Thorsten Teutenberg[1]
Koen Hollebekkers[2]
Steffen Wiese[1]
Andrea Boergers[1]

[1]Institut fuer Energie-und Umwelttechnik e. V. (IUTA), Duisburg, Germany
[2]Shimadzu Benelux B.V., s'Hertogenbosch, The Netherlands

Original Paper

Temperature and pH-stability of commercial stationary phases

Le condizioni sono estreme: 150°C, ma in ogni caso una comparazione può essere indicativa sulla robustezza di una colonna

In this paper, the temperature and pH stability of silica-based RP stationary phases were investigated. Furthermore, nonsiliceous phases like a polymeric column based on polystyrene divinylbenzene and a polybutadiene coated zirconium dioxide column were also included. The columns were heated up to 150°C at dynamic conditions, which means that the eluent consisting of water and methanol (90:10, v/v) was continuously purged through the packed bed. After every 5 h, the columns were cooled down to room temperature and the efficiency was measured by injecting a test sample based on the Neue test. It could be shown that some stationary phases exhibited a very good temperature stability at the test conditions specified above.

Aumentando la T si hanno degli effetti sulla diffusione che influenza la diffusione longitudinale e i processi di trasferimento di massa. Per questo la Temperatura può essere variata ai fini del miglioramento dell'efficienza, ma un aumento della T comporta anche una degradazione della colonna più rapida a causa di attacchi chimici.

1 Introduction

High temperature LC is well known for increasing speed and enabling special hyphenation techniques. Numerous advantages of high eluent temperatures in LC have been described and reviewed extensively over the last years [1–3]. However, new hyphenation techniques based on high temperature LC are mentioned only briefly. They include isotope ratio monitoring MS of stable isotopes [4–8], flame ionization detection [9–11] and biomarker discovery [12]. The implementation of these methods in routine analysis requires the availability of robust stationary phases, which exhibit low bleed at high temperatures. Although silica-based phases are characterized by their high mechanical stability and excellent mass transfer properties, their robustness against aggressive pH and temperature conditions cannot compete with polymeric or metal oxide stationary phases. As was already stated by Neue, '*A packing with all the advantages of silica, but with an expanded pH range is still the holy grail of HPLC*' [13]. In addition, chemical stability at high eluent temperatures is also desirable. When mobile phases are used with pH < 2, the bonded phase is susceptible to hydrolysis, and at pH > 8, particle erosion can occur due to the dissolution of the base silica particle [14]. Moreover, elevated temperatures will accelerate the degradation.

Although some progress has been made to increase the stability of packing materials at very high and low pH, further improvements of silica-based stationary phases regarding dissolution at high temperatures still is a challenge.

Competitive materials for high temperature HPLC include coated metal oxides like *e.g.* coated zirconium dioxide or titanium dioxide stationary phases [15, 16]. Zirconia phases have been introduced by Carr and co-workers some years ago [17]. However, there is still a widespread reluctance to use these columns in industry since the retention mechanism is different to silica-based phases where ionic interactions are concerned [18]. In an excellent review, Nawrocki *et al.* [19] pointed out that the chemistry of a zirconia surface is very complex and ion exchange reactions play an important role. Strong, hard Lewis acid sites, present on a zirconia surface, can interact with hard Lewis bases and these interactions are often considered troublesome. Therefore, silica-based stationary phases with an enhanced temperature range are needed to establish high temperature LC as a routine method in industry.

Although high temperature LC attracts much interest, data on the stability of common stationary phases at high eluent temperatures are limited. Some authors have published valuable information about the ruggedness of different types of columns at elevated temperatures [20–25] and extended pH [26]. However, until now a standardized protocol for a column ageing procedure

Correspondence: Dr. rer. nat. Thorsten Teutenberg, Institut fuer Energie-und Umwelttechnik e. V. (IUTA), Bliersheimer Strasse 60, 47229 Duisburg, Germany
E-mail: teutenberg@iuta.de
Fax: +4902065-418-211

Table 1. Overview of the tested columns

Manufacturer	Column description	Length (cm)	id (mm)	Particle diameter (µm)	Pore size (Å)
Agilent	Zorbax SB C-18	15	4.6	5	
Macherey Nagel	Nucleodur Gravity C-18	15	4.6	5	
Phenomenex	Gemini C18	15	4.6	5	110
Phenomenex	Gemini NX C18	15	4.6	5	110
Polymer Laboratories	PLRP-S	15	4.6	5	100
Restek	pHidelity C-18	15	4.6	5	140
Selerity	Blaze 200 C-18	15	4.6	3	140
Shimadzu	Pathfinder MR	15	4.6	5	100
Supelco	Ascentis C-18	15	4.6	5	
Waters	XBridge C-18	15	4.6	5	135
YMC	Pack Pro C-18 RS	15	4.6	5	80
ZirChrom	PBD	15	4.6	3	300

at high temperatures does not exist. As a result, the data generated by different authors are difficult to compare. Recently, we reported about the temperature stability of some metal oxide stationary phases under aqueous conditions at very high temperatures [22]. The aim of the present study is to extend this test procedure and to evaluate the stability of new silica-based RP stationary phases at high eluent temperatures. Other methods to elucidate the degradation process of the columns like elemental analysis or SEM as well as inductively coupled plasma-optical emission spectroscopy were not employed.

2 Experimental

2.1 Column test study

The columns included in this study are listed in Table 1. Column selection has been made according to product brochures from different vendors and scientific publications indicating that the material might be suitable for high temperature operation.

Test conditions had been adjusted to induce a rapid degradation of the packing material, since the test procedure is very time consuming. The procedure is similar to the test we published recently [22], but was slightly adapted to silica-based stationary phases. In this study, only brand new columns were used. The experiments were carried out under dynamic, nonrecycling conditions using a mobile phase with a high percentage of water, since the loss of the silane bonded phase results from hydrolysis of the siloxane bond that binds the silane to the support. This degradation is accentuated at higher temperatures, low pH and highly aqueous mobile phases [27]. A test mixture as introduced by Neue [28] was used to evaluate the performance of the columns at room temperature.

The test procedure for each column was subdivided into heating cycles. Five heating cycles were comprised

to a heating phase at neutral, acid and basic mobile phase pH conditions, respectively.

After the evaluation of the initial performance of a column, it was heated for 5 h at 150°C. The flow rate of the mobile phase was adjusted to 1 mL/min except otherwise noted. The mobile phase for high temperature elution always consisted of a mixture of water and methanol, 90:10 v/v. Methanol was added to avoid a phase collapse of the bonded phase [29].

We subdivided the procedure into heating cycles, which means that every 5 h, the column was cooled down to 25°C. Then, we completely changed the mobile phase and used a binary mixture of water and methanol which was buffered according to the Neue test procedure. We then measured a test mixture based on the Neue test. After the measurement of the Neue test mixture, the mobile phase was again changed and adjusted to 90 vol% water and 10 vol% methanol. Then, the next heating cycle was performed and the column was purged for another 5 h at 150°C. After five such heating cycles, the pH of the mobile phase was adjusted to 2.2. Please note that this pH adjustment refers to the stress test, not the conditions the test chromatograms were recorded. The test chromatograms were always recorded at 25°C using the Neue test mixture and the respective mobile phase conditions. If the column still showed an acceptable efficiency, the column was purged for another five heating cycles adjusting the pH to 12.0. In case of a complete loss of the efficiency or a total column failure, the test procedure was terminated.

Before a test measurement based on the Neue test was made and the mobile phase was changed from 90:10 water/methanol v/v to 35:65 v/v methanol/phosphate buffer pH 7, a linear solvent gradient from 10 to 100% methanol was run at the end of each heating cycle.

