Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1060 (2004) 9–21
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Development of some stationary phases for reversed-phase high-performance liquid chromatography

J.J. Kirkland*

19 Kendall Court, Wilmington, DE 19803, USA

Abstract

The availability of a variety of stable organic stationary phases for columns has been a key factor in the development of HPLC as a major scientific tool. This paper explores the history and rationale used in the development of some important stationary phases and attempts to identify some of the strengths and limitations of these materials. Some of the author's experiences in stationary phase development illustrate approaches leading to present-day columns that exhibit a broad range of selectivity coupled with a high degree of reproducibility. Suggestions also are made for additional stationary phases that may be needed to complete column selectivity potential for HPLC separations.

Keywords: High performance liquid chromatography; Stationary phases; Stationary phase developments; Stationary phase selectivity; Stationary phase stability

1. Introduction

Since the origin of what is now called high performance liquid chromatography (HPLC) about 35 years ago, there has been a continuous development of stationary phases for columns that provide improved separations with a wide variety of compound types. Early HPLC separations were performed by liquid–liquid-partition chromatography (LLC) [1] or by liquid–solid (adsorption) chromatography (LSC) [2]. While LLC provided a wide range of potential separation selectivity, practical limitations seriously restricted the usefulness of this approach. Maintaining a constant concentration of the stationary liquid on the column support with a flowing mobile phase proved difficult and cumbersome. This method required pre-saturating the mobile phase with the stationary phase and maintaining close column temperature control [3]. Such requirements also effectively eliminated the possibility of conducting gradient elution separations that often were desired for separating complex mixtures. Early LSC separations were limited to separating relatively non-polar samples using non-aqueous mobile phases. Also, gradient elution separations with this method were difficult and generally irreproducible because of problems in maintaining the activity of the adsorbent with changing mobile phase polarity [4].

The development of so-called bonded stationary phases for reversed-phase chromatography (RPC) eliminated many of the early problems in HPLC. Bonded stationary phases with a range of functionalities made possible the rapid growth of RPC for separating a wide range of compound types. Bonded phases for LSC also extended the range of applications, allowed convenient gradient separations and generally made this approach much easier for practitioners.

Histories of the early development of stationary phases for HPLC columns have previously been published by others and will not be attempted here [5]. Rather, this paper explores the rationale for the development of some important HPLC column bonded stationary phases. The advantages and limitations of these phases are examined and some comments are made regarding the areas of best utility for these materials. Some experiences of the author are used to illustrate these points. Suggestions also are made for additional stationary phase functionalities that might be needed for optimum potential of HPLC column selectivity.

E-mail address: jjackkirkland@worldnet.att.net.

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.10.057

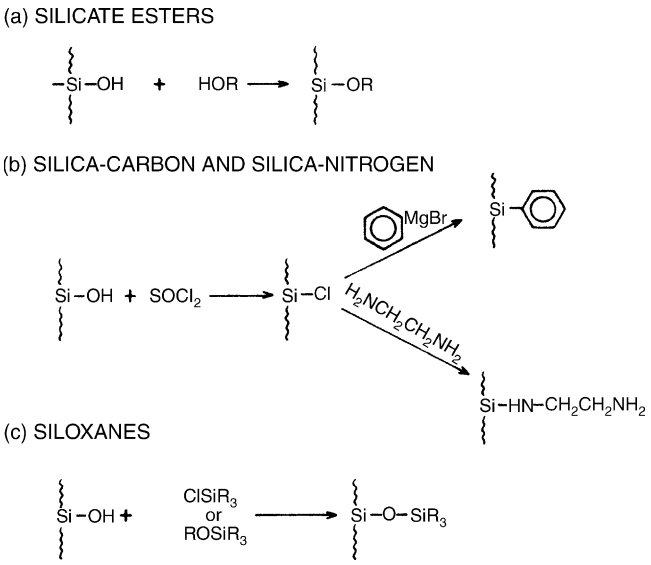

Fig. 1. Reactions for preparing bonded-phase column packings. Reprinted with permission of John Wiley and Sons from [10].

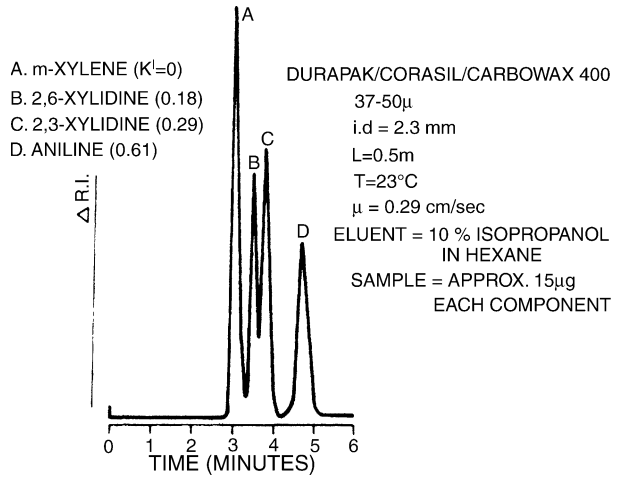

Fig. 2. Separation of amines on porous layer brush packing at room temperature. Reprinted with permission of John Wiley and Sons from [11].

