Review

Review on the chemical and thermal stability of stationary phases for reversed-phase liquid chromatography

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Abstract

At present, in high-performance liquid chromatography (HPLC) for the majority of analyses, reversed-phase liquid chromatography (RPLC) is the separation mode of choice. Faster method development procedures using aggressive eluents under elevated temperature conditions, the need for improved selectivities, efficiencies and resolution, the reduction of solvent consumption and also the decrease of analysis times require reversed-phase (RP) columns of high chemical and thermal stability. Until now, the majority of columns for RPLC separations are manufactured from silica substrates. Silica has many favorable properties making this material nearly ideal as a support for RP columns. However, its solubility, that increases considerably in eluents of pH above ± 7, is a drawback preventing its widespread use over the entire pH range. In addition, also the thermal stability of silica is limited. Recently, however, substantial progress has been made in the synthesis of RPLC silica-based stationary phases showing satisfactory thermal and chemical stability under many different experimental conditions. Also, new substrates mainly based on other inorganic substrates like, e.g. alumina and zirconia have been developed now as a starting material for the preparation of RPLC stationary phases of improved chemical and thermal stability. In addition, for the same reasons, many efforts have also been made to synthesize polymer and also polymer-coated phases. These latter phases, more particularly those based on zirconia, but also polymer phases show a high degree of chemical and thermal stability compared to silica counterparts. In this paper, an overview will be given of the state-of-the-art of the thermal and chemical stability of the different available stationary phases for RPLC.
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Keywords: Reversed-phase stationary phases; Substrates; Chemical and thermal stability; Column longevity

Contents

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1. Introduction

In many analytical laboratories, high-performance liquid chromatography (HPLC) has become an indispensable technique for the analysis of samples, the determination of physical constants and the isolation of purified components from complex mixtures. Now, HPLC has found broad acceptance as the analytical technique of choice in many scientific and application oriented areas like, e.g. life sciences, food, synthetic polymers and environmental chemistry. Additionally, in order to meet legal requirements in a growing number of application areas like, e.g. pharmaceutical and clinical chemistry, HPLC analysis protocols are standardized and validated.

The still continuing development and availability of high-performance equipment and high-quality stationary phases have substantially supported the still enduring growth of HPLC research and applications.

Among the different available HPLC separation modes like, e.g. straight phase (NPLC) or size exclusion chromatography (SEC) definitely reversed-phase liquid chromatography (RPLC) has taken and still takes a dominant position. It is estimated that presently about 80–90% of HPLC separations are performed using RPLC [1]. As already mentioned, the availability of many high quality RP columns together with the large number of tools to control and optimize separations substantially contribute to the great popularity of RPLC.

Although the development of RPLC already started about four decades ago, the technique is still very popular and of great and growing interest. Therefore, RPLC is a continuous subject of substantial research efforts in universities and with manufacturers as well. In turn, this results in a constant appearance of RPLC-oriented books, and scientific and technical papers (see [1–4] and the references therein). In addition, also new and also special equipment and columns for RPLC are continuously developed and marketed. Major theoretical shortcomings of RPLC today are our limited knowledge on the explanation and prediction of underlying retention mechanisms on the molecular level and the role of the eluent therein [1,4–17]; see also the special issue of the Journal of Separation Science dedicated to that subject [18]. Also, we are still lacking fast and adequate method development procedures especially for the separation of larger molecules [19]. Furthermore, also the determination and understanding of the physico-chemical and hence chromatographic properties of RP columns is still limited and subject of ongoing investigations ([2,4,20–26] and references therein). Finally, the chemical and thermal stability of RP columns and thus their longevity under practical eluent and other experimental conditions is also an important issue [2,27–30].

Generally, upon starting the development of an RPLC analysis protocol and anticipating its validation, chromatographers are usually considering four major issues:

 (i) How to select the optimal column for a specific separation from the several hundreds available, and in many cases nominally identical, RP columns?
 (ii) How to perform an objective and fast method development procedure resulting in an optimal and rugged analysis protocol.
(iii) Once an analysis protocol has been established, what will be the repeatability and reproducibility of columns and batches of a specific stationary phase in order to guarantee an undisturbed continuous analysis process.
(iv) What will be the longevity of a column under a specific set of analysis conditions. In other words, how many sample injections can be performed in a column life cycle time.

1.1. Ad (i)

The selection of the best RP column for a specific separation problem is still far from a straightforward process. A substantial number of tests have been discussed in literature. These tests can be roughly distinguished into empirical and model-based testing protocols [31]. For example, three RPLC column tests suggested by Engelhardt and Jungheim [32], Tanaka and coworkers [33] and Neue et al. [34] are well known and often applied in column testing studies.

The limited consistency and poorly correlating results is the major problem of these and also of other tests. For example, hardly any correlation for the important RPLC column parameter "silanols activity" is found between a number of these tests [31]. The differences in testing probes, experimental conditions and calculation procedures substantially contribute to these problems. As a consequence, many chromatographic practioners encounter considerable problems in the objective selection of suitable columns to solve their separation problem. This is even more true for the selection of columns to separate larger molecules [23]. Therefore, in chromatographic practice often many users develop their own in-house testing protocols. Next, these results are then used to

define an internal column quality ranking system [22]. This present situation does not contribute to an objective system of column characterization and quality ranking.

Recently, Snyder and coworkers have developed a column characterization approach based on the so-called "Subtraction Model". This model actually starts from a special form of a linear free energy relationship (LFER) called quantitative structure retention relationship (QSRR). QSRRs treat chromatographic retention as a linear function of a number of different solute–column mobile phase interactions. The modern basis of this approach was laid down, amongst others, by Abraham and coworkers [13] and Kaliszan [35]. In several consecutive papers, Snyder and coworkers have developed this QSRR subtraction model into an interesting and promising method for the characterization and ranking of RP columns [36–41]. In these studies, retention properties of RP columns are measured and described in terms of hydrophobicity, steric resistance, hydrogen bond acidity, hydrogen bond basicity, and cation-exchange activity. This approach allows a much more detailed understanding of the different molecular interactions between solutes, mobile and stationary phases. Furthermore, this subtraction model also facilitates to better distinguish between the chromatographic properties of RP columns and to classify them. In addition, in this approach, RP columns having almost similar properties can easily be divided into subgroups.

Summarizing, it seems that this subtraction model offers an improved and more objective column selection protocol. It also contributes to a more detailed understanding of retention and selectivity in RPLC.

1.2. Ad (ii)

At present, fast and adequate method development is one of the overriding issues in laboratory practice. Apart from the still often applied "trial-and-error" approaches, more and more method development is performed by statistic and/or software supported methods. For example, to arrive fast at rugged and robust analysis protocols, factorial and experimental designs can be used. Such approaches allow to distinguish between the most important separation parameters, e.g. eluent pH and nature of the organic modifier, and to optimize them. A number of good examples of this approach were discussed in the following references ([42–44] and references therein).

Until now, often separation optimization is performed exclusively based on chromatographic criteria. Besides that, business parameters like, e.g. analysis time and/or financial considerations are used as optimization criteria also [45]. Especially, during the last years, several powerful software programs for method development and optimization have become available too. Programs like, e.g. DryLab, ChromSword and others have already shown their benefit in method development and convincing examples can be found in literature [46–50]. However, the use of such programs for the development and optimization of analysis is still legging be-

hind. This is particularly true for the separation of larger molecules as, e.g. peptides and other oligomers and polymers. More convincing examples are needed to further support the widespread use of these programs.

1.3. Ad (iii)

For example, in the food, beverages, pharmaceutical and fine chemicals industries, HPLC is a very important analysis method. In general, but particularly in regulatory analysis, analytical repeatability is of great importance. Therefore, after an analysis protocol has been established and validated chromatographers count on the availability of the same columns, containing exactly the same stationary phase over periods of time from weeks to years. In a number of studies, Guiochon and coworkers have investigated the state-of-the-art of column-to-column and batch-to-batch reproducibility and repeatability of RP columns [51–54]. In these studies for a number of RP columns, the column-to-column and batch-to-batch reproducibility using neutral, acidic and basic test substances was determined. Furthermore, the short- and long-term repeatability of RP columns was also investigated. As an example of that for Symmetry C18 columns, relative standard deviations (R.S.D.s) of retention factors for column-to-column and batch-to-batch reproducibilities were found in the order of 0.1 and 1% range, respectively. On a number of RP columns, however, for basic analytes these values for retention factors, selectivity and plate numbers are generally less favorable. The authors concluded that except for basic compounds the majority of current analysis problems can be satisfactorily solved by the presently available diversity of RP columns.

1.4. Ad (iv)

This paper is focused on the present state-of-the-art of the chemical and thermal stability and hence column longevity of RP columns.

2. Column chemical and thermal stability

For a number of reasons, chromatographers want to improve the chemical and thermal stability and thus longevity of their RP columns. First, in order to adequately and quickly develop analysis protocols, they want to explore the experimentally available space, more particularly with respect to pH and temperature [3,43]. Second, once a separation method has been established and validated, they want to make sure that the selected column will have a certain minimum lifespan. This, in order to estimate the number of sample injections that can be performed on that specific column. Third, working under high pH conditions would allow to analyze basic analytes under ion suppression conditions and also to clean a column on a regular basis from sample matrix constituents [30]. The same is true

for the analyses of anionic analytes performed under low pH conditions. Fourth, in (semi-)preparative RPLC column sample, loadability can greatly be improved by separating the sample components of interest in their non-ionic form [55]. Finally, improved thermal stability of RP columns would be of great interest for the optimization of separations and also to achieve rugged and robust analysis protocols [1,3,43].

In principle, the chemical and thermal stability of RP columns can be enhanced by:

1. The improvement and development of substrates and bonding chemistry.
2. The adjustment and optimization of the mobile phase composition and other experimental conditions.

2.1. The improvement and development of substrates and bonding chemistry

2.1.1. Covalently modified silica substrates

Since authors sometimes report in one and the same paper the results of their chemical stability studies of RP phases for neutral, acidic and basic eluent pH values, the reader may find certain references repeated at different places in this text.

By far, silica is still the most popular substrate to manufacture RP stationary phases. Silica has a high mechanical strength that enables its use under the high-pressure conditions encountered in HPLC. Furthermore, this substrate does not swell or shrink when exposed to organic solvents. Finally, its production and bonding chemistry is well understood and can be performed in many different morphologies and also is very reproducible [1,2,51–54,56–58]. For these reasons, silica seems the perfect starting material for the manufacturing of (bonded) phases for HPLC. During the last decades, many workers have suggested several different approaches for the synthesis of silica-based RP stationary phases and also to improve their chemical and thermal stability. Polymer-coated [59–68], horizontally polymerized [69,70], bidentate [71,72], hybrid organic–inorganic [73] and also polar embedded [74] stationary phases are typical examples in the research for new generations of RP phases of improved selectivity and chemical and thermal stability. Recently, the preparation and properties of different types of RP stationary phases were extensively discussed and reviewed by Nawrocki et al. [57,58].

