prepared in the presence of pore forming solvents, monoliths prepared by polycondensation in the presence of soluble polymers, and particle-based monoliths consolidated either using a sol–gel process or by sintering. Depending on their method of synthesis, the monolithic columns can have either a high external porosity or a porosity similar to that of conventional columns packed with small particles. Obviously, the latter columns confer only part of the practical advantages obtained by the former. Monolithic columns are also often referred to in the literature as rod columns, continuous-bed columns, continuous porous packings or double-pore packing materials. We prefer the first term.

Ross and Jefferson [2] were first to report the preparation of monolithic GC columns from poly-

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urethane in 1970, an idea that was extended to liquid chromatography by Hansen and Sievers [3]. Twenty years later, Hjerten et al. reinvented the concept [4]. Their polymer-based rod-like column was prepared by compression of polyacrylamide gels. The data supplied dealt with proteins that were separated under gradient conditions, making a proper estimate of the column performance difficult. Later, Kumakura et al. [5] and Svec and co-workers [6,7] prepared polymer-rod columns, also using in situ polymerization. The general drawback of these columns, typical for polymeric stationary phases, was an efficiency lower than that of silica-based columns. It may also be expected that polymer shrinking or swelling could have more dramatic effects on the performance of these monolithic columns than on those packed with individual particles of the same polymers. Changes in the apparent volume of the polymeric material may cause losses of performance. The bed may lose contact with the wall and let the eluent by-pass the bed or the external porosity, hence the column permeability, may decrease dramatically [5]. However, the wide range of applications developed by the various groups preparing monolithic columns with polymers [2–11], demonstrate the usefulness of this approach in reversed-phase, hydrophobic interaction, ion-exchange, and affinity chromatography. For example, several groups prepared recently chiral selective polymer-based monolithic columns either by attaching a chiral functional group to the surface [8,9] or by imprinting the polymer [10,11].

In 1979, Pretorius et al. [12] reported briefly two methods (an emulsion and a blowing technique) developed for forming open-pore silica foams suitable for the in situ preparation of a new support for chromatography. These foams had a high porosity, 0.85 and 0.9, respectively, but no data were given regarding the chromatographic performance. The successful preparation of silica-based rod columns was reported by Nakanishi and Soga in 1991 [13,14]. The rods were prepared by the polymerization of tetramethoxysilane in the presence of a water soluble polymer (polyethyleneoxide) and acetic acid. Once formed, the rods were treated with aqueous ammonium hydroxide for pore formation, acid treated with aqueous nitric acid, and dried. In situ derivatization was performed with octadecyldimethyl(*N*,*N*-diethylamino)silane followed by silylation (endcapping) with hexamethyldisilazane [15]. The method lead to rods made of a single piece of porous silica with a well defined pore structure exhibiting both macropores and mesopores. The main feature of the silica monoliths prepared with this method is their high total porosity, approximately 15% higher than that of conventional high-performance liquid chromatography (HPLC) columns. The resulting hydraulic resistance of the column is therefore much lower, allowing their operation at higher flow-rates or permitting the operation of long series of columns.

Silica rod columns prepared with this method in its early stages of development were evaluated by Minakuchi et al. [16–18] and by Ishizuka et al. [19,20]. Later, a prototype column from Merck was studied by Bidlingmeyer et al. [21] and the final product by Cabrera et al. [22]. Applications on these monolith columns were recently developed in HPLC, LC–mass spectrometry (MS) and capillary electrochromatography [18–23].

Bidlingmeyer et al. [21] compared three prototype Merck columns originating from as many different batches. However, the reproducibility of these columns could not be assessed because they were produced during the exploratory phase of the manufacturing process. They reported that the columns used in their study had a bimodal pore size distribution with through-pores or macropores of ca 1.5 to 2 μm in diameter, available for the mobile phase stream, and mesopores of ca. 12 nm, responsible for the specific surface area. The specific pore volume was about 1.0 ml/g, the specific surface area 300–350 m^2/g, and the total bed porosity around 81%. In their evaluation, Bidlingmeyer et al. [21] focused on the column permeability and the plate height, using alkylbenzenes as test solutes. They compared the values obtained for rod columns with those obtained with a particle-based C_{18} column. The plots of the column inlet-pressure versus the flow-rate obtained for three columns had a standard deviation of 3%. The minimum plate height was 13–15 μm, although this low value was achieved on one column only. The plots of the column height equivalent to a theoretical plate (HETP) versus the flow velocity suggested that the performance of the monolithic column corresponded to those of a conventional column packed with 5 μm particles. They concluded

that the low hydraulic resistance of the column is the most advantageous feature of monolithic columns compared with conventional columns.

Cabrera et al. [22] reported values of 8.4 to 9.5 μm for the column plate-height of anthracene in the 0.8 to 2.5 ml/min flow-rate range on one Chromolith column and showed various applications under flow and solvent gradient conditions. Tanaka et al. [23] evaluated monolithic columns prepared inside fused-silica capillary tubings and columns prepared in a mold and then clad with polyether ether ketone (PEEK) by Merck. In both cases, the Nakanishi monolith preparation process [13,14] was used. They achieved column plate heights of 8 to 10 μm on the Merck monolith columns at the optimum flow-rate and values of the column efficiency that were two to three times better at high flow-rates than those observed with columns packed with 5 μm particles, due to the smaller slope of the *H* versus *u* curve on the monolith columns. They compared two columns originating from different batches and observed a fair reproducibility of the hydraulic resistance of the columns. Zöllner et al. [24] compared the performance of a conventional column (12.5×0.3 cm) packed with Superspher 100RP-18 operated at 0.5 ml/min and a Chromolith SpeedROD RP-18 (5× 0.46 cm) operated at 1.6 ml/min for the analysis of ochratoxin A in wines. Similar chromatograms were obtained with the two columns in LC–MS–MS, with a limit of detection of 0.5 ppb in both cases. The packed column gave a narrower peak but four times longer analyses. A sixfold reduction in analysis time ''with no loss in chromatographic performance'' was also reported by Dear et al. [25]. We note, however, that the data supplied show a certain loss in resolution and that a qualitative comparison of the chromatograms of complex mixtures obtained in gradient elution could not be entirely conclusive.

Fields [26] used potassium silicate solutions to prepare continuous xerogel-silica columns. The silica occupied 12% of the column volume, the mean pore diameter was 2 μm, with a distribution ranging from 0.2 to 3 μm. Several columns were produced. The reproducibility of the permeability of these silica-rod columns was 10 to 20%. An evaluation of the reproducibility of the silanization process was not conducted. The low efficiency reported (13 000 plates/m) for naphthalene was explained by the irregular structure of the monolith and the wide distribution of the flow paths across it.

In order to avoid the shrinkage of silica monoliths prepared with the Nakanishi process [13,14], several groups started the monolith formation by filling the columns with chromatographic particles. Dulay et al. [27] reported the immobilization of previously prepared octadecylsilica particles in a sol–gel matrix prepared in situ, in a capillary tube, at high temperature, using tetraethylortho-silicate. Asiaie et al. [28] prepared porous monolithic capillary columns for capillary electrochromatography (CEC) and micro-HPLC applications. They packed conventional capillary tubes (75 μm I.D.) with C_{18}-bonded silica particles that they converted into a porous monolith by agglomerating the particles at 360°C with a $NaHCO_3$ flux. After this agglomeration, a second surface modification step was necessary due to the pyrolytic effects on the C_{18} coating of the high temperature treatment used for the monolith formation. Tang and Lee [29] used a similar approach for the preparation of capillary monoliths. They filled a capillary tube with C_{18} silica particles, treated the material with propylsulfonic acid, which modified the surface of the particles, and bound them together in the bed using a sol–gel formation process (with tetramethoxysilane and ethyltrimethoxysilane). They proved the versatility of this approach by employing them in micro-HPLC and CEC [29,30]. Columns prepared using these last three approaches can be referred to as particle-fixed monolithic columns. This approach has the advantage of increasing the mechanical stability of packed microbore-columns and of allowing their use without frits (a major advantage in CEC). Unfortunately, this procedure has the inconvenience that the interstitial porosity, hence the permeability of the columns obtained is, at best, similar to those of the conventional packed columns. It seems that the coating tend to obstruct part of the channels around the particles and to increase the hydraulic resistance of the columns. Furthermore, the procedure cannot increase the degree of radial homogeneity of the packing and cannot produce more efficient columns that conventional packing procedures.

As opposed to conventional packed columns, which are produced by slurry packing ready-made particles into a suitable piece of tubing, monolithic

columns are filled with a single piece of porous material which adheres to the wall of the tubing. The silica-based Merck monoliths prepared by the Nakanishi process cannot be produced inside what will become the column tubing. Instead, they must be dried and then encapsulated. Also, the surface modifications needed, e.g., the bonding of alkyl ligands, must be performed later, in batches. The interstitial volume of the bed of a conventional packed chromatographic column is practically independent of the particle size and of the particle size distribution, at least for the rather narrow distributions used in chromatography. The packing density varies in only a narrow range since the mechanical stability of the columns requires that the particles be closely packed. The external porosity of chromatographic columns is usually between 0.39 and 0.42 [31] and rarely exceeds 0.45 [32]. By contrast, the preparation of monolithic type columns allows for the independent control of the skeleton size (that controls mass transfer kinetics, hence efficiency) and of the interstitial volume (that controls bed permeability). The volume of the macropores through which flows the stream of mobile phase corresponds in monolithic columns to the volume between the particles or interstitial volume in packed columns. It is the size and the volume of the macropores that controls the column permeability, hence the back pressure required to achieve the operational flow-rate. In both types of columns, the size and the volume of the mesopores controls the surface area, hence the retention volumes. A comparison between the performance of both types of columns becomes complex because there is no convenient length scale to refer the column plate height in monolithic columns. Finally, note that the mechanical stability of conventional columns is tested during their packing (typically done under a pressure twice as high as the maximum pressure achieved during their intended use). By contrast, the mechanical stability of rod columns is tested during their very application. This sets the minimum density of the silica skeleton in the monolith: it should not collapse under the inlet pressure applied to the column.

The goal of this work is to assess the reproducibility of the results obtained in HPLC with monolithic columns. This determination is important with these columns, particularly at this stage of their development. Our study of the Chromolith Performance monolithic columns by Merck is part of our more general investigations of the column-to-column and batch-to-batch reproducibilities achieved by commercial columns packed with silica-based reversed-phase packing materials. It uses the same experimental protocol as previously described [33] and applied to columns packed with Waters Symmetry C_{18} [34], Kromasil C_{18} [35], Luna C_{18} (2) [36], Vydac 218TP54 C_{18} [37], and Hypurity Elite C_{18}. These packing materials are made of 4 to 6 μm spherical silica particles. Besides the nature of the column bed, they differ (or may differ) from the Chromolith Performance material in the average size and size distribution of the mesopores (100–300 Å), the specific surface area of the bed, the type of alkyl ligand bonding, and the degree of surface coverage.

2. Experimental

The experimental protocol followed in the investigation of the repeatability and the reproducibility of the data acquired with the Chromolith Performance columns are the same as those described in detail and discussed earlier [33]. We 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. This instrument includes a binary solvent delivery system, an autosampler, a diode-array UV detector, a column thermostat, and a data station. All these units are 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). Because the expected variations between the performance of columns packed with different batches of a packing material are small, it was critical to keep the experimental errors as low as possible. We adhered strictly to the experimental

protocol [33] and ran equipment performance tests between each chromatographic tests (as should be done under current good laboratory practice (cGLP) conditions).

The column temperature was maintained at 25.0°C by the instrument controller. It was measured with an independent thermometer, as previously described [33], that confirmed 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 through the column was 1.00 ml/min in all tests, except during the determination of the column permeability and the $H(u)$ data. Each column was equilibrated with the required mobile phase for 5 h before the first sample injection.

The injection volume was 10 μl. Each injection was repeated five times. 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.

2.2. Columns

The experimental results reported in this work were acquired with six Chromolith Performance (Merck, Darmstadt, Germany) columns (100×4.6 mm). The reference numbers of these columns are in Table 1. Each column is filled with a single, porous silica-monolith wrapped inside a PEEK tube using a proprietary process that avoids leaks between tube wall and monolith. The silica surface is covered with a monomeric C_{18} layer bonded from monofunctional octadecylsilanes, using a proprietary in-situ surface-modification process. The production of monolithic columns is more complex than that of batches of silica particles since the properties of each individual unit will eventually be assessed separately. It involves successively the production of the silica monolith, its drying, its bonding to octadecyl groups, its endcapping, and its cladding in a PEEK envelope. Because of the large size of the monolith, temperature, reaction time, reagent mixing must be carefully controlled at each step. For better quality control, each rod is individually labeled as soon as made and a cGLP approach adopted for the entire production. This way, when a column is tested at the end of the production line, its entire history is known and statistical correlations may be investigated to improve the reproducibility of the product. The silica monoliths are prepared in small batches. The chemical modifications of the silica surface are carried out in small batches and each monolith is considered as belonging to a different batch.

The reproducibility data acquired in this work represent the batch-to-batch reproducibility.

The columns were prepared by the manufacturer and used as received. The physico–chemical characteristics of the monoliths, as supplied by the manufacturer late Spring 2001, are summarized in Table 1. There were no separate sets of data available for each column studied. However, the manufacturer indicated that the columns that we received were produced between mid-May and mid-July 2000 and had total carbon contents between 17.8 and 18.2% and specific surface areas between 302 and 317 m^2/g. Accordingly, their surface coverages are between 3.4 and 3.6 $\mu mol/m^2$. We observed that one column was significantly curved (like an arc of a wide circle). In spite of that, its performance did not differ significantly from those of the other five columns.

Table 1
Physicochemical characteristics of the monoliths

Specific surface area	280–320 m^2/g
Total pore volume measured by mercury intrusion	3.1 ml/g
Macropore volume measured by mercury intrusion	2.5 ml/g
Mesopore volume measured by BET	0.95 ml/g
Average pore size, macropores	2 μm
Average pore size, mesopores	12–13 nm
Carbon content	17.0–18.5% (monomeric C_{18}, endcapped)

The reference numbers of the columns used were: UM0019 683.03; UM0020 684.02; UM0021 685.01; UM0022 686.01; UM0023 687.03; UM0024 688.01.

2.3. Samples and chemicals

The qualitative and quantitative compositions of the five test mixtures used are given below.