2. Early bonded phases

To eliminate the problem with maintaining liquid stationary phases in LLC, organic ligands that are chemically bonded to the column support were developed [6–9]. One of the first involved the esterification of siliceous supports with a monomolecular organic layer of an alcohol, as illustrated in Fig. 1a. These phases were often prepared by direct esterification of silica silanol groups (Si–OH) with an alcohol (R–OH). Alternatively, the silicate esters were prepared by first chlorinating the silica support with thionyl chloride (shown in the initial reaction of Fig. 1b), then subsequently reacted with an alcohol. The esterification of silica with alcohols resulted in phases called “brushes” with good efficiency for HPLC separations [8]. Materials made by esterifying a totally porous silica, Porasil C, were commercially available as Durapak®. (*Note*: commercial products herein mentioned are summarized in Table 1). Porous thin-layer (pellicular) supports also were used to prepare “brushes” for performing normal-phase separations with non-aqueous mobile phases, as illustrated in Fig. 2. Unfortunately, silicate esters have poor hydrolytic stability, which greatly limited the utility of these materials when RPC with aqueous mobile phase became popular. As a result, the use of esterified silica for HPLC separations rapidly became obsolete.

Simultaneously to the development of esterified silicas, column packings with chemically bonded, non-extractable, hydrolytically and thermally stable polymeric silicone stationary phases were developed. This approach was inspired by silane chemistry studies reported by Abel et al. [12]. Others proposed packing materials for gas chromatography in which silanes were reacted with silica particles to produce bonded phase packings. Such materials also were suggested as potentially useful for liquid chromatographic separations [13]. Working within DuPont, Dr. Paul C. Yates and the author were successful in eliminating the disadvantages of LLC by devising polymeric silicone stationary phases [14]. These polymeric silicone stationary phases were covalently bonded to the porous structure of superficially porous Zipax® particles previously developed by the author and commercially available at that time [15]. These silicone stationary phases were thermally and hydrolytically stable, and a variety of packings with different functionalities were prepared. Initially, normal-phase liquid-partition separations were performed using column packings with polar silicone stationary phases. However, the excellent hydrolytic stability of

Table 1
Commercial products mentioned in text

Commercial name	Column packing type	Manufacturer/supplier
Porasil®-C	Porous silica microspheres	Waters Associates
Durapak®	Esterified silica	Waters Associates
Zipax®	Superficially porous silica microspheres	E. I. DuPont de Nemours
Permaphase®	Silicone stationary phase	E. I. DuPont de Nemours
StableBond®	Sterically protected stationary phase	Agilent Technologies
Zorbax®	Porous silica microspheres	Agilent Technologies
Extend®	Bidentate-C18 stationary phase	Agilent Technologies
Bonus®	Sterically protected amide/C18	Agilent Technologies
XTerra®	Hybrid-organic packing	Waters Associates
Inertsil®-ODS-3	Endcapped C18 stationary phase	G L Science (and others)

these stationary phases quickly led to the realization that reversed-phase separations could be conveniently performed with hydrocarbon silicone phases. As a result, this highly versatile chromatographic mode quickly became the preferred approach for many separations. This was the first bonded silicone stationary phase of this type, and this technology was commercialized by DuPont under the commercial name Permaphase® [16]. These column packings were widely used to reproducibly separate many types of samples, as illustrated in Fig. 3.

However, in spite of the success with Permaphase®, there was a property of this packing that sometimes limited column efficiency. The 1 μm porous outer shell of the Zipax® superficially porous silica support largely was filled with a bonded three-dimensional porous silicone polymer to produce the desired retention range for the column packing. This relatively thick stationary phase sometimes produced slow mass transfer for larger solutes which resulted in reduced column efficiency and less than symmetrical peaks, particularly at higher mobile phase velocities. Nevertheless, this technology served as a good starting point for the development of bonded stationary phases that fulfilled all of the requirements for efficient, reproducible HPLC separations.

Column packings with covalent Si–C or Si–N bonded groups also were made in an attempt to eliminate some of the drawbacks of silicate ester materials. These packings were prepared by first chlorinating the silica silanol groups with thionyl chloride (or phosgene), as illustrated in Fig. 1b. The chlorinated silica then was typically reacted with an appropriate Grignard reagent to produce Si–C bonds [18] or with amines to give Si–N bonds [19]. Compared to silicate ester stationary phases, the Si–C and Si–N materials are superior in the hydrolytic stability desired in reversed-phase chromatography. However, Grignard reactions to prepare the Si–C packings are inconvenient, sometimes result in relatively low concentrations of surface-bonded ligands, and often leave undesired residues on the packing. Si–N stationary phases also are restricted to the pH 4–8 range when aqueous mobile phases are used. These limitations have restricted Si–C and Si–N stationary phases for general use in HPLC, and as a result, these materials are not marketed by major column manufacturers.

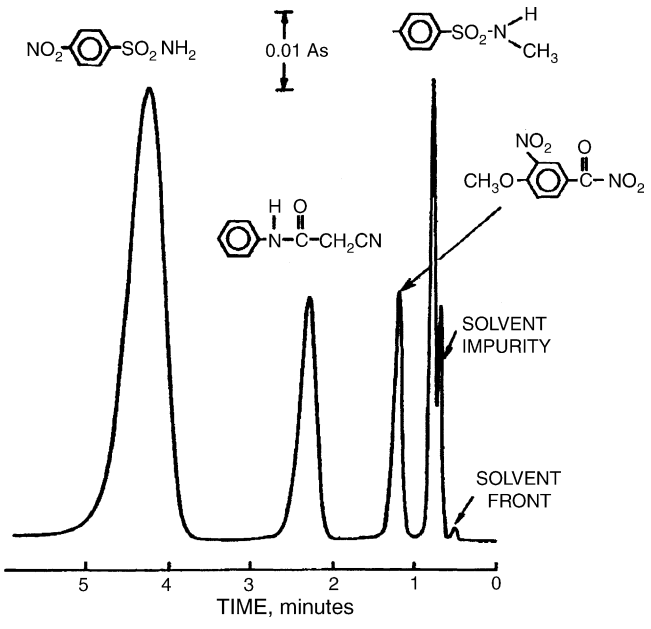

Fig. 3. HPLC separation with bonded "Nitrile" Permaphase® column, 100 mm × 0.21 mm. Mobile phase, 1:1 isopropylchloride/hexane; 0.75% silicone bonded phase on Zipax® support; flow rate, 4.35 mL/min; pressure, 1360 psi; temperature, 27 °C. Reprinted with permission of *Journal of Chromatographic Science* from [17].