However, depending on the physico-chemical properties of the stationary phase, the eluent composition and other experimental parameters silica and silica-based RP stationary phases are vulnerable to deterioration effects. This in turn may result in early column failure. Deterioration of chemically modified silica-based RP phases can be distinguished in two major different processes. Silica already dissolves slightly in the pH range 2–7. The saturation concentration is about 100 ppm, which value is somewhat dependent on the pH [75]. Above approximately pH 7, however, silica dissolution is substantially accelerated. This in turn causes the impairment of the silica backbone of an RP stationary phase. This process generally results in reduced plate numbers and

finally in column clogging [76]. At acidic pH values of the eluent, another process is mainly responsible for the deterioration of RPLC stationary phases. The organic ligands of most of the presently manufactured RP phases are covalently bonded to the silica surface by mono- or polyfunctional Si−O−Si siloxane bond(s) [1,2,4,56,77]. Kirkland et al. provided evidence that in acidic eluents hydrolysis of the covalently bonded Si−O−Si organic ligands is the main responsible mechanism of stationary phase degradation [76].

During the last 10–15 years, a number of reports with focus on the chemical stability of conventional as well as new types of silica-based RP phases have appeared. The conclusions of these studies, however, are not always in agreement with each other. Partly, this can be ascribed to the different conditions like, for example, the nature of the organic modifier, the actual eluent pH and the temperature, under which several groups investigated their RP phases under study. In addition, a major difference between these investigations is also related to how RP columns were subjected to aggressive alkaline or acidic eluents. Generally, workers investigate the chemical stability of RP phases by putting them into contact with aggressive eluents. During that process changes in chromatographic parameters like, for example, plate number, retention factor, and peak asymmetry of specific test components are monitored [73,76]. In addition to that in some studies on purging columns under alkaline eluent conditions, the amount of SiO2 in a column effluent was continuously measured. In these studies, columns were purged with freshly prepared eluents, and were not recycled [72,76]. The SiO2 amount in the column effluents could simply be determined by a silica molybdate complex colorimetric method. The amount of silica in the eluent was then taken as a measure of column deterioration [76].

In summary, several studies have discussed static emerging and also dynamic purging of RP columns under recycling as well as non-recycling conditions [72,73,76,78–81]. Due to saturation effects of stationary phase deterioration products in the eluent, especially chemical stability results from static or recycling eluent experiments must be compared with caution.

Finally, the question of the actual pH in eluents for column testing purposes is another complicating factor. Upon adding an organic modifier to an aqueous buffer, the resulting pH of the mixture may drastically change. As an example of that the addition of 50% MeOH to an aqueous buffer of pH 6.0 causes an increase of the actual pH of about 1 unit. These effects also depend on the nature of the buffering salts and on the nature of the organic modifier [82–84]. Recently, Tindall and Perry [85] pointed out again the substantial influence of the nature of buffering salts on the actual pH in water–organic eluents. For example, upon dilution of an aqueous glycine buffer of pH ≈10.0 with 50% methanol, the pH of that eluent was decreased by only 0.11 pH unit. In contrast, under further similar conditions, using carbonate instead of glycine as the buffer salt, the eluent pH increased by 1.4 pH unit. The results of this study can now also better explain the results of silica

dissolution studies of Claessens et al. at high pH eluent conditions using different buffering salts [86]. In these studies, several buffer salts were used to prepare four different aqueous buffer solutions of pH 10.0. After adding 50% methanol to each of these buffers, RP columns were purged by these eluents and tested. The authors found that the carbonate and phosphate buffers much more deteriorated the RP columns compared to the glycine and borate buffers. Therefore, they concluded that in laboratory practice these latter two buffer salts were to be preferred over carbonate and phosphate. Now at least partly, the observed effects must be ascribed to the different pH shifts caused by the nature of the investigated buffering salts. These latter effects further complicate a fair comparison of the results of chemical stability studies of different workers.

The substantial amount of research effort of universities, but especially of manufacturers, has resulted in a large variety of RP columns with different chromatographic and chemical and thermal stability properties. Unfortunately, however, until now little consensus exists between the different approaches to determine the chemical and thermal stability of RP phases. In turn, this hampers a correct and objective interpretation and comparison of results between columns subjected to different chemical stability studies. It must also be emphasized that chemical stability studies are time consuming. Interestingly, however, Fonseca et al. [67] recently suggested a method for the rapid determination of the chemical stability of RP phases. The authors proposed a specific set of conditions to test the chemical stability study of RP columns. They compared these stability test results with stabilities earlier reported in literature. It was claimed that purging RP columns with 250 ml of an eluent consisting of an aqueous–methanol (50:50, v/v) 0.1 mol bicarbonate buffer, pH 10.0 at 60 °C is equivalent to the consumption of an eluent of pH ≤9 of a salt concentration of 0.01 mol at ambient temperature. The limits of eluent pH and temperature, in between which a specific column can be used, are greatly determined by its substrate properties and the applied bonding chemistry. At present, leading manufacturers prescribe the customers in clear guidelines, which pH, temperature, and other experimental limitations must be taken into account for the use of a particular column. In turn, chromatographers should respect these guidelines in order to prevent early column failure.

2.1.1.1. Intermediate and high pH eluents. Since silica dissolution is substantially accelerated in alkaline environments, this in principle is a serious limitation for the widespread use of silica-based RP phases under high pH eluent conditions. The reported pH limits in neutral and alkaline eluents at which silica and its chemically bonded derivatives can be used safely vary in between pH 7 and 10 ([87–91] and references therein). If one considers that the rate of silica dissolution strongly depends on a large number of different parameters, these large differences in pH recommendations are not surprising.

First of all, silica dissolution is greatly influenced by the nature of the starting substrate material. In principle, silica

can be manufactured by a so-called Silgel or a Solgel process [76,92]. Apart from other differences Solgel silicas have significantly thicker walls in their morphological structures compared to Silgel-based silica types. Kirkland et al. [76] showed that bonded phases prepared from Solgel silicas are significantly more stable towards aggressive alkaline eluents compared to Silgel-based RP phases.

Another approach to improve the chemical stability of silica substrates and thus also of the resulting RP stationary phases is described by Neue and coworkers [77,78] and Wyndham et al. [73]. In this so-called hybrid organic–inorganic technology process, which was first described by Unger et al. [93], a starting silica substrate is obtained, containing inorganic SiO_2 as well as organic $R(SiO)1.5$ groups. R, for example, can be methyl or ethyl. As an example in Fig. 1, the resulting C-18 modified trimethylsilane endcapped stationary phase is schematically illustrated for $R = CH_3$. The authors claim that this approach improves the entire chemical stability of the silica substrate due to decreased hydrolysis.

Furthermore, as shown by Collins and coworkers, titanium grafting or zirconization of silica substrates may also substantially improve the chemical stability under neutral and high pH conditions [63,64]; see also Section 2.1.2.1.

Secondly, also the purity from metal contaminations of silica substrates may strongly influence the resulting stationary phase chemical stability in alkaline eluents. Kirkland et al. [76] showed that metal contaminated type A silica-based RP phases were more stable in alkaline eluents compared to ultra pure type B silica-based counterparts. This is somewhat conflicting with the present strive to manufacture ultra pure silicas in order to obtain a homogenous population of silanols
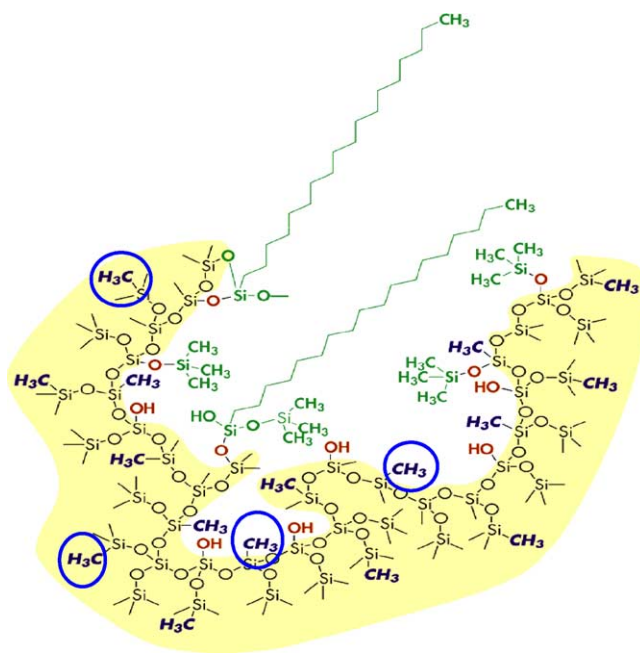

Fig. 1. Schematic picture of a C-18 modified endcapped silica-based stationary phase synthesized by organic–inorganic hybrid technology. (Courtesy of Dr. U.D. Neue, Waters Corp., MA, USA.)
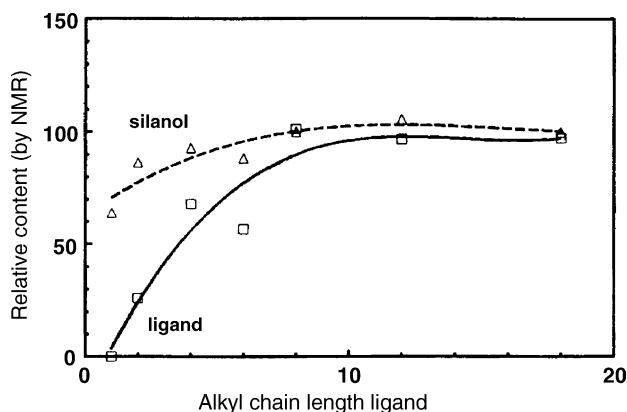
Fig. 2. Percentage ligand m1 and total silanol Q2 and Q3 as a function of the attached alkyl chain length present after a recycling eluent experiment at pH 8.4; measured by ^{29}Si CP-MAS NMR. Columns: methyl, ethyl, *n*-butyl, *n*-hexyl, *n*-octyl and *n*-dodecyldimethylmonochlorosilane modified silica of Merck. Eluent: 0.05 M aqueous phosphate buffer, pH 8.4, and methanol (50:50, v/v); temperature: ambient. (Reprinted with permission of the publisher from [80].)

Fig. 3. General structure of bidentate stationary phases. R = methyl, *n*-butyl, *n*-octyl or *n*-octadecyl; Q = $-CH_2-CH_2-$ or $-CH_2-CH_2-CH_2-$. (Reprinted with permission of the publisher from [72].)

at a silica surface. This in order to improve peak symmetry, efficiency and resolution [77,94,95].