Sample 1: 1, thiourea (6.7 mg/l); 2, phenol (62.9 mg/l); 3, 1-chloro-4-nitrobenzene (11.2 mg/l); 4, toluene (261.0 mg/l); 5, ethylbenzene (217.0 mg/l); 6, butylbenzene (516.0 mg/l); 7, *o*-terphenyl (22.2 mg/l); 8, amylbenzene (518.0 mg/l); 9, triphenylene (6.0 mg/l), in methanol–water (80:20).

Sample 2: 1, thiourea (6.2 mg/l); 2, phenol (66.4 mg/l); 3, aniline (40.9 mg/l); 4, *o*-toluidine (39.9 mg/l), *m*-toluidine (29.7 mg/l), *p*-toluidine (10.1 mg/l); 5, *N*,*N*-dimethylaniline (19.1 mg/l); 6, ethylbenzoate (261.5 mg/l); 7, toluene (435.0 mg/l); 8, ethylbenzene (433.5 mg/l), in methanol–water (55:45).

Sample 3a: 1, thiourea (6.4 mg/l); 2, theobromine (9.3 mg/l); 3, theophylline (15.2 mg/l); 4 caffeine (16.3 mg/l); 5 phenol (82.0 mg/l); 6, 2,3-dihydroxynaphthalene (99.6 mg/l), in methanol–water (30:70).

Sample 3b: 1′, thiourea (6.3 mg/l); 2′, pyridine (49.2 mg/l); 3′, 2,2-dipyridyl (100.4 mg/l), in methanol–water (30:70).

Sample 4a: 1, thiourea (6.4 mg/l); 2, butylparaben (10.1 mg/l); 3, dipropylphthalate (172.3 mg/l); 4, naphthalene (29.9 mg/l); 5, acenaphthene (100.2 mg/l); 6, amitriptyline (low load) (50.0 mg/l), in methanol–water (65:35) buffer (20 m*M*) with potassium phosphate, monobasic/dibasic at pH 7.00.

Sample 4b: 1′, thiourea (6.6 mg/l); 2′, propranolol (199.7 mg/l); 3′, acenaphthene (101 mg/l); 4′, amitriptyline (high load) (149.8 mg/l), in methanol–water (65:35) buffer (20 m*M*) with potassium phosphate, monobasic/dibasic at pH 7.00.

Sample 5a: 1, thiourea (6.6 mg/l); 2, benzylamine (low load) (98.2 mg/l); 3, benzylalcohol (313.2 mg/l); 4, benzoic acid (100.6 mg/l), in methanol–water (30:70) buffer with phosphoric acid/potassium monophosphate buffer (20 m*M*) at pH 2.70.

Sample 5b: 1′, procainamide (6.2 mg/l); 2′, benzylamine (high load) (294.6 mg/l); 3′ phenol (80.4 mg/l), in methanol–water (30:70) buffer with phosphoric acid/potassium monophosphate buffer (20 m*M*) at pH 2.70.

These 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). They were used as received. A new batch of these chemicals was purchased and used for every new column brand investigated. 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 of the same given brand tested. This solution was kept in a well-sealed bottle. 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 atmosphere in the laboratory.

Representative chromatograms (all recorded at 254 nm) obtained for the test mixtures are shown in Figs. 1–5. Because of interferences within the compounds previously selected for the tests, the mixtures for tests 3, 4, and 5 were split into two mixtures that were run separately. The corresponding chromatograms are overlaid in Figs. 3–5. The H versus u curves were measured with the first test solution, at flow-rates of 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 ml/min. The extra-column contribution of the equipment was measured with thiourea (6.0 mg/l), butylbenzene (250 μl/l), and triphenylene (3.0 mg/l), using solutions prepared separately in methanol–water (8:2) mixtures.

2.4. 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) of the results of five consecutive runs carried out with one column over a period of a few hours. Short-term repeatability data on columns of other brands were already published and discussed [33–37]. The values obtained in this study closely match those previously published. This is not surprising because, at least for weakly polar or nonpolar solutes, these results characterize as much the repro-

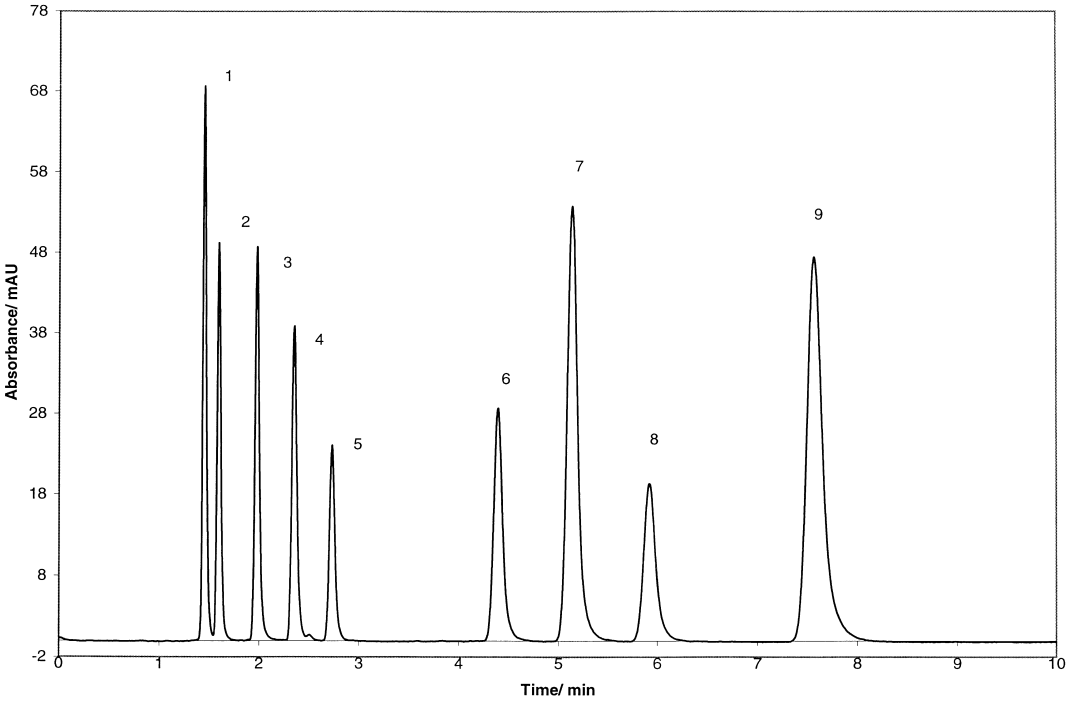

Fig. 1. Chromatogram of the first test mixture. 1, Thiourea; 2, phenol; 3, 1-chloro-4-nitrobenzene; 4, toluene; 5, ethylbenzene; 6, butylbenzene; 7, *o*-terphenyl; 8, amylbenzene; 9, triphenylene. Mobile phase, methanol–water (80:20, v/v) at 1.00 ml/min.

ducibility of the data acquisition procedure as the stability of the stationary phase itself. 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. This interval was typically 5 days. The batch-to-batch reproducibility is the RSD of the 30 injections made on six columns filled with material from the six different batches of the reversed phase studied.

3. Results and discussion

3.1. Absolute retention data

The long-term repeatability and the batch-to-batch reproducibilities of the retention times are plotted in Fig. 6 for the five different tests (data pertaining to tests which were split into two parts are pooled together). The values of the RSDs measured are impressively small, particularly when considering that those are batch-to-batch reproducibility figures for a product just entering the market.

The long-term repeatabilities of the retention times in the first three tests, which are all carried out with unbuffered solutions, are characterized by RSDs lower than 0.1% for all compounds, except for the last four compounds in test 1 (ca. 0.15%). It is hard to explain this slightly higher value for four aromatic hydrocarbons. It is not related to the equipment performance since all the other compounds gave excellent repeatability values (many with a long-term RSDs below 0.05%). Many of the other test compounds (e.g., aniline, toluidines, *N*,*N*-dimethylaniline, pyridine) are strongly basic but still give excellent long term repeatability, which is not always the case with conventional packed columns [33–37]. The long-term repeatability values in the last two test mixtures, run with buffered mobile phases, are not so good as those for tests run with unbuffered solutions. In general, the RSDs are below 0.2%, except for three ionizable compounds (propranolol and ami-

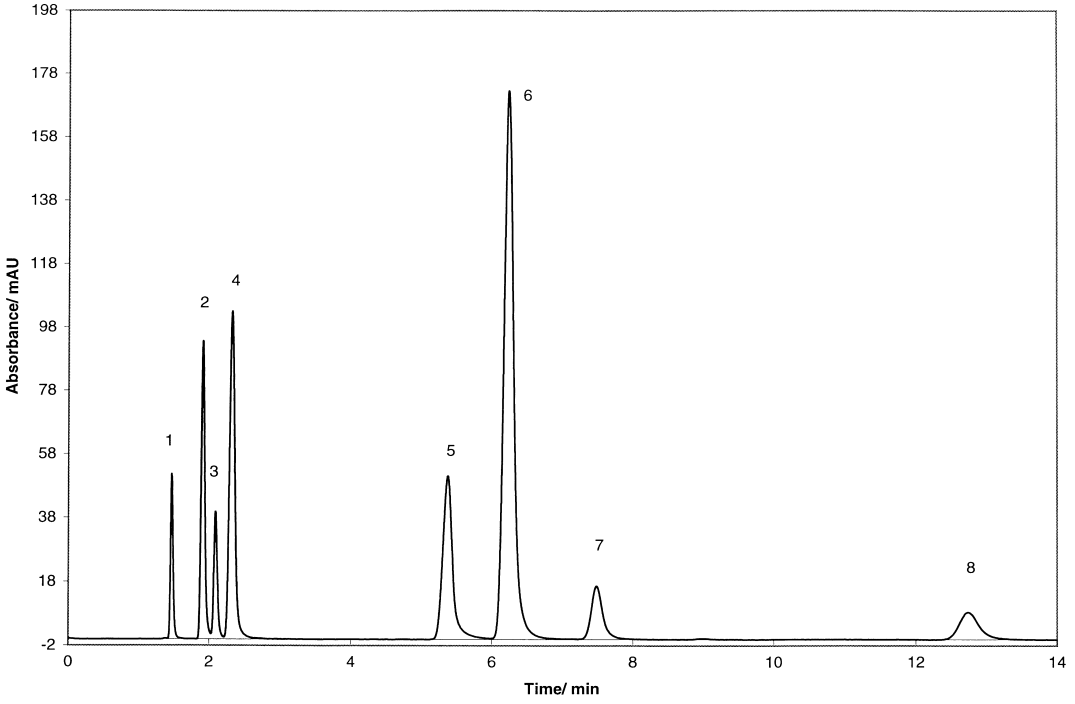

Fig. 2. Chromatogram of the second test mixture. 1, Thiourea; 2, phenol; 3, aniline; 4, toluidines; 5, *N*,*N*-dimethylaniline; 6, ethylbenzoate; 7, toluene; 8, ethylbenzene. Mobile phase, methanol–water (55:45, v/v) at 1.00 ml/min.

triptyline, in test 4 and benzoic acid in test 5). This set of results suggests a very high degree of stability of the surface chemistry.

The very small uncertainties observed for the retention times allow meaningful comparisons between the data obtained with the six columns used with the unbuffered mobile phases (Fig. 6). The RSDs of the retention times measured on these columns (batch-to-batch reproducibility) show no systematic trend but a barely significant tendency toward an increase of the RSD with increasing retention times. The RSD of the hold-up time, taken as equal to the retention time of thiourea, unretained under these experimental conditions, is the same, 1.3%, in all the five tests. This is at least one order of magnitude larger than the long-term repeatability value. Except for three basic solutes, caffeine, pyridine, and 2,2′-dipyridyl in test 3, the RSDs of the retention times of the compounds in tests 1, 2, and 3 are the same as that of the hold-up time, suggesting that this reproducibility problem originates in the difficulties experienced in the reproducibility of the geometrical dimensions (column diameter, bed ''external'' porosity) more than in that of the surface chemistry. This conclusion is not surprising in view of the novelty and the nature of the new procedure of column manufacturing. The same observation, that the fluctuations of the retention times on the six columns are practically the same for retained and unretained components, applies to the components of tests 4 and 5, except for the two components of test 4 that gave poor long-term repeatability (see above and Fig. 6).

3.2. Retention and separation factors

The retention factors derived from the measurements made on the six columns are listed in Table 2. The RSDs of the retention factors are illustrated in Fig. 7. The long-term repeatabilities of the retention factors are all better than 0.1%, except for those of propranolol and amitriptyline in test 4 and those of procainamide, benzylamine, and benzoic acid in test 5 (Fig. 7). The batch-to-batch RSDs of the retention

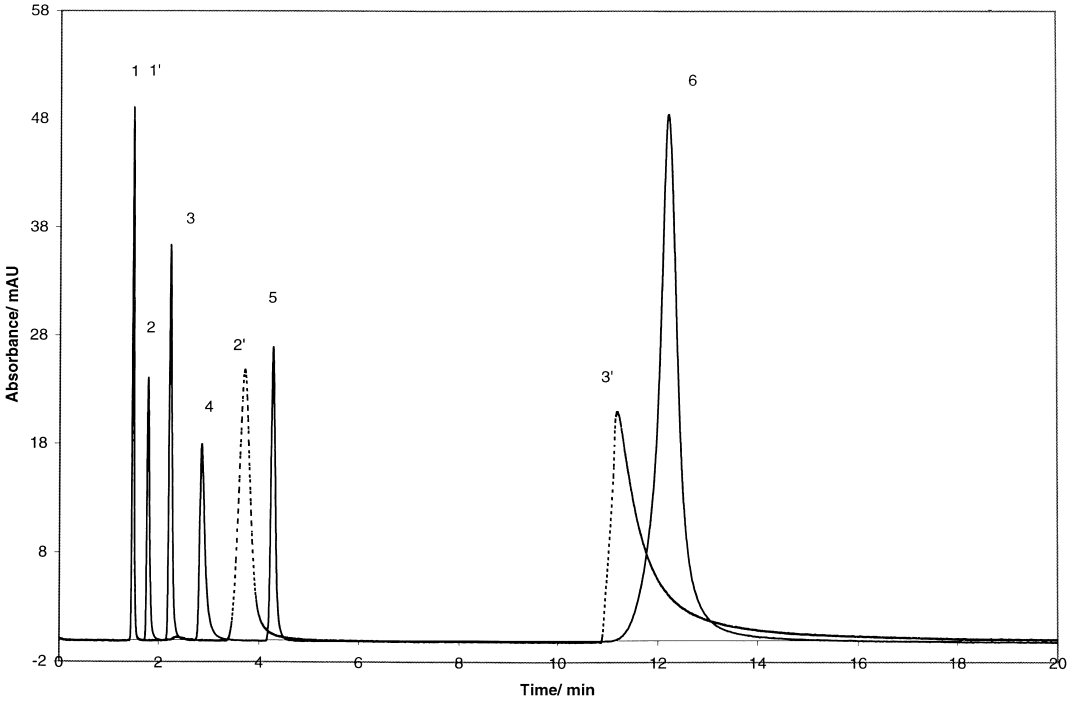

Fig. 3. Chromatogram of the third test mixture. The chromatograms for test mixtures 3a and 3b are overlaid. 1, Thiourea; 2, theobromine; 3, theophylline; 4, caffeine; 5, phenol; 6, 2,3-dihydroxynaphthalene; 1′, thiourea; 2′, pyridine; 3′, 2,2-dipyridyl in methanol–water (30:70).

factors of many compounds are of the order of 1.5%, quite comparable to those of their retention times, in spite of the observation reported above. For a few compounds, caffeine, pyridine, and 2,2′-dipyridyl in test 3, propranolol and amitriptyline in test 4, procainamide in test 5, the RSDs of the retention factors are larger than 3%.