3. Siloxane stationary phases

The most useful and widely available column packings contain stationary phases that are based on siloxanes. Fig. 1c shows that siloxane type (Si–O–Si–C) bonded phase packings are prepared by reacting the silanol groups on silica support with organosilane reagents. These reagents can have reactive groups, containing chloro-, alkoxy, alkylamino or other functions [20], depending on the final R group required. Stationary phases of this type can be quite hydrolytically stable, and column packings with these materials exhibit the highest efficiency because of rapid mass transfer effects.

The most generally available commercial column packings are made with monofunctional reagents, as illustrated in Fig. 4A. Some commercial packings use a lightly polymerized bonded phase resulting from the reaction of bifunctional or trifunctional silanes, as illustrated in Fig. 4 B and C. Fig. 5 shows the various types of covalently bonded silanes used with silica supports. Lightly polymerized phases resulting from the reaction of di- or trifunctional silanes are shown in Fig. 5A. Such polymeric stationary phases sometimes are more stable at low and high pH than traditional monofunctional phases. However, polymeric stationary phases are more difficult to produce uniformly and can be more variable in retention and selectivity than monofunctional phases.

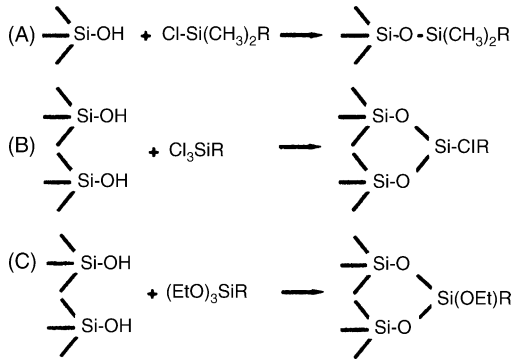

Fig. 4. Chemistry of bonded-phase column packings. (A) Reaction of surface silanols with chlorodimethylsilanes; (B) reaction of surface silanols with trifunctional silane; (C) reaction of surface silanols with trifunctional alkoxysilane. Reprinted with permission of John Wiley and Sons from [21].

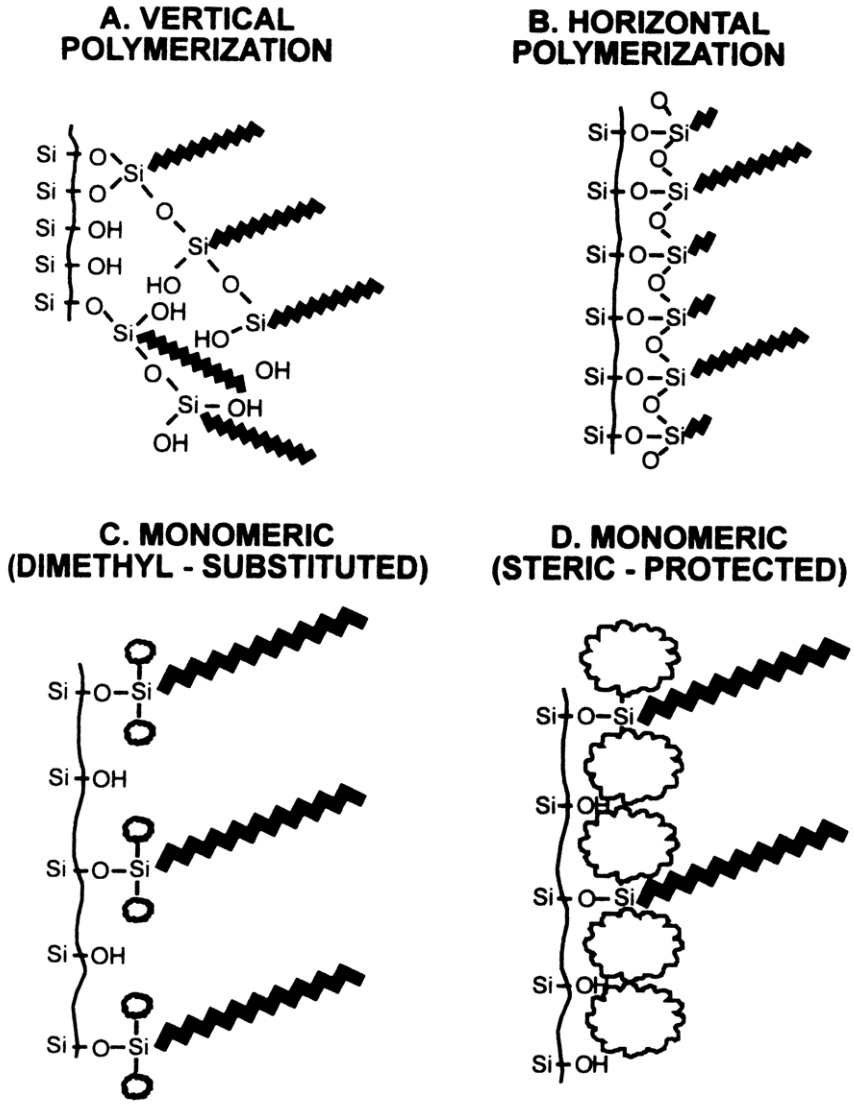

Fig. 5. Types of C18 silane bonded phases. Reprinted with permission of *Journal of Chromatographic Science* from [22].

