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Universal procedure for the assessment of the reproducibility and the classification of silica-based reversed-phase packings I. Assessment of the reproducibility of reversed-phase packings

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Abstract

A method is described that has been used since 1985 to assess the quality and reproducibility of several popular reversed-phase packings. Both the precision of the method and the reproducibility of the packing materials are described. The reproducibility of the newer packings surpasses that of the older packings. In addition, improved results are achieved today for the packings that existed in 1985.

Keywords: Stationary phases, LC; Silica

1. Introduction

Reversed-phase chromatography is by far the most widely used mode of high-performance liquid chromatography (HPLC). The most common type of packing for reversed-phase chromatography is a fully porous silica, whose surface has been derivatized with hydrocarbon chains. The hydrocarbon chains are attached to the silica surface via a Si–O–Si–C bond. Columns packed with fully porous 10-μm reversed-phase packings first became commercially available in 1973. Since that time, there has been a constant concern about the quality and the reproducibility of these bonded phases, and the discussions continue today.

Over the past 25 years, many attempts have been made to develop analytical methods that measure the relevant properties of these packings and allow the assessment of their quality and reproducibility. Early investigators focused their efforts on the understanding of the primary retention mechanism of reversed-phase chromatography. Horváth et al. [1] initially attributed retention in reversed-phase chromatography to the solvophobic effect. However, it was recognized early on [2–5] that silanol groups left on the surface due to incomplete derivatization play an important role in the retention mechanism. Therefore, studies were undertaken to understand and quantify the influence of silanols on retention and peak shape. In some of the early investigations [2], the activity of residual silanols on reversed-phase packings was measured using normal-phase chromatography with hydrocarbons such as heptane as mobile phase. Karch et al. [3] defined arbitrarily a retention factor of less than 0.5 for nitrobenzene in heptane as indicative of a lack of silanol interaction on reversed-phase packings. However, more polar probes exhibited larger retention factors than nitro-

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benzene, indicating a stronger influence of silanol activity than could be ruled out by this test method.

Users of reversed-phase packings generally observed that basic analytes exhibited variable retention, dependent on the age of the packing or its preparation. It became quickly obvious that this phenomenon can be attributed to different levels of silanol activity on the surface of a bonded phase [4,5]. In addition, basic analytes commonly exhibited increased tailing at neutral pH of the mobile phase. It was found that this tailing can often be suppressed using an amine as an additive to the mobile phase. Buffers based on triethylammonium ions were found to be quite effective in suppressing peak tailing for a broad range of compounds. For analytes that exhibit both strong hydrophobic interaction and strong silanophilic interaction, such as tricyclic antidepressants, more hydrophobic amines such as octylamine or the trimethylcetylammonium ion were found to be necessary to suppress tailing [6].

Consequently, the retention of most compounds, particularly compounds with basic functional groups, depends not only on the bonded phase proper, but also on the amount of silanols available for interaction with the analyte. Therefore, a complete characterization of the properties of a reversed-phase packing should test not only the hydrophobic properties of a packing, but also the silanol activity. Daldrup and Kardel [7] described a simple, but well designed test for the characterization of reversed-phase packings using different pharmaceuticals. Two basic compounds, diphenhydramine and diazepam, were chromatographed together with a neutral reference compound, 5-(*p*-methylphenyl)-5-phenylhydantoin, at acidic pH. The relative retention between the bases and the neutral compound was measured together with the absolute retention of the neutral compound. This excellent test was used both for the comparison of different packings and for the comparison of different columns of the same packings. Quite drastic differences were observed between columns of the same brand.

Not only basic analytes were observed to exhibit variable retention. Atwood and Goldstein [8] studied the batch-to-batch variability of 24 batches of a commercially available reversed-phase bonded phase that was commonly used for the separation of polynuclear aromatic hydrocarbons. Another study of the batch-to-batch reproducibility of commercial packings is the investigation by Smith et al. [9], who examined carefully the batch-to-batch variability of the retention properties of a commercial silica used in aqueous mobile phases for the analysis of basic pharmaceutical compounds. The original purpose of the study was the development of ''a database of retention values for interlaboratory comparisons and/or for the identification of basic drugs'' [10]. In a later study [11], the authors found changes in the selectivity of the silica columns with storage over a period of 1 year. While some changes were observed with the storage of dry silica powder, more drastic changes were observed in packed columns. The authors wondered whether these effects were contributing ''to previously observed batch-to-batch differences in the silica''.

A range of tests has been developed to characterize the surface properties of reversed-phase packings [12–21]. The primary goal of these procedures was the characterization of different packings in a fundamental way, with descriptors for the hydrophobicity, the silanol activity and other characteristics of a packing. The reproducibility of a single packing was not the first concern of the authors.

A fairly recent publication by Eymann [22] describes a battery of tests that include neutral analytes, amines, chelators, and acids. The target of the test is the reproducibility of a packing. Unfortunately, no data are presented that indicate the errors of the tests or the reproducibility of packings. Another drawback of the procedure is a lack of internal reference compounds in each test, i.e. all analytes in each test are of the same category.

Based on the early experiences with attempts to characterize reversed-phase packings, Serowik and Neue [23] developed a comprehensive test for the reproducibility of commercial C_{18} packings, specifically μBondapak C_{18} and Nova-Pak C_{18}, The method was derived from the Engelhardt–Jungheim–Dreyer test [14,15]. It employs a mixture of neutral, acidic and basic compounds at neutral mobile phase pH to measure the hydrophobic, silanophilic and ion-exchange properties of silica-based reversed-phase packings [24]. The use of this method for the assessment of the reproducibility of a packing is the subject of this paper, and its subsequent use in the evaluation of the quality of a broad range of packings is the subject of the accompanying paper [25].

Recently, the interest in the reproducibility of

commercial packings has increased, and a related study has been published that characterizes the reproducibility of reversed-phase packings. Kele and Guiochon assembled a group of test procedures [26], including the procedures published in [19,20,23], and tested the reproducibility of a commercial packing [27].

In this publication, we would like to detail the features of the batch-to-batch reproducibility test developed by us and explain the underlying reasoning. We will discuss the influence of external parameters on the test results, including details of the mobile phase composition, test temperature and other variables. In the accompanying paper, we use the same test to characterize different commercial packings.