Thirdly, also the chemical modification of silica substrates substantially influences its solubility and thus the resulting chemical stability in neutral and alkaline eluents. More particularly, the length of the alkyl chains, e.g. C-4 or C-18, the way these ligands are attached to the surface, mono- versus polyfunctional, the surface coverage, nature of side groups and endcapping as well significantly determine the longevity of RP columns.

An example is the following: in a number of eluent recycling experiments at pH 8.4, Hetem and coworkers [80,96,97] investigated the influence of the length of alkyl chains on the chemical stability of monofunctionally modified RP phases. Amongst other findings, the authors concluded that the chemical stability of these phases improved at increasing alkyl chain lengths at the silica surface. This is further illustrated in Fig. 2, where it can be seen that at increasing alkyl chain lengths the loss of ligand, m1, substantially decreases. In another study, Hetem et al. also concluded that the chemical stability of RP phases is substantially influenced by the way of attachment, either mono- or polyfunctional, of C-18 alkyl ligands to silica substrates [98]. These authors concluded, that the best chemical stability was obtained for bi-linked modified silicas. This bi-linked bonding refers to a dual bond from a single silica atom in the ligand to a silica surface. Also in this latter study, it was concluded that the nature of the investigated underlying silicas did not substantially influence the resulting chemical stability of the RP phases.

In a later study, Kirkland et al. investigated a number of RP C-18 phases under non-recycling conditions using freshly prepared eluents at pH 10 [76]. Opposite to the findings of Hetem et al., these authors concluded that the nature of the bonded C-18 phase, viz. mono- or polyfunctional, did not significantly influence the chemical stability of the investi-
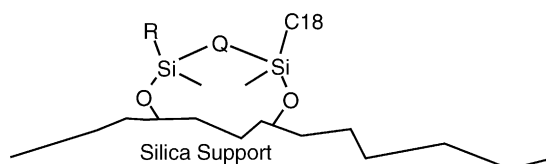
gated RP phases. Also in contrast with the study of Hetem et al. [98], Kirkland et al. found also that under these high pH conditions the nature of the underlying silica substrate strongly influenced the chemical stability of the RP phases [76]. It is emphasized that these before mentioned studies were performed under recycling (Hetem) and non-recycling (Kirkland) conditions. Therefore, comparison of the results of these studies must be performed with caution. Interestingly, following the earlier suggestion from the results of chemical stability studies obtained on bi-linked modified silicas of Hetem and coworkers [80,98], in later studies, Kirkland coworkers developed the concept of so-called bidentate RP phases [72,99,100]. This bonding refers to a dual bond to the surface from two different silicon atoms in the ligand reagent. In Fig. 3, the basic concept of this approach is illustrated. Adjusting the spacing group Q, e.g. ethyl or propyl in the bidentate reagents results in an optimal and stable attachment of the alkyl ligands, e.g. C-18, to the silica substrate. This bidentate type of stationary phases proved to be very stable in the low, intermediate as well as high pH range of eluents. As an example in Fig. 4, the stability of bidentate-based modified silicas for the separation of β-blockers under pH 11 conditions is shown. In this figure, it can be seen that after about 10,000 column volumes, which is equivalent to about 1 month use during 8 h a day, the separation under these aggressive alkaline conditions is still satisfactory [100].

As mentioned earlier, Neue [77] and Wyndham et al. [73] have discussed another interesting concept for the preparation of alkyl-modified silicas of substantially improved chemical stability. Monitoring the changes in USP-tailing factors and plate numbers under aggressive eluent conditions, the authors studied the chemical stability of organic–inorganic hybrid-based RP stationary phases [73,77]. In Figs. 5 and 6, the chemical stability of this type of RP stationary phases under pH 11.5 eluent conditions is demonstrated. In both figures, it can be seen that these RP phases can be used under pH 11.5 conditions for a considerable period of time, viz. minimally 50 days.

Several manufacturers have also attempted to improve the chemical stability and hydrophobicity of their RP phases by endcapping. Therefore, after the first major modification of the silica often a second synthesis step, for example, using monochlorotrimethylsilane, is performed. In contrast to the early days of RPLC, at present more advanced endcapping procedures are also applied now like, for example, double endcapping with dimethylsilyl in some cases together with
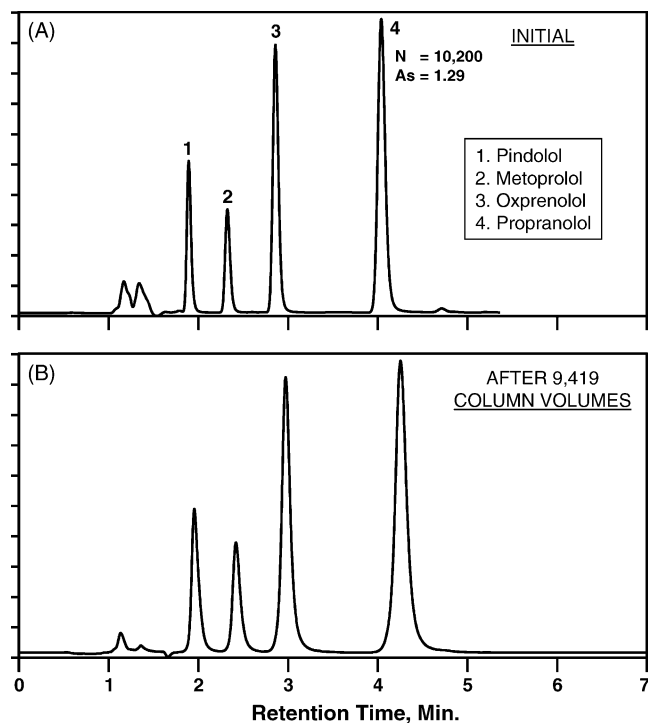
Fig. 4. Stability of bidentate C-18 column at pH 11. Column: Extend® C-18 column 15 cm × 0.46 cm (Agilent Technologies, Newport, DE, USA). Sample: β-blockers; eluent: acetonitrile–0.017 M aqueous potassium phosphate buffer, pH 11.0 (50:50, v/v); flow rate: 1.5 ml/min; temperature: 23 °C. (Reprinted with permission of the publisher from [100].)

Fig. 6. Change in column plate count for nortriptyline at pH 11.5. The period of 50 days corresponds to 3200 sample injections. Column: XTerra® RP18, 4.6 mm × 150 mm, $d_p = 5$ μm, protected by a guard column. Eluent: acetonitrile–50 mM pyrolidine HCl aqueous buffer, pH 11.5 (50:50, v/v); flow rate: 1 ml/min; temperature: 30 °C; test component: nortriptyline ($pK_a = 9.7$). (Reprinted with permission of the publisher from [78].)

trimethylsilyl groups. For obvious reasons, manufacturers have difficulties to disclose firm information about their endcapping procedures. Using non-recycling eluent conditions, Kirkland et al. investigated the effect of endcapping of several nominally identical RP columns under pH 7 conditions at 60 °C [101]. Fig. 7 illustrates the differences in chemi-

cal stabilities for a number of commercially available endcapped C-8 modified silicas. The authors concluded that in general endcapping definitely improves the longevity of RP columns. However, large differences were found between the investigated nominally identical, silica octadecyl-modified RP columns [101]. For example, under these conditions, the Hypersil BDS-C8 column starts to deteriorate after about 7 l of eluent purging. In contrast, on the left-hand side of this figure, it can be seen that the first four C-8 columns show already column failure around or before 1 l of eluent purging. These findings clearly indicate that endcapping alone does not improve chemical stability, but that also other factors like, e.g. the nature of the starting silica substrate, ligand
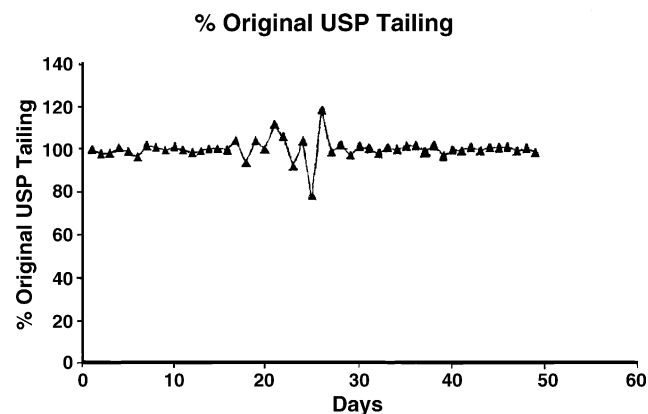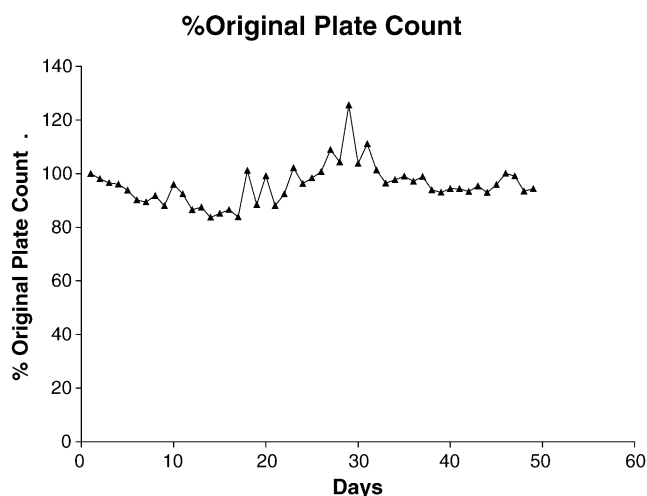

Fig. 5. Change in the USP-tailing factor for nortriptyline at pH 11.5. The column was used continuously for the entire time period of 50 days. Column: XTerra® RP18, 4.6 mm × 150 mm, $d_p = 5$ μm, protected by a guard column. Eluent: acetonitrile–50 mM pyrolidine HCl aqueous buffer, pH 11.5 (50:50, v/v); flow rate: 1 ml/min; temperature: 30 °C; test component: nortriptyline ($pK_a = 9.7$). (Reprinted with permission of the publisher from [78].)
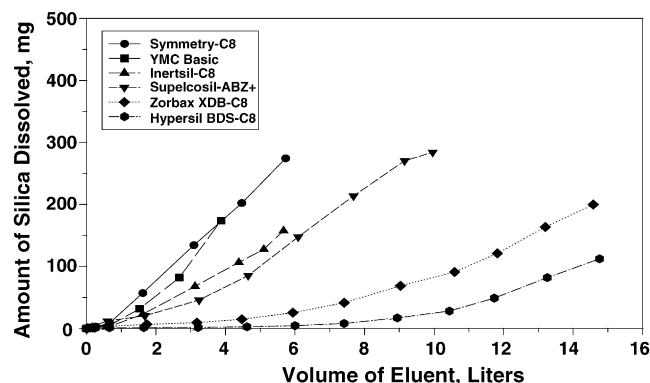

Fig. 7. Silica support dissolution tests. Column: 15 cm × 0.46 cm i.d. Purge eluent: acetonitrile–0.25 M sodium phosphate buffer, pH 7.0 (20:80, v/v); flow rate: 1.0 ml/min; temperature: 60 °C. Silica dissolution measured by molybdate–silicate color reaction. (Reprinted with permission of the publisher from [101].)

attachment and surface coverage also substantially influence the chemical stability of RP stationary phases.

Summarizing, in Fig. 8, the recent progress obtained in the manufacturing of silica-based RP phases of improved chemical stability is further illustrated. The first six RP columns at the left-hand side of this figure show a rather early column failure under these aggressive alkaline eluent conditions. Note, however, that between these six columns larger differences in individual chemical stability occur [76].

In large contrast to that both the Extend® C-18 (Agilent Technologies, Newport, DE, USA) and XTerra® MS C-18 (Waters Corp., Milford, MA, USA) columns based on the bidentate and hybrid organic–inorganic technology, respectively, show a significantly better chemical stability. During the first 15 l of eluent under these conditions, the Extend® C-18 column shows hardly any deterioration. In comparison to that also the XTerra® MS C-18 is only slightly worse with respect to chemical stability under these conditions. Obviously, both these latter columns of the "2000" generation show much better chemical stability compared to the other investigated RP columns. Fig. 8 also shows that substantial progress has been made in the manufacturing of silica-based RP phases of improved chemical stability. It can be seen that the combination of new and improved silica substrates combined with advanced chemical modification methods results in RP phases of high chemical stability in intermediate and alkaline eluents. Examples of that are bidentate and organic–inorganic hybrid silica-based C-18 RP phases. These and comparable RP phases can safely be used for 10–15 l of eluent under strong alkaline pH conditions.
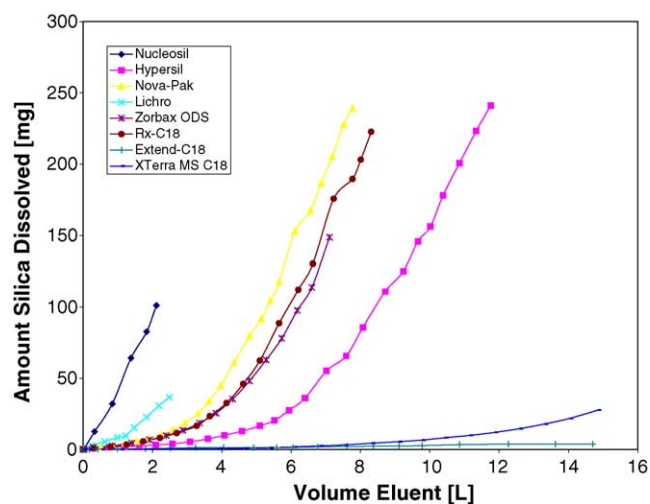

Fig. 8. Comparison of silica support dissolution for some conventional C-18 columns, including two commercial C-18 columns (viz. Extend® of Agilent Technologies, XTerra® MS of Waters) of the "2000" column generation. Columns: 15 cm × 0.46 cm i.d., except 15 cm × 0.39 cm i.d. for Nova-Pak C18; conditions: methanol–sodium carbonate/bicarbonate (0.1 M) buffer, pH 10.0 (50:50, v/v); flow rate: 1.0 ml/min, except 0.72 ml/min for the Nova-Pak column; ambient temperature; molybdate colorimetric analysis for monitoring dissolved silica. (Data reprocessed and reprinted with permission of the publisher from [2].)

As a result of these achievements, chromatographers can now develop rugged HPLC separation methods on specific silica-based columns under intermediate and high pH conditions. For example, Neue et al. described the preparative separation of diphenhydramine, oxybutynin and terfenadine on an XTerra® MS C-18 column under pH 10 eluent conditions [55]. The development of the separation protocol for Vitamin B-1 and its derivatives in whole blood on an Extend® C-18 column under pH 10 conditions by van Landeghem et al. [102] is another example of the benefits of working under high pH eluent conditions. In this latter study, it was shown that within one column life cycle at least 1500 sample injections could be performed without noticeable decrease in column performance. In addition, this latter study also showed that under the applied pH 10 conditions, the fluorescence detectability of the target components and also the ruggedness of the whole method was improved substantially [102].

2.1.1.2. Acidic eluent conditions. In a number of application areas chromatographers may also want to work at low pH conditions. As an example of that many peptide and protein samples are separated in eluents containing a small amount of trifluoroacetic acid (TFA) or another organic acid resulting in a low pH of the eluent [73,103]. Furthermore, also the separation of acidic analytes, for example, organic acids, can be performed efficiently under low pH ion suppression conditions [1,3,4]. Also Neue et al. pointed out that for preparative HPLC the sample loadability of ionic, viz. acidic compounds on RP columns can be substantially improved separating such compounds in their neutral non-ionized form [55]. Finally, for certain applications column cleaning after a separation by strong acidic eluents can also be of great interest.

As mentioned earlier in Section 2.1.1, in the low pH regime the loss of organic ligands from a silica surface by solvolysis and subsequent breaking the Si−O−Si bond is the main cause of column deterioration. In a number of studies, Kirkland and coworkers developed several concepts for the synthesis of RP stationary phases particularly useful and stable in acidic eluents [71,99]. In these two studies, the concept of sterically protected phases, using bulky side groups instead of conventional methyl side groups and their resulting specific stability in acidic eluents, was discussed. Fig. 9 illustrates the principle of sterically protected RP stationary phases.

In Fig. 10, the resulting significantly improved stability of these sterically protected phases is further illustrated. In this figure, these sterically protected bulky phases show a substantially better chemical stability towards acidic eluents compared to their conventional counterparts [71]. In this figure, it can be seen that both conventional RP phases containing methyl side groups start to deteriorate very quickly under these acidic eluent conditions. In contrast to that, after 5000 column volumes both the sterically protected phases containing bulky side groups are still unimpaired.

Applying NMR techniques, Scholten et al. [104,105] could explain and provide evidence for the high chemical stability of these bulky phases in acidic eluents. Fig. 11
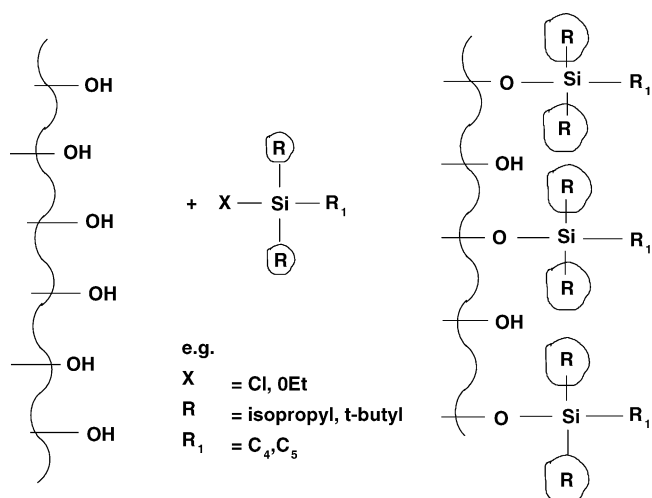
Fig. 9. Schematic representation of the bonding of sterically protecting monofunctional silanes to a hydroxylated surface. (Reprinted with permission of the publisher from [71].)

schematically illustrates the orientation of residual silanols at the surfaces of conventionally and sterically protected modified silicas. The authors explained the improved chemical stability of bulky phases by the decreased interaction be-
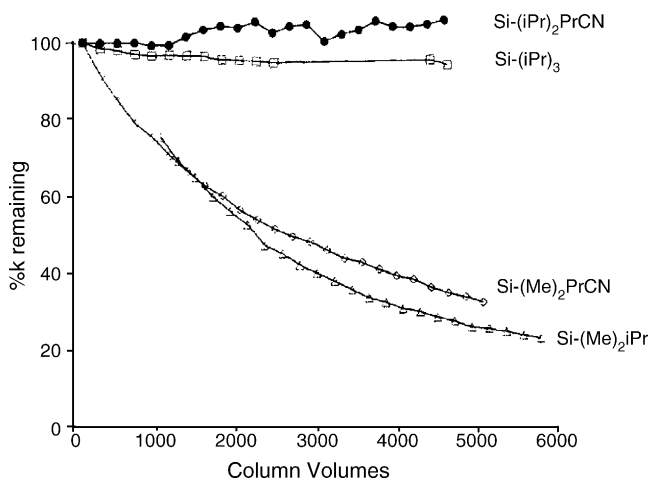

Fig. 10. Loss of retention for two dimethylalkyl bonded phases and two diisopropylalkyl sterically protected bonded phases as a function of column volume of mobile phase: k' measured for 1-phenylheptane. Eluent: acetonitrile–water (both with 0.1% TFA) (50:50, v/v); flow rate: 1.0 ml/min; temperature: 50 °C. (Reprinted with permission of the publisher from [71].)
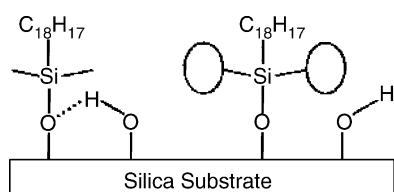

Fig. 11. Schematic drawing of the dimethyl-*n*-octadecylsiloxane (left) and the di-isobutyl-*n*-octadecylsiloxane (right) surface structures, emphasizing the increased steric protection of the ligand siloxane bond by the bulky side groups in the latter. (Reprinted with permission of the publisher from [105].)
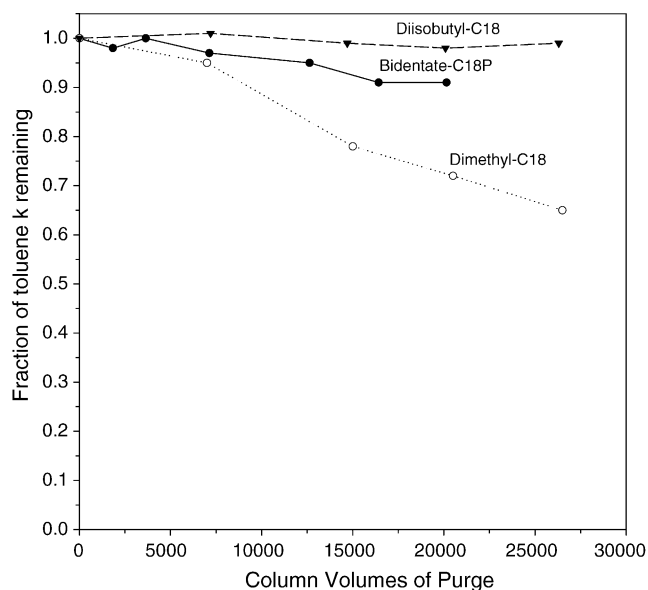

Fig. 12. Stability comparison for C-18 bonded phase columns at pH 0.9 and 90 °C. Column: 15 cm × 0.46 cm. Purge mobile phase (continuous at 1.5 ml/min): methanol–water with 1.0% trifluoroacetic acid (50:50, v/v); temperature: 90 °C. Test mobile phase: methanol–water (60:40, v/v); flow rate: 1.0 ml/min; temperature: ambient; solute: toluene. (Reprinted with permission of the publisher from [72].)

tween residual silanols and the ligand siloxane oxygen atom [104].

In other studies, Kirkland et al. also developed a group of RP phases based on the bidentate attachment concept [72,100]. In Section 2.1.1.1, this type of phases has already been discussed for their high stability in alkaline eluents. These same types of phases, however, also proved to be very stable in acidic eluents. This is illustrated in Fig. 12. In this figure, it can be seen that the bidentate C-18 phase has comparable stability properties compared to the bulky diisobutyl C-18 phase [72]. The superior stability of this latter stationary phase concept towards acidic eluents was already established in earlier studies [71,99]. Also in Fig. 12, opposite to the stability of both the bidentate and diisobutyl bulky phases, the chemical stability of the conventional dimethyl C-18 modified silica is significantly less.

Furthermore, in another study, Kirkland et al. also developed and investigated the chemical stability properties of polar embedded amide groups linking the sterically protecting diisopropyl groups with a C-14 alkyl functionality [106]. This specific type of RP stationary phase also proved to be very stable in acidic as well as in the intermediate pH eluent range.

Other concepts for the preparation of RP phases of improved chemical stability in acidic eluents have been reported as well. For example, the earlier discussed RP stationary phases based on the hybrid organic–inorganic technology have also been investigated for their stability in acidic eluents (see Sections 2.1.1 and 2.1.1.1). Neue et al. [78] and Wyndham et al. [73] have investigated the chemical stability of this type of phases in eluents containing 1% TFA at 50 and 80 °C.

Under these acidic aggressive conditions, the authors found a substantially extended longevity for the C-18 bonded hybrid phases compared to conventional silica-based counterparts. Furthermore, in another study, Ludwig et al. investigated the stability in acidic eluents of a number of commercially available columns of different alkyl functionality, e.g. C-1, C-4, C-6, C-8, C-18, CN and phenyl. These columns were subjected to gradient elution experiments using water and acetonitrile (each containing 1% TFA) at 50 °C. Even for short ligand chain lengths, the authors found an improved chemical stability for these specific RP phases [107].

Summarizing, the development of new and better silica substrates combined with advanced chemical modification methods has provided RP phases that are highly stable in acidic eluents. Examples are bulky, bidentate and organic–inorganic hybrid C-18 modified silica RP phases. These and comparable RP phases can safely be used for 25,000 and more column volumes at temperatures up to 80 °C under highly acidic pH eluent conditions.

2.1.2. Polymer-coated inorganic substrates

The limited chemical stability of silica-based bonded RP phases has strongly stimulated the study for other alternative supports and substrate modifications. Polymer-coated phases usually consist of an inorganic substrate on which a polymer layer is deposited. Catalyzed by γ-radiation or thermal treatment these layers can be physically adsorbed or chemically bonded to the substrate surface. In principle polymer-coated substrates incorporate the mechanical strength of an inorganic support material like silica, alumina and zirconia, and the chromatographic properties, e.g. selectivity and chemical stability of polymers [4,77]. As discussed by Petro and Berek, many different types of polymers like, for example, polysiloxanes and polyethers can be deposited on these substrates [59]. Therefore, polymer-coated stationary phases potentially offer a large variety in chromatographic properties combined with a high degree of chemical stability. Another potential advantage of these phases is the reduction of unwanted residual substrate activity, for example, the decrease of silanols and Lewis sites activity for silica and zirconia substrates, respectively. The deposition and coating of a polymer on a substrate is a complex process and must be carefully controlled to obtain reliable and reproducible phases. On one hand, a sufficient rate of polymer deposition will provide satisfactory retention and selectivity and also shielding of unwanted substrate activity. On the other hand, too high polymer deposition may result in blocking of the pores of the substrate and decreased efficiencies. Recently, Nawrocki et al. have reviewed the various aspects of polymer-coated stationary phases [58].

2.1.2.1. Silica-based polymer-coated phases.
In a number of studies, Hetem et al. [96,108,109] and Schomburg and coworkers [60] investigated the chemical stability and selectivity properties of polymer-coated silicas. More recently, also Collins and coworkers investigated the process of the deposition and polymer coating by polymethyloctylsiloxane (PMOS) and polymethyloctadecylsiloxane on silica [61,62,66,81]. The experiments of Hetem et al. under alkaline pH 8.4 conditions did not provide straightforward conclusions for the chemical stability of the investigated polymer silica coated phases. For example, they concluded that precapping of the silica substrate prior to the final polymer coating process strongly improved the chemical stability of their phases. Furthermore, they also found that for eluents containing high percentages of organic modifier the stability of polymer-coated phases was better compared to conventionally covalently modified counterparts. They also concluded, that the reverse was true for alkaline eluents containing less content of organic modifier. Note that Hetem et al. performed their experiments under recycling eluent conditions. Collins and coworkers [61,81] found that their polymethyl octylsiloxane phases immobilized on silica already started to deteriorate after approximately 300 ml of eluent purging. Opposite to the studies of Hetem et al. [80], Collins and coworkers used a substantially more aggressive aqueous–methanol bicarbonate eluent buffered at pH 8.5 at an elevated temperature of 60 °C under non-recycling conditions. In a later study, however, Collins and coworkers claimed that these latter purging and testing conditions are equivalent to a 6-month routine use of RP columns [67]; see also Section 2.1.1. Also interesting is that Collins and coworkers recently showed that polymethyloctylsiloxane polymer coatings on zirconized silica [63] and titanium-grafted silica [64] could substantially increase the chemical stability of these phases. For example, at pH values of the eluent of 7, 9 and 12, the titanium-grafted silica-based RP phase was stable up to about 10,000 column volumes. This is a considerable increase in chemical stability compared to the findings in an earlier study of the same group for PMOS polymer-coated silicas [81]. These studies suggest that polymer-coated RP phases on zirconium and titanium modified silica substrates offer much better chemical stability compared to phases prepared from unmodified silicas.

2.1.2.2. Polymer-coated phases on alternative substrates.
The application of other more stable inorganic oxides like, e.g. aluminum, zirconium and titanium as potential substrates for RP stationary phases has been investigated already for a long time [1,56,110–112]. All these substrates have in common their much higher pH stability compared to silica making these substrates potential and attractive alternatives. For example, alumina is more stable in aggressive eluents compared to silica. Detectable amounts of alumina were found, however, in low as well as high pH eluents [113,114]. In addition to that, zirconia proved to be very stable over the entire pH range of 0–14 [57,113–115]. From the potentially attractive alternatives for silica until now, alumina and zirconia have been investigated most extensively. Both these inorganic substrates, however, show Lewis basic and acidic activity. Therefore, compared to their silica RP counterparts significantly different retention and selectivity behavior of alumina- or zirconia-based RP phases can be expected. Apart from the stationary phase, this of course strongly depends on the na-

ture of the analytes and also on the composition of the eluent. In addition, due to the ion-exchange properties of (modified) alumina and zirconia substrates this may further complicate the surface interactions and thus retention and selectivity behavior of these phases [1,115–117]. For example, π-electron containing or basic analytes show substantially different retention properties on alumina- or zirconia-based RP phases compared to silica-based counterparts [31,116,118].

A number of attempts were made to modify aluminum and zirconium substrates by covalently bonding of organic ligands similarly to silica [110,117]. For example, Wieserman and coworkers investigated the chromatographic properties of octadecyl ligands covalently bonded aluminum stationary phases for RPLC [110]. In addition, Carr and coworkers [114,115] and Yu and El Rassi [119] investigated the properties of covalently bonded organic ligands on zirconia substrates. These and also other authors concluded that covalently modified zirconia- and alumina-based RP phases did not provide a chemical stability comparable to silica-based RP stationary phases [113–115]. In order to circumvent this problem and attempting to use the improved hydrolytic stability of alumina and zirconia, several workers have investigated the modification of these materials by polymer coating and cladding techniques. Starting from spherical aluminum oxide and applying a coating procedure described by Schomburg et al. [120], Merck GmbH (Darmstadt, Germany) manufactures a RP stationary phase called Aluspher RP-Select B. According to the manufacturer, this polybutadiene-coated aluminum oxide RP stationary phase is very stable over a wide acidic, neutral and high pH range. In addition, this RP phase is also particularly recommended for the separation of basic substances [120–122]. To the authors' best knowledge, however, no sound data on the long-term chemical and thermal stability properties of this RP phase are available.

Carr and other workers have applied zirconia as the starting material for a number of different polymer-coated and also cladded RP phases. In a substantial number of reports, McNeff, Carr and others have described the preparation and also the properties of polybutadiene (PBD), polyethyleneimine (PEI), aromatic polymer-coated and also of carbon-clad zirconia-based RP phases. The preparation of PBD coated zirconia and the chromatographic evaluation of these phases have been described extensively by Carr, McNeff and others [113,119,123,124]. From these studies, the authors conclude that at least for neutral analytes PBD zirconia coated phases behave quite similar with respect to retention and efficiency compared to silica-based RP phases [116]. For polar and ionic analytes, however, substantial differences with respect to retention, selectivity and efficiency have been reported [114,116]. In addition, Weber et al. [125] and Jackson and Carr [126] have described the synthesis and chromatographic evaluation of carbon-clad and carbon-coated zirconia-based RP phases. Furthermore, Zhao and Carr have described the properties of an aromatic polymer-coated zirconia substrate [127]. These latter types of carbon-clad and coated, and also of aromatic polymer-coated phases show
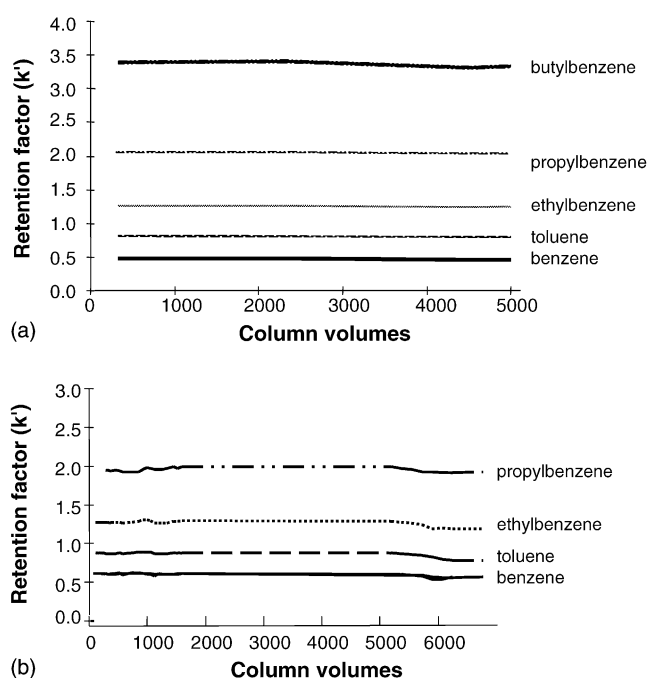

Fig. 13. Analysis of the chemical (a) and thermal (b) stability of a polybutadiene-coated zirconia column. Conditions (a)—wash fluid: 90:10 (v/v) 1 M sodium hydroxide in water–methanol (pH 14); mobile phase: 50:50 (v/v) acetonitrile–water; temperature: 35 °C. Conditions (b)—mobile phase: 15:85 (v/v) acetonitrile–water; temperature: 195 °C. Column (a and b): 150 mm × 4.6 mm ZirChrom-PDB; flow rate (a and b) 1.0 ml/min. (Reprinted with permission of the publisher from [130].)

similarities but also strongly different chromatographic behavior compared to silica-based RP phases [113,125–127]. McNeff and coworkers have investigated the preparation and chromatographic performance of polyethyleneimine porous zirconia as an ion-exchange stationary phase [128,129]. In many of the studies referred to here above, the authors found a very high chemical as well as thermal stability for zirconia-based RP phases (see, e.g. [113,127,130]). For example, the aromatic polymer-coated zirconia was found to be stable under acidic as well as alkaline eluent conditions at 160 °C for a substantial number of column volumes [127]. As another example in Fig. 13, the chemical and thermal stability of PBD-coated zirconia phases is illustrated. In this figure, the ZirChrom-PDB columns (ZirChrom Separations Inc., Anoka, MN, USA) can be used under pH 14 eluent conditions and also up to 195 °C for at least 5000–6000 column volumes without any column deterioration. Chromatographers who want to develop analyses under very aggressive eluent conditions, for example, pH >12 and/or also may want to apply elevated temperatures must seriously consider these alternatives for silica-based RP stationary phases. Furthermore, zirconia-based RP phases offer also attractive additional selectivity properties to solve specific separation problems [125–130]. In turn, however, for these stationary phases depending on the nature of the analytes and also of the eluent usually more effort on method development must be spent [113–116].

2.1.2.3. Polymer RP stationary phases. In view of the earlier discussions on the advantages and drawbacks of the use of silica-based RP columns in acidic and basic eluents substantial effort has also been focused on the replacement of silica-based RP packings by polymer phases. Modern polymer stationary phases for HPLC consist of highly cross-linked copolymers. The particles of these phases are composed of a network of much smaller micro spheres, providing a polymer phase consisting of a continuous pore structure network. In general, non-ionic copolymer, macroporous absorbents for RPLC are obtained by the selection of specific monomers and the application of proper polymerization synthesis conditions. Additional modification can be applied for further improvement of the hydrophobic properties or, for example, to prepare ion-exchangers. Several review articles have discussed the synthesis and properties of polymeric, macroporous stationary phases for HPLC [1,4,131–133].

Copolymers from styrene and divinylbenzene (PSDVB) are probably the most investigated and applied polymer phases for RPLC. But also other underivatized copolymers, for example, of methyl methacrylate and divinyl have been used for the preparation of RPLC phases [4]. A number of polymeric phases have been used for further, for example, alkyl, modification in order to improve the hydrophobicity and selectivity of these packings. Representative examples of this group of RPLC phases are C-18 and also other alkyl modifications of PSDVB, polyvinyl alcohol (PVA), polyvinyl esters (PVE) and alkyl methacrylate–glycerol dimethacrylate [4,131,132]. The present generation of polymer RPLC stationary phases meets the requirements put on high quality HPLC packing materials. Modern polymer phases have sufficient mechanical stability and they are compatible to the organic solvents usually applied in RPLC. Furthermore, these phases show satisfactory selectivity for many solutes, are chemically stable over the entire pH range and can be used at temperatures up to 80 °C and higher. Furthermore, the other chromatographic properties of polymer phases, for example, a large and homogeneous surface area and sample loading capacity are comparable to other more commonly applied RPLC phases [131]. In several studies the chemical and temperature stability of polymer-based RPLC phases was studied. Uchida investigated the chemical stability of a C-18 bonded vinyl alcohol polymer [79]. Based on static immersion experiments, the authors found an excellent chemical stability of that specific polymer phase in the pH range 2–13 during one-half to 2 days at a temperature of 50 °C. However, the long-term chemical stability for this particular phase is difficult to judge because, as discussed earlier in Section 2.1.1, several different approaches to determine the chemical stability of RP phases hamper a fair comparison of the data from different studies. In another study, Tanaka et al. [134] investigated and compared alkyl-modified silica and polymer-based RPLC phases for the separation of polypeptides. Amongst other conclusions the authors found that in an eluent containing 0.1% trifluoro acetic acid (TFA) at 60 °C, the chemical stability of the Asahipak C-4P (esteri-

fied polyvinyl alcohol) and the Shodex D4-613 (alkyl ethers of polyhydroxyalkyl methacrylate) stationary phases showed superior chemical stability over a period of 50 h compared to most of the investigated silica-based counterparts. An exception was the trifunctional modified LiChrospher 500-C18T phase, that showed comparable chemical stability in comparison to the polymer phases [134].

Amongst other issues, Mohammad et al. [135] investigated the chemical stability of a PSDVB-based RPLC phase called Source 5 RPC for the separation of peptides. The authors concluded that this RPLC packing material could be routinely used in between eluent pH values of 2 and 12. Furthermore, the manufacturer of this phase Amersham Pharmacie Biotech, Sweden, claims that this phase can be safely used up to 60 °C. In another study, Ng and Froom discussed the synthesis and application of non-porous and porous poly(divinylbenzene) (DVB)-based RPLC phases [136]. In this study, the authors report a very high chemical stability over the entire pH range for these DVB phases. Furthermore, in the same study, a temperature stability up to 150 °C for the non-porous DVB-stationary phase was claimed [136].

Summarizing, in spite of the somewhat lesser efficiency compared to inorganic-based counterparts and the swelling and shrinking problems with certain organic solvents from a point of view of chemical stability, polymer-based RPLC phases have become an attractive alternative for silica-based counterparts. This is particularly true for the separation of biological macromolecules.

In Table 1, the chemical and thermal stability of several RP stationary phases are summarized. Bare silica is already slightly soluble in acidic solutions. Above pH ≈7, silica starts to dissolve rapidly [56,93]. Silica substrates are thermally stable up to 200 °C [57]. Chemically covalently modified silica-based RP phases may have different chemical stabilities. Specific types of RP phases, for example, C-18 bidentate and C-18 modified organic–inorganic hybrid stationary phases

Table 1

Comparison of chemical and thermal stability of substrates and reversed-phase stationary phases

Phase	Chemical stability		Thermal stability
	Low pH	Intermediate and high pH	
Silica substrate	+	−	++
RP covalently bonded phases	+++	+++	++
Polymer-coated	+	+	+
Zirconized or titanium-grafted and polymer-coated	++	++	++
Polymer	++++	++++	+++
RP modified phases	++++	++++	+++
Alumina substrate	+++	+++	?
RP covalently bonded phases	+	+	?
Polymer-coated	?	?	?
Zirconia substrate	++++	++++	++++
RP covalently bonded phases	+	+	+
Polymer-coated	++++	++++	++++

are stable in the pH range 2–11.5. The thermal stability of these and similar types of phases is limited to about 70 °C. However, the longstanding use of these stationary phases at temperatures of 80–90 °C has also been reported [72,73]. It appears that these and other similar special types of silica-based RP phases can be safely used for a considerable period of time under acidic, neutral and high pH eluent conditions at elevated temperatures. In general, however, for conventional types of covalently bonded silica RP phases more restrictions with respect to these pH and temperature limits must be accepted.

Until now, the chemical stability of RP phases prepared from polymer-coated silica substrates is still limited. This is particularly true at higher eluent pH values [61,81]. Zirconization or titanium grafting of the silica substrate prior to the polymer coating process considerably improves the chemical stability of these RP phases. These latter stationary phases can be safely used in the intermediate and high pH range (up to pH 13) for at least 10,000 column volumes [63,64]. The lack of sound data prevents to set a clear limitation of the temperature stability of these phases.

Polymer RP phases including additional chemically modified phases thereof are chemically stable over nearly the entire pH range 1–14 [131]. Reports have appeared confirming the temperature stability of polymer RP phases to be 80 up to 150 °C [131,135,136].

The chemical stability of alumina is much better compared to silica. Alumina substrates are stable in the range 2 < pH < 13 [4,57].

Many attempts have been made to prepare covalently modified alumina RP phases. These stationary phases, however, show a considerably lower chemical stability compared to their silica-based counterparts ([58] and references therein). Polymer-coated alumina RP phases show a high chemical stability in the acidic, neutral and high pH range. A number of reports have appeared on this issue ([58] and references therein). The scarce data on chemical stability hamper to set clear pH limits for the use of these RP phases. The same is true for the thermal stability of alumina-based stationary phases.

Zirconia substrates are chemically stable over nearly the entire 1–14 pH range and the thermal stability is very high. Similar to alumina covalently modified zirconia-based RP phases also appear to be less chemically stable compared to silica-based counterparts ([58] and references therein). In contrast, the chemical and thermal stability of polymer-coated zirconia-based RP phases is considerably larger compared to silica RP phases. Depending on the nature of the polymer coating zirconia-based RP phases are stable up to 150–200 °C. Furthermore, these stationary phases can be safely used over the 1–14 pH range. A detailed overview of the chemical and thermal stability of these and other zirconia-based RP phases was recently discussed in [57].

In summary, silica and metal oxide-based, and also polymer RP stationary phases offer a large diversity in chemical and thermal stability; each of these particular phases having

its own specific limitations with respect to pH and temperature.

2.2. The adjustment and optimization of the mobile phase composition and other experimental conditions

The former section was mainly focused on the influence of the pH of an eluent on the deterioration of RP columns. Apart from that also other eluent properties like, for example, the nature of the buffering salts and nature and concentration of the organic modifier(s), may substantially influence the longevity of RP columns. The same is also true for other experimental conditions especially the temperature of the column and eluent. For silica-based RP phases, Kirkland and also other workers have shown that the adjustment and optimization of the mobile phase composition and also of other experimental conditions, viz. the temperature, may extend the longevity of RP columns substantially.

In a number of papers, Kirkland and coworkers systematically investigated the influence of the nature of the organic modifier, the nature and concentration of buffering salts and the temperature on the deterioration of silica-based RP stationary phases under neutral and high pH conditions [72,76,86,100,101].