Another type of silane bonded phase surface called horizontal polymerization is illustrated in Fig. 5B. These self-assembled silane monolayers are reported to be superior in both low and high pH stability [23]. However, limited data are available regarding the reproducibility of preparations for repeatable retention and selectivity.

The monomeric stationary phase shown in Fig. 5C is the most widely available silane bonded phase. (The stationary phase shown in Fig. 5D is discussed later). Reaction of chlorodimethylsilanes with silica surface silanol groups is the most commonly used preparation. An advantage of monofunctional silane reactions is high reproducibility; one silanol group reacts with one silane molecules, as represented in Fig. 4A. Packings made by this approach also demonstrate the highest column efficiency because of the favorable kinetics involving fast diffusion in and out of the thin monomeric stationary phase layer.

Some manufacturers attempt to “densely” (completely or fully) react the silanol groups on the silica surface with the silane. However, because of the bulk of the silane groups, even “densely” reacted packings leave more than 50% of the silanol groups unreacted. Early studies in the author’s laboratory indicated that the reaction of surface silanol groups at high yields is promoted by (a) using a large excess of the reactant; (b) carrying out the reaction in the neat liquid reactant or in a dry liquid with a relatively high boiling point; and (c) removing the reaction biproducts during the reaction [24].

Other manufacturers make products with partially reacted silanes. Here the percentage of residual unreacted silanol groups is much higher. “Densely” reacted column packings often have a distinct advantage of reproducibility because partially reacted surfaces are more difficult to reproduce from batch to batch. As might be expected, significant variations

in separation selectivity can result between the two types of packings as a result of differences in solute interaction with varying concentrations of unreacted silanol groups. Unreacted acidic silanol groups on partially reacted column packings also can cause tailing chromatographic peaks, especially for basic compounds.

"Densely" reacted long-chain alkyl bonded phases (especially C18 and longer chains) can present a special problem when highly aqueous mobile phases are used. In such media, the long alkyl chains apparently tend to bind or clump together causing what is often termed a "phase collapse" [25]. The result of "phase collapse" is significantly reduced column efficiency, changes in retention and enhanced opportunity for unsymmetrical peaks. A possible alternative mechanism to the proposed "phase collapse" is that for long-chain alkyl bonded phases with highly aqueous conditions, the mobile phase is inhibited from entering the ligand-reacted pore structure [26]. This condition would result in decreased solute contact between the stationary and mobile phases. Regardless of the true mechanism, the effect of "phase collapse" is real and must be taken into account for reproducible separations.

Re-equilibration of a column that has experienced "phase collapse" during a gradient run also occurs slowly. Some fully reacted C18 packings require as much as 60% organic for re-equilibration after a gradient run to ensure that "phase collapse" does not occur [27]. Other manufacturers have addressed the C18 "phase collapse" problem by structuring the packing surface to prevent phase collapse even at very low organic modifier concentrations. Some manufacturers use low surface coverage (with endcapping) to minimize or eliminate the "phase collapse" effect. In other cases, it is claimed that polar functional groups are introduced into the stationary phase to improve aqueous wettability and reduce the possibility of hydrocarbon phase aggregation. These approaches often are proprietary so that the nature of the treatment for these C18 packings often is not defined. These special reversed-phase packings often are useful for separating compounds that require a high aqueous mobile phase concentration to ensure adequate retention. "Phase collapse" generally is not as serious for shorter hydrocarbon ligands as for C18 packings.

Some silane-based column packings are treated by a process called endcapping to further react residual silanol groups that can cause unwanted solute interactions [21]. In this case, small silanes such as trimethylchlorosilane usually are reacted subsequently to the main reaction with long-chain silanes. These small endcapping reagents scavenge some residual reactable silanol groups so that the packing has a lowered tendency to interact deleteriously with some solutes. Unfortunately, the small endcapping groups on such packings can be more readily hydrolyzed at low pH than long-chain ligands, sometimes making this approach less useful for long-term, reproducible separations performed at low pH (<3) [28]. Densely bonded and exhaustively endcapped hydrocarbon bonded phase packings are especially useful in $pH > 6$ applications, since the more coherent hydrophobic stationary phase also acts to minimize dissolution of the silica support for increased column stability [29].

An often-asked question is, "Why are the most-used column stationary phases based on octadecylsilane (ODS) or C18 groups?" There are several reason for this, but one of the strongest is tradition. Early column packings were based on C18 because C18-based silanes were readily available at that time and reasonable in cost. C18 silanes were used commercially for other purposes and were supplied by several sources. It was then natural for early investigators to use these available materials in their experiments, and this experience was carried over into early commercial products for HPLC. Another reason for the popularity of C18 is the relatively high organic content that can be reacted onto silica supports. This was especially important for pellicular and superficially porous silica supports that were used early in the history of HPLC [3]. These materials had relatively low surface areas, which limited solute retention, so a high organic content was often required for desired retention of lightly held solutes. Another advantage of the long-chain C18 ligand is its better stability at both low and higher pH, compared to shorter chain ligands. Separation reproducibility has been of prime consideration to chromatographers, so the stability advantage of C18 packings always has been held important.

However, there are some disadvantages to C18 bonded phases packings. As described above, densely bonded packings can exhibit phase collapse when mobile phases contain a high aqueous content. For some solutes, shorter bonded phases can show slightly higher column efficiency. Also, column packings with shorter functional groups can re-equilibrate more rapidly after a gradient elution separation. The question of which alkyl stationary phase has the best overall properties for reversed-phase separations has been addressed. Kováts and co-workers [30] determined that the bonded C14 group actually produces the optimum overall properties of efficiency, stability, peak shape and retention characteristics for reversed-phase separations. Although this and other studies suggest that column packings with C14 (or C12) groups exhibit superior overall characteristics, C18 (octadecylsilane or ODS) packings still are the most popular materials currently in use. Curiously, "optimum" C12 and C14 phases are not generally used today, but the section below on Polar-embedded phases gives one exception.