2. Experimental

Since the investigations described in this paper cover a time span of over 14 years across different departments, a variety of different HPLC instruments were used. In the early studies, a typical instrument comprised a Waters M6000 pump, a Waters U6K injector and a Waters 440 UV detector. Current standard equipment consists of a Waters Alliance system and a Waters 486 UV detector operating under Waters MILLENNIUM chromatography manager. The temperature of the column and of the mobile phase entering the column is controlled with a Euramark EU255 column thermostat or a Neslab RTE-111D digital refrigerated bath. The standard column test temperature is 23.4°C. The preequilibration of the mobile phase to the operating temperature is accomplished via a 60–75 cm length of 0.25 mm I.D. stainless steel tubing immersed in the thermostat.

The test mixtures used in this study contained uracil (16 mg/l) or dihydroxyacetone (3 g/l) as marker for the column dead volume, toluene (300 μl/l) or naphthalene (60 mg/l) and acenaphthene (200 mg/l) as hydrophobic markers, propylparaben (20 mg/l) or butylparaben (20 mg/l), and dipropylphthalate (340 mg/l) or dibutylphthalate (400 mg/l) as polar probes, and propranolol (400 mg/l) and doxepin (100 mg/l) or amitriptyline (100 mg/l) as basic probes. The tests for different packings use different test mixtures, but the classes of test compounds remain the same for all tests. For example, in the test for μBondapak C_{18} doxepin is used as an example of a tricyclic antidepressant. However, the commercially available standard contains a stereoisomer that elutes closely to the parent compound and makes it impossible to measure the tailing of a packing. Therefore, doxepin was substituted with amitriptyline in the procedure used for Symmetry C_{18}. For parabens and phthalates, the appropriate chain length is chosen for each packing to ensure good peak spacing in the test chromatograms. For the same reason, naphthalene replaces toluene in some test mixtures. Every test mixture is dissolved in the mobile phase. The injection volume is 20 μl on a 150×3.9 mm column. Uracil, propranolol, dipropyl- and dibutylphthalate, and amitriptyline are obtained from Aldrich, propyl- and butylparaben, naphthalene, acenaphthene and doxepin from Sigma. The mobile phase consists of 65.0% (v/v) methanol and 35.0% (v/v) of a 20 m*M* K_2HPO_4–KH_2PO_4 buffer at pH 7.00. The buffer is prepared by adding a solution of the dibasic form of the buffer to a solution of the monobasic form. The pH is adjusted to 7.00 before the addition of methanol using a freshly calibrated pH meter. For maximum precision, the mobile phase is prepared by combining carefully weighed quantities of the buffer and methanol. After the methanol is added, the mobile phase is degassed for less than 30 s using a vacuum pump and an ultrasonic bath simultaneously. The columns are equilibrated with 60 to 90 ml of the test eluent followed by three injections of the test mixture.

3. Physicochemical measurements

The carbon content of a packing was measured using a CM-5010 by UIC. The specific surface area and the pore size distribution of a packing were determined by multipoint nitrogen adsorption using a Micromeritics ASAP 2400. The particle size distribution was measured with an Elzone Model 280PC from Particle Data.

Physicochemical measurements are a prerequisite for, and an adjunct to, chromatographic test methods. The specific surface area and the specific pore volume of the silica determine its retentivity. Both parameters are linked to each other through the pore-size distribution. From the carbon content, %C,

and the specific surface area, SA, the surface concentration χ of the bonded phase can be determined according to the following equation:

$$\chi = \frac{\%C}{100 \cdot nC \cdot 12 \cdot SA \cdot \left(1 - \frac{\%C}{100} \cdot \frac{M_r - 1}{nC \cdot 12}\right)} \quad (1)$$

where nC is the number of carbon atoms in the bonded phase, and M_r is the molecular mass of the ligand.

Reproducible surface concentrations are important for the consistency of the selectivity of a packing. While chromatographic test methods can be designed to be more sensitive than physicochemical measurements, the latter provide a rapid first view of the success of the synthesis procedure for a chromatographic packing. Table 1 contains some physicochemical measurements obtained for 20 different batches of Nova-Pak C_{18}, Symmetry C_8 and Symmetry C_{18} as examples. The average particle diameter, the specific surface area and the average pore diameter are measured for the silica particles just prior to bonding. The average particle diameter is important for the backpressure of a column, which varies with the inverse square of the particle diameter [24]. The carbon content is the primary physicochemical measurement for the success of a derivatization procedure. The actual selectivity properties of the packings are better reflected in the surface coverage. The data for Symmetry C_8 and Symmetry C_{18} demonstrate the reproducibility of a modern reversed-phase packing. The data for Nova-Pak C_{18}, which was introduced in the early 1980s, are included as a reference for an older packing. For this packing, the reproducibility of the particle size is very good, with a SD around 1.5%. Similarly, the surface area is well controlled, with a SD under 2%. The reproducibility of the bonding procedure, measured by the surface coverage, has a standard deviation of about 7%. For the newer Symmetry packings, the SD of the average particle size is slightly higher

Table 1
Physicochemical properties of 20 batches of Nova-Pak C_{18}, Symmetry C_8 and Symmetry C_{18} packings

Batch no.	Nova-Pak C_{18}			Symmetry C_8					Symmetry C_{18}				
	Surface area (m^2/g)	%C	Coverage ($\mu mol/m^2$)	Particle diameter (μm)	Pore size (Å)	Surface area (m^2/g)	%C	Coverage ($\mu mol/m^2$)	Particle diameter (μm)	Pore size (Å)	Surface area (m^2/g)	%C	Coverage ($\mu mol/m^2$)
1		7.53		4.94	91	336	11.99	3.47	5.00	91	344	19.80	3.16
2		7.17		4.97	88	331	11.98	3.52	5.00	91	344	19.81	3.18
3	124	7.15	2.65	4.97	93	338	12.10	3.48	5.07	89	343	20.03	3.19
4	126	7.11	2.59	4.99	90	340	12.09	3.50	5.07	89	343	19.82	3.21
5	125	7.13	2.62	5.06	91	335	11.89	3.49	4.96	91	340	19.66	3.16
6	123	7.29	2.73	5.05	90	340	12.07	3.48	5.09	90	337	19.47	3.16
7	127	6.91	2.49	4.95	93	343	12.19	3.46	4.95	93	332	19.37	3.21
8	129	7.16	2.55	5.02	92	335	12.11	3.58	4.99	93	337	19.57	3.20
9	123	7.16	2.67	4.97	93	341	12.35	3.57	5.07	89	343	19.83	3.24
10	126	7.04	2.56	4.97	93	341	12.24	3.51	5.02	92	335	19.48	3.22
11	118	7.15	2.78	4.97	93	341	12.29	3.54	4.99	91	340	19.22	3.13
12	124	7.23	2.68	5.09	90	338	12.04	3.52	4.99	91	340	19.62	3.16
13		7.48		5.09	90	335	12.12	3.54	5.09	91	337	19.82	3.19
14	123	6.84	2.54	5.09	90	335	12.12	3.53	5.09	90	335	19.58	3.18
15	124	6.94	2.56	5.08	90	335	12.09	3.54	5.07	88	347	19.83	3.14
16	124	7.02	2.59	5.07	89	347	12.28	3.50	4.95	88	344	19.71	3.14
17	123	7.10	2.65	4.95	88	341	12.14	3.45	4.95	88	344	19.68	3.15
18	124	8.80	3.34	4.95	90	335	11.84	3.46	4.95	90	337	19.75	3.20
19	123	7.13	2.66	4.95	90	335	11.91	3.48	4.95	90	334	19.04	3.12
20	125	7.12	2.61	5.06	90	343	12.16	3.50	5.06	90	343	19.73	3.13
Average	124	7.22	2.66	5.01	91	338	12.10	3.51	5.02	90	340	19.64	3.17
SD (%)	1.85	5.62	7.06	1.14	1.79	1.15	1.11	1.04	1.10	1.64	1.24	1.19	1.06