The pH adjustment was made using a 0.02 mol/L phosphate buffer. For the acid pH value of 2.2, crystalline phosphoric acid and potassium phosphate dibasic were weighed and dissolved in 400 mL deionized water. Subse-

Table 2. Chemicals used for the evaluation of the column efficiency

Chemicals	CAS No	Manufacturer	Purity
Acenaphthene	83-32-9	Fluka	≥99.0%
Amitriptyline hydrochloride	549-18-8	Sigma – Aldrich	≥98.0%
Butyl paraben	94-26-8	Fluka	≥99.0%
Dihydroxyacetone	96-26-4	Merck	98%
Dipropyl phthalate	131-16-8	Fluka	≥98.5%
Naphthalene	91-20-3	Fluka	≥98.0%
Phosphoric acid	7664-38-2	Fluka	≥99.0%
Potassium phosphate dibasic	7758-11-4	Fluka	≥99.0%
Potassium phosphate monobasic	7778-77-0	Fluka	≥99.5%
Propranolol hydrochloride	318-98-9	Sigma – Aldrich	99%
Propyl paraben	94-13-3	Fluka	≥99.0%
Toluene	108-88-3	Promochem	Picograde
1.0 N potassium hydroxide solution	1310-58-3	Sigma – Aldrich	1.043 mol/L

quently, the pH of 2.2 was adjusted with a 1.0 mol/L potassium hydroxide solution. Then, the buffer solution was filled up to 500 mL. For the basic pH value of 12.0, potassium phosphate and potassium phosphate dibasic were weighed and dissolved in 400 mL deionized water. Then the pH value was adjusted with a 1.0 mol/L potassium hydroxide solution and the buffer solution was filled up to 500 mL.

2.2 HPLC system

All measurements were performed using a Shimadzu LC2010C HPLC system (Duisburg, Germany). For data acquisition and analysis the Shimadzu LCsolution software (version 1.21 SP1) was used. To maintain the temperature of the mobile and stationary phase, a Metalox Model 200-C high temperature column oven (Systec, New Grighton, USA) was used.

2.3 Chemicals

Table 2 contains all relevant information about the chemical standards used in this study. Deionized water was produced in house using an Elix 10-Milli-Q Plus water purification system (Millipore, Eschborn, Germany). Methanol and ACN were of HPLC Optigrade from LGC Promochem (Wesel, Germany).

3 Results and discussion

The results are discussed separately for each column. For most of the columns, only the test chromatograms obtained before the columns were exposed to high eluent temperature and after the fifth heating cycle are given, since most columns were already seriously affected by the high eluent temperature at neutral conditions. The decision to stop the test procedure was mainly based on the visual inspection of the resulting test chro-

matograms, because a chromatogram already contains the most important parameters. Data on retention factors, asymmetry and pressure were collected but is not presented in detail in this paper. Otherwise, the scope of the paper would be too large for publication. In order to highlight important results, a reference to these data is made in the text.
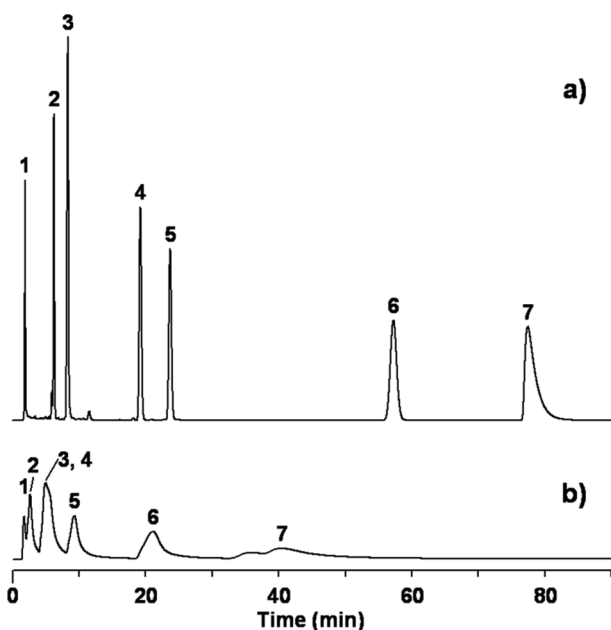
3.1 PackPro C-18 RS

This material is made from highly purified silica gel, followed by a high-coverage C18 bonding and finished with an endcapping procedure utilizing Lewis acid – base chemistry. As can be seen from Figs. 1a and b, the column is degraded very rapidly after 25 h of operation under neutral elution conditions. The retention time dramatically decreased for naphthalene from 23.8 to 9.3 min. The system pressure decreased from 175 to 140 bar indicating that there is no clogging of the system but a loss of bonded phase. Therefore, the test procedure was not continued after the neutral heating phase.

3.2 Gemini C18

This phase is synthesized by grafting a silica-organic layer to create a composite particle. According to the manufacturer, the internal base silica is unaltered by this manufacturing process and the particle retains the mechanical strength and rigidity of silica, while the silica-organic shell protects the particle from chemical attack.

However, our results very clearly indicate that the column has totally collapsed after 25 h of operation (Figs. 2a and b). The efficiency is completely reduced and there is virtually no retention of test compounds, indicating that the temperature has a pronounced impact on the hydrolysis of the bonded phase. While for the YMC Pack Pro column the peaks are at least partially resolved after 25 h of

Figure 1. (a) Test chromatogram obtained before the column was heated up to 150°C; (b) test chromatogram obtained after the fifth heating cycle. Column: YMC Pack Pro C18; mobile phase: 35:65 v/v methanol/phosphate buffer pH 7; flow rate: 1 mL/min; detection: UV@254 nm; temperature: 25°C. Analytes: 1 = dihydroxyacetone, 2 = propyl paraben, 3 = propranolol, 4 = dipropyl phthalate, 5 = naphthalene, 6 = acenaphthene, 7 = amitriptyline.

Esempio di cosa significa che una colonna non funziona più

operation at 150°C, for the Gemini phase all compounds elute as two broad peak clusters at the beginning of the chromatogram. Therefore, the silica-organic layer is not an effective protection against high eluent temperatures.

3.3 Gemini C18 NX

This phase differs from the original Gemini stationary phase. Here, the silica-organic layer is additionally protected by ethylene bridges between the silicon atoms. According to the manufacturer, this will enhance the stability.

As our results clearly point out, the stability of this phase is significantly higher than for the Gemini phase (Fig. 3). However, a slight decrease in the retention is observed for the hydrophobic markers acenaphthene and naphthalene. The retention factors decrease from 6.4 and 13.4 at the beginning of the test period to 4.0 and 8.8 at the end of the acid heating phase. Also, a fronting is observed for most of the compounds after the acid heating phase indicating that the degradation has proceeded. Applying the basic elution, the column is completely degraded after three more heating cycles. This means that the silica is rapidly dissolved at alkaline conditions, indicating that the protection of the silica-
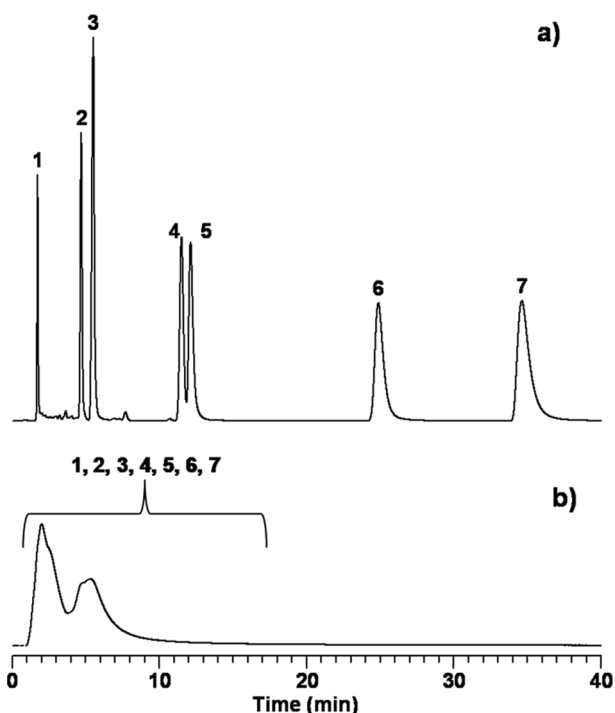
Figure 2. (a) Test chromatogram obtained before the column was heated up to 150°C; (b) test chromatogram obtained after the fifth heating cycle. Column: Phenomenex Gemini C18; mobile phase: 35:65 v/v methanol/phosphate buffer pH 7; flow rate: 1 mL/min; detection: UV@254 nm; temperature: 25°C. Analytes: 1 = dihydroxyacetone, 2 = propyl paraben, 3 = propranolol, 4 = dipropyl phthalate, 5 = naphthalene, 6 = acenaphthene, 7 = amitriptyline.