Table 3 reports the average values of the relative retention data (i.e., the separation factors, α) for the pairs of successively eluted peaks, for the five tests carried out, and their RSDs. In the first test, these RSDs are all below 0.5%. In the other tests, the RSDs of the separation factors of the neutral compound pairs are comparable to those observed in the first test. The RSDs on the α values are at least one order of magnitude larger for the neutral/basic or basic/basic pairs of compounds. The highest RSD value (11%) was obtained for the relative retention of the pair benzylamine/procainamide (test 5). Out of a total of 28 separation factors or relative retention calculated in Table 3, 16 have reproducibilities within 0.5%, 19 are better than 1.5%, and 26 better than 4%.

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 [38–43] for the characterization of surface properties of different brands. These values are now discussed.

3.3. Hydrophobic selectivity

As in previous papers [33–37], we derived the hydrophobic selectivity of different batches using the retention data measured in two different tests. First, we calculated $\alpha(CH_2)$ as the ratio of the retention factors of the three following pairs of compounds, ethylbenzene/toluene (tests 1 and 2, Fig. 8a and b), amylbenzene/butylbenzene (test 1, Fig. 8c), and butylbenzene/ethylbenzene (test 1, Fig. 8d). Then, from the data measured in the fourth test, we derived

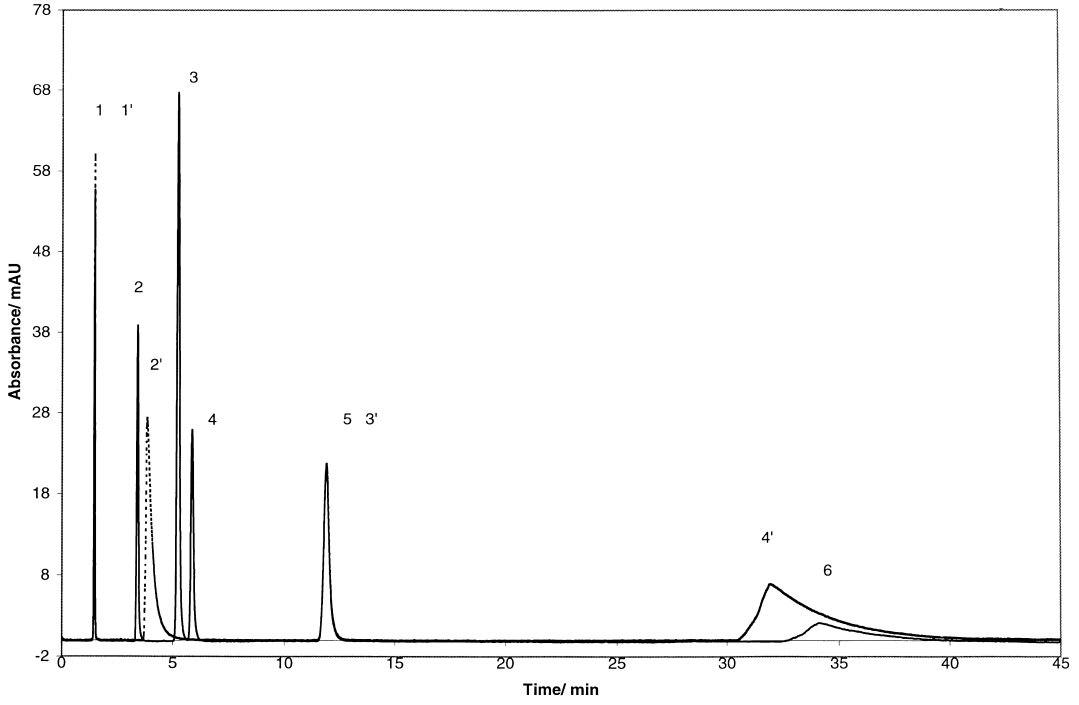

Fig. 4. Chromatogram of the fourth test mixture. The chromatograms for test mixtures 4a and 4b are overlaid. 1, Thiourea; 2, butylparaben; 3, dipropylphthalate; 4, naphthalene; 5, acenaphthene; 6, amytriptyline (low load); 1′, thiourea; 2′, propranolol; 3′, acenaphthene; 4′, amitriptyline (high load). Mobile phase, methanol–water (65:35, v/v) buffer with potassium phosphate, monobasic/dibasic at pH 7.00 at 1.00 ml/min. Note that peaks 3′ and 5 overlay exactly.

the separation factor of the pair acenaphthene/naphthalene (test 4, Fig. 8e). The figures report the values obtained in the long-term repeatability and the batch-to-batch reproducibility tests. They also give the RSDs of the data. The long-term repeatability values are close to 0.1% or better. With RSD values of 1.5 to 1.8%, the batch-to-batch reproducibility of the various definitions of the column hydrophobic selectivity is of the same order of magnitude as that for the retention factors of these same compounds (Fig. 8a–e). The correlation between the values derived under different test conditions is more than satisfactory. There is a certain similarity in the patterns of the separation factors exhibited in Fig. 8a–e, but this similarity is not sufficient to establish a relationship between the hydrophobic selectivities of the six columns.

Attempts at deriving a correlation using the much broader data base that we have acquired lead to a rather simple conclusion. The correlation coefficients between the retention factors of ethylbenzene in test 1, toluene in test 2, and acenaphthene in test 4 on the one hand and the phase ratio calculated for the bare silica [32], the specific surface area, the carbon content of the phase, and the surface coverage by the C_{18} ligands were calculated. Only the correlation coefficient of the retention factors with the phase ratio is important, at 0.92. Since retention factors are proportional to the phase ratio, this suggests that there are little differences between the hydrophobicities of the C_{18} silica surfaces studied in our different measurements. This result is consistent with previous findings [39,44,45] that the carbon content of a monomeric-type reversed-phase liquid chromatography (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 [39], the hydrophobic selectivity, expressed as the selectivity of ethylbenzene and

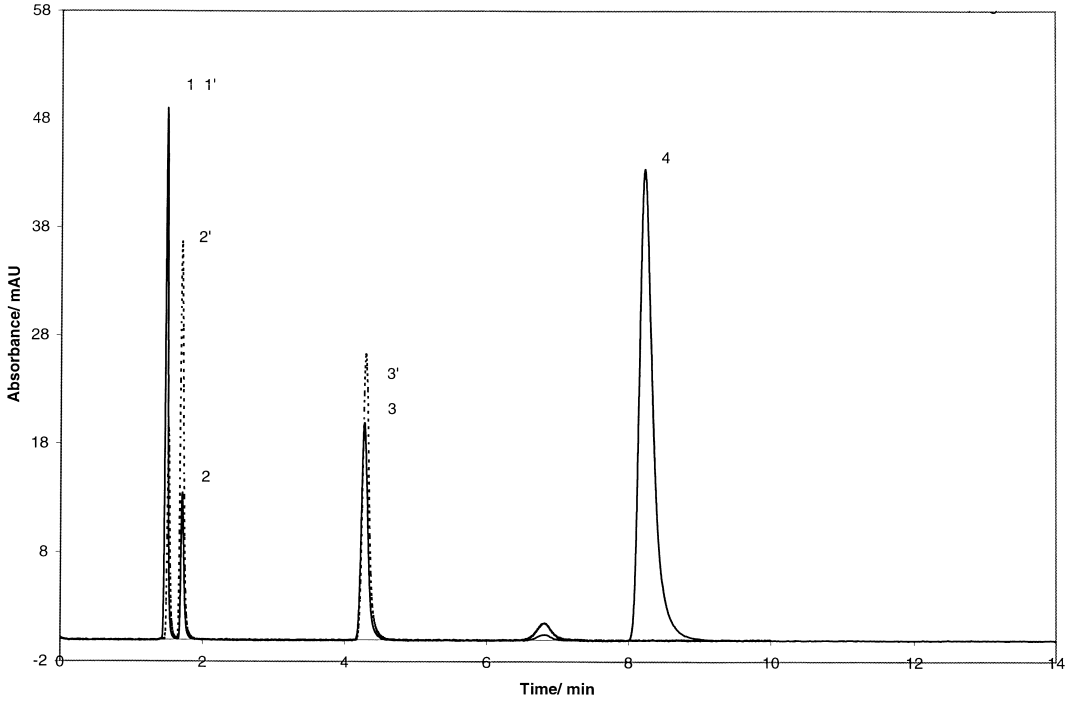

Fig. 5. Chromatogram of the fifth test mixture. The chromatograms for test mixtures 5a and 5b are overlaid. 1, Thiourea; 2, benzylamine (low load); 3, benzylalcohol; 4, benzoic acid; 1′, procainamide; 2′, benzylamine (high load); 3′ phenol. Mobile phase, methanol–water (30:70, v/v) buffer with phosphoric acid/potassium monophosphate buffer at pH 2.70 at 1.00 ml/min.

toluene, is a quasi-linear function of the carbon content up to 12% carbon, above which value the dependence is less pronounced.

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 [38]. All the values of the steric selectivity measured are plotted in Fig. 9. Each set of five successive data points correspond to one column. The long-term steric selectivity of a column is highly reproducible (RSD=0.05%). That of the six columns is also well reproducible (RSD=0.4%) if we consider that the six columns belong to six different batches. This last RSD is approximately half as large as the one previously reported for batches of conventional packed columns of different brands. Admittedly, the production of these batches spanned several years, allowing for drifts of the product characteristics. Finally, we note that the steric selectivity is more sensitive to fluctuations of the surface chemistry than the hydrophobic selectivity.

3.5. Separation factors of the basic compounds

In our test protocol we use four different sets of test conditions, nine test compounds, and three chromatographic parameters to assess the interaction of residual surface silanols with basic compounds. It is certainly necessary to use a broad variety of parameters in any attempt at assessing this most debated property of stationary phases. In previous papers [33–37], we discussed the complexity of the retention of basic compounds and listed the various factors which may affect the separation of their mixtures. Here we summarize these major factors again. The parameters that affect the repeatability of the experimental results are also summarized.

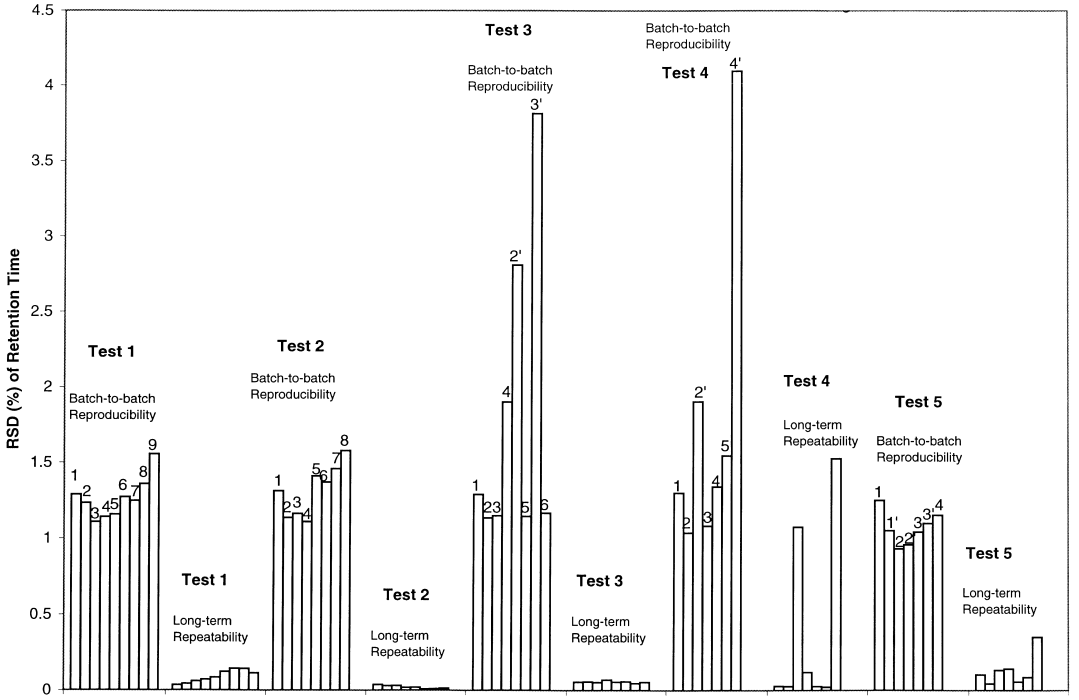

Fig. 6. Long-term repeatability and batch-to-batch reproducibility of the retention times measured for the components of the five test mixtures. The bars represent the relative standard deviation of retention times in the following order: test 1: 1, thiourea; 2, phenol; 3, 1-chloro-4-nitrobenzene; 4, toluene; 5, ethylbenzene; 6, butylbenzene; 7, *o*-terphenyl; 8, amylbenzene; 9, triphenylene. Mobile phase, methanol–water (80:20, v/v). Test 2: 1, thiourea; 2, phenol; 3, aniline; 4, toluidines; 5, *N*,*N*-dimethylaniline; 6, ethylbenzoate; 7, toluene; 8, ethylbenzene. Mobile phase, methanol–water (55:45, v/v). Test 3: 1, thiourea; 2, theobromine; 3, theophylline; 4, caffeine; 2′, pyridine; 5, phenol; 3′, 2,2-dipyridyl; 6, 2,3-dihydroxynaphthalene. Mobile phase, methanol–water (30:70, v/v). Test 4: 1, thiourea; 2, butylparaben; 2′, propranolol; 3, dipropylphthalate; 4, naphthalene; 5, acenaphthene; 4′, amytriptyline. Mobile phase, methanol–water (65:35, v/v) buffer with potassium phosphate, monobasic/dibasic at pH 7.00. Test 5: 1, thiourea; 1′, procainamide; 2, benzylamine (low load); 2′, benzylamine (high load); 3, benzylalcohol; 3′, phenol; 4, benzoic acid. Mobile phase, methanol–water (30:70, v/v) buffer with phosphoric acid/potassium monophosphate buffer at pH 2.70.

3.5.1. Stationary phase factors

The most important stationary phase factors are the acidity and the absolute and relative concentrations of the different silanol groups (e.g., isolated, acidity-enhanced, vicinal, geminal, etc.) on the surface, the concentration of the metal impurities, and the accessibility of the corresponding sites. Examination of the published literature shows that enormous efforts were invested in the determination and interpretation of the silanol types, their reactivity, and their acidity on many different silica surfaces and for various purposes. There is a general agreement that isolated free silanols are responsible for the secondary interactions of basic compounds with silica surfaces. However, there is a paucity of reliable data on the actual chemical composition of the surface of adsorbents used in chromatography [31]. There is little agreement on the definition and the proper method of determination of the acidity of a silica surface. Four terms are commonly used to describe the surface acidity, the pK_a, the isoelectric state or pH at which the surface silanols are completely undissociated, the point of zero charge or pH at which the numbers of positively and negatively charged sites are equal, and the pH of a 5 or 10% slurry of the silica in water. The literature values of

Table 2
Retention factor values data of the components of the five test mixtures on six columns

	Column 19	Column 20	Column 21	Column 22	Column 23	Column 24
Test 1						
Phenol	0.100	0.101	0.104	0.100	0.101	0.102
1-Chloro-4-nitrobenzene	0.363	0.370	0.380	0.365	0.364	0.370
Toluene	0.620	0.634	0.652	0.627	0.627	0.635
Ethylbenzene	0.880	0.901	0.925	0.891	0.891	0.902
Butylbenzene	2.028	2.079	2.132	2.055	2.056	2.079
o-Terphenyl	2.541	2.601	2.664	2.568	2.572	2.599
Amylbenzene	3.084	3.164	3.244	3.127	3.130	3.162
Triphenylene	4.222	4.317	4.456	4.314	4.285	4.327
Test 2						
Aniline	0.298	0.302	0.313	0.300	0.297	0.302
Phenol	0.420	0.424	0.436	0.422	0.423	0.427
Toluidines	0.576	0.585	0.605	0.580	0.574	0.584
N,*N*-Dimethylaniline	2.654	2.731	2.818	2.690	2.675	2.721
Ethylbenzoate	3.232	3.316	3.418	3.266	3.251	3.305
Toluene	4.094	4.209	4.327	4.154	4.157	4.212
Ethylbenzene	7.674	7.901	8.120	7.795	7.807	7.905
Test 3						
Theobromine	0.206	0.213	0.222	0.209	0.207	0.211
Theophylline	0.507	0.521	0.540	0.514	0.510	0.516
Caffeine	0.932	0.975	1.040	0.961	0.918	0.946
Pyridine	1.508	1.598	1.694	1.554	1.443	1.521
Phenol	1.890	1.913	1.964	1.904	1.905	1.921
2,2-Dipyridyl	6.574	6.872	7.377	6.791	6.372	6.598
2,3-Dihydroxynaphthalene	7.269	7.349	7.533	7.313	7.327	7.389
Test 4						
Butylparaben	1.335	1.352	1.386	1.337	1.349	1.360
Propranolol	1.630	1.675	1.781	1.629	1.560	1.668
Dipropylphthalate	2.562	2.609	2.680	2.573	2.588	2.621
Naphthalene	2.988	3.062	3.155	3.030	3.039	3.070
Acenaphthene	7.131	7.330	7.556	7.247	7.276	7.344
Amitriptyline	20.820	21.966	23.287	20.879	19.863	21.533
Test 5						
Procainamide	0.021	0.020	0.021	0.017	0.016	0.023
Benzylamine load 1	0.154	0.154	0.158	0.149	0.146	0.158
Benzylamine load 2	0.144	0.144	0.148	0.140	0.136	0.148
Benzylalcohol	1.890	1.914	1.968	1.901	1.898	1.921
Phenol	1.900	1.922	1.973	1.915	1.917	1.932
Benzoic acid	4.560	4.610	4.739	4.592	4.584	4.613

these terms are contradictory, which is not surprising as these values depend on the silica sample and on the method of determination.

3.5.2. Mobile phase factors

These factors are the pK_a of the solute, the mobile phase pH, the buffer composition, possible steric effects (affecting the penetration of the analyte through the bonded layer toward the silica surface), and the nature of the organic solvent modifier. The nature of the solute is obviously important and overloading effects vary from solute to solute. Some compounds may show unexpected results for this reason. Note that the pK_a of the solute, the pK_a of the

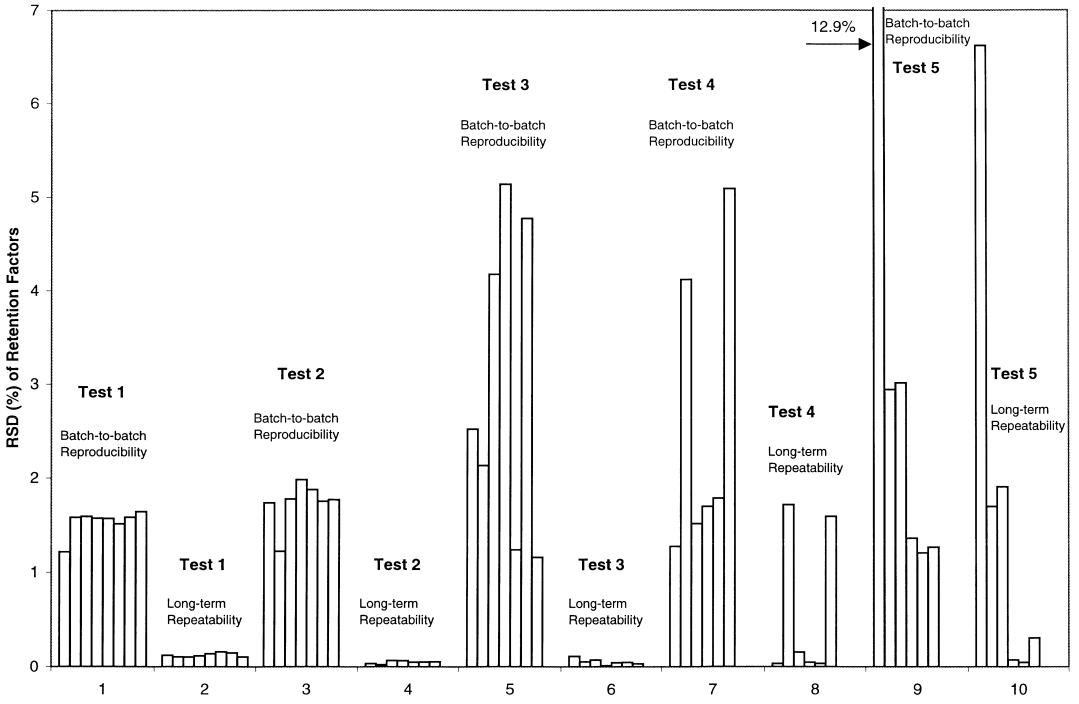

Fig. 7. Long-term repeatability and batch-to-batch reproducibility of the retention factors measured for the components of the five test mixtures. The bars represent the test compounds in the same order as in Fig. 6. There are no bar for thiourea ($k' = 0$).

buffer's conjugated acid or base and the pK_a of surface silanols vary depending on the nature and% content of the organic solvent in the mobile phase.

Further description of silanol properties can be found in the review of Nawrocki [46] and in the references therein. The effect of the organic modifier on the pK_a values was studied by Canals et al. [47], the chromatographic behavior of basic compounds under reversed-phase conditions by McCalley [48]. Recently Neue et al. [49] measured the ion-exchange and ion-exclusion properties of both reversed-phase bonded phases and their underlying base materials. Their experimental values indicated that the bonding of the silica surface shifted the pK_a values of the residual silanols by two units (from 7.0 for the initial silica to 9.2 for the fully endcapped C_{18} phase). They also observed that the ion-exchange properties of high-purity silica and conventional silica were very similar. They could confirm that the pK_a of ionizable analytes changes with the organic composition of the mobile phase, but, under the experimental conditions used, they could not capture the pK_a shift of residual silanols with changes in the organic content of the mobile phase.

3.5.3. Repeatability of the measurements

When we compare the repeatability of the retention parameters obtained for neutral and for ionizable analytes, we must take into account that some minor changes in the experimental conditions (e.g., small variations in the organic content of the mobile phase, temperature fluctuations) may affect not only the hydrophobic retention of the ionizable analytes but also the ionization state of the buffer, of the surface silanols, and of the analytes. Through these ionization effects, the intensity of the secondary interactions is modified, hence their contribution to the retention of these ionizable analytes. These relative changes will define the repeatability of the experiment. In some cases, the fluctuations may cancel out, due to parallel effects, but the general trend is an increase of the RSD characterizing the repeatability of the measurement.

Table 3
Reproducibility of the relative retention data of the components of the five test mixtures

	Average value of relative retentions on six columns	RSD (%) of relative retentions
Test 1 (MeOH–water, 8:2)		
1-Chloro-4-nitrobenzene/phenol	3.642	0.430
Toluene/1-chloro-4-nitrobenzene	1.716	0.221
Ethylbenezene/toluene	1.420	0.038
Butylbenzene/ethylbenzene	2.306	0.078
o-Terphenyl/butylbenzene	1.251	0.088
Amylbenzene/*o*-terphenyl	1.216	0.122
Triphenylene/amylbenzene	1.371	0.356
Test 2 (MeOH–water, 55:45)		
Phenol/aniline	1.409	0.711
Toluidines/phenol	1.373	0.726
N,*N*-Dimethylaniline/toulidines	4.649	0.414
Ethylbenzoate/*N*,*N*-dimethylaniline	1.215	0.123
Toluene/ethylbenzoate	1.271	0.355
Ethylbenzene/toluene	1.876	0.058
Test 3 (MeOH–water, 3:7)		
Theophylline/theobromine	2.452	0.407
Caffeine/theophylline	1.856	2.137
Pyridine/caffeine	1.614	1.312
Phenol/pyridine	1.236	4.184
2,2-Dipyridyl/phenol	3.529	3.757
2,3-Dihydroxynaphthalene/2,2-dipyridyl	1.091	3.819
Test 4 (MeOH–pH 7.0 buffer, 65:35)		
Propranolol/butylparaben	1.224	3.177
Dipropylphthalate/propranolol	1.574	2.952
Naphthalene/dipropylphthalate	1.173	0.340
Acenaphthene/naphthalene	2.392	0.116
Amitriptyline/acenaphthene	2.924	3.760
Test 5 (MeOH–pH 2.7 buffer, 3:7)		
Benzylamine load 1/procainamide	7.916	10.791
Benzylamine load 2/procainamide	7.400	10.739
Benzylalcohol/benzylamine load 2	13.376	2.404
Phenol/benzylalcohol	1.006	0.247
Benzoic acid/phenol	2.396	0.219

3.5.4. Discussion of our results

The behavior of the columns studied toward basic compounds was characterized by the relative retentions of seven basic test compounds with respect to neutral, nonpolar or moderately polar compounds (phenol was used in one case). Fig. 10a–g to 10g illustrate the results obtained, showing the separation factors of the following pairs: aniline and toluene (Fig. 10a), toluidine and toluene (Fig. 10b), *N*,*N*-dimethylaniline and toluene (Fig. 10c) – all three from test 2 – pyridine and phenol (Fig. 10d) from test 3; amitriptyline and acenaphthene (Fig. 10e) and propranolol and acenaphthene (Fig. 10f) – both from test 4 – and benzylamine and benzylalcohol (Fig. 10g) from test 5. 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 mixture. Unfortunately, none of these two

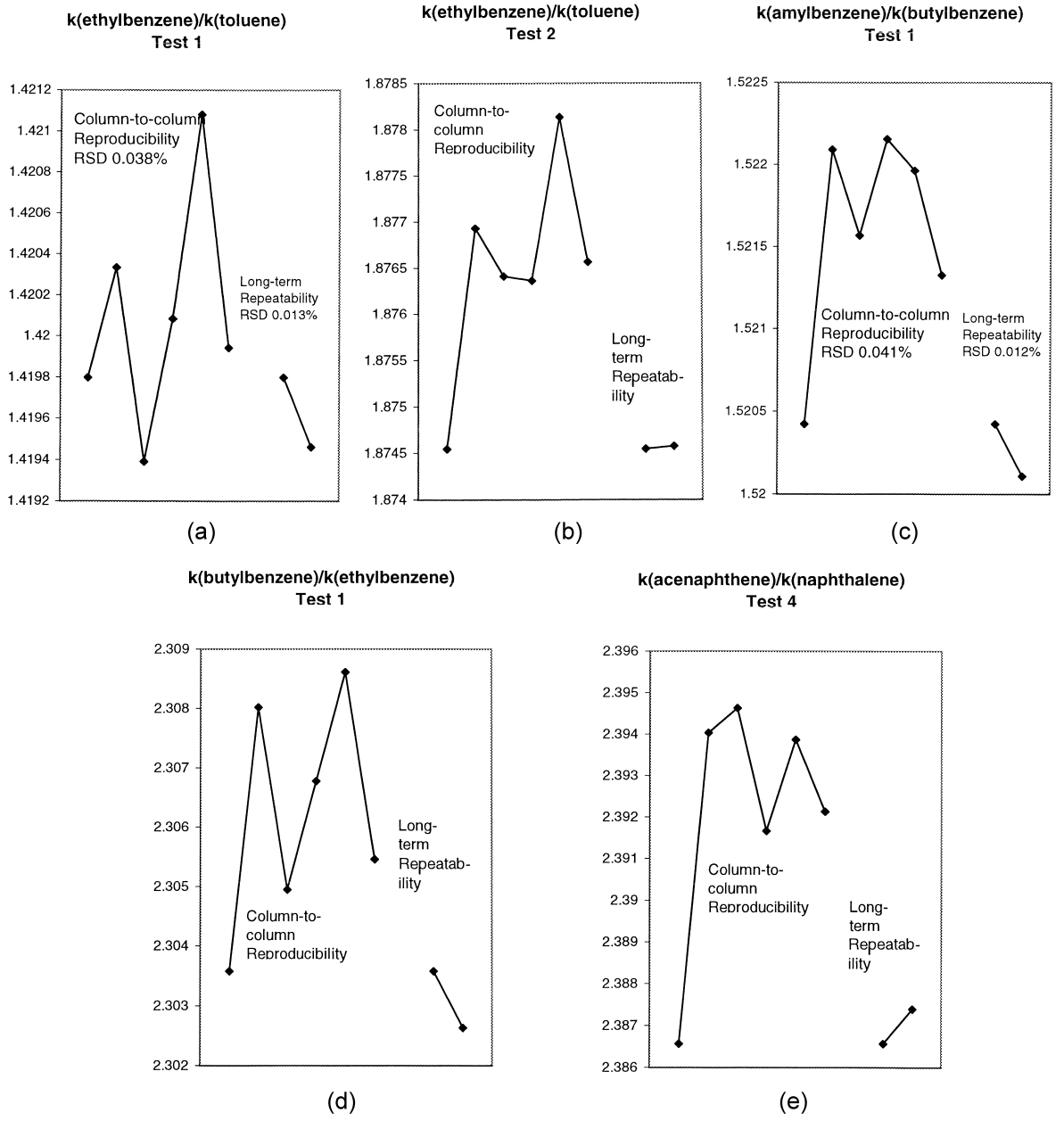

Fig. 8. Values measured for different parameters characterizing the hydrophobic selectivity of the columns. (a) Separation factor of ethylbenzene and toluene (test 1), (b) separation factor of ethylbenzene and toluene (test 2), (c) separation factor of amylbenzene and butylbenzene (test 1), (d) separation factor of butylbenzene and ethylbenzene (test 1), (e) separation factor of acenaphthene and naphthalene (test 4).

methods can take into account the possible effect of the stationary phase on the actual pH of the solution inside the column.

In tests 2 and 3, 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 are 4.63 for aniline,

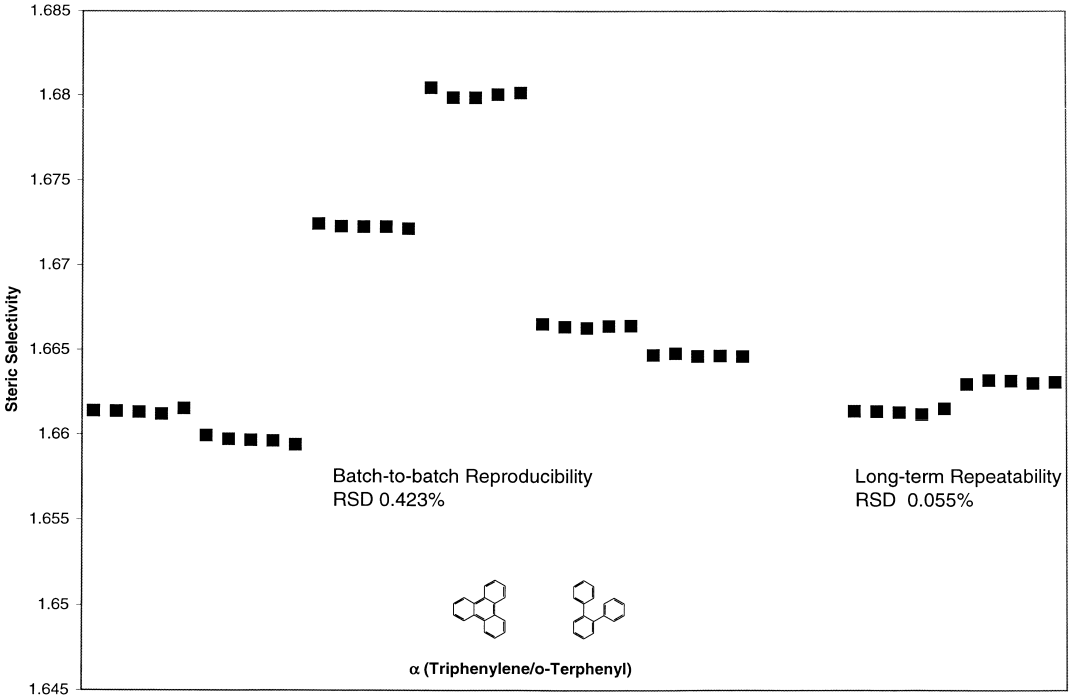

Fig. 9. Repeatability and reproducibility of the steric selectivity of the columns.

4.45 for *o*-toluidine, 5.15 for *N*,*N*-dimethylaniline, and 5.5 for pyridine. Under these conditions, the most acidic silanols (the acidity enhanced silanols that are often blamed for secondary interactions with basic compounds) are dissociated but the bulk of the silanol groups, with pK_a probably between 7 and 9, might be present under both forms. The analytes dissolved in water (or in a methanol–water mixture) dissociate according to their dissociation constant in the sample because the unbuffered sample solvent does not have any buffer capacity to shift the equilibrium. On the other hand, when the sample is injected into the column, this equilibrium is shifted depending on the acidity of the stationary phase. The retention and peak shape will be defined by the ratio of the concentrations of the two analyte forms and by the ratio of the protonated and unprotonated silanol groups. Without proper knowledge of the concentration and acidity of the silanol groups in the monolith columns and of the effects of the stationary phase on the pH changes in the column, any further elaboration would be speculative. We must accept the empirical observations of Engelhardt and Junghelm [39] who suggested to use unbuffered mobile phases to assess the silanol activity of stationary phases because he found that the differences between the stationary phases that he studied were more pronounced under these conditions.

The RSDs of the measurements of the relative retentions of the pairs aniline/toluene toluene/toluidine, and *N*,*N*-dimethylaniline/toluene on the six columns were 0.69, 0.62, and 0.38%, respectively (Fig. 10a–c). The RSDs for the long-term repeatability of these separation factors were approximately thirty times lower, at 0.02%. The RSD of the measurements of the relative retention of the two neutral compounds ethylbenzene and toluene was 0.06% on the same columns, under the same test conditions (Fig. 8b). The difference between the patterns in Fig. 10a and b on the one hand, Fig. 10c on the other hand confirms a previous observation that the interactions of aniline and *N*,*N*-dimethyl-

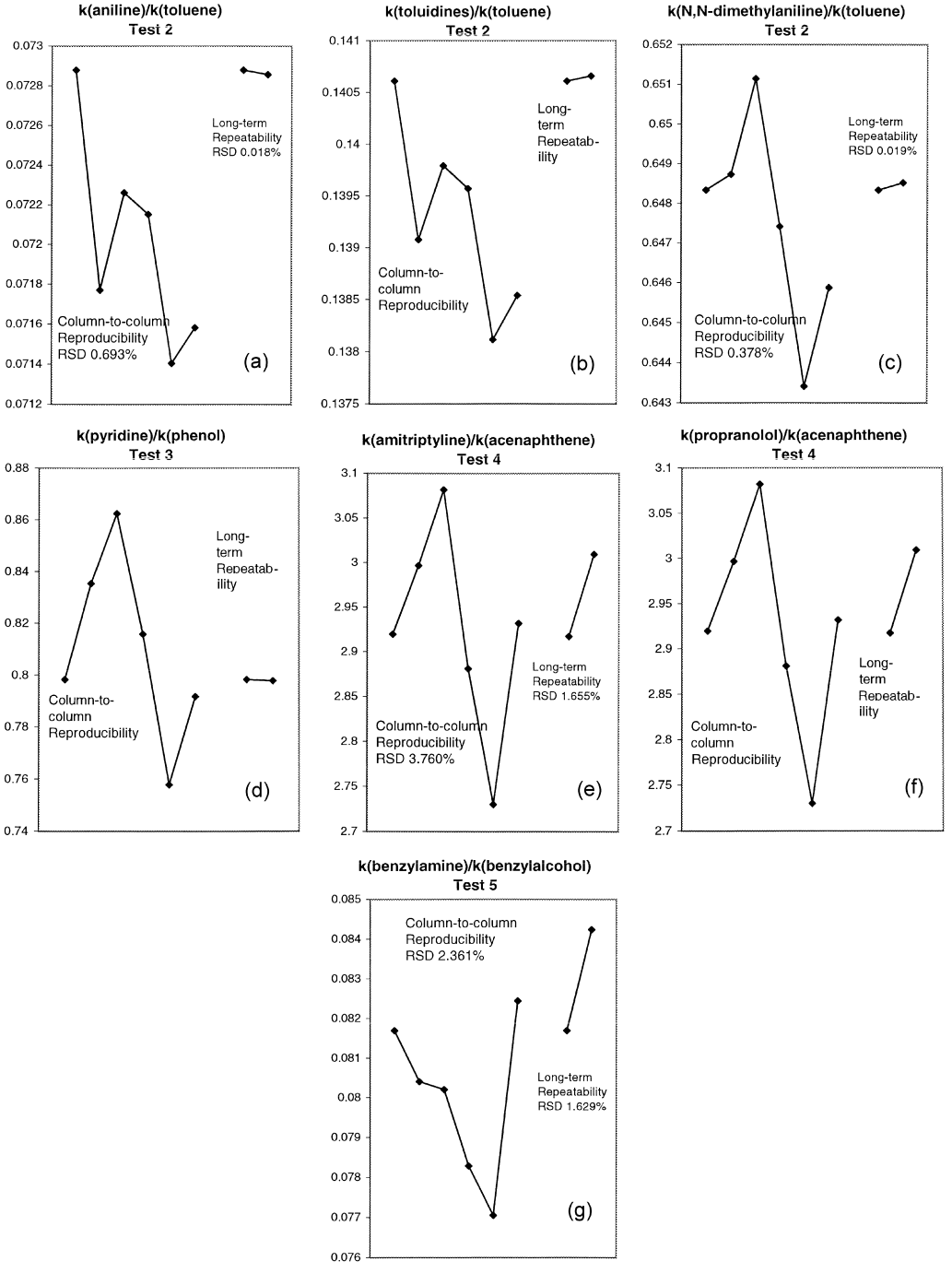

Fig. 10. Values measured for the separation factors of different pairs of basic and neutral compounds, characterizing the silanol activity of the columns. (a) Aniline and toluene (test 2), (b) toluidine and toluene (test 2), (c) *N*,*N*-dimethylaniline and toluene (test 2), (d) pyridine and phenol (test 3), (e) amitriptyline and acenaphthene (test 4), (f) propranolol and acenaphthene (test 4), (g) benzylamine and benzylalcohol (test 5).

aniline with the silica surface are most probably different. The RSD of the measurements of the relative retention of the pair pyridine/phenol was 4.18% in test 3 which also uses an unbuffered mobile phase, this one with 30% methanol.

Buffered mobile phases allow the assessment of the reproducibility of the interactions between the stationary phase and basic compounds, as suggested by several authors [32,38]. These conditions allow a clearer interpretation of the experimental data. In test 4, the mobile phase is buffered at pH 7.0. The pK_a of the basic compounds amitriptyline and propranolol in water are 9.4 and 9.5, respectively. With these pK_a values, both amines are expected to be completely protonated in the mobile phase. Strong ion-exchange interactions are expected to take place between these protonated amines and the silica surface. One would expect to see more pronounced differences between the retention data on the different batches than in the previous test and this is indeed the case, by contrast with previous observations [34–36]. The RSDs of the measurements of the separation factors of these two basic compounds relative to the neutral acenaphthene are approximately ten times larger than those found in the second test, 3% for propranolol/acenaphthene and 3.8% for amitriptyline/acenaphthene (Fig. 10e and f). However, if we use the apparent pH scale of the methanol–water mixture and assume that the apparent pK_a of the amines decreases by 0.3 unit for each 10% increase in the organic modifier concentration in the mobile phase [46], the pK_a values of the two compounds become only 7.5 while the pH* of the mobile phase is around 8 [50]. Then, the amines would be only partially protonated, casting some doubts on the rational just presented. Note the very similar patterns in Fig. 10e and f, suggesting that the two basic solutes interact very similarly with the silanol groups.

In tests 3 and 5, the methanol content of the mobile phase is 30%. In test 5, the mobile phase is buffered at pH 2.7, a pH that 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 under these conditions. This conclusion holds true no matter which pH scale is used for the explanation. The RSD of the measurements of the relative retention of the pair benzylamine/benzylalcohol on the six batches of packing material was 2.4%, not much larger than the long-term repeatability (RSD= 1.6%). We observe no correlation between the results obtained with the pair benzylamine/benzylalcohol and any other pair investigated (cf. Fig. 10a–g), by contrast with correlations observed previously [34,35] with conventional columns packed with other brands of C_{18}-bonded silica.

3.6. Column efficiency

The ChemStation supplies the efficiencies of each recorded peak, derived from the detector data using five different algorithms. In this study we report only the RSDs of the measurements of the peak efficiencies derived from the peak width at half-height. The values obtained are presented in Fig. 11. The long-term repeatabilities are similar to those found in previous studies [33–37]. The RSDs are all between 1 and 4%, except for 2,3-dihydroxynaphthalene in test 3.

The RSDs corresponding to the batch-to-batch reproducibility for the neutral compounds were between 4 and 6% in test 1, below 6% in test 2 (except phenol, 7%), and below 4% in test 5, a remarkable result. They are less good for the polar compounds in tests 3 and 4, although all reproducibility data are below 15%. Only the values for pyridine, 2,2′-dipyridine and 2,3-dihydroxynaphthalene in test 3 are above 10%, however.

It could be that the columns were overloaded with the sample sizes injected, at least for some of the basic compounds. Then, fluctuations of peak efficiency could arise from fluctuations of the injected amount. There is a general belief [46,51–55] that the surface of RPLC packing materials contains strongly acidic ion-exchange sites that, because of their low density, are easily overloaded, causing a decrease of the capacity factor and of the efficiency and an increase of the peak asymmetry with increasing sample size. Literature values [46,51–55] suggest that the concentration range within which the onset of column overloading takes place depends on the specific compound and stationary phase studied, as well as on the pH and the composition of the mobile phase. This is related to the dependence of the column loadability on the initial curvature of the isotherm. The determination of the reproducibility of

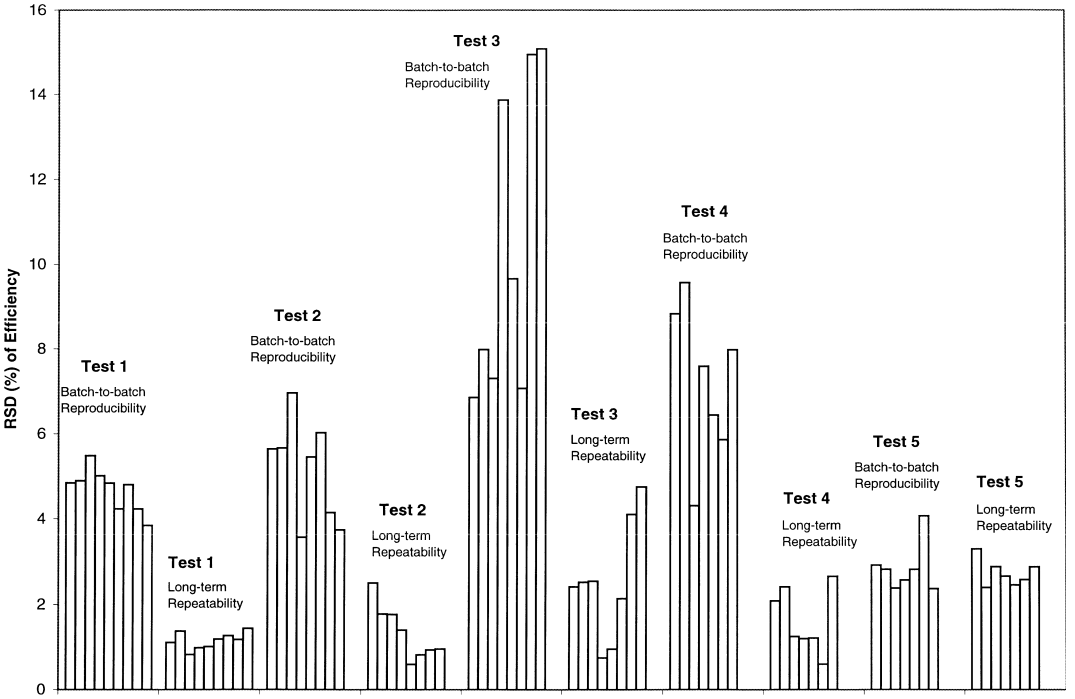

Fig. 11. Long-term repeatability and batch-to-batch reproducibility of the column efficiency measured for the components of the five test mixtures. Same numbering as in Fig. 6.

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 (normalized for the length and cross-section area of the column and for the specific surface area of the packing material [33]).

The influence of the sample size on the profile of the peak of amitriptyline is illustrated in Fig. 4 in which the chromatograms obtained with the test mixtures 4a and 4b are overlaid (the ratio of the amounts injected is 3). However, even under overloading conditions, random fluctuations of the peak efficiency for a constant size sample could only be explained by different batches having different specific surface areas or different surface chemistries (e.g., different density of strongly interacting silanol sites). Other experiments have shown that a 10-fold decrease in the loaded amount resulted in a 10% increase of the peak efficiency of *N*,*N*-dimethylaniline on one of the Symmetry columns [34]. This result suggests that, although there is an effect of the sample size on the efficiency, it is not important (at least not with the sample sizes used here, sizes that are below 1 μg/g packing material with the unbuffered mobile phases and below 4 μg/g for the buffered ones).

The relatively high values of the RSDs observed for the reproducibility of peak efficiency on all the column brand studied 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. Column efficiency and flow velocity

The dependence of the column efficiency on the velocity was measured on the six columns, using three compounds, thiourea ($k'=0.0$), butylbenzene ($k'=2.0$), and triphenylene ($k'=4.3$) in methanol-water 8:2. The results are reported in Figs. 12–14, as

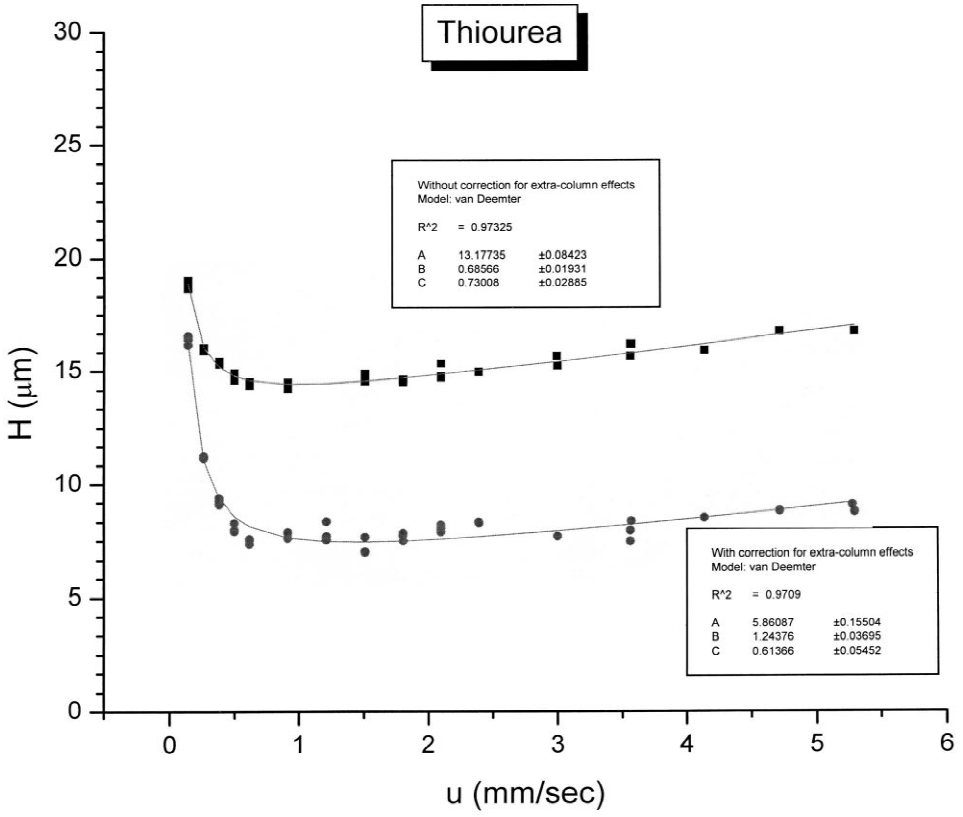

Fig. 12. Efficiency of one of the Chromolith columns investigated. Plot of the HETP for thiourea ($k'=0$) versus the mobile phase flow velocity. Best values of the Van Deemter parameters: before correction for extra-column effects: $A=13.2$, $B=0.7$, $C=0.7$ (based on 1 μm as length unit); after correction for these effects: $A=5.9$, $B=1.2$, $C=0.6$.

plots of the HETP versus the mobile phase velocity. With monolithic columns, there is no reliable estimate of the particle diameter, hence no particle Peclet number nor reduced velocity can be calculated. The data are plotted before and after correction for the extra-column variance of the instrument. The correction is extremely important for the nonretained compound (Fig. 12) and negligible for the most retained compound (Fig. 14). In each case, the data were fitted to a simple Van Deemter equation and the best numerical values of the coefficients *A*, *B*, and *C* are given in the figure captions. The minimum value of the HETP increases with increasing retention factor, from about 7.5 to 13 μm. Those are extremely high values for a column efficiency, comparable to those achieved with conventional columns packed with spherical particles having average diameters between 3 and 5 μm. Thus, we could assign to these columns a ''chromatographic apparent particle size'' of 4 μm, a value not inconsistent with an average size of 2 μm for the macropores, albeit probably on the high side for an estimate of the ''poron'' dimensions. We have no interpretation for the variations of *A* and *B* with the nature of the compound. There are too few data points for an accurate estimate of *B*, which should decrease with increasing molecular mass. *A* is a geometrical factor which may be influenced by the nature of the probe. The increase of *C* with increasing retention factor (and presumably decreasing molecular diffusivity) was expected.

The reproducibility of the column efficiency data is an important aspect of the reproducibility of the column performance. It is illustrated in Fig. 15 which shows the HETP plots for triphenylene ($k'=4.3$) obtained with the six columns studied. Two sets of

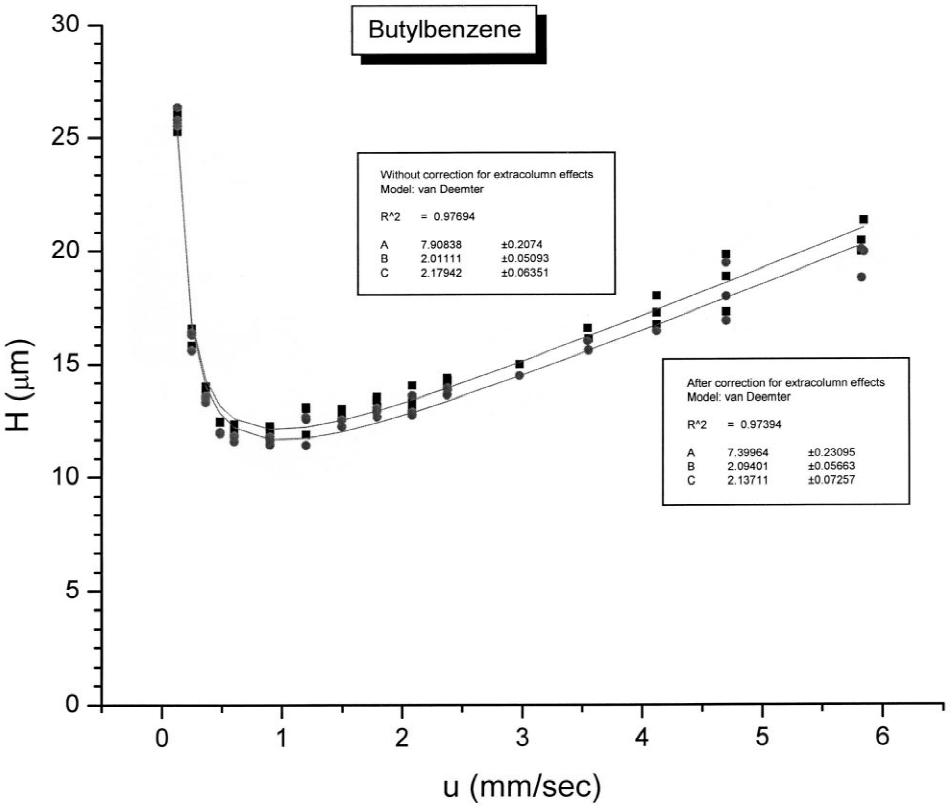

Fig. 13. Efficiency of one of the Chromolith columns investigated. Plot of the HETP for butylbenzene ($k'=2.0$) versus the mobile phase flow velocity. Best values of the Van Deemter parameters: before correction for extra-column effects: $A=7.9$, $B=2.0$, $C=2.2$ (based on 1 μm as length unit); after correction for these effects: $A=7.4$, $B=2.09$, $C=2.1$.

data are supplied, corresponding to the efficiencies derived from the peak width at half-height and from the second centered moment of the peaks, respectively. It is not surprising that the data in the first set (peak width at half-height) are more reproducible and lower than those in the second one (second moment of the peak). Second moments are notorious for their poor reproducibility, arising from the random errors caused by the signal noise in the end of the moment calculations.

3.8. 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 the ratio of this total peak width to twice the forward half-width. The values obtained for this asymmetry factor are illustrated in Fig. 16. The average values for the six columns, the repeatability and reproducibility of the tailing factors are listed in Table 4.

All the compounds used in our study gave elution peaks that tail to a degree. For most of them (24 compounds out of 34, Table 4), however, the tailing factor is below 1.4. For 18 of them (but not for thiourea) it is below 1.3. The extent of this tailing is independent of the retention factor, indicating that it is not or, at least, cannot be entirely due to extra-column effects (these effects explain probably the tailing of thiourea and of compounds with $k'<1$). We can also rule out overloading effects as a general cause because the peaks of neutral, nonpolar compounds are also affected and the columns were certainly not overloaded for these compounds. This leaves us with two possible reasons, a column-bed

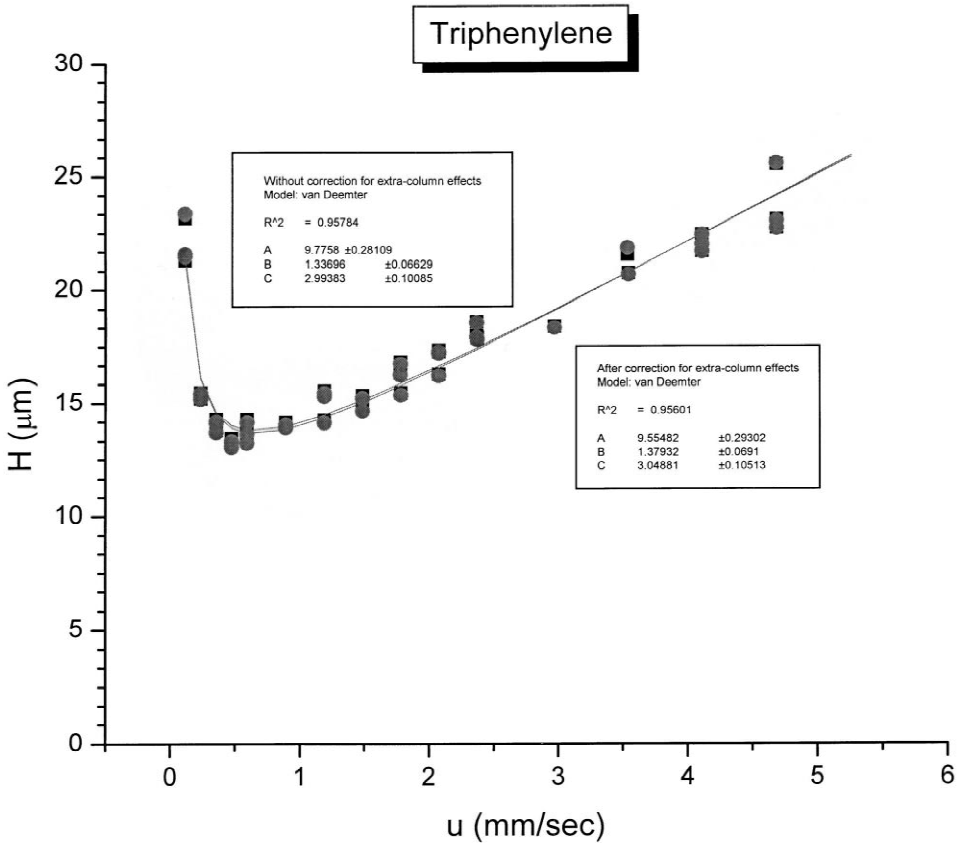

Fig. 14. Efficiency of one of the Chromolith columns investigated. Plot of the HETP for triphenylene ($k' = 4.3$) versus the mobile phase flow velocity. Best values of the Van Deemter parameters: before correction for extra-column effects: $A = 9.8$, $B = 1.3$, $C = 3.0$ (based on 1 μm as length unit); after correction for these effects: $A = 9.6$, $B = 1.4$, $C = 3.0$.

heterogeneity and/or a slow kinetics of mass transfer. It is a rather challenging exercise to define and measure the bed heterogeneity of monolith columns. This was entirely out of the scope of this study.

Three basic compounds give the highest tailing factor (Fig. 16), 2,2-dipyridyl in test 3, propranolol and amitriptyline in test 4. The average tailing factor of 6.1 for 2,2-dipyridyl (chelate forming compound) is a surprisingly high value taking into account the fact that the monolith is made of synthetic silica and the column hardware is glass in the monolith preparation and PEEK after the cladding step. The metal content of the monolith was reported to be <10 ppm by the manufacturer. 1,3-Dihydroxynaphthalene, the other chelate forming compound, gives peaks exhibiting a more moderate degree of tailing or fronting on the different columns and as a consequence an average value of tailing factor nearly equal to 1.

The reproducibility of the tailing factor varies between 1.2 and 2.6% for neutral compounds and between 3.4 and 22.6% for basic ones on the six columns. These values are comparable with the reproducibility values obtained with traditional particle based packing materials for which surface modification is performed in a batch process.

3.9. Column permeability

A remarkable property of monolithic columns is their high permeability [1–26]. This is illustrated in Fig. 17 which compares the pressure drop required to achieve the same flow-rate or flow velocity through

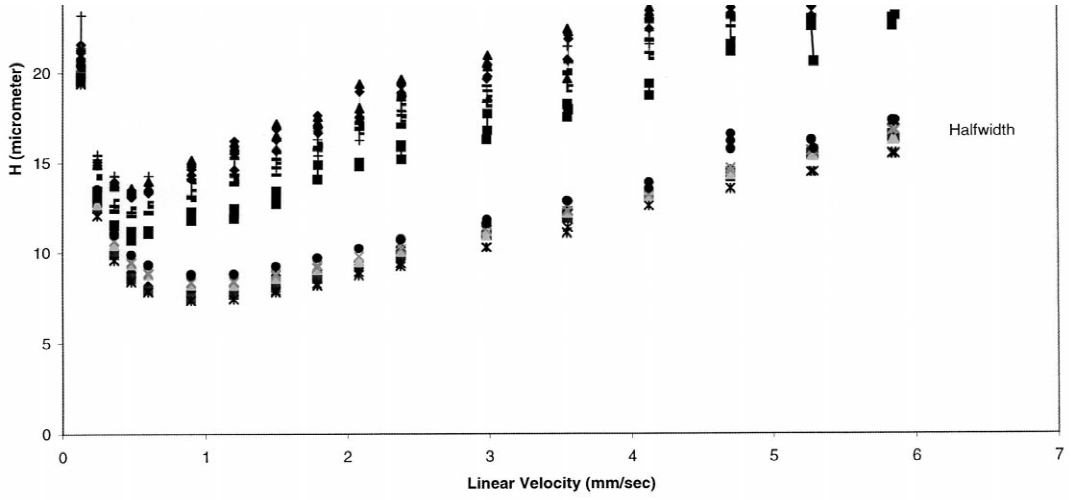

Fig. 15. Reproducibility of the plot of the HETP for triphenylene ($k' = 4.3$) versus the mobile phase flow velocity. Data obtained for the six columns, after correction for the extra-column effects. The lower set of data is derived from the efficiency calculated by the HP Chemstation after the peak width at half-height. The higher set corresponds to the efficiency derived from the second moment.

columns packed with particles of different brands or filled with a monolith. The relative standard deviation of the pressures reported in Fig. 17 is 6% for the six Chromolith columns studied. It was between 1 and 7% for the conventional packed columns investigated. The pressures required to operate the six columns during the first three tests (unbuffered mobile phases) are listed in Table 5 (the values of the pressure measured during five consecutive runs are reported separately in Table 5). The error of measurement due to the limited precision of the pressure gauge is of the order of 0.4%, causing most of the short-term reproducibility (noise of ± 1 of the last digit). The column permeabilities were calculated from these pressures, using the conventional relationship between pressure, flow-rate, and column dimensions [31]. The values are listed in Table 5. The differences between values of permeability obtained for the same column during different tests can be explained in part by the low precision of the pressure gauge, in part by errors made in the estimation of the viscosities of the different mobile phases. Extrapolating the classical relationship between particle size and column permeability ($k = d_p/1000$), values of the ''hydrodynamic apparent particle size'' can be derived. They are reported in Table 5. The estimates of the average particle diameters of the conventional packed columns investigated previously and the calculated apparent particle size of monolith are reported in Fig. 18. The values obtained on particle based columns are in good agreement with the nominal particle diameters supplied by the manufacturers [33–37].

The permeability advantage of monolith columns over packed columns is explained by the larger diameter of the channels open for percolation of the mobile phase through the bed of stationary phase in the former type of columns. A certain ambiguity in this comparison, however, arises from the different values of the total and external porosities and of their ratio. Experimental data show that the monolith in the Chromolith columns has a larger specific porosity

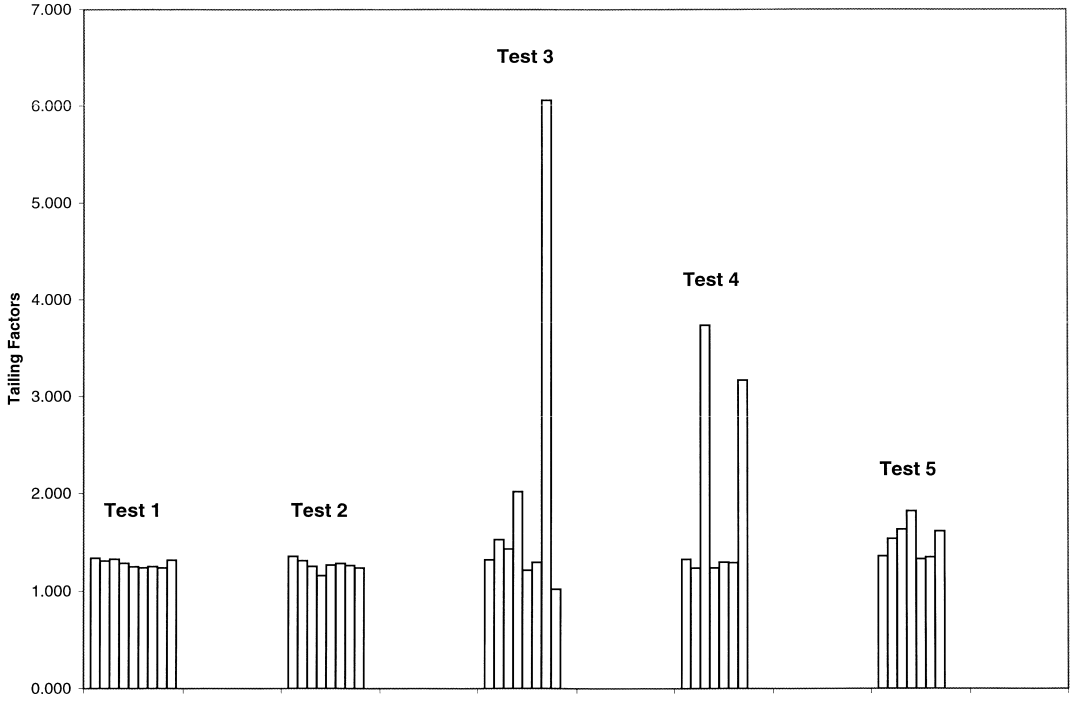

Fig. 16. Long-term repeatability and batch-to-batch reproducibility of the asymmetry factors measured for the components of the five test mixtures. Same numbering as in Fig. 6.

and a much larger total porosity than packed columns. It has also a much larger external porosity. In order to achieve the same velocity in the external pore space, hence the same amount of eddy dispersion and mass transfer resistances, a larger flow-rate is necessary with a monolith than with a packed column, hence the difference between the last two sets of data.

In order to compare the performance of the monolith and the packed columns, we need to estimate the average size of the free channels in a packed column and compare this size to that of the macropores in a monolith column. Note that, in both types of columns, the channels are heavily anastomozed, exhibiting a high degree of tortuosity and constriction which makes the comparison of their size more difficult and less straightforward to interpret. An estimate of the average size of the macropores in a packed bed is such that, on the one hand, under the same pressure drop, the volume flow-rates through the packed bed and through a bundle of parallel capillaries are the same and, on the other hand, the total volume of the capillaries is the same as the external pore volume of the bed. Let d_p and r_c be the diameter of the particles in a packed column of radius R_c and the radius of the capillary channels in a bundle of parallel capillary tubes, respectively. The flow-rate through the packed bed is:

$$F_v = uS = \frac{k_0 d_p^2 \Delta P}{\eta L} \cdot \pi R_c^2 \epsilon_T \quad (1)$$

where u is the mobile phase velocity, S the cross section available to the mobile phase in the column, k_0 the permeability coefficient, ΔP and L the inlet pressure and the length common to the column and the equivalent bundle of capillaries, η the mobile phase viscosity, and ϵ_T the column porosity. The flow-rate through a bundle of n parallel capillaries is:

$$F_v = uS = n\pi \cdot \frac{r_c^4 \Delta P}{8\eta L} \quad (2)$$

Equalling the two flow-rates in Eqs. (1) and (2) gives a first relationship between n and r_c. A second

Table 4
Tailing factor of the components of the five test mixtures

	Average value of tailing factors on six columns	RSD (%) of tailing factors on six columns	Long-term repeatability of tailing factors, RSD (%)
Test 1 (MeOH–water, 8:2)			
Thiourea	1.338	1.375	0.352
Phenol	1.310	1.224	0.390
1-Chloro-4-nitrobenzene	1.329	1.712	1.121
Toluene	1.288	1.921	0.329
Ethylbenzene	1.253	2.172	0.439
Butylbenzene	1.242	2.435	0.285
o-Terphenyl	1.254	2.657	0.224
Amylbenzene	1.240	2.501	0.568
Triphenylene	1.318	2.368	0.239
Test 2 (MeOH–water, 55:45)			
Thiourea	1.359	1.718	2.889
Aniline	1.314	4.945	2.924
Phenol	1.255	1.366	2.689
Toluidines	1.161	3.421	0.888
N,*N*-Dimethylaniline	1.269	10.645	0.675
Ethylbenzoate	1.285	2.774	0.527
Toluene	1.266	2.227	0.469
Ethylbenzene	1.239	2.553	1.100
Test 3 (MeOH–water, 3:7)			
Thiourea	1.322	1.869	1.626
Theobromine	1.529	3.293	1.055
Theophylline	1.434	1.867	1.553
Caffeine	2.020	3.797	2.333
Pyridine	1.216	4.791	3.166
Phenol	1.296	2.804	1.697
2,2-Dipyridyl	6.059	22.590	5.482
2,3-Dihydroxynaphthalene	1.023	11.968	5.489
Test 4 (MeOH–pH 7.0 buffer, 65:35)			
Thiourea	1.329	1.618	1.054
Butylparaben	1.238	1.461	1.640
Propranolol	3.736	10.498	4.777
Dipropylphthalate	1.241	2.089	1.403
Naphthalene	1.300	1.675	1.249
Acenaphthene	1.293	2.011	1.171
Amitriptyline	3.169	19.386	8.460
Test 5 (MeOH–pH 2.7 buffer, 3:7)			
Thiourea	1.360	1.165	2.899
Procainamide	1.538	3.628	4.749
Benzylamine load 1	1.633	2.803	4.029
Benzylamine load 2	1.821	2.985	5.245
Benzylalcohol	1.331	1.992	1.065
Phenol	1.348	1.964	0.925
Benzoic acid	1.615	3.287	7.342

Average values and their reproducibility.

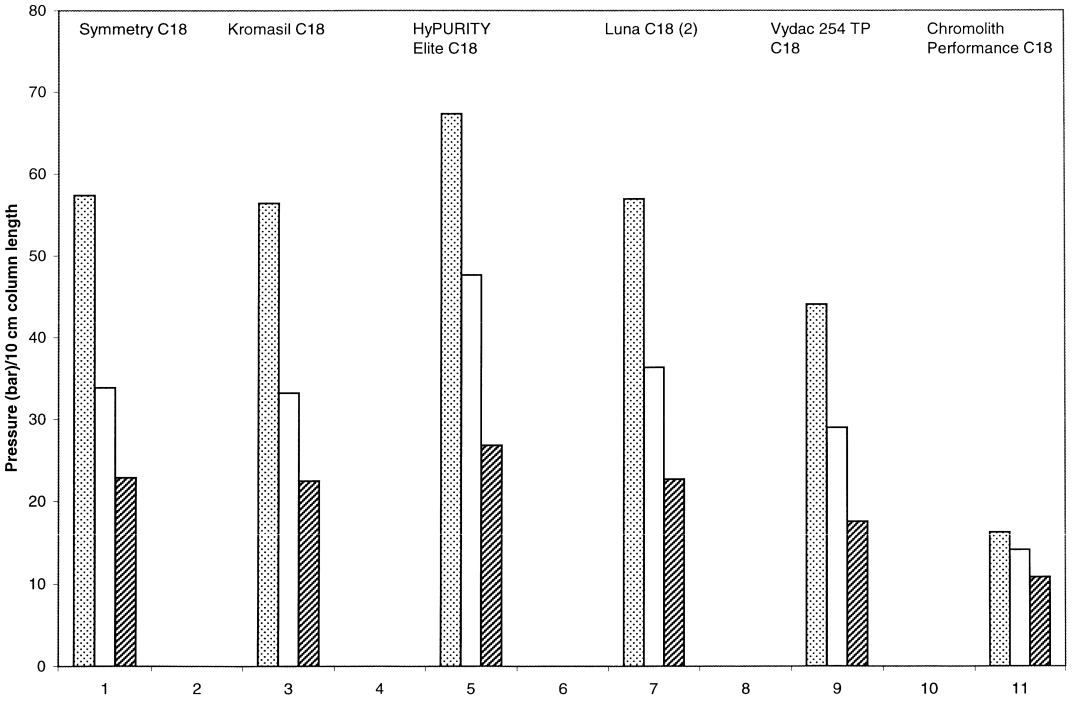

Fig. 17. Permeability of different column brands. Dotted bars: pressure drop required to achieve a 1 ml/min flow-rate of a methanol–water (80:20) solution through a 10 cm long column. Open bars: pressure drop required to achieve a 1 mm/s flow velocity of a methanol–water (80:20) solution through a 10 cm long column (velocity calculated on the basis of the total porosity). Hatched bars: pressure drop required to achieve a 1 mm/s flow velocity of a methanol–water (80:20) solution through a 10 cm long column (velocity calculated on the basis of the interstitial or external porosity).

is provided by the condition that we also want that the volumes of the two systems to be equal, which gives:

$$\pi R_c^2 L \epsilon_T = n \pi r_c^2 L \quad (3)$$

Proper simplifications and combination of these two equations gives:

$$r_c = \sqrt{8 k_0} d_p \quad (4)$$

In practice ($k_0 = 0.001$), this means that the average diameter ($2r_c$) of the equivalent channels is equal to $0.18 d_p$. Conversely, 2 μm channels would give a hydrodynamic behavior equivalent (i.e., same flow-rate, hold-up time and volume, and flow velocity) to that of a conventional column packed with 11 μm particles. This result is in excellent agreement with the experimental observations and the results in Fig. 18 (the small difference may be ascribed to errors of measurement of the average diameter of the macropores, the simplicity of the model, and the fact that hydrodynamic considerations do not average the channel diameters in the same way as the method of measurement of the channel dimensions).

In conclusion, the high performance of monolith columns, that afford both a high permeability and a high efficiency, results from the design of a column combining small porons and large convective channels. In a way, this material appears to be the utmost perfusive material: the column is made of a single particle but has a dense network of relatively wide channels that allows the rapid convection of the mobile phase and its percolation around fine porons through which mass transfer appears to be fast. This certainly vindicates the original concept of Knox [1].

Table 5
Pressure measured on six chromolith performance columns, permeability of six chromolith performance columns and apparent particle size calculated from the pressure measured at different eluent compositions

	Pressure (bar)			Permeability (cm^2)			Apparent particle size (μm)		
	Test 1 (MeOH–water, 8:2) $F=1.0$ ml/min	Test 2 (MeOH–water, 55:45) $F=1.0$ ml/min	Test 3 (MeOH–water, 3:7) $F=1.0$ ml/min	Test 1 (MeOH–water, 8:2) $F=1.0$ ml/min	Test 2 (MeOH–water, 55:45) $F=1.0$ ml/min	Test 3 (MeOH–water, 3:7) $F=1.0$ ml/min	Test 1 (MeOH–water, 8:2) $F=1.0$ ml/min	Test 2 (MeOH–water, 55:45) $F=1.0$ ml/min	Test 3 (MeOH–water, 3:7) $F=1.0$ ml/min
	16.0	23.3	21.7	$7.84\cdot10^{-10}$	$7.71\cdot10^{-10}$	$8.09\cdot10^{-10}$	8.9	8.8	9.0
	16.1	23.5	21.7	$7.79\cdot10^{-10}$	$7.64\cdot10^{-10}$	$8.09\cdot10^{-10}$	8.8	8.7	9.0
	15.9	23.4	21.6	$7.89\cdot10^{-10}$	$7.68\cdot10^{-10}$	$8.13\cdot10^{-10}$	8.9	8.8	9.0
	16.1	23.4	21.6	$7.79\cdot10^{-10}$	$7.68\cdot10^{-10}$	$8.13\cdot10^{-10}$	8.8	8.8	9.0
	15.9	23.5	21.7	$7.89\cdot10^{-10}$	$7.64\cdot10^{-10}$	$8.09\cdot10^{-10}$	8.9	8.7	9.0
Average on column 19	16.00	23.42	21.66	$7.84\cdot10^{-10}$	$7.67\cdot10^{-10}$	$8.11\cdot10^{-10}$	8.85	8.76	9.00
RSD (%)	0.625	0.357	0.253	0.625	0.358	0.253	0.313	0.179	0.127
	15.5	21.3	20.5	$8.09\cdot10^{-10}$	$8.43\cdot10^{-10}$	$8.61\cdot10^{-10}$	9.0	9.2	9.3
	15.5	21.4	20.4	$8.09\cdot10^{-10}$	$8.39\cdot10^{-10}$	$8.57\cdot10^{-10}$	9.0	9.2	9.3
	15.4	21.5	20.5	$8.09\cdot10^{-10}$	$8.35\cdot10^{-10}$	$8.57\cdot10^{-10}$	9.0	9.1	9.3
	15.4	21.4	20.5	$8.14\cdot10^{-10}$	$8.39\cdot10^{-10}$	$8.48\cdot10^{-10}$	9.0	9.2	9.3
	15.5	21.3	20.7	$8.09\cdot10^{-10}$	$8.43\cdot10^{-10}$	$8.57\cdot10^{-10}$	9.0	9.2	9.2
Average on column 20	15.46	21.38	20.56	$8.11\cdot10^{-10}$	$8.40\cdot10^{-10}$	$8.56\cdot10^{-10}$	9.01	9.17	9.25
RSD (%)	0.354	0.391	0.534	0.355	0.391	0.532	0.177	0.195	0.266
	17.0	23.5	22.2	$7.38\cdot10^{-10}$	$7.64\cdot10^{-10}$	$7.91\cdot10^{-10}$	8.6	8.7	8.9
	16.8	23.8	22.3	$7.47\cdot10^{-10}$	$7.55\cdot10^{-10}$	$7.87\cdot10^{-10}$	8.6	8.7	8.9
	17.0	23.7	22.3	$7.38\cdot10^{-10}$	$7.58\cdot10^{-10}$	$7.87\cdot10^{-10}$	8.6	8.7	8.9
	16.9	23.6	22.3	$7.42\cdot10^{-10}$	$7.61\cdot10^{-10}$	$7.87\cdot10^{-10}$	8.6	8.7	8.9
	16.8	23.6	22.0	$7.47\cdot10^{-10}$	$7.61\cdot10^{-10}$	$7.98\cdot10^{-10}$	8.6	8.7	8.9

Table 5 *(cont.)*

	Pressure (bar)			Permeability (cm^2)			Apparent particle size (μm)		
	Test 1 (MeOH–water, 8:2) F=1.0 ml/min	Test 2 (MeOH–water, 55:45) F=1.0 ml/min	Test 3 (MeOH–water, 3:7) F=1.0 ml/min	Test 1 (MeOH–water, 8:2) F=1.0 ml/min	Test 2 (MeOH–water, 55:45) F=1.0 ml/min	Test 3 (MeOH–water, 3:7) F=1.0 ml/min	Test 1 (MeOH–water, 8:2) F=1.0 ml/min	Test 2 (MeOH–water, 55:45) F=1.0 ml/min	Test 3 (MeOH–water, 3:7) F=1.0 ml/min
Average on column 21	16.90	23.64	22.19	$7.42\cdot10^{-10}$	$7.60\cdot10^{-10}$	$7.90\cdot10^{-10}$	8.61	8.72	8.89
RSD (%)	0.592	0.482	0.587	0.592	0.482	0.590	0.296	0.241	0.295
	14.7	19.7	19.2	$8.53\cdot10^{-10}$	$9.12\cdot10^{-10}$	$9.15\cdot10^{-10}$	9.2	9.5	9.6
	14.6	19.8	19.0	$8.59\cdot10^{-10}$	$9.07\cdot10^{-10}$	$9.24\cdot10^{-10}$	9.3	9.5	9.6
	14.8	19.9	19.2	$8.47\cdot10^{-10}$	$9.03\cdot10^{-10}$	$9.15\cdot10^{-10}$	9.2	9.5	9.6
	14.6	19.8	19.1	$8.59\cdot10^{-10}$	$9.07\cdot10^{-10}$	$9.19\cdot10^{-10}$	9.3	9.5	9.6
	14.7	19.9	19.1	$8.53\cdot10^{-10}$	$9.03\cdot10^{-10}$	$9.19\cdot10^{-10}$	9.2	9.5	9.6
Average on column 22	14.68	19.82	19.13	$8.54\cdot10^{-10}$	$9.06\cdot10^{-10}$	$9.18\cdot10^{-10}$	9.24	9.52	9.58
RSD (%)	0.570	0.422	0.438	0.569	0.423	0.438	0.285	0.211	0.219
	17.2	23.7	21.8	$7.29\cdot10^{-10}$	$7.58\cdot10^{-10}$	$8.05\cdot10^{-10}$	8.5	8.7	9.0
	17.4	23.8	21.8	$7.21\cdot10^{-10}$	$7.55\cdot10^{-10}$	$8.05\cdot10^{-10}$	8.5	8.7	9.0
	17.5	23.9	21.8	$7.17\cdot10^{-10}$	$7.51\cdot10^{-10}$	$8.05\cdot10^{-10}$	8.5	8.7	9.0
	17.4	23.7	21.5	$7.21\cdot10^{-10}$	$7.58\cdot10^{-10}$	$8.17\cdot10^{-10}$	8.5	8.7	9.0
	17.3	23.7	21.6	$7.25\cdot10^{-10}$	$7.58\cdot10^{-10}$	$8.13\cdot10^{-10}$	8.5	8.7	9.0
Average on column 23	17.36	23.76	21.68	$7.23\cdot10^{-10}$	$7.56\cdot10^{-10}$	$8.09\cdot10^{-10}$	8.50	8.69	9.00
RSD (%)	0.657	0.376	0.652	0.658	0.375	0.654	0.329	0.188	0.327
	16.9	22.4	21.6	$7.42\cdot10^{-10}$	$8.02\cdot10^{-10}$	$8.13\cdot10^{-10}$	8.6	9.0	9.0
	17.1	22.6	21.4	$7.33\cdot10^{-10}$	$7.95\cdot10^{-10}$	$8.21\cdot10^{-10}$	8.6	8.9	9.1
	17.2	22.4	21.6	$7.29\cdot10^{-10}$	$8.02\cdot10^{-10}$	$8.13\cdot10^{-10}$	8.5	9.0	9.0
	17.1	22.5	21.7	$7.33\cdot10^{-10}$	$7.98\cdot10^{-10}$	$8.09\cdot10^{-10}$	8.6	8.9	9.0
	17.0	22.4	21.6	$7.38\cdot10^{-10}$	$8.02\cdot10^{-10}$	$8.13\cdot10^{-10}$	8.6	9.0	9.0
Average on column 24	17.06	22.46	21.67	$7.35\cdot10^{-10}$	$8.00\cdot10^{-10}$	$8.14\cdot10^{-10}$	8.57	8.94	9.02
RSD (%)	0.668	0.398	0.508	0.669	0.397	0.510	0.335	0.199	0.255
Average ΔP on six columns	16.243	22.413	21.147	$7.75\cdot10^{-10}$	$8.05\cdot10^{-10}$	$8.33\cdot10^{-10}$	8.80	8.97	9.12
RSD (%)	5.999	6.462	4.910	6.160	6.817	5.265	3.061	3.365	2.599

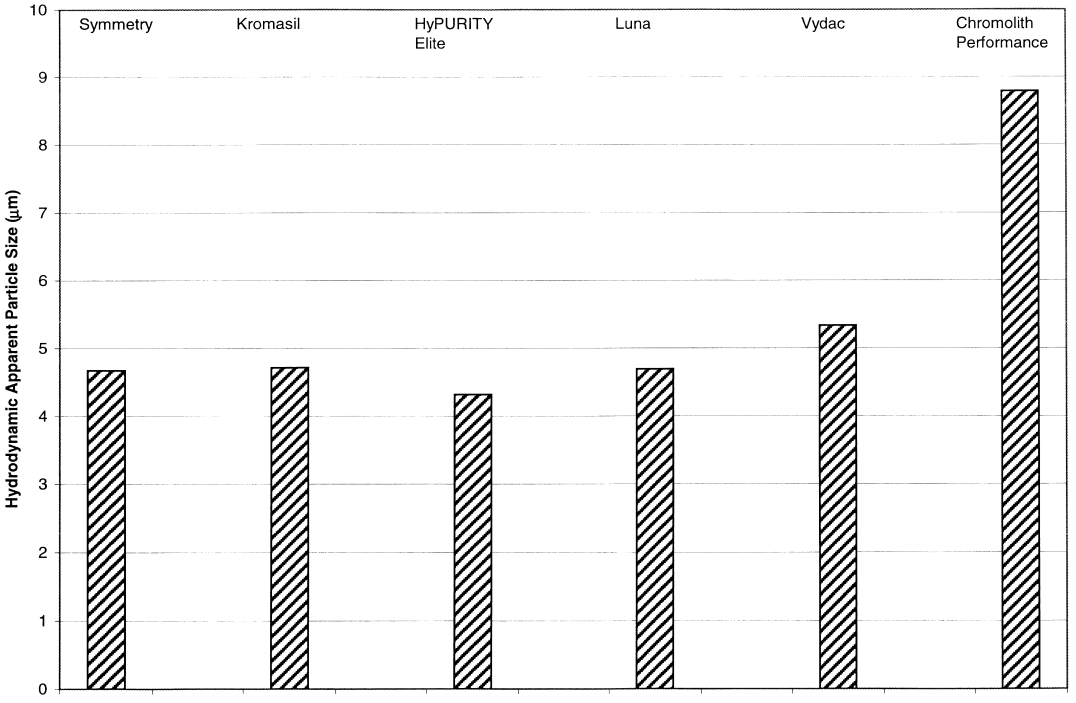

Fig. 18. Apparent average particle size derived from column permeability.

4. Conclusions

Surprisingly for a product hardly out of the development stage, the Chromolith Performance columns exhibit a high degree of reproducibility. The reproducibility figures obtained on the six Chromolith Performance C_{18} columns are better or closely match the values obtained previously on five brands of particle based columns.

The major advantages of these monolith columns compared with traditional particle based columns originate from the higher interstitial volume present as macropores. The high permeability combined with a high efficiency makes these columns suitable for high speed separations or for the injection of high viscosity samples. However, we observed also some surprising, unexpected drawbacks for these columns. Tailing peaks were recorded under all conditions, even for neutral non-ionizable compounds (with a tailing factor around 1.2). Strong loading effects and high tailing factors were obtained in the buffered mobile phase at pH 7.0 with basic compounds. A high tailing factor was obtained with one of the chelate forming compounds (2,2-dipyridyl) but symmetrical peak with the other (2,3-dihydroxynaphthalene). The origin of these effects remains unknown to us.

Finally, as amply illustrated in this report, this new type of column has important technical advantages over conventional packed columns. We may anticipate further improvements in the performance of these analytical columns for HPLC brought by new technical progress and by developments in the production of more homogeneous porous silica monoliths. The excellent results obtained now with this entirely new type of columns that, five years ago, few thought could ever be reduced to practice illustrate how deleterious could have been the implementation in 1998 of the concept of standard column, even under the guise of a reference column. At that time, the obstacles expected in bringing this new product to market were still considered as nearly insurmountable. We could have missed, for political reasons [56], the opportunity of achieving faster and/or more difficult separations under similar experimental conditions.

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