than 1%. Since the column permeability depends on the inverse square of the average particle diameter, the batch-to-batch reproducibility of column backpressure can be expected to vary by no more than 5%. The SD for the specific surface area for Symmetry silica is 1.25%. The variability of the carbon content of the packing parallels this value: 1.11% for Symmetry C_8 and 1.19% for Symmetry C_{18}. However, the most important value for the reproducibility of the selectivity of a packing is the surface coverage. For both Symmetry C_8 and Symmetry C_{18}, the reproducibility of the surface coverage is just about 1%. From the physical data, one can expect excellent batch-to-batch reproducibility of the chromatographic results for Symmetry packings.

4. Description of the chromatographic test methods

A good chromatographic method for the characterization of a reversed-phase packing should attempt to measure several parameters [28]. First and foremost is the measurement of the hydrophobic retentivity of the packing. This can easily be accomplished using the retention time or the retention factor of simple hydrophobic compounds, such as aromatic hydrocarbons. We selected toluene or naphthalene and acenaphthene for this purpose (Fig. 1, first row). In addition, one can use the relative retention between two of these compounds to measure the purely hydrophobic selectivity of a packing,

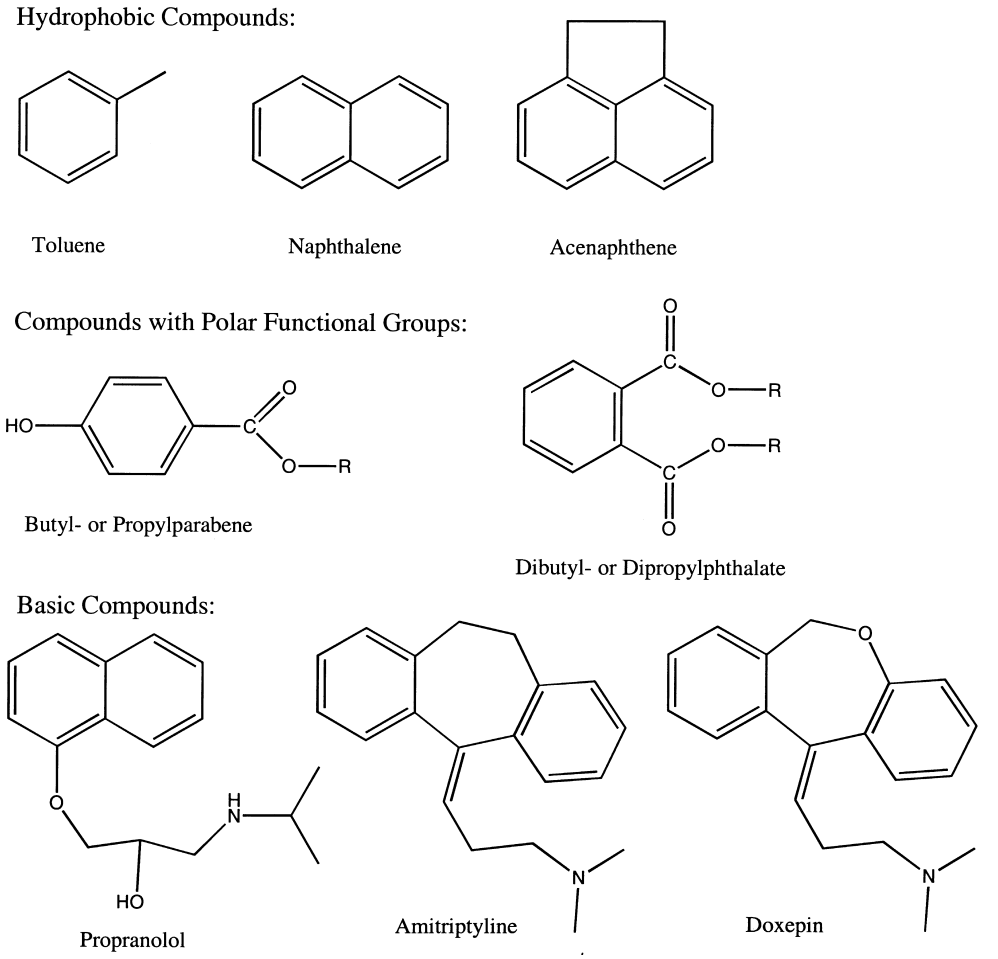

Fig. 1. Structure of test compounds.

somewhat in analogy to the steric selectivity measured by the method of Sander and Wise [12,13]. Secondly, one desires to measure the interaction of the packing with simple polar compounds. We selected phthalate esters and parabens as representatives for this group (Fig. 1, second row). In addition, parabens are proton donors. Thirdly, the most interesting parameter of reversed-phase packings is the interaction of basic analytes with the residual surface silanols of a packing. Once again, two compounds were chosen after an initial screening of a range of basic compounds that exhibited tailing on reversed-phase packings: one compound is propranolol, the other one is a tricyclic antidepressant, either doxepin or amitriptyline (Fig. 1, third row). The primary reasons for the selection of these compounds was the amount of tailing shown on classical reversed-phase packings and the variability of the retention between different preparations of the packings under investigation at the time that the protocol was established. Commercial doxepin is a mixture of stereoisomers that can be partially resolved under the chromatographic conditions of the batch tests. This phenomenon prohibits the use of doxepin for the determination of the tailing factor of a packing. Recently, Kirkland et al. [29] have adopted the use of tricyclic antidepressants for the measurement of silanol interactions.