4. Polymeric stationary phases

Some column packings contain stationary phases prepared by polymerizing various monomers on the support [31]. These phases are not covalently bound and are most useful with supports for which the covalent bonding of silanes is not practical. For example, polybutadiene-modified alumina [32] and zirconia column packings [33] and other polymeric stationary phases have been reported and commercialized. Modified alumina packings have not reached any significant level of usage since they have no distinct advantages over

conventional silica-based bonded column packings. On the other hand, polymer-modified zirconia column packings have proved to be useful for certain separations, particularly those that must be performed at high pH and high temperatures. Polymer-modified zirconia packings are quite stable at high pH as a result of the very low solubility of zirconia and the relative inertness of the modifying polymeric stationary phase. Early polymer-modified packings suffered from lower column efficiency mainly due to the slower kinetics of mass transfer caused by thick, uneven support coatings. However, more recent offerings have largely overcome this problem with better coating and polymerization methods [34]. A possible limitation of polymer-modified column packings is the difficulty of reproducing the organic nature and support coverage from one preparation to the next. (This is in contrast to the excellent coverage reproducibility that can be obtained by reacting monofunctional silanes with the surface of silica supports.) Definitive data on the reproducibility of columns with polymer-modifying chromatographic supports is sparse.

5. Some special stationary phases

In recent years, special organic stationary phases have been developed to eliminate some of the disadvantages of traditional bonded-phase materials. These special phases have demonstrated increased capability to provide stable, reproducible separations in instances where conventional materials may have limitations. As a result, these new materials have had significant commercial impact and increasingly wide acceptance as important separating media.

5.1. Sterically protected silane bonded phases

DuPont's emerging interest in the life sciences in the middle 1980's led Dr. Joseph L. Glajch and the author to embark on research studies to develop new stationary phases for separating materials of pharmaceutical and biomedical interest such as peptides and proteins. At that time, unprotected peptides and proteins usually were separated by gradient elution using reversed-phase packings with short (C3 and C4) hydrocarbon ligands in conjunction with acetonitrile/low pH aqueous trifluoroacetic acid mobile phases. Our studies had demonstrated that typical bonded-phase columns used for reversed-phase separations at low pH < 3, are not stable over relatively short periods of time [35]. It was found that short-chain ligands (C3, C4) were especially labile. The goal of our subsequent work was to develop new bonded-phase packings with much-improved stability at low pH conditions, while maintaining good separation quality for peptides, proteins and other biomacromolecules. The fact that long-chain, bulky ligands such as C18 were more hydrolytically stable than short chain groups led us to speculate that bulky groups surrounding the siloxane Si–O–C bond might give the needed steric protection against hydrolytic degradation. Studies then led to the development of covalently bonded silane stationary

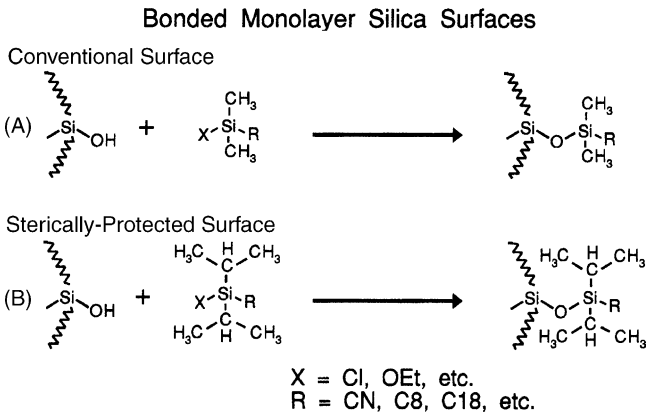

Fig. 6. Monofunctional silane bonded stationary phases. (A) Conventional dimethyl-substituted monofunctional stationary phase and (B) sterically protected monofunctional stationary phase.

phases with bulky side groups (Fig. 5D) that sterically protect the siloxane bond against hydrolysis [28]. Fig. 6A shows the surface structure of a traditional dimethyl-substituted monofunctional bonded phase, while Fig. 6B gives the structure of a monofunctional bonded phase with bulky sterically protecting groups. Fig. 7 shows a lowest-energy molecular model of diisopropyloctylhydroxysilane that illustrates how the bulk

