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Review

Chromatographic characterization of silica-based reversed phases

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ABSTRACT

Test procedures for high-performance liquid chromatographic columns are reviewed. Most of the proposed tests in the literature are only applicable for the evaluation of the hydrophobic properties of stationary phases. The criteria for the description of "good" columns should be symmetrical peaks for both neutral and basic solutes, and independence of retention on sample size. A test in methanol–water (49:51, w/w) with phenol, aniline and the three isomeric toluidines can be used to select columns suitable for the separation of basic solutes.

CONTENTS

1. INTRODUCTION

Since the introduction of reversed phases (RPs) in high-performance liquid chromatography (HPLC) [1,2] 20 years ago, the discussions on their properties and their characterization have been manifold and controversial [3]. In the early years, methods of characterizations were established by the manufacturers with the only aim of making their stationary phases and columns look better than those of the competitors. This was not too difficult, because the properties of RPs are a function of the competition of many processes of the interaction of solutes with the different sorptive sites on the surface of the stationary phase. By proper selection of the test solutes, the contributions of individual sorption mechanisms can be diminished or exaggerated. The main problem with RP systems stems from the presence of surface silanols and their interaction with basic solutes, leading to peak asymmetry, bad

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reproducibility, mass dependence of retention times and possibly irreversible adsorption.

Before going further into the discussion of the properties of RPs, the features for a "good" RP column should be summarized. There is no discussion on column efficiency (measured as plate height, H, or plate number, N), which is a clear function of the stationary phase particle diameter. A good column should exhibit at the optimum linear velocity of *ca.* 1 mm/s a plate height of *ca.* of 2–4 times the particle diameter (reduced plate height 2–4), Usually these values are measured in a low-viscosity eluent (mainly pure methanol or acetonitrile) with non-polar solutes (mainly aromatic hydrocarbons, phenylalkanes or esters of aromatic acids). With these types of solutes there is no problem with peak asymmetry. However, basic solutes are often eluted with tailing peaks (peak asymmetry above 1.3). From "good" stationary phases basic solutes should elute with symmetrical peaks and the retention time should be independent of sample size, below an arbitrarily selected value of 0.1 mg per gram of stationary phase. It has been shown [4] that peak asymmetry and dependence of retention on sample size are a function of the concentration of silanols present. If the silanol concentration is high (extremely low coverage), also strongly basic solutes are eluted from RPs with symmetrical peaks. An increased alkyl group concentration first leads to increased peak asymmetry and, after passing through a maximum at a coverage of *ca.* 2 μmol/m^2, more and more symmetrical peaks are achieved. This is in good agreement with the findings of Kirkland and co-workers [5,6], who obtained the best phases for the separation of basic solutes when starting the preparation of RPs from silica with a totally hydroxylated surface.

Despite causing problems with the elution of basic solutes, silica is by far the most widely used carrier for RPs. The main reason is that silica can easily be prepared with the required precision as small particles. The physical properties of silica, such as specific surface area, average pore diameter and pore volume, can easily be adjusted to the chromatographic requirements [7,8]. Many methods have been described for improving the properties of RPs by varying and optimizing the binding procedure, by selecting different and more reactive silanes or by coating the silica surface with polymeric films [9–13]. However, the standard nomenclature commonly used, assigning to the RP the name of the longest bonded alkyl chain, is insufficient for describing the properties of stationary phases totally. It is, therefore, not surprising that the properties of the RP-8 and RP-18 phases differ widely between the various manufacturers [14–19] and within different batches from a single manufacturer [17].