2.2.1. Nature of organic modifier(s)
In Fig. 14, the effect of the influence of the nature of the organic modifier on column deterioration is illustrated. In a series of duplicating tests, a number of conventional Zorbax RX-C18 columns were purged under pH 10 eluent conditions. Under such high pH eluent conditions, column deterioration is mainly caused by silica dissolution. Column deterioration was determined by the continuous monitoring of the amount of silica in the effluent and plotted on the Y-axis as a function
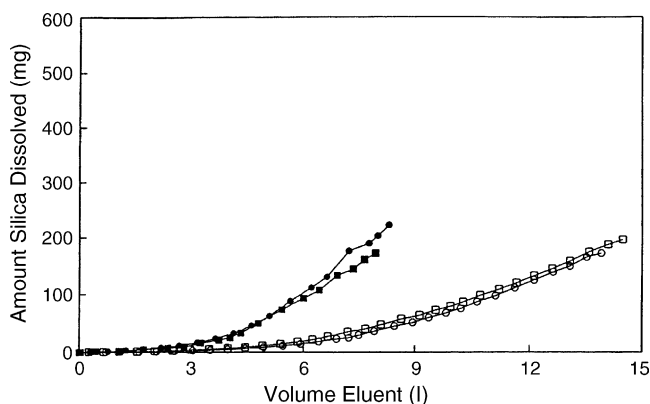

Fig. 14. Reproducibility of tests for silica support dissolution. Column (A = ○; B = □): 15 cm × 0.46 cm i.d. Zorbax RX-C18 (dimethyl-C18); duplicate tests with mobile phases: methanol–sodium carbonate/bicarbonate (0.1 M) buffer, pH 10.0 (50:50, v/v) (solid symbols) and acetonitrile–sodium carbonate/bicarbonate buffer (0.084 M) buffer, pH 10.0 (40.6:59.4, v/v) (open symbols); flow rate: 1.0 ml/min; ambient temperature; molybdate colorimetric analysis for dissolved silicate. (Reprinted with permission of the publisher from [76].)
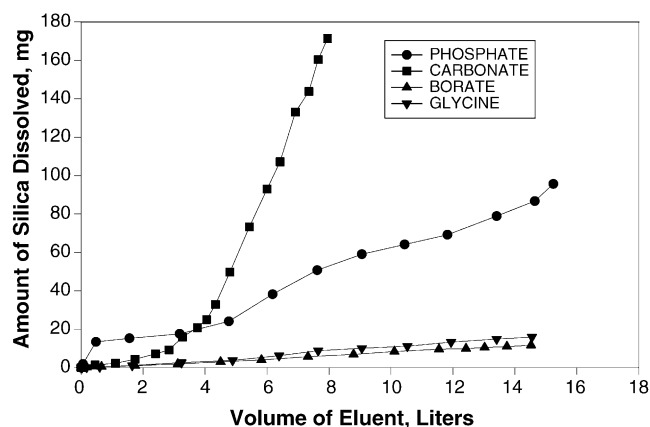
Fig. 15. Effect of pH 10 purge buffer anions on silica support dissolution. Column: Zorbax RX-C18, 15 cm × 0.46 cm i.d. Purge: methanol–0.1 M buffers, pH 10 (50:50, v/v); flow rate: 1.0 ml/min; temperature: 25 °C. (Reprinted with permission of the publisher from [86].)

Fig. 17. Effect of pH 7 phosphate buffer on silica support dissolution. Column: Zorbax RX-C18, 15 cm × 0.46 cm i.d. Purge: acetonitrile–buffer, pH 7.0 (20:80, v/v); flow rate: 1.0 ml/min; temperature: 60 °C. (Reprinted with permission of the publisher from [86].)

of the eluent volume purged through the column. Fig. 14 shows that under high pH eluent conditions acetonitrile in the aqueous mobile phase is preserving column longevity much better than methanol. It appears that acetonitrile substantially better prevents the dissolution of the silica backbone compared to methanol. Therefore, from a chemical stability point of view, at least for silica-based RP phases, acetonitrile is to be preferred over methanol as the organic modifier in RPLC [76].

2.2.2. Type and concentration of buffering salts

In Figs. 15 and 16, the effect of the nature of the buffering anion and cation salts in the eluent under high pH 10.0 conditions on column deterioration is illustrated [2,30,86]. In Fig. 15, it can be seen that borate and glycine buffers improve column longevity substantially better compared to the carbonate and also to the widely applied phosphate buffer. Presumably, the column deterioration caused by these two
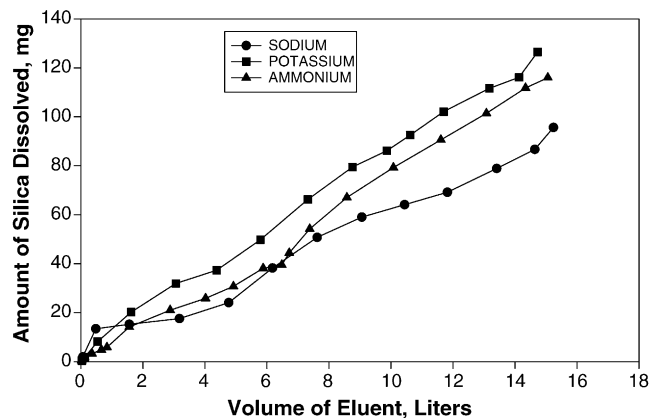

Fig. 16. Effect of pH 10 phosphate buffer cation on silica support dissolution. Conditions same as for Fig. 15 except different buffer cations. (Reprinted with permission of the publisher from [86].)
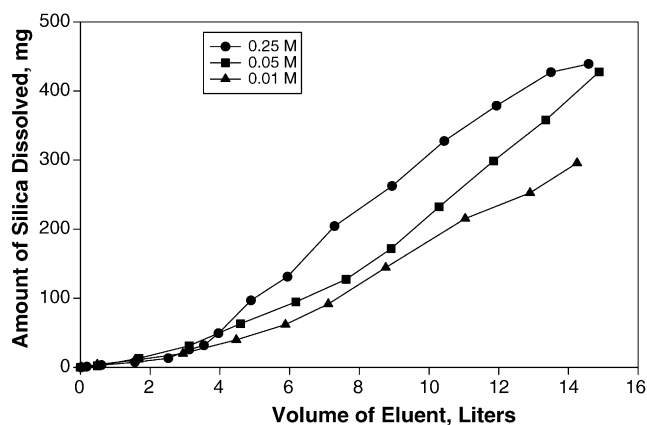
latter buffering anions can be explained by their complex formation abilities with silica [76]. In addition, and already earlier discussed in Section 2.1.1. Tindall and Perry [85] found a substantial influence of the nature of buffering anions on the pH of water–organic eluents. As a consequence from this latter study, the dissolution effects of these different buffers on the deterioration of the silica backbone of the investigated RP phases observed by Kirkland et al. may be caused by the combined effects of complexation and pH shifts [76].

In addition to that in this latter study [76], it was found that the nature of the cations in an eluent buffer also substantially influences the longevity of RP columns. For example, from the investigated cations in Fig. 16, column deterioration is best prevented by sodium as the buffering cation. In contrast, potassium is the most aggressive buffering cation compared to sodium and ammonium. Therefore, for optimal chemical stability under these experimental conditions sodium is the most favorable buffering cation compared to both the other cations [86]. Furthermore, the longevity of silica-based RP columns also depends on the ion strength, viz. buffer concentration in the eluent. Fig. 17 illustrates this effect of buffer concentration on column deterioration. Note that these experiments were carried out under often applied neutral buffered pH 7 eluent conditions. Obviously, in order to prevent early column failure low buffer concentrations should be used. In Fig. 18, the combined effects of the nature and the concentration of different buffers in the eluent on column deterioration of silica-based RP columns are further illustrated. In this study, dimethyl C-18 columns were purged under pH 7.0 eluent conditions at a temperature of 60 °C. In that figure, it can be seen that the 50 mM organic-based Tris buffer hardly causes any column deterioration up to at least 15 l of freshly prepared not-recycled eluent. In contrast to that, upon increasing the Tris buffer concentration to 0.25 M after about 5 l of eluent purging column failure can be observed. The replacement of Tris for a phosphate buffer under further similar conditions results in the deterioration of the column already
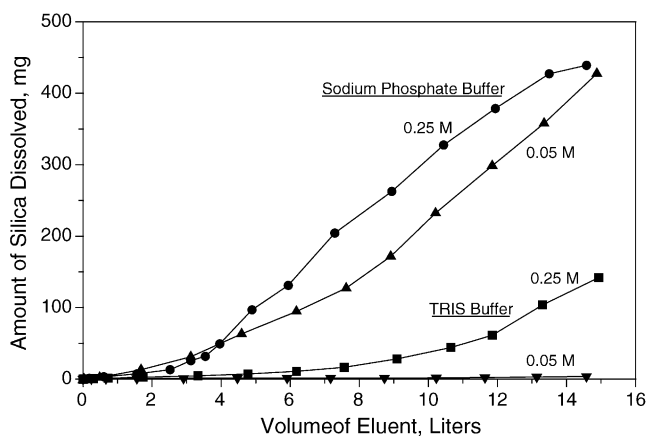
Fig. 18. Effect of buffer type and concentration on silica support dissolution. Column: Zorbax RX-C18, 15 cm × 0.46 cm i.d. Purge: acetonitrile–sodium phosphate and Tris buffers, pH 7 (20:80, v/v); flow rate: 1.0 ml/min; temperature: 60 °C. (Reprinted with permission of the publisher from [86].)

after about 1 l of eluent purging [86]. In this latter case, however, also the additional effect of the increase of the eluent pH by phosphate further accelerates silica dissolution and thus column failure [85] (see also the discussion in Section 2.1.1).

In order to preserve the longevity of silica-based RP columns apart from the choice of the proper column, also the selection of the nature and concentration of the buffer in the eluent is of great importance. Under the applied experimental conditions in the latter study [86], borate and organic-based buffers like, e.g. glycine at low concentration combined with the proper counterion substantially prevent early column failure of silica-based RP columns. This can be explained by the relatively small shifts in the actual eluent pH caused by these buffering salts upon addition of an organic modifier. Opposite to that, the use of phosphate and carbonate as buffering anions causes an increase of the eluent pH of 0.65 and 1.4 pH units, respectively [85], resulting in a faster dissolution of the silica. Among the investigated cations sodium appears to be the most favorable buffering cation. In summary, together with the selection of the proper column, and following a number of well-established rules, under optimized conditions the longevity of special as well as conventional types of silica-based RP columns can be extended considerably [137].

2.2.3. Temperature

The influence and importance of the role of the temperature in HPLC has been neglected or underestimated for a long time. This is surprising, because in HPLC temperature influences many relevant physical parameters like, e.g. viscosity, diffusivity of the analytes in the mobile and stationary phases, and also sample solubility. In turn, these parameters determine, for example, back pressure, efficiency, retention and selectivity of a particular HPLC column. At best, until recently most standard HPLC equipment host a column oven to achieve reproducible analysis results. During recent years, however, a number of workers have (re)investigated the role

of the temperature in HPLC. In a number of studies, they have shown the benefits of using HPLC columns at lower, below ambient and also at elevated temperatures or temperature programs. Tran et al. investigated the temperature effects on retention in RPLC [138]. The authors concluded that, next to the percentage organic modifier in the eluent within the investigated temperature range of 10–90 °C, temperature is an important tool to control and optimize retention and selectivity in RPLC. Furthermore, extrapolating their results the authors also predicted that higher temperatures above 90 °C may further improve the quality of RPLC separations [138].

Greibrokk and Andersen studied the effects of temperature programming in the temperature range of approximately 25–120 °C in miniaturized RP columns [139]. In this study, the authors discussed the great and positive influence of temperature elevation on the reduction of analysis time, the improvement of peak shapes and the achievement of higher efficiencies. For basic analytes, McCalley also reported similar conclusions with respect to improved efficiencies and peak shapes at increased temperatures of columns and eluents [140]. In another study, Sander and Wise investigated the influence of column temperature of RP columns on shape selectivity [141]. These authors concluded that selectivity and more particularly also shape recognition for most of the RP stationary phases is continuously changing with the temperature. Furthermore, they also found that in most cases shape recognition is enhanced at lower column temperatures [141].

Working under controlled, fixed temperatures or temperature programming conditions is a strong tool to adjust and to improve retention and selectivity. In addition, higher temperatures also decrease eluent viscosity allowing significantly higher linear eluent velocities. As a result of that retention times can be drastically reduced. Concomitantly at higher temperatures also the diffusivity of analytes in the mobile and stationary phases increases, resulting in much better efficiencies and improved peak shapes. Combining these latter effects, higher temperatures in RPLC may provide better resolution and faster analysis. In addition, at higher analysis temperatures also lesser amounts of organic modifiers in eluents are needed to achieve the same separation. In turn, this contributes to reduce the use of toxic organic solvents and hence contribute to the appearance of "greener" laboratories. Also opposite to the change of the amount of organic modifier(s) in an eluent, which needs a certain equilibrium time, temperature is a more flexible and easily adjustable parameter to optimize chromatographic separation. Finally, temperature can also be used to induce specific effects like, for example, on column sample focusing.

Summarizing, from the here above-mentioned studies and references therein, it can be concluded that working at low but especially at elevated temperatures and also using temperature programming the quality and speed of RPLC separations can be substantially improved [138,141,142].

With a few exceptions, however, most studies in [138–141] were carried out on silica-based RP phases. In Sections 2.1.1.1 and 2.1.1.2, the column chemistry of chemically sta-
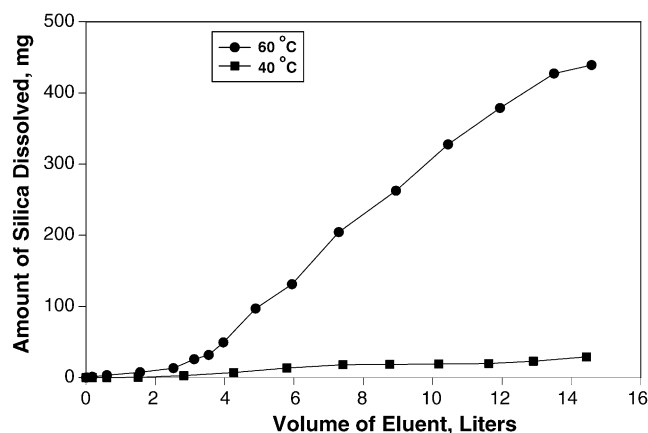
Fig. 19. Effect of temperature with pH 7.0 phosphate buffer on silica support dissolution at 60 and 40 °C. Column: Zorbax RX-C18, 15 cm × 0.46 cm i.d. Purge: acetonitrile–aqueous buffer (0.25 M), pH 7.0 (20:80, v/v); flow rate: 1 ml/min. (Reprinted with permission of the publisher from [86].)

ble silica-based RP phases for acidic and alkaline eluents has been discussed. These improved chemical modifications have also improved the thermal stability of these RP phases.

For example, Kirkland et al. investigated the effect of temperature on the deterioration of a conventional dimethyl C-18 column under neutral pH 7.0 eluent conditions [86]. In Fig. 19, the effects on column failure at two different temperatures, viz. 40 and 60 °C are illustrated. At 40 °C, only minor deterioration effects of that particular RP phase were observed. In contrast to that at 60 °C after about 2 l of eluent purging column deterioration is progressing [86]. In another study, Kirkland et al. investigated and compared the thermal stability of conventional and also of new concepts of modified silica RP phases, viz. sterically protected and bidentate phases, under highly acidic conditions at 90 °C [72]. In Fig. 12, both the sterically protected diisobutyl C-18 and also the bidentate C-18 RP phase show substantially higher thermal stability compared to the conventional dimethyl C-18 counterparts. Especially, the diisobutyl C-18 modified silica can easily be used up to at least 30,000 column volumes under these aggressive conditions [72]. Wyndham et al. also reported similar thermal stability results for RP columns based on the silica hybrid organic–inorganic technology [73]. In this study, the authors report the use of these columns at 80 °C under acidic pH eluent (1% TFA in the eluent) conditions for a considerable period of time.

Although substantial progress has been made in the manufacturing of better thermally stable silica-based RP columns ([72,73] and references therein), until now the practical application of these phases appears to be restricted to temperatures of about 30–60 °C above ambient [143].

For several reasons, chromatographers may want to use higher temperatures for their RP columns and eluents, for example, in order to optimize their separations and/or reduce analysis time. Zirconia-based RP phases must be considered now as attractive thermal and chemically stable alternatives for silica-based counterparts. Zirconia-based phases can be
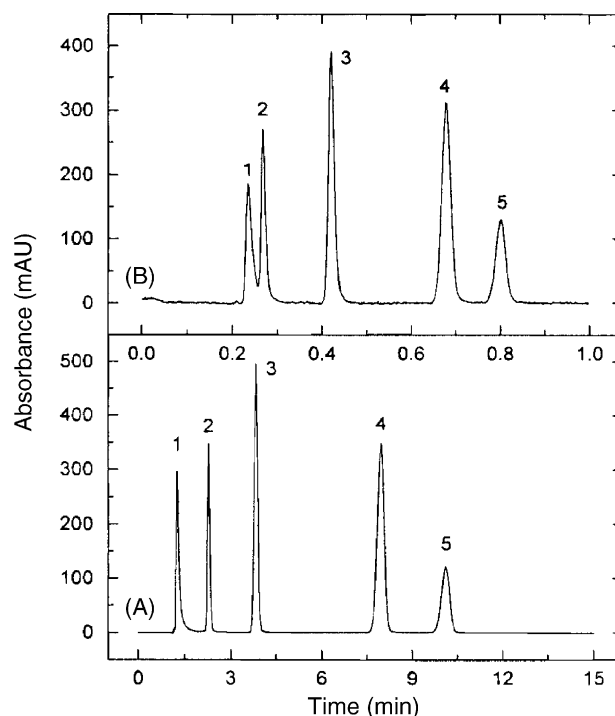

Fig. 20. Chromatograms of a reversed-phase test mixture. Column: polybutadiene-coated zirconia (PDB-ZrO$_2$), 100 mm × 4.6 mm (ZirChrom Separations Inc., Anoka, MN, USA). Mobile phase acetonitrile–water (40:60, v/v). Plot A is the chromatogram at 30 °C and 1 ml/min and plot B is the chromatogram at 100 °C and 5 ml/min. Solutes: (1) uracil; (2) ρ-nitroaniline; (3) methyl benzoate; (4) phenetole; (5) toluene. (Reprinted with permission of the publisher from [145].)

used at substantially higher temperatures, at the same time they also offer specific selectivities. Li et al. [144,145] and Carr and coworkers [146,147] investigated the influence of the temperature on the thermodynamics, kinetics, and stability of polybutadiene-coated zirconia RP phases. Moreover, in these studies the influence of temperature on the analysis speed was investigated also. The authors found that for a neutral eluent of methanol and water (50:50, v/v) at 100 °C zirconia PDB phases could be used for at least up to 7000 column volumes [144]. In Fig. 13, the thermal stability of zirconia PDB phases is illustrated under neutral eluent conditions at 195 °C. Under these harsh eluent conditions, these columns could be used for at least 6000–7000 column volumes. Furthermore, these studies also convincingly showed that temperature increase resulted in a considerable efficiency improvement and also in a substantial reduction in analysis time. For example, upon increasing the temperature of a 50 mm × 4.6 mm column packed with 3 μm particles from 25 °C up to 150 °C the analysis time was decreased by a factor 50. In Fig. 20, this latter effect is illustrated. In this figure, an increase of the column temperature from 30 to 100 °C results in a decrease in analysis time from 10 to <1 min [145]. Furthermore, these studies also proved that working at elevated column temperatures may substantially reduce the consumption of organic modifiers [147]. Finally, these latter studies also showed that selectivity optimization can substan-

tially benefit from working at higher temperatures. In turn, however, this requires the availability of thermally stable RP columns ([148] and references therein).

In summary, a substantial number of studies have convincingly shown that in HPLC and especially RPLC chromatographers can benefit much more from temperature as an efficient and flexible tool to improve and to speed up their separations. In turn, this demands RP columns with improved chemical as well as thermal stability towards aggressive eluent conditions. It appears that such RP columns are available now for chromatographic practice.

3. Conclusions

Until now and for good reasons, silica-based RP columns are and will be used to solve many separation problems. The great variety in selectivities of the available silica-based RP columns, their high efficiencies and reproducibilities are yet unsurpassed. Furthermore, for many separation problems in practice, specific silica RP phases offer sufficient chemical and also thermal stability over a wide range of experimental conditions. These columns can safely be used in a pH range from 1 up to 12 and from sub-ambient to 90 °C.

However, faster method development methodologies, shorter analysis times, reduced organic solvents consumption and further improvement of selectivities demand for RP columns with even higher thermal and chemical stability.

RP stationary phases based on other inorganic substrates rather than silica, for example, zirconia and also polymer stationary phases, meet these demands and are commercially available.

To date, chromatographers can select RP columns meeting many of their requirements with respect to chemical and thermal stability. Apart from these latter properties, however, one must bear in mind that simplicity of method development and the stationary phase reproducibility and repeatability are also important criteria for column selection. In laboratory practice, an acceptable compromise must be found between these considerations.

Acknowledgements

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