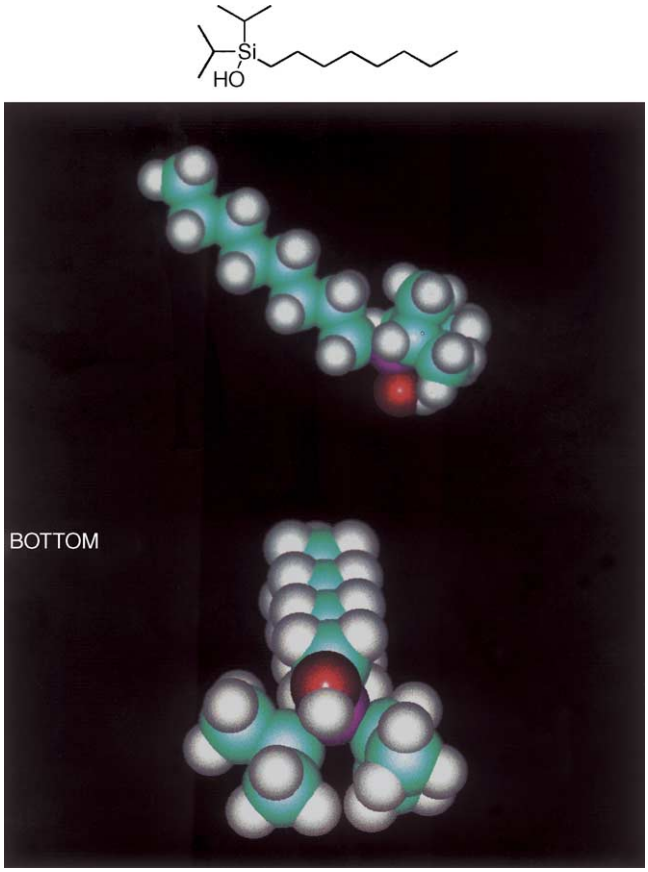

Fig. 7. Lowest energy molecular model of diisopropyloctylhydroxysilane. Model courtesy of Dr. B. E. Boyes.

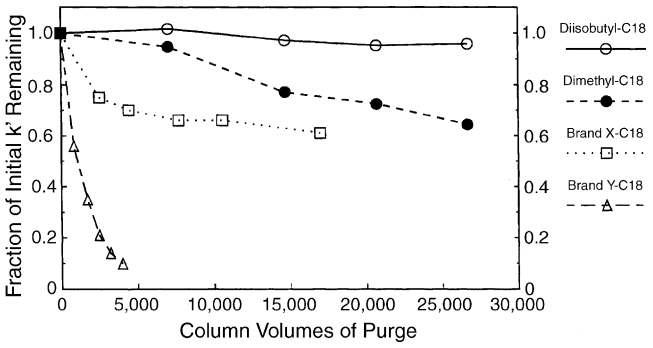

Fig. 8. Stability of C18 silane stationary phases at pH ∼0.9 and 90 °C. See text for details.

of isopropyl groups surround the Si–O function, thereby protecting this bond against hydrolysis when it is attached to a silica support. Fig. 8 shows the rate of stationary phase loss at low pH for a column with a diisobutyl-C18 stationary phase compared with conventional monofunctional C18 column, a polymeric C18 column and a column with a mixed phase of C18 and shorter silanes. For this test, these columns were purged continuously using highly aggressive methanol 1.0% trifluoroacetic acid mobile phase (pH ∼0.9) at 90 °C. The loss of stationary phase was then measured periodically by noting the retention of a neutral, hydrophobic test solute (toluene). Non-endcapped sterically protected silane columns specifically designed for use in low pH environments demonstrate excellent long-term ruggedness under such aggressive operating conditions [36]. These hydrolysis-resistant sterically protected stationary phases can be readily used at higher column operating temperatures that significantly increase separation efficiency, especially for macromolecules such as peptides and proteins [37]. Sterically protected stationary phases on Zorbax® silica microsphere support are available as StableBond® columns with a variety of functional groups to provide differences in selectivity and retention: C18, C8, C3, CN, phenyl, etc.

5.2. Bidentate stationary phases

The separation of highly basic compounds (e.g., basic drugs) on silica-based column packings has always been problematic because of the tendency of such solutes to interact deleteriously with unreacted acidic silanol groups to produce inefficient, tailing peaks. As a result, most separations of basic solutes are performed at low pH (< 3) where both silanol groups and basic solutes are protonated and unwanted ionic interactions are unlikely. However, to gain desired selectivity effects, some separations of basic compounds are carried out in the intermediate pH range of 6–9. Here, fully reacted, endcapped C18 column packings generally are preferred since they often provide better peak shapes and superior column stability than partially reacted and non-endcapped materials [38]. An alternative to these approaches is to carry out the separations at high pH where basic solutes

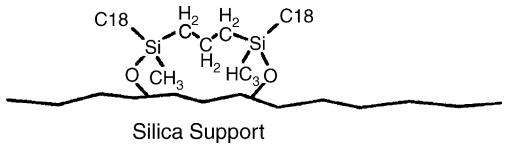

Fig. 9. Schematic of bidentate-C18 stationary phase. Reprinted with permission of *American Laboratory* from [29].

are not ionized and the silica support silanol groups are fully ionized; unwanted ionic interactions again are not possible. Early reports of high pH separations with silica-based packings were promising [39]. However, silica-based supports can dissolve readily at high pH [40], creating a potentially serious problem for column stability and lifetime.

To bring about a better understanding of how separations might better be performed with silica-based columns at high pH (up to 12), the author participated in a series of studies to define the effects of operating parameters on column stability: silica support type; effect of buffer type and concentration; pH and temperature effects; bonding and endcapping effects; ligand length effects, etc. [41–44,46]. From these studies, insight was developed for a new silane stationary phase and preferred operating conditions that make separations at high pH practical. Earlier work had indicated that bonded silanes with a bidentate configuration possessed unusual stability at low pH [28]. It was postulated that the ring structure of such materials might also have stability at high pH, especially if bulky C18 groups could be configured in a manner to shield the silica support from dissolution. Subsequent studies proved that this approach was viable. We determined that a C18 bidentate ring structure with a propylene bridge (Fig. 9) provided not only a high degree of stability at high pH, but also exhibited the desired column efficiency and retention characteristics both at high and low pH [29,45]. This bidentate-C18 stationary phase was double-endcapped to minimize exposure of a bare silica surface and resist dissolution at high pH. Fig. 10 shows retention factor k and plate height values for a column ageing study carried out at pH 11 with an organic buffer at ambient temperature. Fig. 10A shows that no change occurred in k values after almost 40,000 column volumes of continuously flowing mobile phase for several highly basic drugs (pK_a 9.5–9.7) and the neutral solute, toluene. Fig. 10B shows that there also was no change in toluene plate height during this ageing. The slight increase in plate height for the basic drugs suggest small changes in the unreacted silica support surface during this rugged ageing test. A high pH separation of some basic drugs on the C18 bidentate column is shown in Fig. 11. Studies had shown that high pH phosphate and carbonate buffers aggressively dissolve silica supports so organic amine buffers are preferred for high pH separations [43,44]. Note the excellent peak symmetry and column efficiency for these highly basic compounds on a silica-based column at high pH.