More than 100 different RP-8 and RP-18 stationary phases are commercially available as such or packed by different suppliers in columns. It is therefore difficult to compare the stationary phases and to select an appropriate one for a defined separation problem. The manufacturers' descriptions are poor and insufficient for characterizing the stationary phases. In addition to the bonded alkyl group, some properties of the base silica can sometimes be obtained. The overall retention behaviour of packed columns depends on the physico-chemical properties of the silica (specific surface area, packing density), the chemical surface properties (type and concentration of surface silanols, surface concentration of metal oxides), the bonding procedure (whether mono, di- or trialkoxy-or chlorosilanes have been used), the surface concentration of bonded groups achieved, the concentration and type of residual silanols and whether part of the residual silanols have been end-capped or not. The

wide range of achieveable selectivities with RPs is on the one hand a great advantage for selecting and optimizing HPLC separations, but on the other it is sometimes difficult to find a second column of identical selectivity. Therefore, methods of RP characterization have been described for evaluating stationary phases and packed columns with respect to their specific selectivities.

2. CHARACTERIZATION OF BULK MATERIAL

It is no problem to determine from bulk material the specific surface area, the pore volume and, with mercury porosimetry, the average pore diameter. The carbon content determined by CHN analysis gives a measure of the retentive properties. A linear relationship of between the capacity factor k', and the weight percentage of carbon has been found. With a knowledge of the surface concentration, Boszewski *et al.* [20] were able to predict the elution behaviour of basic solutes such as pyridine and lutidine in a methanol–water eluent. At surface coverages of 4.24 μmol/m^2, *ca.* 85% of the silanol groups are blocked and with these stationary phases the influence of silanols on the retention of basic solutes is no longer noticeable. With stationary phases with alkyl group surface concentrations below 3.8 μmol/m^2, silanophilic interactions contribute to retention, producing asymmetric peak shapes of the basic solutes.

By diffuse reflectance infrared fourier transform (DRIFT) spectroscopy, differentiation between the different surface silanols, the CH valence absorptions and the trimethylsilyl groups from end-capping procedures is possible [21,22]. Isolated silanols with a sharp absorption at 3470 cm^{-1} are the reactive groups in surface silanization and are the centres for strong adsorption of bases.

The silicas differ in their pH values owing to metal oxide impurities enriched on the silica surface [23,24]. Sodium concentrations in the range 0.1–0.3% [23], 20 metals in the ppm range and 15 metals in the ppb range [24] have been determined. The concentration of silanol groups can be determined by methyl-red adsorption [25]. Solid-state NMR spectroscopy has also been used for the characterization of bonded stationary phases [20,26,27]. All these measurements require bulk material either available from the manufacturer or obtainable by unpacking a column. Further, most of these testing methods are not sensitive enough to compete with chromatographic measurements, which are always based on a molecular surface process by selective interactions of the solute and eluent molecules with the active surface adsorption centres of the stationary phase.

3. CHROMATOGRAPHIC TEST PROCEDURES

To describe the selectivity of stationary phases, various test procedures have been described. Mainly isocratic conditions have been used. The test solutes were either chosen arbitrarily or were related to the authors' work. Consequently, a variety of test procedures have been reported in the literature. Only a few of them will be reviewed here. A good summary can be found in a recent book [28]. Using only neutral and non-polar solutes, extremely large plate numbers for packed columns can be achieved. In routine chromatographic work, polar solutes often containing basic functional groups have to be separated.

Because of the mixed influence of hydrophobic and hydrophilic stationary phase properties on solute retention, test solutes from different groups of organic compounds should be selected. Homologous series as test solutes only give an insight into hydrophobic properties, but can be used to develop a retention index system for LC [29]. Test procedures based only on hydrocarbons [30] or other non-polar test solutes cannot be used to examine the properties of RP systems totally.

A special test for evaluating RPs for their suitability for separating sixteen polynuclear aromatic hydrocarbons (PAHs) proposed for testing drinking water according to the requirements of the Environmental Protection Agency (EPA) has been described [31]. Only a few RP-18 materials are able to separate these hydrocarbons within 30 min. Especially suitable for this separation are polymeric phases, prepared by the addition of water during the silanization process [32]. With most of the RP-18 phases it is only possible to separate 12–14 PAHs. This test is, of course, only suitable for studying the hydrophobic properties of RPs and not silanophilic interactions.

Sophisticated test procedures apply gradient elution and about 20 different solutes to characterize RP systems for solvophobic effects [33], or apply solutes that are not generally available, such as barbiturates [34] or penicillins [35].

In the following, some test procedures will be described in more detail, which permit the characterization not only of hydrophobic properties but also hydrophilic interactions. The aim of these tests was additionally to evaluate the suitability of stationary phases for the separation of basic solutes.

For selecting columns for forensic and toxicological analysis, a test has been described by Daltrup and Kardel [16] for characterizing RP-8 and RP-18. As the eluent a mixture of acetonitrile and phosphate buffer (pH 2.3) was proposed. Three solutes, diphenhydramine, 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH) and diazepam, were used and detected at 220 nm. The retention of the basic pharmaceutical diphenhydramine is extremely sensitive to the presence of surface silanols, whereas MPPH serves as a neutral solute to determine relative retention times (RRT) and to measure column efficiencies. The retention of diazepam depends strongly on the carbon content of the stationary phase, *i.e.*, the concentration of alkyl groups on the surface.

With this test two types of stationary phases can be distinguished. With one group of phases, diphenhydramine is eluted after diazepam. In this instance the peak of diphenhydramine is strongly tailed. With the second group, diphenhydramine is eluted before diazepam and MPPH. Usually with these phases diphenhydramine elutes with a symmetrical peak, especially if its relative retention time is half that of MPPH. To be able to separate in a single isocratic run most of the toxicologically important drugs, a certain carbon content is required. For a suitable stationary phase, diazepam is eluted with an RRT of 1.5–1.6. The disadvantage of this test is its limitation to forensic analysis and the problems with the test solutes, which are not commonly available.

The silanophilic characters of sixteen RPs has were determined and compared by studying the retention behaviour of dimethyldiphenylcyclam (DMDPC), a tetraazo macrocyclic solute, relative to that of chrysene [36] in pure methanol. The retention of DMDPC relative to chrysene varied between 1.2 and 2450 for the phases studied. This test is extremely sensitive for surface silanols, but no recommendations for the suitability of stationary phases for the separation of basic solutes can be derived.

Five different tests were proposed by Goldberg [15] to compare RP columns. The hydrophobic properties were determined by measuring the retentions of toluene, naphthalene and anthracene in methanol–water (85:15). Polar interactions were studied with retention measurements of dimethyl and diethyl phthalate in methanol–water (65:35). The elution of the more polar solutes caffeine, benzoic and *p*-toluic acid was studied in acetonitrile–0.01 *M* acetate buffer (pH 4.5) (20:80). The most polar solutes were measured at relatively low pH and with a relatively high salt content. Under these conditions at least part of the silanophilic interactions can be suppressed. Additionally, it was not stated whether volume or weight relationships were used for the preparation of eluent mixtures.

A mixture of naphthalene, 1-nitronaphthalene and acetylacetone was used by Verzele [37] to characterize RPs, especially for their content of trace metals. Naphthalene was used to determine the kinetic properties of the column and its hydrophobic characteristics. From the relative retentions of 1-nitronaphthalene and naphthalene it should be possible to distinguish between end-capped and non-end-capped phases. With end-capped RP-18 the relative retention of this pair is 1.4 or larger, whereas with non-end-capped RP-18 values between 1.1 and 1.2 were measured. With RP-8 smaller but still noticeable differences in the relative retentions were observed. Acetylacetone is normally not retained. Only with totally metal-free silica surfaces was acetylacetone eluted as symmetrical peak. With all commercially available RPs acetylacetone was eluted with tailing peaks, and with several phases it was irreversibly adsorbed. For demineralization complexation of the metal ions with 0.5% phosphoric or citric acid was recommended. The elution behaviour of most of the β-diketones is a good and sensitive indicator for metal impurities in RPs. For chromatographic measurements methanol–0.5% sodium acetate (60:40 or 50:50) was recommended. This test is suitable for characterizing stationary phases for their trace metal content.