organic layer is not given when both a high eluent temperature and a basic pH are applied.
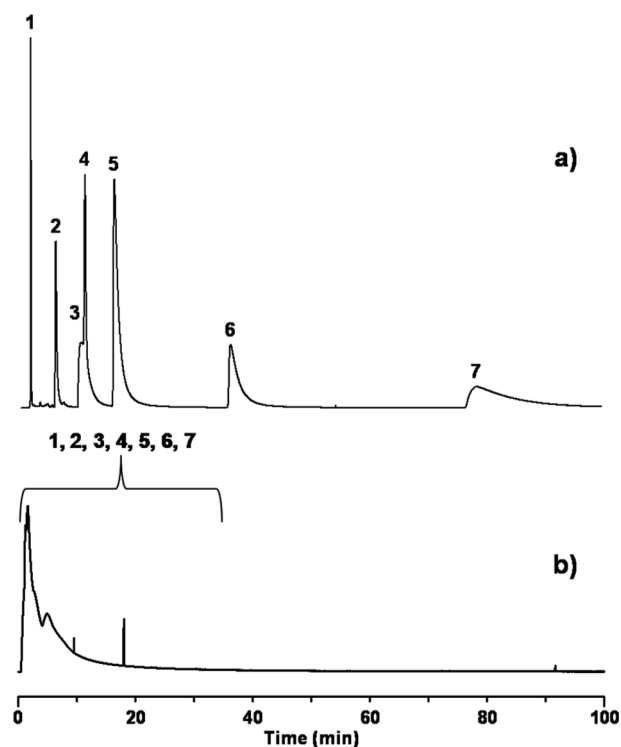
3.4 Pathfinder MR

This material is also known as polymer encapsulated silica, made from organic and inorganic building blocks, one forming the internal silica core and another forming the external polymer capsule. According to the manufacturer, the potential value of polymer-encapsulated silica is that the external capsule provides resistance to hydrolysis of ligands.

The data presented in Fig. 4 clearly show that there is a complete loss of retention after just 25 h of operation. The retention of naphthalene decreased from 16.2 to 5.6 min indicating that there is a dramatic loss of the bonded phase. Also, the system pressure decreased from 186 to 123 bar.

It is interesting to note that Guiochon and Riddle reported about the use of a Pathfinder column for the separation of free sterols at temperatures up to 150°C [30]. In their study, the mobile phase always contained
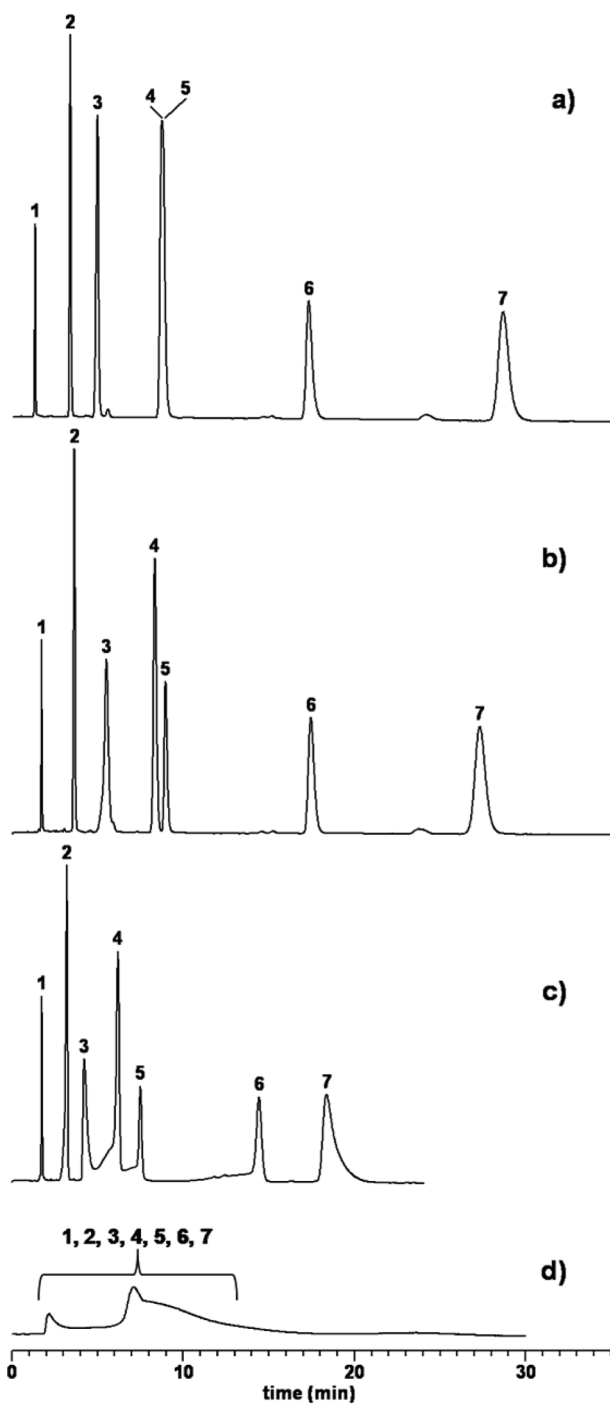
Figure 3. (a) Test chromatogram obtained before the column was heated up to 150°C; (b) test chromatogram obtained after the fifth heating cycle, (c) test chromatogram obtained after the tenth heating cycle, (d) test chromatogram obtained after the thirteenth heating cycle. Column: Phenomenex Gemini NX; mobile phase: 35:65 v/v methanol/phosphate buffer pH 7; flow rate: 1 mL/min; detection: UV@254 nm; temperature: 25°C. Analytes: 1 = dihydroxyacetone, 2 = propyl paraben, 3 = propranolol, 4 = dipropyl phthalate, 5 = naphthalene, 6 = acenaphthene, 7 = amitriptyline.

Figure 4. (a) Test chromatogram obtained before the column was heated up to 150°C; (b) test chromatogram obtained after the fifth heating cycle. Column: Shimadzu Pathfinder MR; mobile phase: 35:65 v/v methanol/phosphate buffer pH 7; flow rate: 1 mL/min; detection: UV@254 nm; temperature: 25°C. Analytes: 1 = dihydroxyacetone, 2 = propyl paraben, 3 = propranolol, 4 = dipropyl phthalate, 5 = naphthalene, 6 = acenaphthene, 7 = amitriptyline.

only a low concentration of water. At 150°C, the percentage of water was 30%. Unfortunately, no data are available showing the initial performance of the column. Nevertheless, the findings given by Guiochon do not contradict our own results. Increasing the amount of the organic solvent at high temperature should prevent a rapid dissolution of the silica. However, as was also pointed out by Guiochon, raising the temperature of the mobile phase changes its physicochemical properties resulting in a lower surface tension and static permittivity. This means that less organic solvent is required at elevated temperatures to achieve the same retention. In RP chromatography this effect can be exploited to completely replace the organic solvent as was demonstrated by many authors [31–36]. Consequently, temperature stability of stationary phases should also be viewed from the perspective of a mobile phase consisting mainly of water. In this respect, the Pathfinder phase does not seem to be appropriate for its use in high temperature LC in RP mode. However, it might be used successfully for applications requiring a high organic content of the mobile phase at elevated temperatures.

3.5 Nucleodur Gravity C-18

The Nucleodur Gravity column is a high purity silica RP column. The separation mechanism is based on purely hydrophobic interactions since it consists of a nonpolar high density monomeric octadecyl modification with a carbon content of approximately 18%.

After the fourth heating cycle, the efficiency of the column was totally reduced. In fact, it was not possible to continue with the test procedure after the fourth heating cycle since the column was clogged resulting in a very high back-pressure exceeding the pressure limit of the HPLC-system.