In order to establish the nature of the interaction of the polar compounds with the surface of the packing, one can either measure the retention of these compounds or their relative retention with respect to a neutral hydrophobic reference compound such as acenaphthene, naphthalene or toluene. Since the absolute retention of a polar, especially a basic analyte can be influenced by both the silanophilic and the hydrophobic interaction, it is more prudent to look at the relative retention between a hydrophobic compound and a polar compound as a measure of the extra-hydrophobic interactions. Specifically, the relative retention between a basic analyte and a purely hydrophobic analyte can be used as a measure of the silanophilic interaction.

A point of contention in the literature is the question, whether such a test should be carried out in the presence of a buffer or not. The presence of buffer ions suppresses some of the interaction of surface silanols with basic analytes. If weak basic probes such as anilines are used, even small amounts of buffer ions suppress the activity of surface silanols [30], and the retention of such probes can no longer be used for the assessment of silanol activity. On the other hand, the reproducibility of a method based on a non-buffered mobile phase can be questioned. In our protocol, we opted for the selection of basic analytes that exhibit a strong interaction with surface silanols in conjunction with the use of a mobile phase buffered to pH 7.00 with a phosphate buffer. We have found that under our test conditions with our probes the control of the pH of the mobile phase is of utmost importance to obtain reproducible results (see below).

5. Validation of the test methods

In order to validate the test method, two elements need to be established: (1) the reproducibility of the results obtained by the method, and (2) the ability of the method to measure the targeted influences. If a method is to be used to measure the reproducibility of a packing, one needs to make sure that the external influences that can cause errors in the test results are minimized, and that the errors of the method are smaller than the measurement targets, i.e., the reproducibility of the preparation of the packing itself. Whenever the test method described above is implemented as a production control procedure for a new packing, the dependence of the test on external variables is established. An example is shown in Tables 2 and 3 for Symmetry C_8, and the results can be taken as being typical for the method. A single column is used, and the mobile phase composition and the test temperature are varied. Changing the methanol concentration by 1% results in a shift of the retention time by typically around 8%, a shift in the relative retentions between 3.55% for the pair propranolol/acenaphthene and an immeasurably small shift for the pair amitriptyline/acenaphthene. A change in the buffer pH value of 0.1 units results in a change in the relative retention between a neutral and a basic compound by nearly 8.5%, while its influence on the relative retention between the two hydrophobic compounds is within the experimental error. The strong shift in retention of the basic analytes as a function of the pH of the

Table 2
Retention time shifts with a change in a test variable – column: Symmetry C_8

	Retention time shift (%)			
	Buffer conc. per 1 m*M*	Buffer pH per 0.1 pH units	% Methanol per 1%	Temperature per 1°C
Propranolol	0.48	5.91	4.49	0.18
Toluene	0.12	0.21	5.71	1.27
Dipropylphthalate	0.11	0.05	8.93	1.68
Acenaphthene	0.16	0.20	8.52	2.20
Amitriptyline	0.31	7.89	8.88	0.91

Table 3
Shifts in relative retention with a change in a test variable – column: Symmetry C_8

	Relative retention shift (%)			
	Molarity per 1 m*M*	pH per 0.1 pH units	% Methanol per 1%	Temperature per 1°C
Propranolol/acenaphthene	0.48	8.38	3.55	2.64
Toluene/acenaphthene	0.03	0.05	2.50	0.79
Dipropylphthalate/acenaphthene	0.05	0.16	1.20	0.48
Amitriptyline/acenaphthene	0.15	8.48	0.06	1.44

mobile phase is due to the shift in pK_a of the analytes and the buffer in the presence of 65% methanol [31,32]. In neat aqueous solution, the pK_a values of amitriptyline and propranolol are close to 9. However, retention studies (e.g. Fig. 2) show that the apparent pK_a values of these analytes in the methanol–buffer mobile phase are around 6.5 to 7, i.e. around the pH of the aqueous buffer of the mobile phase. Therefore, the control of the pH of the mobile phase is especially important for achieving

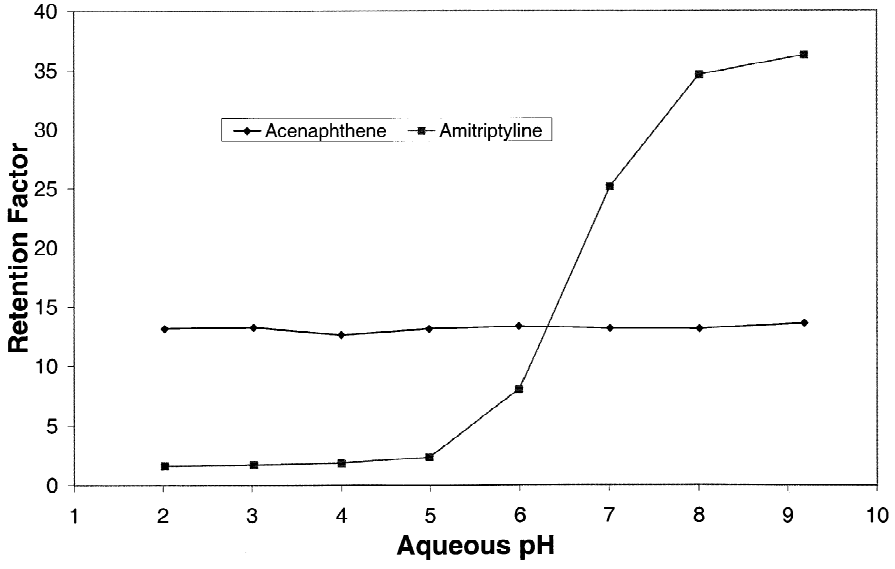

Fig. 2. Dependence of retention on the pH of the aqueous component of the mobile phase. Column: Nova-Pak C_{18}. Buffer 40 m*M* in K^+, either acetate (for pH 4 and 5) or phosphate.

reproducible results. Changes in both retention and relative retention caused by a concentration change of the buffer are negligibly small: less than 0.5% for a 1 m*M* change in the buffer concentration. Temperature shifts influence retention as well: the decline in retention is smaller than 2.5%/1°C, while the change in relative retention is always less than 2.7%/1°C, quite in line with expectations.

A similar data set is shown in Table 4 for Symmetry C_{18}, which shows the changes in relative retention between the compounds in the table and acenaphthene as a function of changes in mobile phase pH, the methanol concentration and the temperature. Again, the major effect is the shift in the relative retention of the basic analytes as a function of the mobile phase pH (around 8% per 0.1 pH units). The relative retention of the weakly acidic analyte, butylparaben, also shows a significant dependence on the pH of the mobile phase (just under 3% per 0.1 pH units). As observed for the Symmetry C_8 column, the relative retention between the strong base propranolol and acenaphthene shifts by about 3% per 1% change in the methanol concentration in the mobile phase. Also, the shift of the relative retention with temperature parallels the results obtained for Symmetry C_8: 2.4–3.4%/1°C for the basic analytes and under 1%/1°C for the neutral analytes. As expected, the results for the Symmetry C_{18} packing and for the Symmetry C_8 packing are generally very similar.

Consequently, if one compares these influences with the batch-to-batch differences of a modern packing (see below), one needs to realize that the pH needs to be controlled to better than 0.01 units, the mobile phase composition to better than 0.1% and the temperature to better than 0.1°C, if one wants to obtain reliable data on the batch-to-batch reproducibility of a packing [26]. For example, the test method for Symmetry C_{18} specifies a mobile phase consisting of 65.0% methanol and 35.0% of a 20 m*M* K_2HPO_4–KH_2PO_4 buffer at pH 7.00 (±0.01) and a column temperature of 23.4°C (±0.1°C). Such an accurate control of mobile phase parameters and temperature is necessary to assess the batch-to-batch reproducibility of a highly reproducible packing. The accuracy required substantially surpasses that needed for typical HPLC methods. For example, the control of the pH value to an accuracy of ±0.01 ensures that the relative retention between the basic analytes and the neutral reference compounds is reproducible to better than 1%. Similarly, the control of the methanol concentration to better than 0.1% enables us to control the absolute retention of all analytes to better than 1% and the relative retention of a base–neutral pair to better than 0.4%. Also, if the temperature is controlled to better than 0.1°C, the relative retention of basic and neutral analytes can be reproduced to within 0.4%. Furthermore, the amounts injected are kept below the loadability of a packing.

The task of such a test method is not only to provide a measurement of the reproducibility of a packing, but also to indicate the influence of the composition of the packing on its chromatographic behavior. A simple example is the relationship between the hydrophobicity of a packing, as measured by the retention factor of a neutral analyte such as acenaphthene, and the carbon content of the packing. Another, more complex, but expected relationship is that between the relative retention of a base/neutral pair of analytes and the surface coverage of the packing material. This relationship is shown in Fig. 3: as the surface coverage increases, the relative influence of silanols on the retention of the basic analyte decreases and therefore the base/

Table 4
Shifts in relative retention with a change in a test variable. Column: Symmetry C_{18}

	Relative retention shift (%)		
	pH per 0.1 pH units	% Methanol per 1%	Temperature per 1°C
Butylparaben/acenaphthene	2.70	0.14	0.29
Propranolol/acenaphthene	9.14	3.24	3.41
Naphthalene/acenaphthene	0.00	0.68	0.49
Dipropylphthalate/acenaphthene	0.09	0.09	0.90
Amitriptyline/acenaphthene	7.40	1.35	2.36

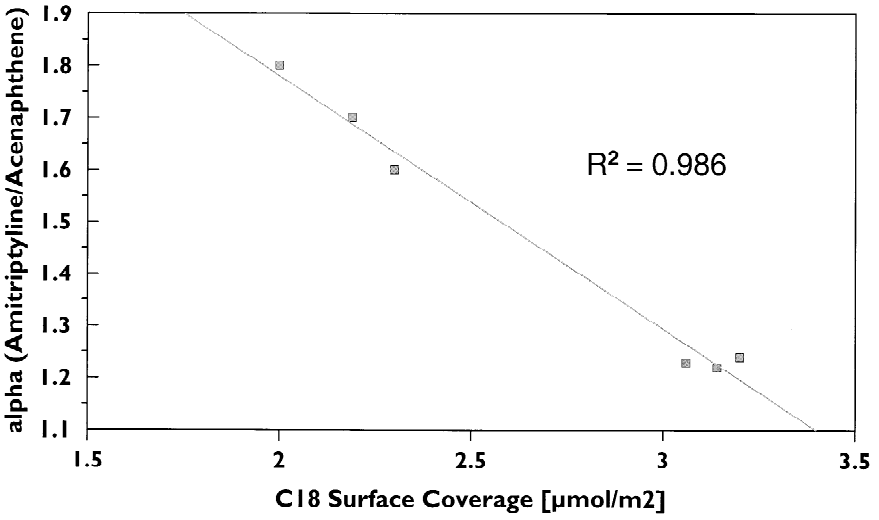

Fig. 3. Relative retention between amitriptyline and acenaphthene as a function of the coating level of the packing.

neutral relative retention decreases. This result demonstrates the usefulness of the alpha value of a base/neutral pair for the measurement of the accessibility of surface silanols.

Silanols are also removed during the endcapping process, which is a secondary reaction of the bonded phase with a trimethylsilyl reagent. One can follow the success of the endcapping process by measuring the carbon content of a packing before and after endcapping, but the measurement is hampered by the fact that the packing contains already a large amount of carbon from the primary silanization procedure. A more effective measurement is offered by a chromatographic evaluation of the packing, using the test method described here. A comparison of the relative retention factors of acidic, basic and neutral test compounds before and after endcapping is shown in Fig. 4. Endcapping reduces all relative retention values. However, while the shift for the neutral hydrophobic reference compounds is small (1.0%) and close to the noise of the measurement, the reduction in relative retention for the base/neutral pairs is substantial, i.e. between 8.8% for propranolol/acenaphthene and 17.4% for amitriptyline/acenaphthene. This is another indication that the relative retention of the base/neutral pairs selected for this test is a good indication of silanol activity. It also demonstrates that the chromatographic test method described here is an excellent tool to measure the properties of a packing, including the efficacy of the endcapping process, which is difficult to measure reliably by physicochemical means.

6. Batch-to-batch reproducibility

The original goal of the procedure outlined above was the measurement of the batch-to-batch reproducibility of reversed-phase packings manufactured by Waters [23]. Previous tests used at Waters prior to 1985 either simply assessed the hydrophobicity of a packing by measuring the retention factor of a simple aromatic hydrocarbon, or measured the retention factors of acids, bases and neutral compounds in separate independent tests [33]. An alternative procedure were a plethora of actual use tests, such as the separation of water-soluble vitamins [34]. Unfortunately, a correlation of the results obtained through the use tests with the properties of the packing remained a daunting task. The establishment of a single reliable test with known external influences represented a significant step forward in the characterization of packings at Waters. Currently, versions of the test are used for the measurement of the batch properties of μBondapak C_{18}, μBondapak Phenyl, Nova-Pak C_{18}, Nova-Pak C_8, Nova-Pak Phenyl, Nova-Pak CN, Symmetry C_{18}, Symmetry C_8, SymmetryShield RP_{18}, and SymmetryShield RP_8 pack-

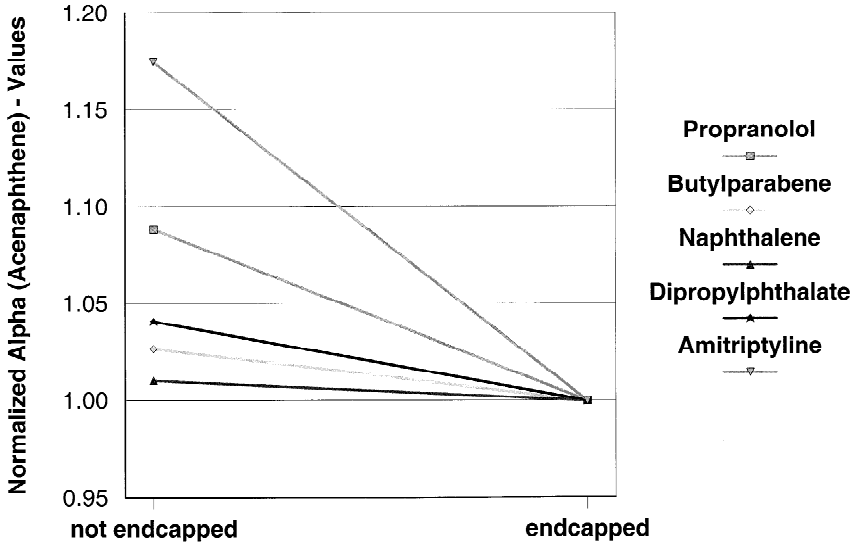

Fig. 4. Influence of endcapping on the relative retention values; the strongest influence is seen for the relative retention of the base/neutral pairs.

ings. Current results of the batch-to-batch reproducibility tests are shown in Figs. 5–7 for the classical C_{18} packings produced by Waters and tested by this method. The figures represent the production data obtained over a period of several years. Plotted are the relative retention values of the neutral, acidic and basic test compounds. The details of the tests are described in the legends to the figures. In order to simplify the visual comparison of the variability of different measurements and different values, the data are plotted on a logarithmic axis of equal length for the three different packings. In such a representation, an equal relative variability of a particular value can be compared by simple visual inspection of the graph without regard to the absolute value.

In Fig. 5, the results are shown for 19 batches of μBondapak C_{18}, which was developed in 1973. One can clearly see that the variability of the relative retention of the neutral hydrophobic test compounds toluene and acenaphthene is very low, i.e. smaller than 1.5%. The variability of the relative retention of polar, non-basic compounds and the hydrophobic reference compound ranges from 1.1% for the pair butylphthalate/toluene to 2.8% for the pair propylparaben/toluene. The worst variability is obtained for the relative retention between the basic analytes propranolol and doxepin and the neutral reference compound acenaphthene: 7.0 and 7.3%, respectively. One can also see that the retention data for both basic compounds change for the most part in parallel to each other. This indicates that both are influenced by the same properties of the packing. As shown above, the property under question is the silanol activity of the packing.

The second graph (Fig. 6) shows the corresponding figure for 19 batches of Nova-Pak C_{18}. Nova-Pak was developed in the early 1980s. In general, one can see an improvement of the variability of all data compared to μBondapak C_{18}. As was the case for μBondapak C_{18}, the variability of the relative retention between the basic analytes and the neutral analytes, 4.3% for the doxepin/acenaphthene pair and 3.4% for the propranolol/acenaphthene pair, is larger than the variability of the relative retention of the neutral analytes, which was 1.1% for the toluene/acenaphthene pair. In addition, the changes in relative retention between the basic analytes parallel each other, demonstrating once again the influence of silanols.

Fig. 7 shows the data for 35 batches of Symmetry C_{18}, a packing that was introduced in 1994. It is based on a high-purity silica, which eliminates the influence of metal contamination of the silica on the activity of surface silanols [35]. A significant effort

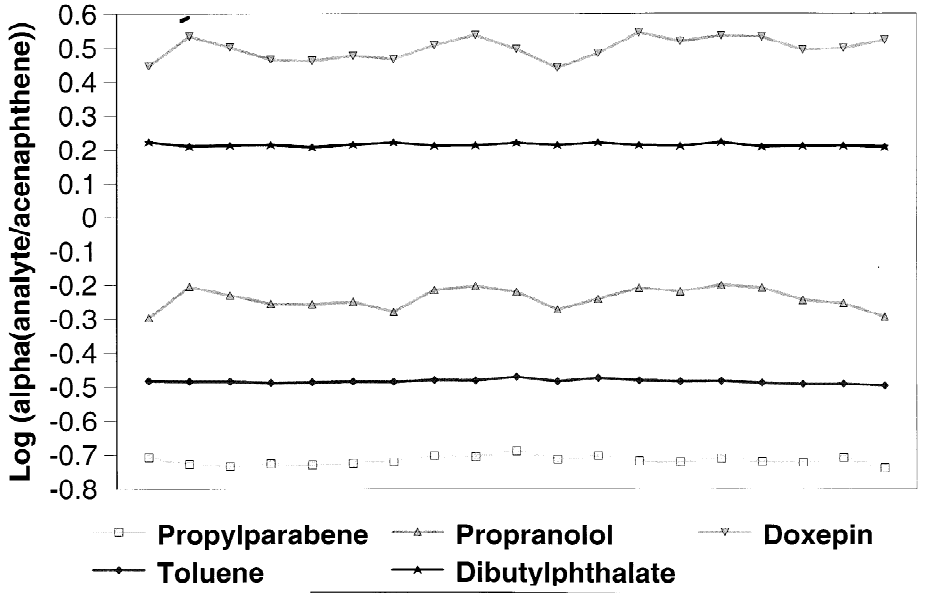

Fig. 5. Batch-to-batch reproducibility of the chromatographic test results for μBondapak C_{18}.

was put into the production procedure of the packing to minimize the variability of the silica and the bonded phase. Consequently, the batch-to-batch variability of Symmetry C_{18} is reduced significantly compared to the older packings. This is true for both the relative retention between the neutral hydrophobic reference compounds naphthalene and acenaphthene, 0.44% and the relative retention between

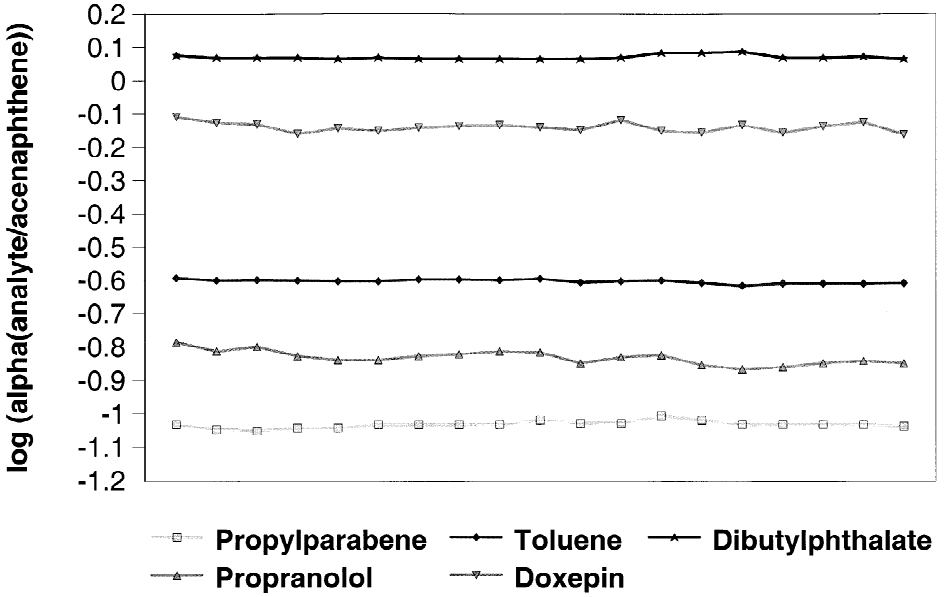

Fig. 6. Batch-to-batch reproducibility of the chromatographic test results for Nova-Pak C_{18}.

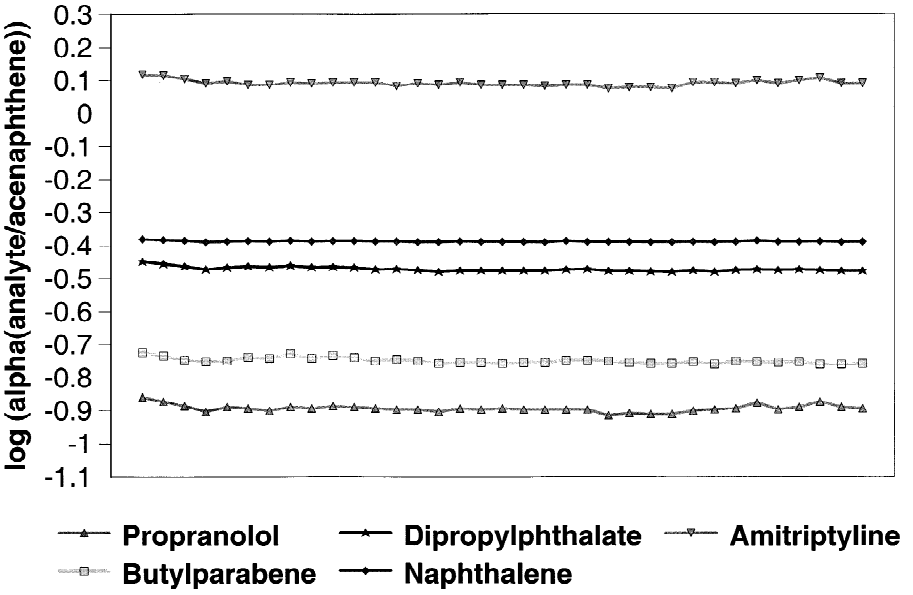

Fig. 7. Batch-to-batch reproducibility of the chromatographic test results for Symmetry C_{18}.

basic and neutral analytes, 2.5% for the pair propranolol/acenaphthene and 2.2% for amitriptyline/acenaphthene. The batch-to-batch reproducibility obtained for Symmetry C_{18} is better than the selectivity reproducibility of most chromatographic methods.

The data presented here are in general agreement with the data reported in ref. [27] for Symmetry C_{18}. However, the results in Ref. [27] were obtained within a few days on a single instrument with a single operator. Consequently, the variability reported in [27] is even lower than the variability reported here, which includes the measurement of the data set over a time frame of several years.

An example of the batch test chromatogram is shown in Fig. 8. In this figure, four chromatograms of four different preparations of the SymmetryShield

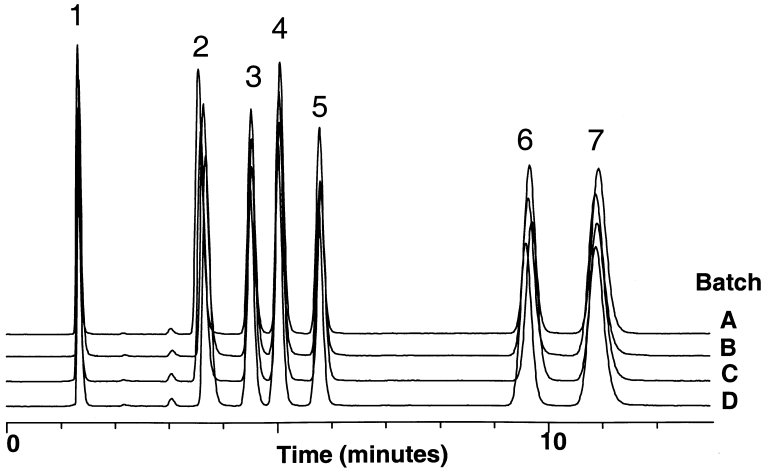

Fig. 8. Overlay of the batch test chromatogram for four different batches of SymmetryShield RP_8. Peaks: 1=uracil; 2=propranolol; 3=butylparaben; 4=dipropyl phthalate; 5=naphthalene; 6=amitriptyline; 7=acenaphthene.

RP_8 packing are shown. The batch-to-batch differences of retention times are barely visible, demonstrating the excellent batch-to-batch reproducibility of Symmetry and SymmetryShield packings. In addition, tailing of the peaks obtained for propranolol and amitriptyline (peaks 2 and 6) is barely visible.

It is worthwhile to compare the reproducibility of the three C_{18} packings to each other and to the state of the art in 1985, before the test procedure described here was established. Such a comparison is shown in Fig. 9a for the relative retention of neutral hydrophobic analytes and in Fig. 9b for the relative retention of a pair of basic and neutral analytes. In all cases, one sees an improvement in the reproducibility of the test results from the time prior to

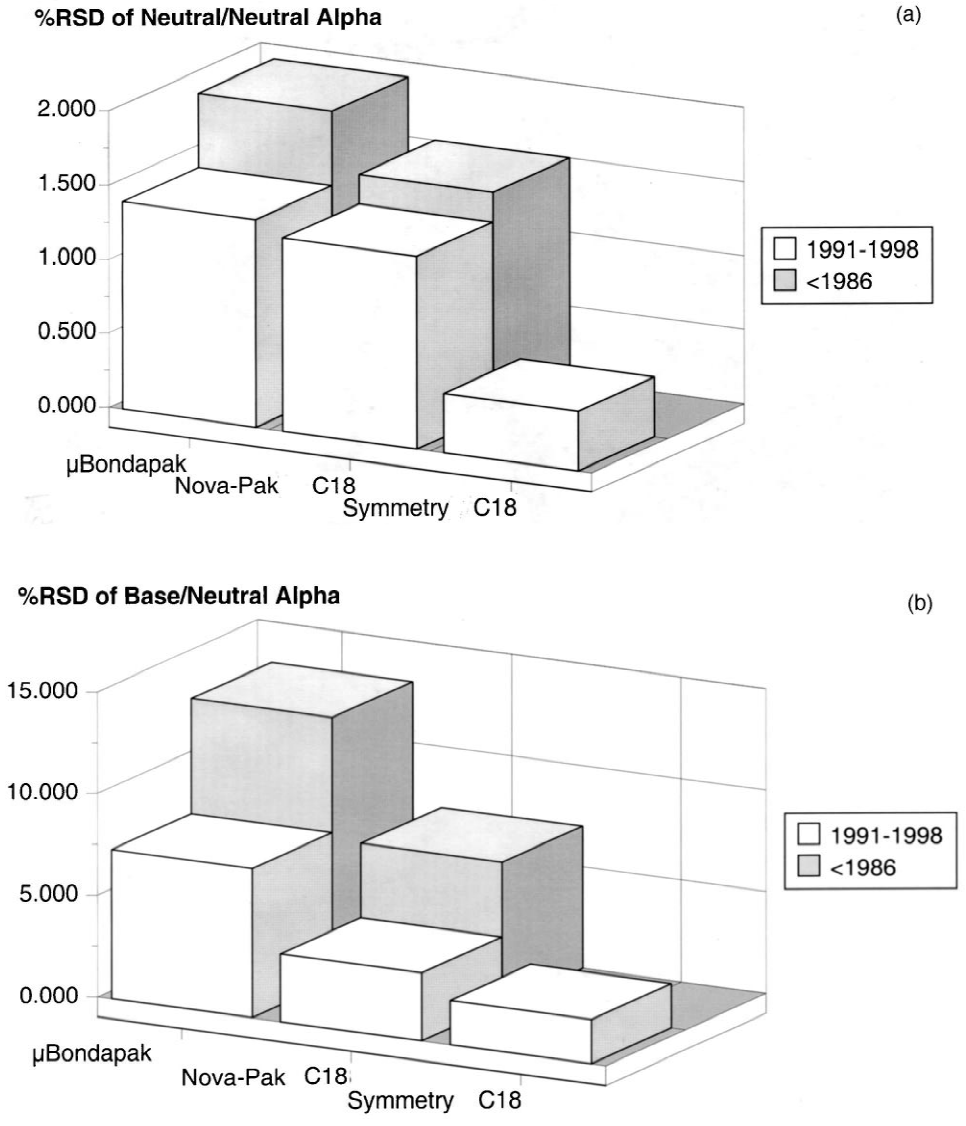

Fig. 9. Comparison of the batch-to-batch reproducibility of µBondapak C_{18}, Nova-Pak C_{18}, and Symmetry C_{18}. For the older packings, the chart also contains the reproducibility data measured in 1985, when the test procedure was established. (a) Reproducibility of the relative retention of a pair of neutral hydrophobic analytes. (b) Reproducibility of the relative retention of a pair of a base and a neutral analyte.

1985 to the current period. For example, the relative standard deviation of the relative retention between a base and a neutral compound was reduced from 13% prior to 1985 to 7.3% today for μBondapak C_{18} and from 7% to 3.3% for Nova-Pak C_{18}. At the same time, one can see the improvement in the reproducibility from the older packings to the newer packings. The variation of the relative retention between a base and a neutral analyte is now only 2.2% for Symmetry C_{18}. The data in Fig. 9a for neutral hydrophobic analytes parallel the improvements obtained for the base/neutral compound pairs: the reproducibility improves both from the time period prior to 1985 to today, and from μBondapak C_{18} to Nova-Pak C_{18} to Symmetry C_{18}. The batch-to-batch reproducibility of the relative retention between neutral analytes for Symmetry C_{18} is as low as 0.4% today.

7. Conclusion

In this paper, we have described the state of the art of the reproducibility of chromatographic packings. We have examined both the physicochemical and the chromatographic properties of several packings. A chromatographic test procedure is described that has been used since 1985 in the quality control of reversed-phase packings. This procedure is an excellent tool for the assessment of the chromatographic properties of a packing. Its sensitivity exceeds by far the sensitivity of classical physicochemical measurements, especially with respect to the silanol activity of a packing. The use of this procedure has contributed to the improvement in the reproducibility of Waters packings, especially during the development of the newer packings such as Symmetry and SymmetryShield. The chromatographic test described here can also be used to characterize different reversed-phase packings. The results of this investigation are presented in a separate publication [25].

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