In addition to physico-chemical methods, Danielson and Kirkland [38] used the elution behaviour of phenylhexane, phenylheptane and N,N-diethylaniline in methanol–water (60:40) to characterize stationary phases. Only stationary phases prepared by the author were used and no commercially available stationary phases were studied.

4. TEST PROCEDURE FOR HYDROPHOBIC AND SILANOPHILIC PROPERTIES

A general test procedure should not only reveal the hydrophobic properties of a packed column but also give an insight into the applicability of columns for the separation of polar and especially basic solutes. Everyone should be able to perform this test, and consequently simple, generally available UV-absorbing test solutes should be selected. For the following test procedure, monosubstitued aromatic compounds were chosen. For an optimum column test, their retentions should be in the usual range of $0.5 < k' < 10$ for most of the columns. Additionally, it has been shown [19] that the properties of bonded phases differ with varying eluent composition. The influence of silanol groups on solute retention is most obvious at eluent compositions where the RP is totally wetted (less than 60% water;) and sufficient water is present in the eluent for polar interactions. With the test solutes used and described below, methanol–water [49:51 (w/w) or 55:45 (v/v)] was found to be optimum for both RP-8 and RP-18 column evaluation [39]. No buffer or salt solutions were used in the aqueous component.

The following test solutes were found to be sufficient to describe hydrophobic and silanophilic stationary phase properties:

hydrophobic interactions: toluene, ethylbenzene;

neutral polar: phenol, ethyl benzoate;

basic: aniline, *o*-, *m*- and *p*-toluidine, N,N-dimethylaniline;

inert: thiourea.

A typical chromatogram of the test solutes obtained with a "good" column is shown in Fig. 1. As can be seen, all basic solutes are eluted with symmetrical peaks. The three isomeric toluidines of identical hydrophobicity but different pK values (4.4, 4.7 and 5.1) are hardly separated. Aniline is eluted in front of phenol, both closely after the inert peak.

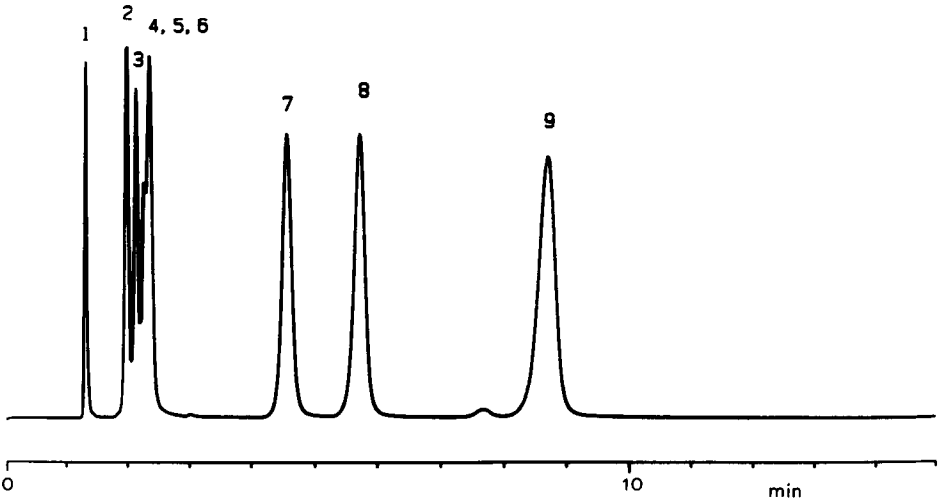

Fig. 1. Chromatogram of the standard test mixture with a "good" column. Conditions: Polyencap A 120, d_p 5 μm (Bischoff, Leonberg, Germany) (150 × 4 mm I.D.); eluent, methanol–water (49:51, w/w); flow-rate, 1 ml/min. Peaks:: 1 = thiourea; 2 = aniline; 3 = phenol; 4–6 = *o*-, *m*- and *p*-toluidine; 7 = N,N-dimethylaniline; 8 = toluene; 9 = ethylbenzene.

For fast column characterization it is not necessary to inject all these components. The elution behaviour of ethyl benzoate can serve as a test of whether an RP-8 or RP-18 column is used: with RP-8 ethyl benzoate is always eluted after toluene, whereas with RP-18 columns it is eluted in front of toluene. A differentiation between these two columns can also be made by determination of k' values or relative retention of toluene and ethylbenzene: with RP-8 the relative retention of these two solutes was 1.7 ± 0.1 and with RP-18 it was 1.8 ± 0.1.

N,N-Dimethylaniline, the strongest base in the mixture, is not such a sensitive tracer for silanophilic interactions as are the isomeric toluidines. Statistical data evaluation showed [40] that N,N-dimethylaniline is a much better tracer for hydrophobic interactions than for silanol groups.

Columns can be considered "good" for the separation of basic solutes if the following two requirements are fulfilled [39]: (1) aniline should elute in front of phe-

nol, and the ratio of the asymmetry of the aniline peak to that of the phenol peak should be 1.3 or smaller; and (2) the three isomeric toluidines should coelute, or the ratio of their k' values should be below 1.3.

Columns evaluated as "good" with these test conditions also showed "normal" behaviour in the RP chromatography of peptides and proteins [41]. With such columns protein retention does not increase at high concentrations of organic modifier.

The test can also be used to determine column stability, as shown in Fig. 2. The upper chromatogram shows the separation of the test solutes obtained with the new column and the lower chromatogram was measured after storage of the column for 1 year in a water–methanol eluent. As can easily be seen, the peak shapes of phenol, ethyl benzoate, toluene and ethylbenzene were hardly affected during storage, but the peak shape and retention of the basic solutes were altered significantly.

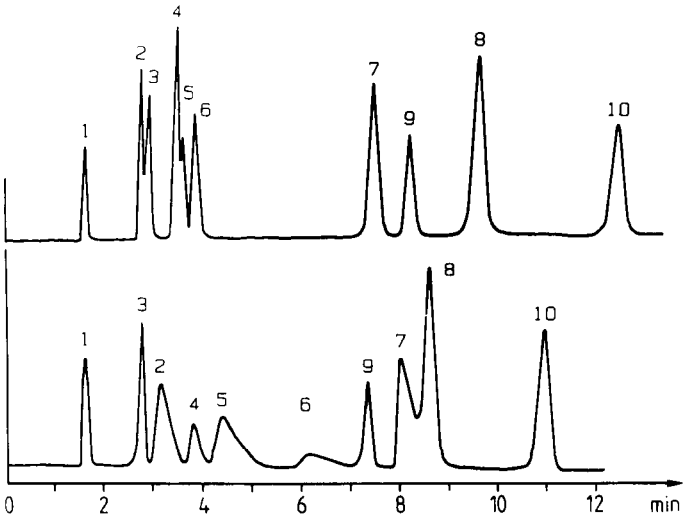

Fig. 2. Destruction of a stationary phase. Conditions: RP-8 (Macherey, Nagel & Co., Düren, Germany) (150 × 4 mm I.D.). Upper chromatogram, new column; lower chromatogram, same column after prolonged use. Other conditions as in Fig. 1. Peaks: 1 = thiourea; 2 = aniline; 3 = phenol; 4 = *o*-toluidine; 5 = *m*-toluidine; 6 = *p*-toluidine; 7 = N,N dimethylaniline; 8 = ethyl benzoate; 9 = toluene; 10 = ethylbenzene.

5. MAKING BAD COLUMNS LOOK GOOD

The test described here places strong requirements on column performance, because pure water is used as an eluent component. Through the addition of strong bases such as trimethylamine to the eluent or by the use of salt or buffer solutions, the silanophilic interactions can be reduced. This is demonstrated in Fig. 3. The chromatogram obtained with the proposed test procedure is shown on the left. According to the conditions discussed above, this column is not suitable for the separation of basic solutes. However, if a buffer of pH 4.8 is used, the column appears to be good. The

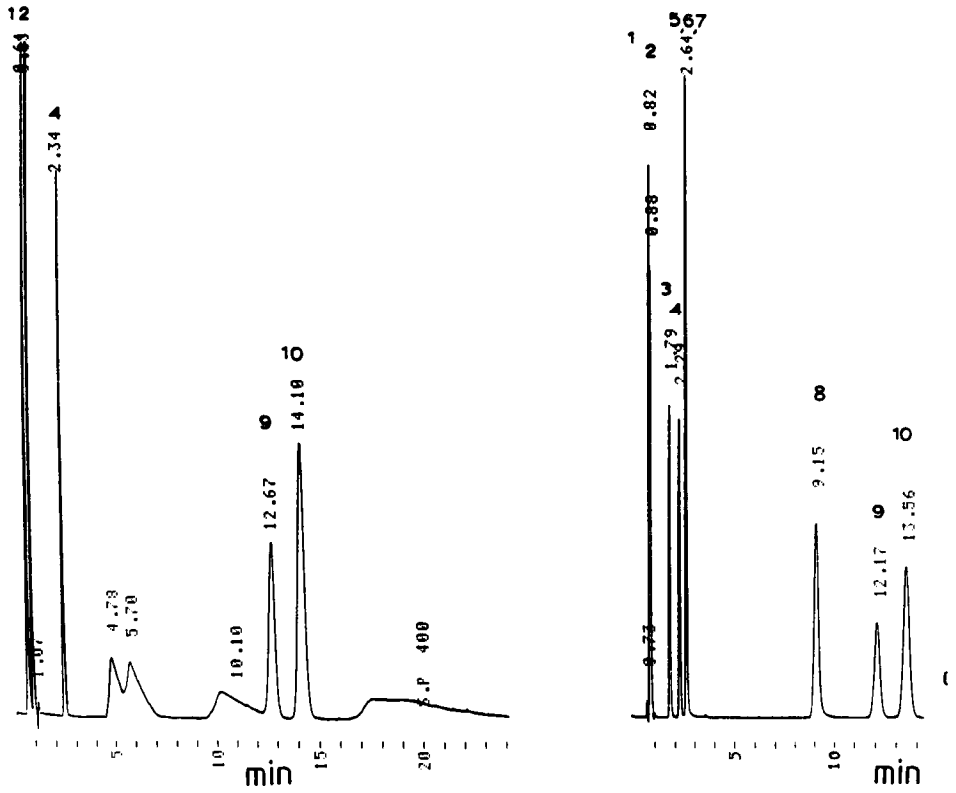

Fig. 3. Alteration of column properties by addition of buffer. Conditions: Superspher RP-8 (Merck, Darmstadt, Germany) (125 × 4 mm I.D.). Eluent: left, as in Fig. 1; right, methanol–0.05 *M* phosphate buffer (pH 4.8). Peaks: 1 = *p*-toluenesulphonic acid; 2 = thiourea; 3 = aniline; 4 = phenol; 5–7 = toluidines; 8 = N,N-dimethylaniline; 9 = toluene; 10 = ethyl benzoate.

decrease in pH reduces the dissociation of the surface silanols and their interaction with the basic test solutes. Depending on the type and amount of accessible surface silanols, much harsher conditions are required. Higher concentrations of strong bases or lower pH values (<2.5) are sometimes necessary to achieve spearations with symmetric peaks also for basic solutes. Of course, the last alternative is to use ion-pairing reagents. In every instance, however, the selectivity of the separation is also affected, but this may be an additional advantage.

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