However, from the scientific literature it could be assumed that this column might be suitable for high temperature chromatography. Cabooter et al. [37] used a Nucleodur Gravity column in a recent study to compare the separation speed potential of a critical pair on commercially available high temperature supports at elevated temperatures. These supports comprise a ZirChrom-CARB, a ZirChrom-PBD and a Nucleodur Gravity column. The latter column was only used at a maximum temperature of 90°C, whereas the metal oxide stationary phases were used at temperatures up to 120°C. However, no chromatograms are shown to demonstrate the performance of the columns and to compare the performance before and after the study. It is only briefly mentioned that the measurements on the Nucleodur Gravity column were limited to 90°C due to coating stability reasons, which means that a degradation at higher temperatures was expected by the authors.

Also, Albert et al. [2] used a Nucleodur Gravity column at temperatures up to 90°C in order to investigate the beneficial effects on the separation and detection of basic compounds using ESI-MS. However, a comparison of the column performance before and after the study is not given. Nevertheless, their results seem to indicate that at a temperature of 90°C the column is stable.

3.6 Zorbax SB C-18

The Zorbax StableBond columns are made using bulky silanes that sterically protect the siloxane bond. According to the manufacturer, this results in an extended pH and temperature stability. The temperature limit of this column is specified at 90°C by the manufacturer.

The results we obtained after the neutral elution at 150°C clearly indicate that the column has a superior performance over the other columns tested so far except the Gemini NX phase (Fig. 5). This means that the sterical protection by the isopropyl or isobutyl groups is effective to prevent a total loss in efficiency. Nevertheless, a loss in retention and a severe peak broadening is observed and the column performance was not sufficient to go ahead with the test procedure after the neutral heating phase,
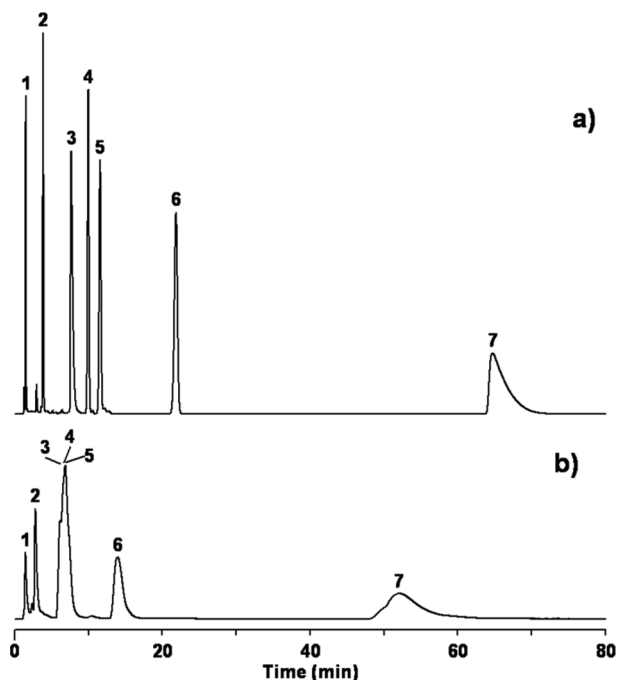
Figure 5. (a) Test chromatogram obtained before the column was heated up to 150°C; (b) test chromatogram obtained after the fifth heating cycle. Column: Agilent Zorbax StableBond; mobile phase: 35:65 v/v methanol/phosphate buffer pH 7; flow rate: 1 mL/min; detection: UV@254 nm; temperature: 25°C. Analytes: 1 = dihydroxyacetone, 2 = propyl paraben, 3 = propranolol, 4 = naphthalene, 5 = dipropyl phthalate, 6 = acenaphthene, 7 = amitriptyline.

since a rapid breakdown was expected at acidic conditions.

It has to be noted that we very successfully use these columns at temperatures as high as 100°C for an extended period of more than 100 h of operation with only a minimal loss in retention and efficiency, even when the pH is adjusted in the range of 2.5 and 3.5. However, at temperatures above 100°C a rising baseline is observed when using UV-detection (data not shown here), which is a clear sign of column bleed and thus a loss of the bonded phase. These results are in full agreement with the findings given by He and Yang [24], who conducted a study about the long-term stability of selected stationary phases with superheated water as the purging eluent. The comparison of the test chromatograms obtained after purging a Zorbax RX-C$_8$ column with 1565 and 6180 column volumes of water at 100°C clearly demonstrates that there is virtually no loss in efficiency. It is interesting to note that the RX-C$_8$ column shows a very high resistance to hydrolysis although it consists of octyl-modified silica. As Hetem et al. [38] have shown, the ligand alkyl chain length has a major influence on the stability of the column. The longer the alkyl ligands, the better the protection against the dissolution

of the underlying silica matrix. These results also underscore that the column lifetime of the Zorbax SB C-18 column we have investigated in this study should be far greater if the temperature is not raised above 100°C.

3.7 PLRP-S

The PLRP-S column consists of a rigid, spherical styrene/divinylbenzene copolymer. This type of column has been used frequently for high temperature LC [39–41]. The columns are also well established for gel permeation chromatography using pure organic solvents like THF at 150°C. However, the use of mobile phases with a high water content is often problematic, even if a solvent gradient is applied since swelling and shrinking of the polymer can occur.

The overall performance of the column is still very good after 25 h of operation. There is only a minor loss in retention and no severe peak broadening is observed. However, we also noticed that there was a leakage as the column was cooled down to room temperature after each heating cycle. This means that the fittings had to be retightened. This presents a severe problem, especially if the columns are used for temperature programming, since a safe operation cannot be guaranteed. This clearly demonstrates that not only the stability of the packing bed needs to be taken into account, but also a perfect seal of the hardware is mandatory for high temperature gradient operation.

Nevertheless, we successfully employed this type of column for the elution of thalidomide using either a pure water or a water-organic mobile phase at a maximum temperature of 185°C [42]. The only problem we encountered in that study was that there was a steady increase in the pressure when the column was flushed with a pure water mobile phase for a period of more than 5 h.

However, we observed a complete column failure for the column employed in this study when the pH was adjusted to 2.2 due to a co-elution of all compounds of the test mixture after one heating cycle at acidic conditions.

The extreme pH and temperature stability of these materials is highlighted in many scientific publications. Smith *et al.* [43] used these phases for the separation of model drugs employing superheated heavy water. They also used a phosphate buffer at a pH of 3 for the elution of salicylamide and encountered no problems with the stability of the stationary phase. However, detailed information about the initial and final column performance is missing. In contrast to this, He and Yang [24] give experimental evidence for the stability of a Hamilton PRP-1 column, also consisting of polystyrene-divinylbenzene. The test chromatograms they obtained after a PRP-1 column was exposed to 482 and 8821 column volumes of superheated water at 150°C are in full agreement with

our own results after the neutral elution phase, since there is hardly any change in the overall performance of the column. All these findings underline that polymeric columns can be quite effectively used at high temperatures and extreme pH. However, problems arise if the pressure limit of the column is approached, which might lead to a sudden clogging of the column. Furthermore, the efficiency of polymeric phases is still very low compared to silica-based phases as was also observed in this study.

3.8 Ascentis C-18

This material is made from a high purity type B silica. The bonding process is aimed at optimizing the bonded phase coverage and maximizing stability while minimizing bleed and unwanted secondary interactions.

However, the efficiency of the column dropped significantly after finishing the neutral heating phase. Therefore, the column cannot be recommended for its usage at very high temperatures.

3.9 Blaze 200 C-18 and pHidelity C-18

The Blaze column from Selerity and the pHidelity column from Restek are in effect the same stationary phases. The Selerity Blaze 200 column consists of a polydentate silica phase that is said to be stable at extreme temperatures. The bonding chemistry integrates multiple point attachments to the silica with a high degree of cross-linking. The temperature limit as specified by the manufacturer is dependent on the fraction of the organic solvent in the mobile phase. Although the packing material is identical for both columns, the temperature limit as specified by the column manufacturer for the Blaze column is at 150°C for a pure water mobile phase, while for the pHidelity column the temperature limit is set at 80°C for all mobile phase compositions.

The results we obtained using the Selerity Blaze 200 column were encouraging in terms of maintaining a high efficiency after the neutral elution phase at 150°C. As can be seen from Fig. 6, there is a loss in retention after the column was flushed for 25 h at 150°C. The retention factor of acenaphthene and naphthalene decreased from 8.9 to 6.5 and from 4.1 to 3.0, respectively. Surprisingly, the efficiency still remained very high. In effect, the plate count of naphthalene even increased after the first heating cycle from 12.284 to 14.791. This was fairly unexpected since usually the efficiency for all compounds either decreased or remained constant. This means that although there appears to be a slight loss of the bonded phase due to reduced retention, the initial efficiency of the column is maintained up to the fourth heating cycle. This is also underlined by the peak asymmetry of naphthalene, which improves from 2.39 to 1.37
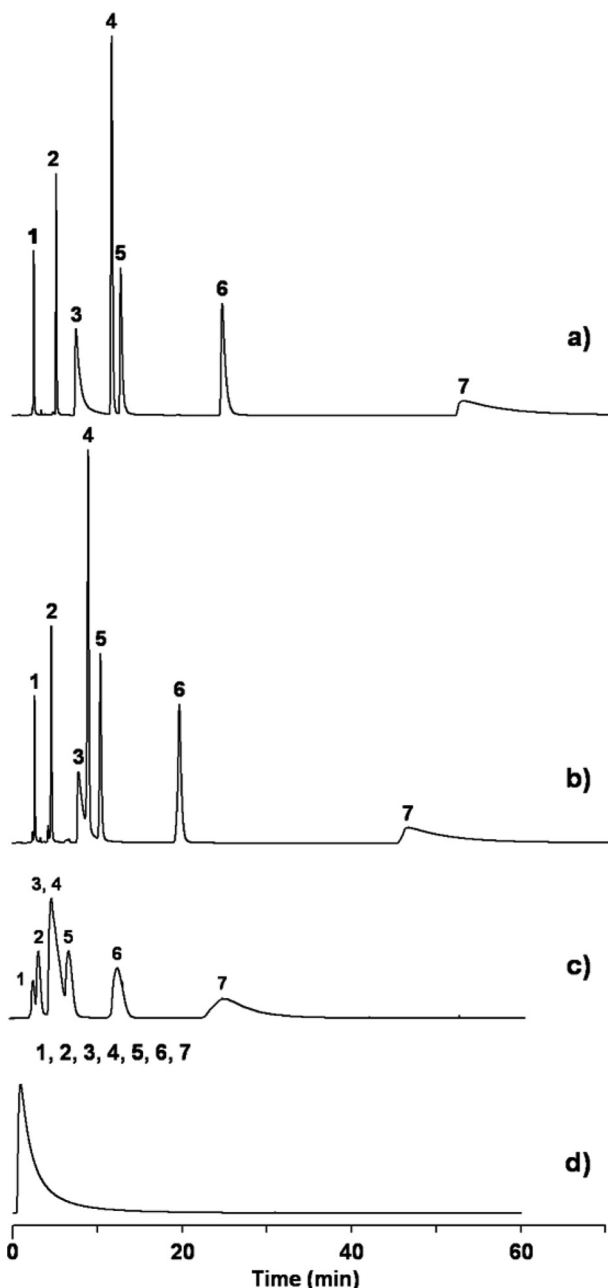
Figure 6. (a) Test chromatogram obtained before the column was heated up to 150°C; (b) test chromatogram obtained after the fifth heating cycle, (c) test chromatogram obtained after the tenth heating cycle, (d) test chromatogram obtained after the eleventh heating cycle. Column: Selerity Blaze 200; mobile phase: 35:65 v/v methanol/phosphate buffer pH 7; flow rate: 0.7 mL/min; detection: UV@254 nm; temperature: 25°C. Analytes: 1 = dihydroxyacetone, 2 = propyl paraben, 3 = propranolol, 4 = dipropyl phthalate, 5 = naphthalene, 6 = acenaphthene, 7 = amitriptyline.

during the same period. It is only after the fifth heating cycle that there is a loss in efficiency indicating that the column is slowly degraded. A similar effect was observed for a different column by Li [44], who reported about an

increase of the initial efficiency measured at 40°C after a polybutadiene coated zirconium dioxide column was operated at 150°C for about 1350 column volumes.

Due to the fact that the Blaze 200 column proved to be stable at neutral elution conditions, the pH of the mobile phase was adjusted to 2.2 with phosphate buffer and the column was flushed for another 25 h. The comparison of the test chromatogram obtained after 50 h with the initial performance of the column as depicted in Figs. 6 (a and c) demonstrate that a combination of a high eluent temperature and a low pH favours the loss of bonded phase. The retention of naphthalene has now decreased to 6.9 min. However, in order to evaluate the effect of a basic mobile phase at high temperature, the pH was adjusted to 12.0 and the column was flushed for 5 h. The test chromatogram given in Fig. 6d shows that after one basic heating cycle, there is a complete loss of retention and the stationary phase has totally collapsed.

The results clearly demonstrate that the Blaze column is suitable for high temperature operation, which is also partly supported by other authors. Marin *et al.* [45] evaluated the stability of six commercially available columns under temperature-programmed high temperature LC conditions. They concluded that a Blaze C8 column can be used at maximum temperatures between 100 and 200°C with temperature programming without evidence of column degradation. They further stated that acidic and basic pH conditions combined with high column temperatures did not appear to cause any collapse of the stationary phase. Clearly, we cannot support these findings, since our results unambiguously reveal that there is a tremendous loss in efficiency when the column is exposed to low and high pH in combination with a high eluent temperature. Therefore, the recommendation of the column manufacturer to increase the organic fraction in the mobile phase when working at high temperatures in order to reduce the dissolution of the silica should be followed. However, as was already pointed out for the Pathfinder column, in RP mode an increase of the temperature means that the elution strength of the mobile phase will also increase. Therefore, a higher eluent temperature leads to a reduction of the content of the organic solvent to achieve the same retention.

The results we obtained for the pHidelity column from Restek were similar to the results obtained for the Blaze column. After the first five heating cycles, a loss in retention is observed but the overall performance of the column is still very good. Also, the efficiency increases for acenaphthene from 6909 to 8215 plates after the column was exposed for 5 h at 150°C. Since the results were very similar to those obtained for the Selerity column, the test procedure was stopped after the neutral elution phase because it was not expected to gain new information.

3.10 XBridge C-18

The XBridge phase from Waters is made up of ethyl-bridged hybrid and porous silica particles [14]. According to the manufacturer, these stationary phases have a superior mechanical stability which allows their use in ultra high pressure chromatography up to 1000 bars. Also, the hydrolytic stability is claimed to be greatly enhanced enlarging the pH range.

The results we obtained using this column were quite unexpected. As can be seen from the chromatograms before the first and after the fifth heating cycle, there is virtually no change in the chromatographic conditions (Figs. 7a and b). The retention of all compounds is unchanged and the peak asymmetry remains constant. Also, the pressure does not change. This means that this stationary phase turned out to be most suited for its use at high eluent temperatures.

Our results are in full agreement with the findings of Liu *et al.* [46]. They used a hybrid C18 stationary phase for the separation of alkyl benzenes and aromatic alcohols and applied temperatures up to 200°C. They also commented on the stability of this column, reporting that over a 1-month period the column was flushed with pure water as the mobile phase at 200°C and a flow rate of 5 mL/min. The change of the retention factor during the test period was only 10%, although no data are given on the efficiency and peak asymmetry. Also, Shen *et al.* [47] come to similar findings. They used an XBridge column for the separation of substituted anilines. The mobile phase consisted of pure water at temperatures as high as 200°C, applying temperature programming from 165 to 200°C. Although there is no reference chromatogram which compares the initial and final retentivity of the column, from the chromatograms presented in that paper it can be deduced that the findings well agree with our own observations and the results obtained by Liu.

Therefore, we adjusted the pH of the mobile phase to 2.2 and the column was flushed for another 25 h (Fig. 7c). Surprisingly, there were only little changes in the retention, peak asymmetry and efficiency as becomes obvious from the test chromatogram obtained after 50 h of operation and the data given in Table 3. However, a close analysis of the peak symmetry of acenaphthene reveals that

Table 3. Overview of the chromatographic parameters of acenaphthene

Duration (h)	Retention time (min)	Asymmetry[a]	Theoretical plates of column
0	19.362	1.099	12 134
25	19.258	1.043	12 734
50	18.988	0.760	10 501

[a]   Tailing factor in 5% peak height.
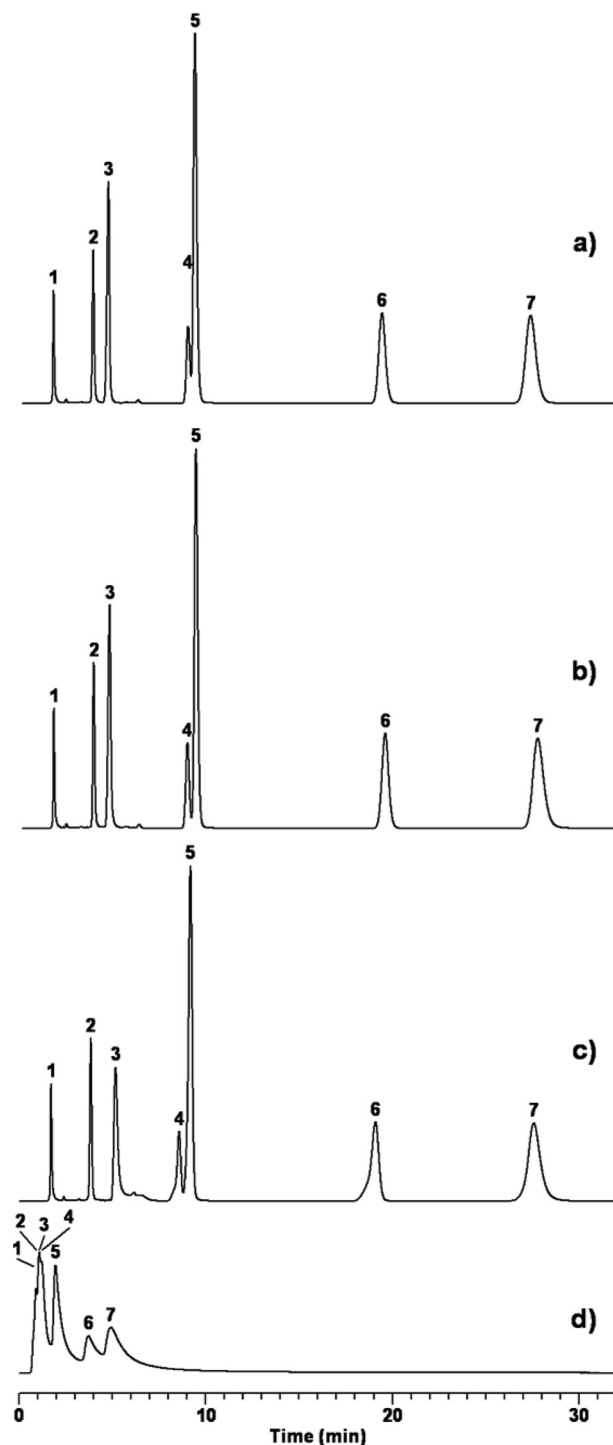
Figure 7. (a) Test chromatogram obtained before the column was heated up to 150°C; (b) test chromatogram obtained after the fifth heating cycle, (c) test chromatogram obtained after the tenth heating cycle, (d) test chromatogram obtained after the fifteenth heating cycle. Column: Waters XBridge; mobile phase: 35:65 v/v methanol/phosphate buffer pH 7; flow rate: 1 mL/min; detection: UV@254 nm; temperature: 25°C. Analytes: 1 = dihydroxyacetone, 2 = propyl paraben, 3 = propranolol, 4 = dipropyl phthalate, 5 = naphthalene, 6 = acenaphthene, 7 = amitriptyline.

Esempio di una colonna estremamente stabile!

the column performance begins to deteriorate, since a distinct fronting becomes visible. However, the performance of the column after the acid heating phase even allowed the elution at highly basic conditions. As can be seen from Figs. 7a and d, after 25 h at basic pH, the efficiency of the column has dropped markedly. Nevertheless, the peaks are still partially resolved which again underlines that the stability of this phase is superior compared to all other silica-based RP stationary phases investigated in this study.

3.11 PBD coated zirconium dioxide

Polybutadiene coated zirconia is prepared by depositing polybutadiene on bare zirconia. The stability of coated metal oxide stationary phases has been unparalleled so far and there are numerous papers which report on the use of these materials at high eluent temperatures and extreme pH [48–52]. Therefore, a polybutadiene coated zirconium dioxide column was also included in this investigation as a reference column. Due to its extraordinary stability, the pH could be adjusted to 2.2 and 12.0. After the first five heating cycles at neutral elution conditions, there is only a slight loss in retention (Figs. 8a and b). Even after the acidic (Fig. 8c) and basic heating phase (Fig. 8d), the retention of acenaphthene has only decreased from 10.2 to 9.2 min. Also, the column backpressure has only marginally changed from 230 to 220 bar at the end of the complete test procedure, although there are some fluctuations during the acidic and basic elution period. However, the efficiency has dropped markedly, since the asymmetry for all compounds has increased. During the acidic elution, a pronounced tailing became visible for all compounds. Therefore, the column was regenerated according to the manufacturer's guidelines after the ninth heating cycle, since it was thought that strongly adsorbing contaminants had been enriched either on the inlet frit or on the head of the column. After this procedure, the tailing could be partly reduced for the hydrophobic compounds. Nevertheless, after the first basic heating cycle, there was again a sharp increase in the tailing of these compounds and the column was again regenerated. In order to further investigate this effect, we opened the column at the end of the study and found out that there was a void at the top of the column, indicating that the column bed had been collapsed.

The results clearly underline that the stability at extreme temperatures and pH is unparalleled for this type of stationary phase. Nevertheless, the efficiency of the zirconium column is significantly affected by these conditions. During the past it was always concluded that zirconia columns suffered from a low efficiency when compared to silica-based materials. Li noted that this might be due to an inhomogeneous distribution of the
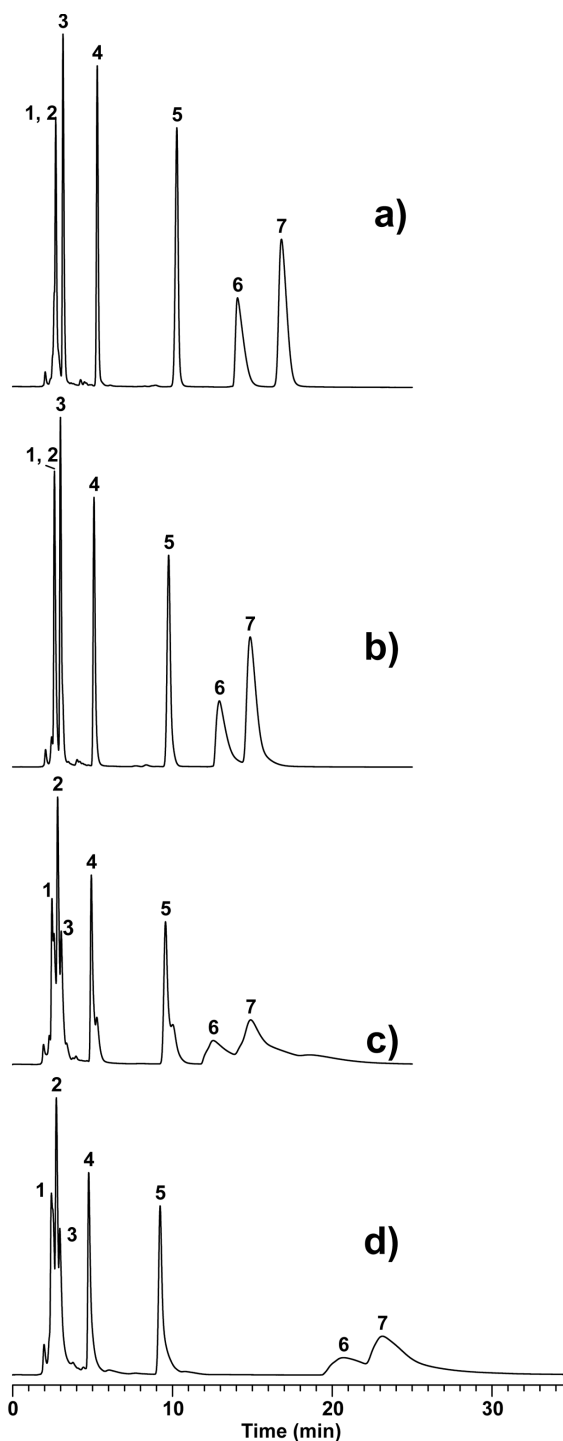
Figure 8. (a) Test chromatogram obtained before the column was heated up to 150°C; (b) test chromatogram obtained after the fifth heating cycle, (c) test chromatogram obtained after the tenth heating cycle, (d) test chromatogram obtained after the fifteenth heating cycle. Column: ZirChrom-PBD; mobile phase: 35:65 v/v methanol/phosphate buffer pH 7; flow rate: 0.7 mL/min; detection: UV@254 nm; temperature: 25°C. Analytes: 1 = dihydroxyacetone, 2 = propyl paraben, 3 = dipropyl phthalate, 4 = naphthalene, 5 = acenaphthene, 6 = propranolol, 7 = amitriptyline.

polymer on the surface of the pores [17]. However, the test chromatogram we obtained before the column was exposed to high temperatures reveals that the manufacturer has significantly improved the coating chemistry, since the efficiency is comparable to the silica-based columns. Unfortunately, this optimization means that at high temperatures the efficiency decreases due to void formation at the column inlet. This effect is clearly seen when the asymmetry of the hydrophobic markers is evaluated. Before the test procedure, naphthalene and acenaphthene elute as very symmetrical bands, whereas the asymmetry increases from 1.21 to 2.78 and 0.96 to 2.50 for both compounds during the test procedure.

3.12 General assessment of the results

In this section, the results are summarized and compared to other approaches of column test studies. The experimental design of the study described here was chosen because the column was not only exposed to high eluent temperatures and extreme pH, but also to alternating heating and cooling cycles. By repeatedly heating the column up and cooling it down, thermal stress is exposed to the bed and to the column hardware. This thermal stress can lead to a swelling or shrinking of the packing and the column hardware [53, 54]. Both effects might contribute to a degradation of the initial performance of the column.

In contrast to the long-standing and widespread notion that at temperatures above 100°C the stability of silica-based RP stationary phases is very limited, our results unambiguously show that new materials based on ethyl bridged hybrids exhibit an extremely high stability at high eluent temperatures. In fact, the stability of the XBridge phase under neutral elution conditions was even higher than the stability of the polybutadiene coated zirconium dioxide stationary phase since there was virtually no alteration of the column's overall efficiency. Also, the stability of the Gemini NX phase is significantly higher than that of the 'conventional' Gemini phase, because the silica-organic layer has also been stabilized by cross-linking silicon atoms with ethylene bridges.

Although it is often assumed that packings consisting of a polymer which is coated on a substrate such as silica are very robust when compared to bonded silanes, our results do not support these presumption. Both the Gemini and the Pathfinder phase suffer from a very low stability at these high temperatures, even if the pH is not adjusted. A much better way is to employ hybrid materials based on a covalent linking of silicon atoms in the silica matrix. However, running silica-based columns at elevated temperatures and a highly basic pH will inevitably facilitate a rapid degradation. Here, the stability of coated metal oxide stationary phases is unparalleled.

Clearly, the test conditions we employed resemble a worst-case scenario since there is still a widespread reluctance in industry to increase the temperature even beyond 60°C. However, as Claessens also noted, column testing can be extremely time consuming [55]. The complete study presented in this paper took more than half a year. With this approach we were able to generate a benchmark for future improvements of column stability, which is considered very important for column manufacturers. If the test procedure would have been carried out at a much lower temperature, the differences might not be that pronounced. Furthermore, our test procedure was carried out under dynamic conditions, which means that the eluent was not recycled but continuously pumped through the column. This prevented the effluent from becoming saturated with dissolved silica, which might have lead to an over assessment of the actual column stability.

The fact that we did not adjust the pH during the neutral elution phase was due to the common practice that the eluents are usually not spiked with acidic or basic modifiers if there are no ionizable compounds in the mixture. The high water content of the mobile phase was chosen since water is a very aggressive eluent. As was also pointed out, increasing the temperature changes the polarity of the mobile phase. The static permittivity of the eluent decreases with increasing temperature resulting in a much lower polarity of the mobile phase. In some cases this effect can also be exploited to fully replace the organic co-solvent. However, purging a column with a pure water mobile phase can lead to an unwanted retention loss [56]. In order to circumvent this problem we added 10% of methanol to the purging eluent.

The decision behind the use of phosphate buffer for pH adjustment was made for practical reasons since the Neue test required a buffered mobile phase based on phosphate. Using a phosphate buffer also allows for a pH adjustment in the acidic and basic region.

Although the results are very encouraging, column stability still needs to be improved. Also, the discussion is on-going about what can be considered an acceptable column lifetime. If we assume that the columns are used for ultra fast chromatography and the run time for the separation of a low complex sample mixture can be decreased to 60 s, then at least theoretically 1440 injections can be made within 24 h of operation. If the column needs to be replaced routinely after 1000 injections as is often the case in a regulated environment like the pharmaceutical industry, the stability of the silica-based columns should be sufficient when the column temperature is adjusted around 100°C. At this temperature, the viscosity of the mobile phase is significantly lower compared to room temperature resulting in a higher diffusivity of analytes so that the advantages of high tempera-

Importanza di avere dei criteri per la valutazione della riproducibilità nel tempo di un metodo cromatografico

ture LC can be fully exploited for fast separations. However, if the organic solvent needs to be replaced by water as is the case for the aforementioned special hyphenation techniques, then even higher operation temperatures up to 200°C have to be employed.

4 Conclusions

The test procedure revealed that new silica-based stationary phases have a much higher stability at high eluent temperatures than was anticipated. It could be shown that the XBridge column was virtually not affected by a mobile phase consisting of 90:10 water/methanol v/v at 150°C during a period of 25 h. Even after adjusting the pH to 2.2 using phosphate buffer and purging the column for another 25 h, the column performance was virtually unchanged except the fact that for some peaks a fronting became visible. Also, the stability of the Gemini NX phase is significantly higher than the stability of the 'conventional' Gemini column. This clearly underlines that ethylene bridges which are used to stabilize the silica-matrix are very effective to enhance the temperature stability of the stationary phase. In contrast to this, materials which are based on an organic layer deposited over the silica-matrix seem to combine the effects of both the low mechanical strength typically encountered for polymeric phases and the low temperature stability of conventional silica-based columns.

5 References

[1] Plumb, R., Mazzeo, J. R., Grumbach, E. S., Rainville, P., Jones, M., Wheat, T., Neue, U. D., Smith, B., Johnson, K. A., *J. Sep. Sci.* 2007, *30*, 1158–1166.

[2] Albert, M., Cretier, G., Guillarme, D., Heinisch, S., Rocca, J. L., *J. Sep. Sci.* 2005, *28*, 1803–1811.

[3] Yang, Y., *J. Sep. Sci.* 2007, *30*, 1131–1140.

[4] Godin, J. P., Hau, J., Fay, L. B., Hopfgartner, G., *Rapid Commun. Mass Spectrom.* 2005, *19*, 2689–2698.

[5] Godin, J. P., Fay, L. B., Hopfgartner, G., *Mass Spectrom Rev* 2007, *26*, 751–774.

[6] McCullagh, J. S., Juchelka, D., Hedges, R. E., *Rapid Commun. Mass Spectrom.* 2006, *20*, 2761–2768.

[7] Adair, C. G., Burns, D. T., Crockard, A. D., Desai, Z. R., Harriott, M., *J. Chromatogr.* 1984, *336*, 429–433.

[8] Krummen, M., Hilkert, A. W., Juchelka, D., Duhr, A., Schluter, H. J., Pesch, R., *Rapid Commun. Mass Spectrom.* 2004, *18*, 2260–2266.

[9] Yang, Y., Kondo, T., Kennedy, T. J., *J. Chromatogr. Sci.* 2005, *43*, 518–521.

[10] Guillarme, D., Heinisch, S., Gauvrit, J. Y., Lanteri, P., Rocca, J. L., *J. Chromatogr. A* 2005, *1078*, 22–27.

[11] Yarita, T., Nakajima, R., Otsuka, S., Ihara, T. A., Takatsu, A., Shibukawa, M., *J. Chromatogr. A* 2002, *976*, 387–391.

[12] de Boer, A. R., Alcaide-Hidalgo, J. M., Krabbe, J. G., Kolkman, J., van Emde Boas, C. N., Niessen, W. M., Lingeman, H., Irth, H., *Anal. Chem.* 2005, *77*, 7894–7900.

[13] Neue, U. D., *HPLC Columns*, Wiley-VCH, Weinheim 1997, 109.

[14] Wyndham, K. D., O'Gara, J. E., Walter, T. H., Glose, K. H., Lawrence, N. L., Alden, B. A., Izzo, G. S., Hudalla, C. J., Iraneta, P. C., *Anal. Chem.* 2003, *75*, 6781–6788.

[15] Nawrocki, J., Dunlap, C., McCormick, A., Carr, P. W., *J. Chromatogr. A* 2004, *1028*, 1–30.

[16] Nawrocki, J., Dunlap, C., Li, J., Zhao, J., McNeffe, C. V., McCormick, A., Carr, P. W., *J. Chromatogr. A* 2004, *1028*, 31–62.

[17] Li, J., Carr, P. W., *Anal. Chem.* 1997, *69*, 2193–2201.

[18] Dai, J., Yang, X., Carr, P. W., *J. Chromatogr. A* 2003, *1005*, 63–82.

[19] Nawrocki, J., Rigney, M. P., McCormick, A., Carr, P. W., *J. Chromatogr. A* 1993, *657*, 229–282.

[20] Fonseca, D. A., Gutierrez, H. R., Collins, K. E., Collins, C. H., *J. Chromatogr. A* 2004, *1030*, 149–155.

[21] Wilson, I. D., *Chromatographia* 2000, *52*, 28–34.

[22] Teutenberg, T., Tuerk, J., Holzhauser, M., Giegold, S., *J. Sep. Sci.* 2007, *30*, 1101–1114.

[23] Teutenberg, T., Tuerk, J., Holzhauser, M., Kiffmeyer, T. K., *J. Chromatogr. A* 2006, *1119*, 197–201.

[24] He, P., Yang, Y., *J. Chromatogr. A* 2003, *989*, 55–63.

[25] Yang, Y., *LC-GC Eur.* 2003, *16*, 37–41.

[26] Claessens, H. A., van Straten, M. A., Kirkland, J. J., *J. Chromatogr. A* 1996, *728*, 259–270.

[27] Snyder, L. R., Kirkland, J. J., Glajch, J. L., *Practical HPLC Method Development*, Wiley-Interscience, New York 2001, 193ff.

[28] Neue, U., Van Tran, K., Iraneta, P. C., Alden, B. A., *J. Sep. Sci.* 2003, *26*, 174–186.

[29] Przybyciel, M., Majors, R. E., *LC-GC Eur.* 2002, *15*, 652.

[30] Riddle, L. A., Guiochon, G., *J. Chromatogr. A* 2006, *1137*, 173–179.

[31] Kephart, T. S., Dasgupta, P. K., *Talanta* 2002, *56*, 977–987.

[32] Chienthavorn, O., Smith, R. M., Saha, S., Wilson, I. D., Wright, B., Taylor, S. D., Lenz, E. M., *J. Pharm. Biomed. Anal.* 2004, *36*, 477–482.

[33] Coym, J. W., Dorsey, J. G., *Anal. Lett.* 2004, *37*, 1013–1023.

[34] Yarita, T., Nakajima, R., Shimada, K., Kinugasa, S., Shibukawa, M., *Anal. Sci.* 2005, *21*, 1001–1003.

[35] Smith, R. M., *Anal. Bioanal. Chem.* 2006, *385*, 419–421.

[36] Dugo, P., Buonasera, K., Crupi, M. L., Cacciola, F., Dugo, G., Mondello, L., *J. Sep. Sci.* 2007, *30*, 1125–1130.

[37] Cabooter, D., Heinisch, S., Rocca, J. L., Clicq, D., Desmet, G., *J. Chromatogr. A* 2007, *1143*, 121–133.

[38] Hetem, M. J. J., de Haan, J. W., Claessens, A., van de Ven, L. J. M., Cramers, C. A., *Anal. Chem.* 1990, *62*, 2288–2296.

[39] Yarita, T., Nakajima, R., Shibukawa, M., *Anal. Sci.* 2003, *19*, 269–272.

[40] Tajuddin, R., Smith, R. M., *Analyst* 2002, *127*, 883–885.

[41] Smith, R. M., Burgess, R. J., *Anal. Commun.* 1996, *33*, 327–329.

[42] Giegold, S., Holzhauser, M., Kiffmeyer, T., Tuerk, J., Teutenberg, T., Rosenhagen, M., Hennies, D., Hoppe-Tichy, T., Wenclawiak, B., *J. Pharm. Biomed. Anal.* 2008, *46*, 625 – 630.

[43] Smith, R. M., Chienthavorn, O., Wilson, I. D., Wright, B., Taylor, S. D., *Anal. Chem.* 1999, *71*, 4493 – 4497.

[44] Li, J., Hu, Y., Carr, P. W., *Anal. Chem.* 1997, *69*, 3884 – 3888.

[45] Marin, S. J., Jones, B. A., Felix, W. D., Clark, J., *J. Chromatogr. A* 2004, *1030*, 255 – 262.

[46] Liu, Y., Grinberg, N., Thompson, K. C., Wenslow, R. M., Neue, U. D., Morrison, D., Walter, T. H., Gara, J. E. O., Wyndham, K. D., *Anal. Chim. Acta* 2005, *554*, 144 – 151.

[47] Shen, S., Lee, H., McCaffrey, J., Yee, N., Senanayake, C., Grinberg, N., *J. Liq. Chromatogr. Relat. Technol.* 2006, *29*, 2823 – 2834.

[48] Teutenberg, T., Goetze, H. J., Tuerk, J., Ploeger, J., Kiffmeyer, T. K., Schmidt, K. G., Kohorst, W. G., Rohe, T., Jansen, H. D., Weber, H., *J. Chromatogr. A* 2006, *1114*, 89 – 96.

[49] Sanagi, M. M., See, H. H., Ibrahim, W. A., Naim, A. A., *Malays. J. Chem.* 2004, *6*, 55 – 66.

[50] Xiang, Y., Yan, B., Yue, B., McNeff, C. V., Carr, P. W., Lee, M. L., *J. Chromatogr. A* 2003, *983*, 83 – 89.

[51] Fields, S. M., Ye, C. Q., Zhang, D. D., Branch, B. R., Zhang, X. J., Okafo, N., *J. Chromatogr. A* 2001, *913*, 197 – 204.

[52] Li, J., Carr, P. W., *Anal. Chem.* 1997, *69*, 837 – 843.

[53] Gritti, F., Guiochon, G., *J. Chromatogr. A* 2006, *1131*, 151 – 165.

[54] Andersen, T., Nguyen, Q. N., Trones, R., Greibrokk, T., *J. Chromatogr. A* 2003, *1018*, 7 – 18.

[55] Claessens, H. A., van Straten, M. A., *J. Chromatogr. A* 2004, *1060*, 23 – 41.

[56] Walter, T. H., Iraneta, P., Capparella, M., *J. Chromatogr. A* 2005, *1075*, 177 – 183.