The superior resistance of the C18 bidentate silica-based column packing to dissolution at high pH is illustrated in Fig. 12. When purged with a highly aggressive pH 11 phos-

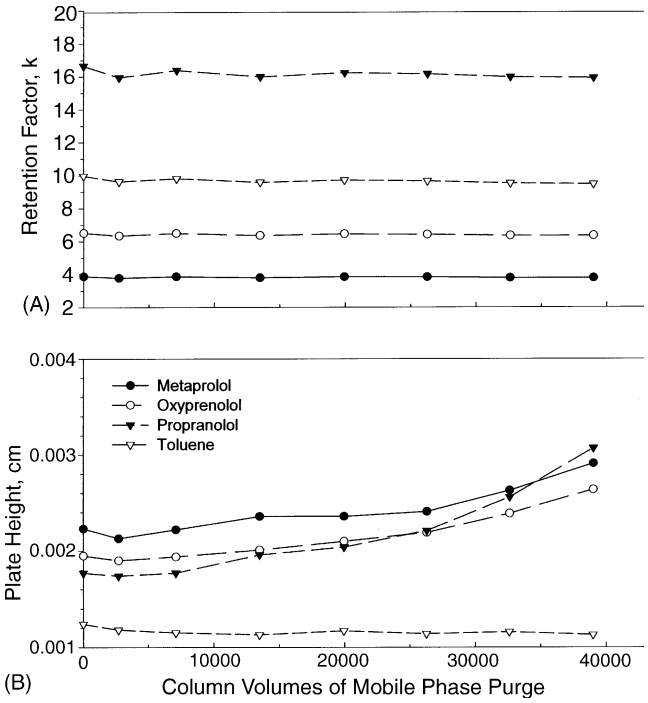

Fig. 10. Aging of bidentate-C18 column at pH 11. Column, 150 mm × 4.6 mm bidentate-C18; mobile phase, 55% methanol/50 mM 1-methyl-piperidine buffer, pH 11; flow rate, 1.0 mL/min; temperature, 23 °C; UV detector, 215 nm. (A) Retention factor *k* values and (B) plate height values. Reprinted with permission of *American Laboratory* from [29].

phate/acetonitrile mobile phase, the C18 bidentate packing shows a much lower rate of silica support dissolution compared to a densely reacted endcapped monofunctional C18 column. These data indicate that the bidentate-C18 stationary phase, especially when used with organic buffers, can be an effective approach for stable, efficient separations at high pH. The bidentate-C18 stationary phase is available in Extend® columns. This type of packing is an attractive alternative for separating basic compounds when low or intermediate pH

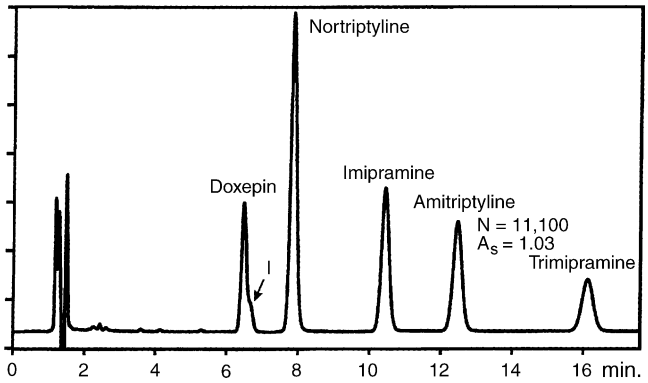

Fig. 11. Separation of highly basic tricyclic antidepressant drugs at pH 11.5. Column, 150 mm × 4.6 mm bidentate-C18; mobile phase, 75% methanol/25% 50 mM pyrrolidine buffer, pH 11.5; flow rate, 1.0 mL/min; temperature, 23 °C; UV detector, 215 nm. Reprinted with permission of *American Laboratory* from [29].

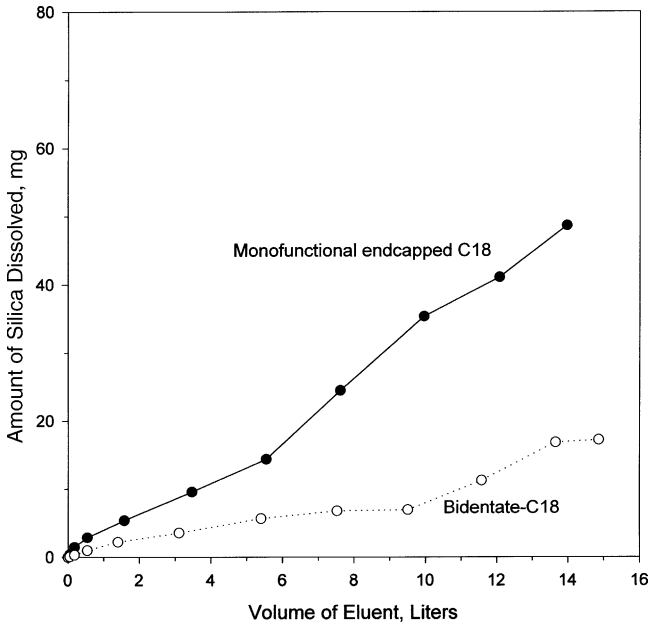

Fig. 12. Effect of bonded phase on silica support dissolution. Columns, 150 mm × 4.6 mm; eluent: 50% acetonitrile/50% 0.02 M potassium phosphate, pH 11.0; flow rate, 1.5 mL/min; temperature, 25 °C; dissolved silica measured by the silicomolybdate colorimetric method [40].

conditions do not provide the required selectivity or peak shapes.

5.3. Polar-embedded stationary phases

Traditional C18 and C8 alkyl stationary phases have widespread applications for reversed-phase separations. However, column packings with these phases have disadvantages for some applications; phase collapse can occur at high aqueous mobile phase concentrations, as indicated earlier. This effect results in lowered column performance and changes in retention. In addition, poor peak symmetry often occurs for some basic compounds, especially with intermediate pH mobile phases. As a result of these shortcomings, researchers have devised alkyl stationary phases with polar-linked or embedded polar groups for reversed-phase separations [47–49]. Columns have been developed with a variety of embedded polar groups such amides, carbamates, sulfamides and ureas. Packings with these stationary phases often exhibit good peak shapes and efficiencies for polar compounds, especially for ionizable solutes that can be particularly troublesome in some separations. Another advantage of most columns with embedded polar group stationary phases is that they can be used with highly aqueous mobile phases without the phase collapse problem. These columns often are useful for separations of compounds that are at least partially ionized at intermediate pH (6–9) to produce efficient peaks with good symmetry.

However, when used for low pH separations, particularly at higher column temperatures, Polar-embedded columns can show long-term stabilities that are less than desired

for some applications. As a result of this shortcoming, a study was completed in the author's laboratory to produce an embedded polar-alkyl stationary phase that would display good stability both with intermediate and low pH applications. The approach was to use sterically protecting groups to produce at alkyl-amide stationary phase that would resist hydrolysis at low pH, and at the same time, also provide good performance and stability with intermediate pH mobile phases. The structure of the developed diisopropyl-amide/C14 stationary phase is shown in Fig. 13. Ageing data for this sterically protected stationary phase and other polar-embedded columns at pH 7 and pH 2 is shown in Fig. 14. In Fig. 14A, stability data are shown for three embedded-phase columns continuously purged with acetonitrile/pH 7 phosphate buffer at 23 °C, as measured by plate heights for the basic drug, amitriptyline. After about 25,000 column volumes of purging, the diisopropyl-amide/C14 and the dimethyl-carbamate/C18 columns appear less affected than the dimethyl-amide/C18 column. The former columns also showed little effect after almost 40,000 column volumes of purging when the experiment was arbitrarily terminated. In Fig. 14B, the stability advantage for the sterically protected diisopropyl-amide/C14 column at low pH clearly is noted. The *k* value for toluene is unchanged after purging with almost 40,000 column volumes of methanol/pH 2 trifluoroacetic acid mobile phase at 23 °C. Contrarily, the dimethyl-amide/C18 column lost almost half of its retention after about 12,000 column volumes of purge, indicating extensive hydrolytic loss of the stationary phase. Therefore, the use of a sterically protecting groups again inhibited ligand hydrolysis as was also the case for the purely alkyl stationary phases discussed above. The amide/C14 stationary phase is available in Bonus® columns. Polar-embedded stationary phases are increasingly finding applications because of a combination of desirable properties for reversed-phase HPLC separations.

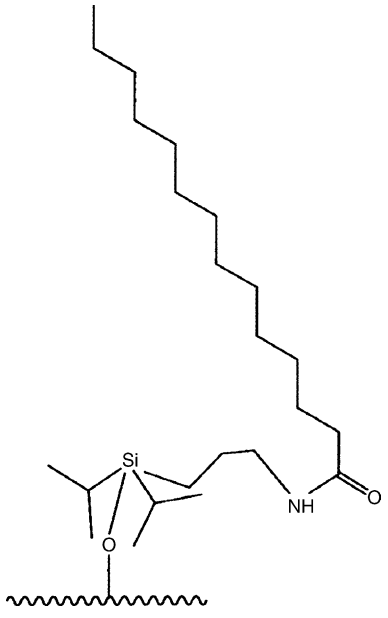

Fig. 13. Structure of the diisopropyl-C14-amide stationary phase. Reprinted with permission of *LC-GC* from [52].

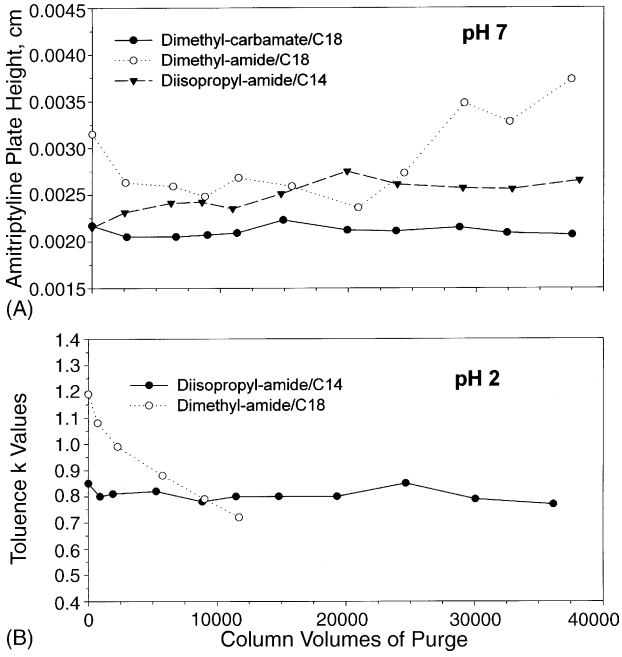

Fig. 14. Aging of amide-embedded alkyl columns at pH 7 and 2 (continuous non-recycled flow). (A) