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Journal of Chromatography A, 830 (1999) 41–54

JOURNAL OF CHROMATOGRAPHY A

Repeatability and reproducibility of retention data and band profiles on reversed-phase liquid chromatography columns I. Experimental protocol

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Received 24 July 1998; received in revised form 7 September 1998; accepted 14 September 1998

Abstract

A procedure is described for the determination of the short-term and long-term repeatabilities and of the column-to-column and lot-to-lot reproducibilities of the retention and profile characteristics of the peaks obtained with a number of different reversed-phase liquid chromatography (RPLC) C_{18}-bonded silica columns of several commercial brands. Data characterizing the retention, the steric selectivity, the hydrogen bonding capacity, the hydrophobic interaction selectivity, the column efficiency and the peak asymmetry will be acquired for all the probe compounds. These include 30 neutral, acidic and basic compounds distributed into five groups to be eluted under as many different sets of chromatographic conditions. The data will be obtained using an HP 1100 liquid chromatograph. The compounds and experimental conditions selected were similar to those previously used by different authors for comparison purposes. Careful attention will be paid to minimization of external error contributions by adhering to a strict operational procedure. The precision expected will be high because preliminary results gave standard deviations of around 0.04% for the separation factors, below 0.15% for the retention times and around 1% for the column efficiency in short-term repeatability experiments performed with one column.

Keywords: Band profiles; Stationary phases, LC; Retention data

1. Introduction

In principle, the chromatographic properties of a stationary phase should be completely characterized by the numerical values of a few physico-chemical parameters describing the properties of the bulk material and, for an adsorbent, of the solid–solution interface. This approach might be conceivably successful for well-defined adsorbents such as zeolites or graphitized carbon black, although it would be fraught with enormous difficulties related to the huge variety of mixtures amenable to separation by liquid chromatography. In the case of silica-based adsorbents, this approach is completely precluded by the extreme complexity of the structure and of the chemistry of the internal surface of the porous particles of this amorphous material, by the wide variety of bonding reagents available and by the considerable changes in the properties of the adsorbent resulting from relatively minor adjustments in the synthesis process. The only approach available

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0021-9673/99/$ – see front matter
PII: S0021-9673(98)00757-2

to us for an adequate characterization of silica-based high-performance liquid chromatography (HPLC) stationary phases is the use of appropriate tests related to the expected analytical performance and comparison of the results obtained with the different chromatographic phases available. However, most of the data currently available for these materials are related to their production process.

The physico-chemical properties, which are reliably measured and supplied by most manufacturers of chemically bonded silica phases, are particle size and particle size distribution, particle shape, average pore size, fractional pore volume and internal porosity distribution, specific surface area, the nature of the bonded ligand, the carbon loading, degree of surface coverage, concentration of metal impurities, phase type and whether the stationary phase was endcapped or not. Certainly, this is a wide collection of data. Unfortunately, it is neither easy nor straightforward to predict relationships between these physico-chemical properties and the chromatographic behavior expected from a new stationary phase.

For example, (a) the retention factors of neutral analytes follow a general correlation with the phase ratio and the carbon loading. (b) The number of free silanol groups on the surface can be correlated with its silanophilic properties, although it was shown [1] that, at high silanol concentrations, strongly basic compounds are eluted with symmetrical peaks, an observation which suggests that the validity of the correlation is less general than it is often considered to be. Furthermore, the surface concentration of the residual silanols is not available from the manufacturers as a measured physico-chemical property. (c) The particle size correlates excellently with the backpressure of the column and reasonably well with its number of theoretical plates, although the particle shape may interfere, insofar as it contributes to determination of the packing ability of the material. However, the influence of the particle size distribution can bring some degree of confusion to this picture. According to Dewaele and Verzele [2], column efficiency is not affected by a wide particle size distribution when the solvent flow-rate is close to its optimum value. Since an actual sample is a mixture of compounds with diffusion coefficients that are often quite different, the reduced velocity for each of them corresponds to different values of the actual flow-rate, leaving the analyst with a difficult compromise to ponder when the mobile phase velocity is selected. (d) Finally, metal impurities contained in the initial silica tend to concentrate at the solid surface, providing its acidity, causing undesirable secondary retention mechanisms, and giving strong interactions with chelating reagents and with some basic compounds [3]. Thus, the metal impurity content of a silica could be a valuable guideline for those analysts who want to separate these compounds. This could apply only, however, if they use metal-free instruments (pump, sampling valve, connecting tubes) and columns (tube and frits) ([4], p. 200). It seems probable that metal atoms embedded in the silica matrix have a different effect on the chromatographic properties (e.g., by influencing the acidity of closely bonded OH groups) than those trapped on the surface by e.g., ion exchange. Thus, after 30 years of failed attempts, we may conclude that characterization of stationary phases by process-related data is not a successful approach.

Although this is a more promising approach, the characterization of these phases by performance-related data became popular only in recent years. Unger collected the test procedures developed and used before 1990 [[5], p. 389]. He concluded that ‘the wide application of reversed phase columns necessitates several tests to judge the selectivity of a column’. Kimata et al. [6] developed a series of test procedures that allow characterization of the hydrophobicity, steric selectivity, hydrogen bonding capacity and ion-exchange capacity of stationary phases. They used a series of samples of different composition and methanol–water solutions of different relative concentrations, buffered at different pH values, as the eluent. Engelhardt and his coworkers [7–9] developed a general test method for characterization of the hydrophobicity and silanophilic activity of RPLC phases and used it to compare the properties of different brands of commercially available stationary phases. Serowik and Neue [[4], p. 198] developed a derivative of the Engelhardt test, using neutral, basic and acidic compounds in the test sample and a buffered solution as the mobile phase. McCalley [10,11] evaluated stationary phases by comparing the performances of commercial brands

of RPLC columns with several basic test probes, using isoeluotropic mixtures of different organic modifiers in buffered solutions at pH 3.0 and pH 7.0. An automated testing procedure was developed by Eymann [12] who used aqueous solutions of acetonitrile, buffered also at pH 3 and at pH 7, and an accelerated ageing process. Note that this last work considered two different properties of stationary phases, i.e., their retention spectrum and their stability, which would be better studied separately. Cruz et al. [13] classified 30 commercial brands of columns using Kimata et al.'s test procedure [6] and chemometric methods.

As interesting as they are for the characterization of RPLC stationary phases and for comparison of their retention spectra, these studies leave unaddressed one aspect of column performance that is of critical importance for the analyst, i.e. the repeatability and reproducibility of chromatographic data. The reproducibility of the retention data obtained in the HPLC analysis of basic drugs with columns packed with bare silica of the same brand but coming from different batches was studied by Smith et. al. [14], more than ten years ago. The probe compounds used and the test conditions selected were specifically designed for the pharmaceutical industry. So far, this work remains the only more or less systematic study made on this topic.

We report here on the development of a series of tests designed specifically for the systematic investigation of the repeatability and reproducibility of chromatographic data, characterizing either the retention or the column efficiency in RPLC. As an illustration of our purpose, we present preliminary results obtained regarding the repeatability of the chromatographic data acquired with one column from an important commercial brand, Symmetry C_{18} (Waters, Milford, MA, USA). Further reports will be published later, discussing the complete sets of results obtained on this and other important brands of alkyl bonded silica columns. The companion paper [15] reports on the results obtained with Symmetry C_{18}. This work was carried out in order to provide a much needed baseline for an improved understanding of the quantitative performance of chromatography, the suitability of modern stationary phases to the current needs of regulated analyses, and the potential problems encountered in validating data or in developing 'reference' columns.

2. Experimental

2.1. Instrument

The data were acquired using a Hewlett-Packard (Palo Alto, CA, USA) HP 1100 liquid chromatograph, which includes a solvent delivery system with a binary pump, an autosampler, a diode array detector, a column thermostat and a data station. All of these units are 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 temperature of the column was maintained at 25.0°C by the instrument controller and was measured periodically with an error of less than 0.1°C using a high function thermometer model HFT-82 with a surface probe, purchased from Cole-Parmer (Niles, IL, USA). The mobile phases (see composition later) were obtained by instructing the solvent delivery system to mix the two required streams (pure water or buffer and pure methanol) in the proper ratio, using the binary pump. The two components of the mobile phase were stored in the containers of the solvent delivery unit. The vacuum degasser of the equipment was used to remove the air and carbon dioxide that had dissolved in the solutions from the laboratory atmosphere.

2.2. Tests of the chromatographic equipment

Obviously, the first step in a study of the reproducibility of column performance is to make sure that the chromatographic system is sufficiently stable. All determinations were made using the most modern HPLC equipment available to us, a new HP 1100 (see description above). No efforts were made to improve on its performance. In order to complete, in a reasonable period of time, the planned study, which involved similar tests on different brands of column, we had to be able to test a new column

Table 1
List of instrument performance tests

Method	
Lamp intensity test	*Flow-rate stability*
Holmium oxide test	Diode array detection wavelength accuracy
Thermostat temperature accuracy	Injector precision
Thermostat temperature stability	Detector linearity and injector carryover
Noise	*Eluent composition*
Drift	*Extracolumn band-spreading of the equipment*
Flow-rate accuracy	

every day for several months. The consistent maintenance of a high degree of accuracy over the period of the study was necessary. This required periodic tests of the instrument.

Note that almost[1] all of the data measured were based on time (retention times) or on the relative detector response (efficiency, peak asymmetry and peak profiles). To be accurate, these data do not require accurate detector calibration. They are not affected by significant fluctuations of the detector response factor nor even by noise or drift of the baseline, provided that they remain moderate. We needed to operate in the linear range of the column (negligible curvature of the equilibrium isotherm, hence, with retention time being independent of sample size) and of the detector (no profile distortion), with a signal-to noise-ratio of a few hundred, which is not a demanding specification. Since no quantitative measurements were involved in this project, we did not calibrate the detector for any of the probes studied. We carried out the tests in the analytical range of concentrations, injecting small volumes of low concentration solutions (see below). The detector signal was always less than 170 milliabsorbance units, a range within which the UV detectors are considered to be linear.

The tests on the instruments that were carried out before the beginning of each test procedure are summarized in Table 1. These tests correspond to the operational qualification and performance verification procedures for the HP 1100 series HPLC modules [16], which use a holistic approach. The acceptance limits were determined and used according to the values specified for new equipment. Additional instrument tests were introduced, or a modified version of the test was also carried out in cases where verification of a particular parameter had special importance for our study. These parameters are highlighted in Table 1 and are discussed below. A few times during the series of experiments, when instrument performance fell outside the specification levels, the experiment was interrupted until the specified performance was regained.

2.2.1. Flow-rate accuracy and stability

Flow-rate accuracy was measured by pumping pure water with each of the pump heads, successively, collecting the eluent for 5.00 min and weighing the collected fractions with a semimicro balance. Repeated experiments showed a 0.15% relative standard deviation of the measured flow-rate at 1 ml/min. When mixtures of methanol and water or a buffer were mixed with the pump, the mass differences between successive collected fractions of mobile phase characterize the medium-term flow-rate stability. The values obtained varied between 0.05% (methanol–water; 3:7, v/v) and 0.20% (methanol–water; 55:45, v/v). A large part of this error is explained not by flow-rate instability but by errors of the measurement itself. From these experiments, we estimate the accuracy of the flow-rate at ± 2 μl/min at flow-rates of around 1 ml/min. From the repeatability of the retention time of thiourea (non-retained), the flow stability over periods of between 1 min and a few hours is better than 0.05%.

2.2.2. Eluent composition

To measure the accuracy of the eluent composition, the equipment manufacturer suggests a method that is described in the document ASTM E-19.09.07:

[1] Peak areas were measured and recorded by the data station but the tests described here make little use of it. The possibility of incorporating them in a test will be investigated later.

Proposed Standard Practice for the Evaluation of Gradient HPLC pumps. Based on these measurements, the composition accuracy, the ripple within each run and the composition precision across multiple runs are determined by recording the detector baseline while pumping mixtures of different composition ratios of solvent A (isopropanol) with one pump head and solvent B (isopropanol–acetone; 99.5:0.5, v/v) with the other head, through a restriction capillary. This method had some marked disadvantages in the present study. We use the pump to mix methanol with water or aqueous buffers. These two solvents have different compressibilities and viscosities. Furthermore, during their mixing, a volume contraction takes place (mixing volume). To ensure the accuracy of our test data, it is most important to keep the mixing ratio stable, even if its exact value is not known accurately.

To measure the stability of the mixing, we used water as solvent A and a solution of thiourea in methanol as solvent B. A staircase breakthrough curve was generated by increasing the thiourea concentration in a stepwise manner, i.e., by increasing the concentration ratio of solvent B to solvent A in the mobile phase, in steps of 10%. This experiment was repeated a number of times over a few hours. Such a series of experiments was carried out a few times a month. The repeatability of the relative step heights in the region between 30 and 80% methanol characterizes the short- or long-term repeatability of the composition of the stream of mobile phase generated by the pump. The R.S.D. of the step heights was found to be 0.06% for the short-term and 0.34% for the long-term experiments.

2.2.3. Effect of extracolumn band-spreading

The additional contribution to band-spreading caused by differing extracolumn volumes of the equipment can be characterized by the volumetric peak variance measured without any chromatographic column connected to the instrument. This contribution was measured by excluding the column, replacing it with a zero dead volume connection unit, and injecting a 10-μl sample of a thiourea solution. The band-spreading contribution was derived from both the 5σ peak width and the second moment of the thiourea peak. The values found, 10 and 11 μl, respectively, are in excellent agreement.

Alternately, the contribution of these connecting tubes can be calculated from Taylor's [17] solution for the dispersion of a non-compressible plug of fluid in a straight tube

$$\sigma_c^2 = \frac{\pi r^4 FL}{24 D_m} \quad (1)$$

where r is the tube radius, L is its length, F is the flow-rate and D_m is the molecular diffusivity of the probe compound used. This equation overestimates the dispersive effects because the connecting tubes used in HPLC equipment are rarely straight. A secondary flow, generated by the centrifugal forces, causes radial mixing and limits the extent of axial dispersion due to the Poiseuille flow profile. This secondary flow is not taken into account in Eq. (1), which assumes a straight tube.

Neue [[4], p. 57] proposed an alternate procedure, allowing a 'better estimate'. He observed that the HETP of connecting tubes is constant and equal to 10 cm in the normal flow-rate range. We will assume this value, which is consistent with the work of Atwood and Golay [18] on the peak spreading contribution of short lengths of tubing. The standard deviation (in volume units) of the band at the column outlet is equal to

$$\sigma_c = \sqrt{HL}\,\pi r^2 \quad (2)$$

The injection- and the detector-cell volumes are treated as rectangular signals, the standard deviation of which is classically calculated by the equation

$$\sigma_c = \frac{V}{\sqrt{12}} \quad (3)$$

Based on the length and diameter of the connecting capillaries (400×0.25 and 180×0.17 mm), the volume of the detector flow cell (5 μl), the injection volume (10 μl) and the volume of the heat exchanger (3 μl), the estimated extracolumn band-spreading contribution was 12 μl, which is in good agreement with the measured value.

2.3. Column

The experimental results reported in this work for the purpose of illustration were acquired with one

150×3.9 mm column packed with 5 μm Symmetry C_{18} stationary phase from Waters. This column was packed by the manufacturer and used as received. The study involved the determination of the short-term repeatability of the data acquired with this column (during one day). Symmetry C_{18} is a porous silica that is chemically bonded with octadecylsilane. Its main characteristics are described in the companion paper [15]. The whole set of data reported here was obtained with an initially virgin column.

2.4. Samples and chemicals

The samples required for each of the five tests carried out were prepared by dilution of the selected chemicals in the corresponding mobile phase. Fresh sample solutions were prepared once a week, using filtered solvents, in carefully cleaned glassware. They were kept in a refrigerator between use. Sample volumes of 10 μl were injected successively, using the autosampler. The injection period is discussed later. The qualitative and quantitative compositions of the five test mixtures are given below. The actual amounts loaded on the columns to obtain the test chromatograms are reported in the captions of the corresponding figures.

Sample **1**: thiourea (12 mg/l), phenol (120 mg/l), 1-chloro-4-nitrobenzene (20 mg/l), toluene (522 mg/l), ethylbenzene (433.5 mg/l), butylbenzene (1032 mg/l), *o*-terphenyl (44 mg/l), amylbenzene (1036 mg/l) and triphenylene (12 mg/l) in methanol–water (80:20, v/v).

Sample **2**: thiourea (12 mg/l), aniline (81.7 mg/l), phenol (120 mg/l), *o*-toluidine (79.8 mg/l), *p*-toluidine (20 mg/l), *m*-toluidine (59.3 mg/l), *N*,*N*-dimethylaniline (38.2 mg/l), ethylbenzoate (523 mg/l), toluene (870 mg/l) and ethylbenzene (867 mg/l) in methanol–water (55:45, v/v).

Sample **3**: thiourea (12 mg/l), theobromine (18 mg/l), theophylline (30 mg/l), caffeine (32 mg/l), phenol (160 mg/l), pyridine (98.3 mg/l), 2,2-dipyridyl (200 mg/l) and 1,3-dihydroxynaphthalene (200 mg/l) in methanol–water (30:70, v/v).

Sample **4**: thiourea (12 mg/l), propranolol (400 mg/l), butylparaben (20 mg/l), dipropylphthalate (340 mg/l), acenaphthene (200 mg/l) and amitriptyline (100 mg/l) in methanol–water buffer with potassium phosphate, monobasic/dibasic at pH 7.00 (65:35, v/v).

Sample **5**: procainamide (12 mg/l), benzylamine (196.4 mg/l), benzyl-alcohol (626.4 mg/l), phenol (160 mg/l) and benzoic acid (200 mg/l) in methanol–water buffer with phosphoric acid/potassium monophosphate buffer at pH 2.70 (30:70, v/v).

The chemicals were obtained from Fluka, a Sigma–Aldrich Company (Milwaukee, WI, USA), except for *o*-toluidine, benzylamine, methanol and water, which were from Fisher Scientific (Pittsburgh, PA, USA). They were used as received. In order to avoid any possible errors caused by fluctuations of the buffer composition due to lack of reproducibility of the buffer preparation, the same buffer solution was used for all of the columns of a given brand that were tested. This solution was in a well-sealed bottle.

Typical chromatograms obtained with these five test mixtures are shown in Figs. 1–5. All of the compounds were resolved with a resolution of two or more, except for phenol and benzyl alcohol in the fifth test, in which case, the resolution was close to 1.0. The only peak that exhibited a strong degree of tailing was that of pyridine (5) in the third test

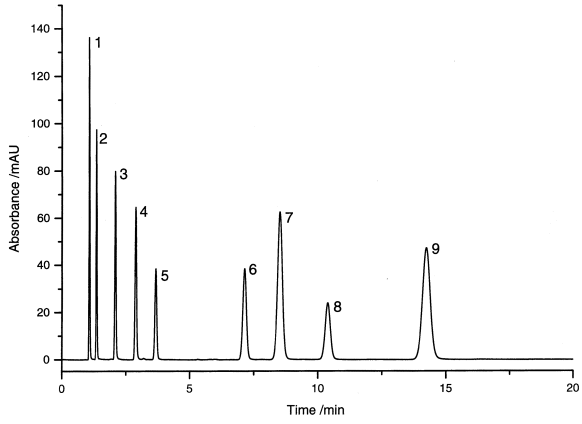

Fig. 1. Chromatogram of the first test mixture. Test compounds and loaded amounts: 1, thiourea (0.12 μg); 2, phenol (1.20 μg); 3, 1-chloro-4-nitrobenzene (0.20 μg); 4, toluene (5.22 μg); 5, ethylbenzene (4.33 μg); 6, butylbenzene (10.32 μg); 7, *o*-terphenyl (0.44 μg); 8, amylbenzene (10.36 μg) and 9, triphenylene (0.12 μg). Mobile phase, methanol–water (80:20, v/v) at 1 ml/min. Temperature, 25.0°C. Detection, 254 nm, UV.

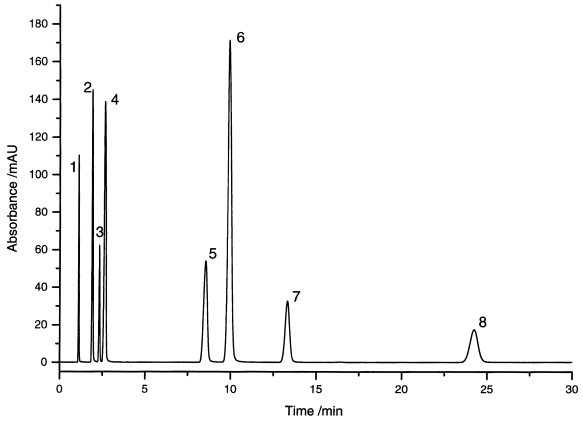

Fig. 2. Chromatogram of the second test mixture. Test compounds and loaded amounts: 1, thiourea (0.12 μg); 2, aniline (0.82 μg); 3, phenol (1.20 μg); 4, *o*-toluidine (0.89 μg), *p*-toluidine (0.20 μg) and *m*-toluidine (0.59 μg); 5, *N*,*N*-dimethylaniline (0.38 μg); 6, ethylbenzoate (5.23 μg); 7, toluene (8.70 μg) and 8, ethylbenzene (8.67 μg). Mobile phase, methanol–water (55:45, v/v) at 1 ml/min. Temperature, 25.0°C. Detection, 254 nm, UV.

mixture (Fig. 3). The differences from chromatogram to chromatogram recorded during this test were far too small to be illustrated by overlaying them. They could be characterized only by the R.S.D.s of the various parameters of the elution peaks.

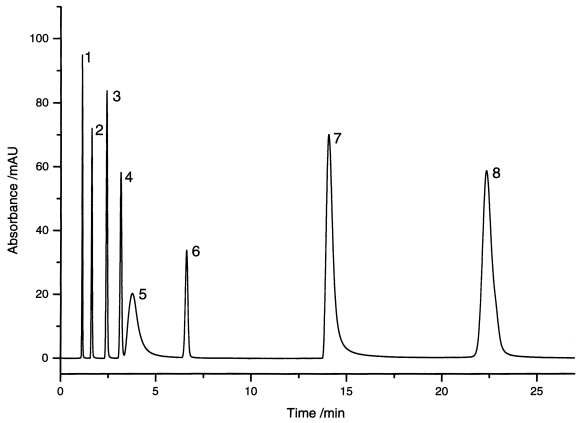

Fig. 3. Chromatogram of the third test mixture. Test compounds and loaded amounts: 1, thiourea (0.12 μg); 2, theobromine (0.18 μg); 3, theophylline (0.30 μg); 4, caffeine (0.32 μg); 5, pyridine (0.98 μg); 6, phenol (1.60 μg); 7, 2,2-dipyridyl (2.00 μg) and 8, 1,3-dihydroxynaphthalene (2.00 μg). Mobile phase, methanol–water (30:70, v/v) at 1 ml/min. Temperature, 25.0°C. Detection, 254 nm, UV.

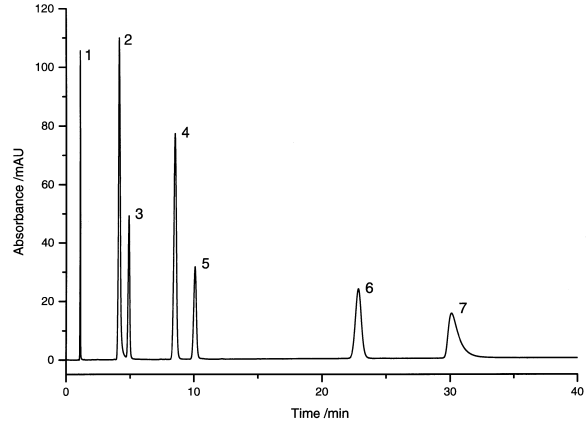

Fig. 4. Chromatogram of the fourth test mixture. Test compounds and loaded amounts: 1, thiourea (0.12 μg); 2, propranolol (4.00 μg); 3, butylparaben (0.20 μg); 4, dipropylphthalate (3.40 μg); 5, acenaphthene (2.00 μg) and 6, amitriptyline (1.00 μg). Mobile phase, methanol–water buffer with potassium phosphate, monobasic/dibasic at pH 7.00 (65:35, v/v). Temperature, 25.0°C. Detection, 254 nm, UV.

2.5. Procedures

The procedure described here is the one used in the study of a given brand of column. Only the part dealing with the short-term repeatability of the data on one column was followed for the experiments

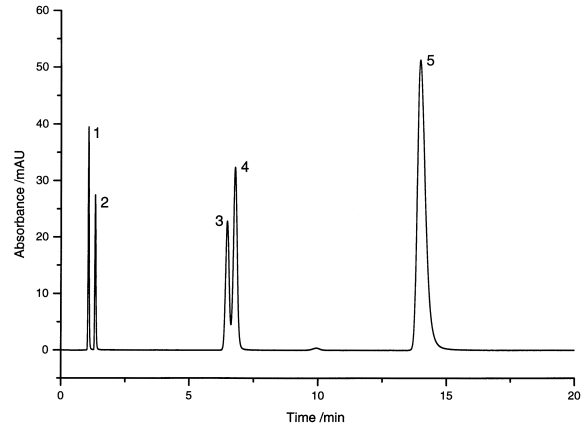

Fig. 5. Chromatogram of the fifth test mixture. Test compounds and loaded amounts: 1, procainamide (0.12 μg); 2, benzylamine (1.96 μg); 3, benzyl alcohol (6.26 μg); 4, phenol (1.60 μg) and 5, benzoic acid (2.00 μg). Mobile phase, methanol–water buffer with phosphoric acid/potassium monophosphate buffer at pH 2.70 (30:70, v/v). Temperature, 25.0°C. Detection, 254 nm, UV.

described later in this paper. Each day, in principle, a new column was connected to the instrument, placed in the temperature-controlled oven (at 25.0°C), and the pump was switched on, with the flow-rate set at 1 ml/min. The column was flushed with the mobile phase for 300 min before any test was performed. After 300 min, the inlet pressure was noted.

The elution of each sample was carried out for a time equal to the elution time of the most retained compound plus 5 min. Five successive injections of each sample were made. Immediately after the five runs of a sample had been finished, the column was flushed with 20 column volumes of acetonitrile (the storage solvent recommended by the stationary phase manufacturer) and was stored filled with this solvent, until the time came for the acquisition of data with the next test sample. In this way, all of the columns were exposed to identical contact times with an aqueous environment and very similar storage times.

The chromatograms were recorded at wavelengths of 254, 220, 230, 270 and 290 nm. The column temperature and inlet pressure were recorded before each injection was made. The chromatograms were stored as files labeled with the column identifier number and the sample number for the test performed. The data files were uploaded to the main computer of the University of Tennessee for further processing. A copy of the data files was made daily and kept in the laboratory, on a WORM ('write-once-read-many') CD-ROM.

In the case of sample 1 only, the same procedure (involving the successive injection of five replicate samples) was repeated at flow-rates of 2.0 and 0.5 ml/min, successively, after completion of the whole series of five tests at 1 ml/min. A period of 30 min was allowed for the flow-rate to stabilize after each new adjustment of the flow-rate. The other samples were run only at 1 ml/min.

In order to minimize the integration error for the first-eluted peaks, the frequency of data acquisition was set at 20 Hz. This allowed the acquisition of 20 points/sigma for the first peak, which is a reasonable value, as suggested by earlier studies on the error contribution due to the discretization of the chromatographic signals [19]. As a consequence, however, the latter peaks are oversampled. This does not affect the accuracy or precision of the corresponding data but results in large data files. Note that the peak shape and the signal noise also contribute significantly to the errors in the measurements [20], however, a discussion of their effect is beyond the scope of this report.

2.6. Presentation of the data

All of the data measured or calculated and reported in this and further works are the average of five successive measurements carried out under identical conditions. Fig. 6 illustrates the short-term repeatability of the retention times of the probe compounds in the five different tests on the column studied. These data are the average values of the parameters measured for five consecutive injections into the column over a period of a few hours.

The words repeatability and reproducibility are defined in many textbooks and in the literature on errors, precision and accuracy. They are, obviously, used with this meaning. However, some qualifiers are required because of the nature of the tests made. The repeatability is characterized by the R.S.D. of a series of consecutive measurements made under the same experimental conditions, using the same products. In most cases, experiments are carried out without interruption. In our case, they were made within a few hours and we refer to that as the short-term repeatability. Similar experiments were carried out periodically, over several weeks, still using the same column and chemicals, under the same conditions. We refer to these results as characterizing the long-term repeatability. Finally, two different reproducibilities were determined, using either different columns from the same batch or columns from different production batches (a definition of a production batch will be given in each application paper). We refer to these data as characterizing the column-to-column and the batch-to-batch reproducibility, respectively.

3. Results and discussion

3.1. Discussion of the selected test compounds and conditions

The test compounds and the corresponding experimental conditions were selected after careful

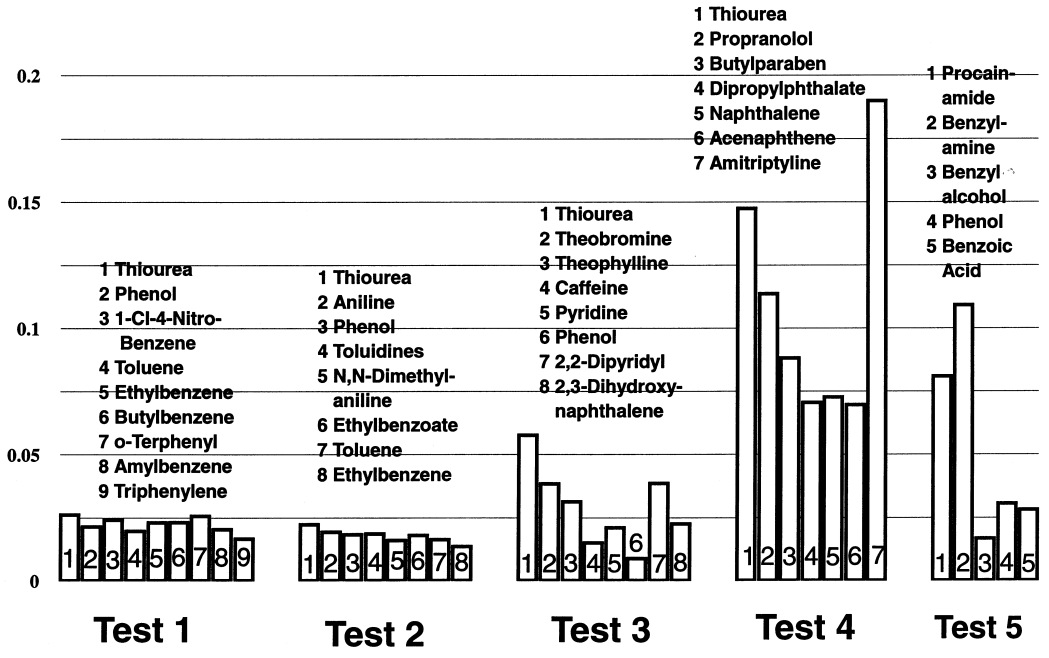

Fig. 6. Short-term repeatability of the retention times of the different probe compounds.

consideration of the literature [5–13]. The tests developed by several manufacturers for the characterization of high quality RPLC packings were also taken into account. Some of the compounds that they used will be recognized in our list of test components. We sought results that could be related to the tests currently available for the characterization and comparison of columns. Thus, our results indicate how precise the results of these tests can be and what degree of reproducibility analysts could expect in most applications. For this reason, compounds having a wide range of properties were selected. Obviously, the only relevant test for an analyst would be one involving compounds representative of those that are analyzed routinely. In trying to achieve some degree of generality by using standard tests, one loses track of the specificity of each analytical case.

The first test mixture contained only neutral compounds, except phenol. Substituted aromatic hydrocarbons with increasing alkyl chain lengths provide information on the hydrophobicity of the stationary phase. The two polyaromatic hydrocarbons allow the determination of one kind of steric selectivity.

The second test is now known as the Engelhardt test. It was originally described by Engelhardt and Jungheim [7]. Nonbuffered water was used to prevent the ‘suppression effect’, i.e., ‘to manifest the presence and influence of silanol groups’ [9]. The test mixture contained toluene and ethylbenzene, to measure the hydrophobic properties of the adsorbent, five basic compounds (including the three toluidine isomers), to characterize the silanophilic interactions, and ethylbenzoate, to test for polar interactions.

The third test is part of the test procedures developed by Kimata et al. [6] for the full characterization of packing materials. According to these authors, the caffeine/phenol selectivity expresses the hydrogen bonding capacity of the material, a property that is affected by the amount of unreacted silanols, the degree of endcapping and the surface coverage. The test uses a low (30%) methanol concentration and nonbuffered water. The authors of

this test also believe that, under these experimental conditions, the elution times of the amines are affected by the density of both the residual silanols and the ion-exchange sites. Pyridine was used in this test mixture because of an earlier suggestion made by McCalley [21] that this compound could be used as a measure of the suitability of RP columns for the analysis of basic compounds ('Pyridine in unbuffered mobile phases does appear to give a reasonable indication of the likelihood of success for analysis of basic alkaloids in buffered mobile phases. This suggests that pyridine is a useful simple test compound' [21]). The statement was based on data obtained with four brands of stationary phase. In view of the results that we obtained (see later), this statement should be checked by investigating the behavior of a few other simple molecules related to pyridine (e.g., quinoline, imidazole). Systematically, in the measurement of all chromatographic properties, pyridine yielded the poorest reproducibility of the 30 compounds studied, with a R.S.D. that was often much larger than the second largest value measured.

The two chelate-forming compounds, 2,2-dipyridyl and 2,3-dihydroxynaphthalene, were added to the third test mixture, in order to obtain valuable information regarding the amount of metal impurity in the packing material studied. Recent results suggest, however, that the heavy metals detected by such tests in RPLC columns originate from the leaching of the parts of the chromatographic instrument upstream of the column [[4], p. 200].

The fourth test had the same general aim as the second one. The sample contained neutral hydrocarbons to measure the hydrophobic properties, strong bases (propranolol and amitriptyline) to provide information on the hydrophilic interactions and two polar compounds. The solution was buffered at pH 7.00 using a 20-m*M* buffer. This ensured that the data obtained with this test had better reproducibility than those obtained with the Engelhardt test. The quality of the water and the contaminations found in a particular laboratory affect the quantitative results to a much lesser degree.

The fifth test was designed to measure the ion-exchange capacity of the stationary phases [6]. An eluent with a low (30%) methanol concentration, buffered at pH 2.7, was used. At this pH, the silanols are protonated, so the elution of basic amines is affected by the ion-exchange sites present on the surface. In order to use this eluent to acquire information about the elution properties of acid solutes, a weak acid (phenol) and a strong one (benzoic acid) were included in the sample.

The first test used a high methanol concentration eluent (80%). The second and fourth tests use also relatively high concentrations of methanol (45 and 65%, v/v, respectively). The third and fifth tests use the lowest methanol concentration (30%). If there was a problem of wettability of the stationary phase by the mobile phase, it would be noticed first using these last two tests. However, concentrations above 20% methanol are generally considered to be sufficient for complete wetting of most C_{18} bonded phases [22], at least those for which the interactions between the bonded alkyl groups are insufficiently strong to cause their collapse into a film covering the surface and shielding the residual silanol groups.

3.2. Influence of the sample size

The total amount of probe compounds in the sample injected corresponded to a loading that was much lower than 1 mg/g of packing, the value suggested by Karch et al. [23] as the safe estimate of the linear capacity of reversed-phase systems. According to Bristow and Knox [24], the amount of each solute in the sample should be kept below 1 μg/g of packing. The data published by Kirkland and Henderson [25] on nortriptyline show a continuous decrease of the capacity factor when the loading was increased from 0.01 to 10 μg solute/g of packing and a slight (2–5%) increase in the reduced plate height in the range of 0.01 to 0.1 μg/g, followed by a large increase (100%) in the range of 1 to 10 μg/g. The range in which linear behavior disappears depends on the stationary phase being studied and, in particular, on its specific surface area and surface chemistry. Data reported by Engelhardt et al. [26] and Gruner and Engelhardt [27] show that the capacity factor of *p*-ethylaniline on a conventional RPLC column remains constant up to a loading of approximately 10 μg in a buffered solution but decreases rapidly above ~0.5 μg in a nonbuffered solution. McCalley [21,28,29] concluded from his studies that the overloading by basic compounds takes place much less readily using mobile phases buffered at pH 7.0 than with mobile phases buffered

at pH 3.0. He also found that, in some cases, peak shape can improve with increasing sample size.

These different results appear at first glance to be somewhat contradictory, largely because we collected the above data pertaining to different compounds and obtained on different stationary phases. The retention mechanisms involved may even be different (e.g., there seems to be an important difference between results obtained with buffered and nonbuffered solvents). We know that the loadability depends on the initial curvature of the isotherm, a property that varies widely from one retention mechanism to another and, within a given mechanism, from one compound to the next [30]. Nevertheless, these data as a whole suggest caution in the selection of the sample size.

From the point of view of this work, that is, the determination of the batch-to-batch reproducibility of the chromatographic data, a continuous change in the retention factors with sample size would be of little importance, except for the minor contribution to the error caused by the fluctuation of the amount injected in successive analyses. By contrast, this effect is important if one wants to compare data measured on different brands of stationary phase and packed into columns having different sizes (standard column diameters and lengths vary from vendor to vendor). It was also important for our study to keep a sufficiently large signal-to-noise ratio (preferably several hundred), even for those compounds that have relatively low molar absorptivities (e.g., phenol, alkylbenzenes, propranolol). The effect of this ratio on the precision of chromatographic data varies considerably from parameter to parameter (e.g., it is different for a retention time and for a centered moment). As a compromise, we decided to keep the amount of each basic compound injected into the column at less than 4 μg in the buffered solutions and at less than 1 μg in the nonbuffered solutions.

3.3. Comments on the preliminary results

The short-term repeatability of the retention times of the neutral and acidic compounds are shown in Fig. 6, in which the repeatability of the retention time of each compound is presented. The data are

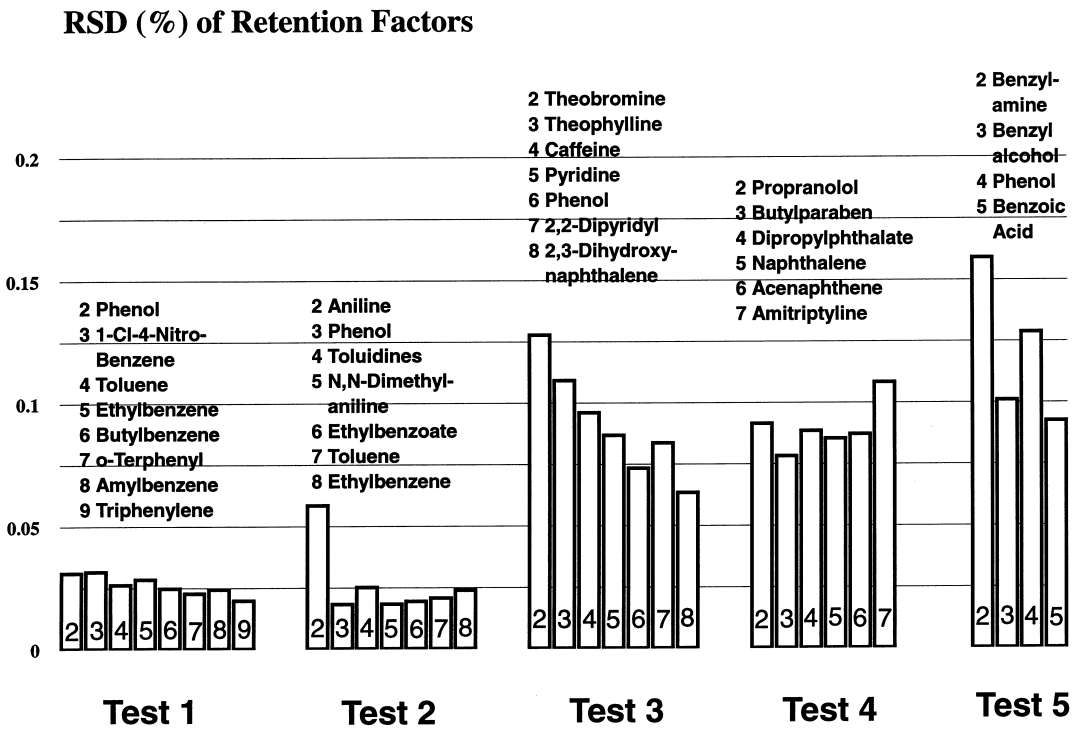

Fig. 7. Short-term repeatability of the retention factors of the different probe compounds.

characterized by a R.S.D. that is lower than 0.1% for all five tests and for the 30 compounds, except for the three basic ones, propranolol, amitryptiline (test 4) and benzylamine (test 5). The experiments were carried out during a three-month period. The column was kept filled with acetonitrile when not in use between the experiments but the equipment itself was used extensively. The precision of the data measured was better than the value (0.3% R.S.D.) defined by the manufacturer as its specification for a new HPLC instrument. This high measurement precision will allow accurate comparisons of the data obtained with five columns packed from the same batch of packing material and with columns of different batches. A large proportion of the R.S.D.s observed is between 0.03 and 0.06%. These results illustrate the high degree of precision that currently can be achieved in liquid chromatography, a precision that is nearly two orders of magnitude better than that claimed in other separation methods or than that achieved in HPLC twenty years ago. This remarkable improvement originates mainly from the progress made in instrument design and from the incorporation of computer controls in all of the functions of the instrument. These data suggest that the use of a standard commercial instrument, without any modifications, is probably sufficient to carry out our project successfully.

The repeatability of the retention factors and the separation factors are shown in Fig. 7 Fig. 8, respectively. The R.S.D.s of the retention factors are higher than those of the retention times because two determinations of retention times and the calculation of their difference are required, causing a larger random error in spite of a partial cancellation of the influence of flow-rate fluctuations. The selectivity factors give lower R.S.D. values than the retention times, except for the benzylalcohol/benzylamine pair in test five. This is explained by cancellation of the errors due to the (small) fluctuations of the column

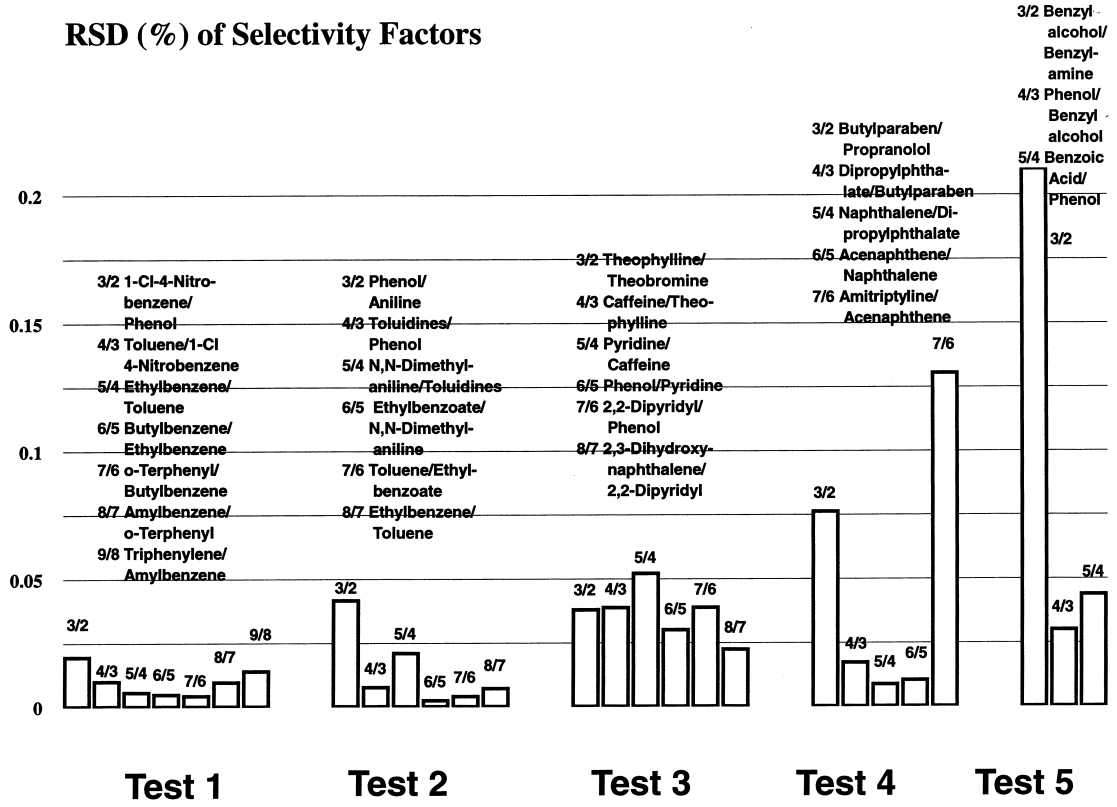

Fig. 8. Short-term repeatability of the separation factors of the different probe compounds.

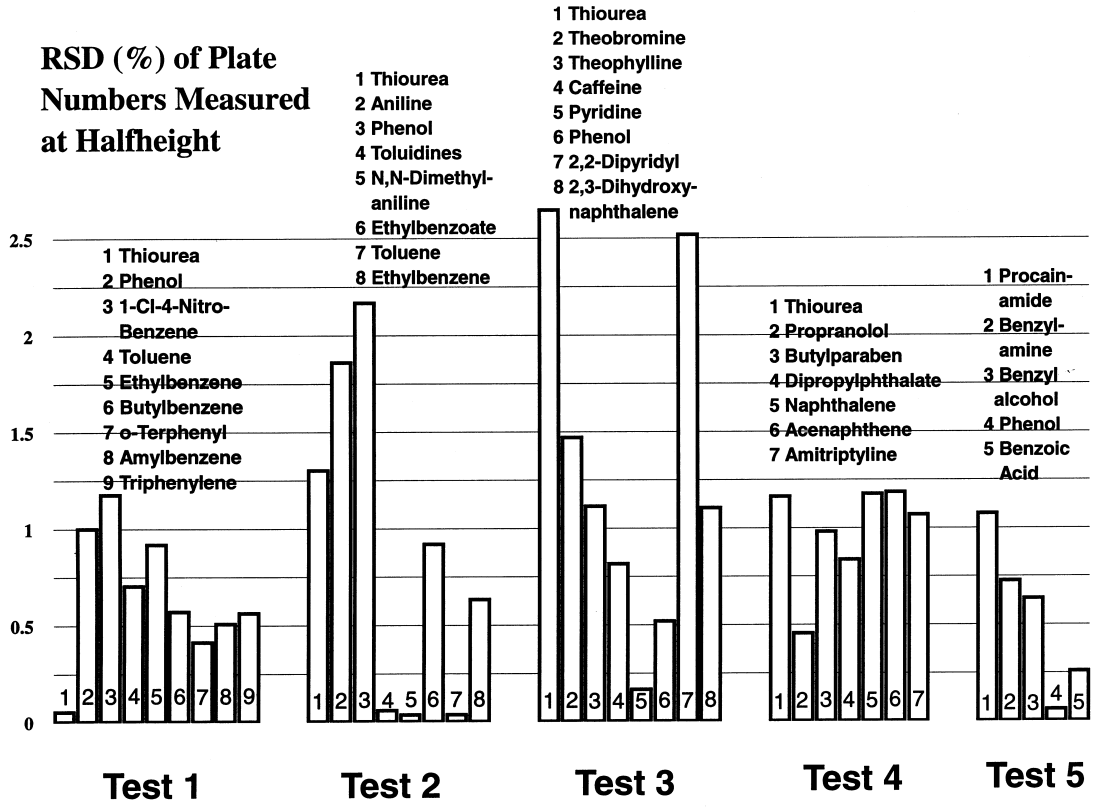

Fig. 9. Short-term repeatability of the column efficiency of the different probe compounds.

temperature and mobile phase composition. Finally, the repeatability of the column efficiency is shown in Fig. 9. Again, an excellent measurement precision, with R.S.D.s in the order of 1%, is demonstrated.

4. Conclusion

To the extent of our knowledge, this work is the first comprehensive protocol for a study on the batch-to-batch reproducibility of commercially available stationary phases by an independent group of investigators. Although some similar data are available from several vendors, the different test conditions used make their comparison nearly impossible. Until the last few years, the degree of reproducibility achieved by commercial columns was such that this lack of attention on the part of professional chromatographers was justified. For those who have worked for more than ten years in analytical chromatography, the degree of reproducibility of column and instrument performance observed in the experiments reported here is impressive. Spurred by the need of their customers to fulfil regulatory requirements and because the economic force of the present column market could supply the necessary resources, some instrument manufacturers have made great strides in improving their HPLC instruments. Column manufacturers have followed suit and dramatically improved the control of the process of synthesis of porous silica particles, of the process of bonding silica with octadecylsilane, and of the column packing process, as following studies will show [15,31].

It seems to us that, for all practical purposes, the batch-to-batch reproducibility of the properties of C_{18} silica stationary phases for RPLC has now reached such a degree as to satisfy most users. Thus, it is time to address this issue systematically and collect a sufficient amount of data to assess the reproducibility of the columns currently available on

the market. In the present paper, we describe the experimental conditions of the test that we plan to use for such an investigation, justify the choices made and provide a few examples to demonstrate that our experimental set-up is capable of achieving low R.S.D.s of the chromatographic data measured. In this work, we will not address the issue of the long-term stability of the retention data. This is an entirely different problem, one that is very specific to the user, as it depends largely on the nature of the samples being analyzed and on their pretreatment. This is not within the scope of this study. We simply want to determine the performance that can be expected from modern columns.

Thus, this report will be followed by a series of studies dealing with the precision of the analytical performance achieved with several commercially available packing materials. The companion paper [15] is the first such study. It deals with Symmetry C_{18}. The next ones will deal with Kromasil (BTR Separations, Wilmington, DE, USA) and with columns from Vydac (Hesperia, CA, USA), Luna (Phenomenex, Torrance, CA, USA) and others. It is not our intention to rank or even compare the products of the different manufacturers, but rather to provide a report on the present state of the art, as a reference for analysts, for column manufacturers and for others interested in this topic.

Acknowledgements

This work was exclusively supported by the cooperative agreement between the University of Tennessee and the Oak Ridge National Laboratory. We thank Uwe Neue (Waters, Milford, MA, USA) for the gift of the column used in this work.

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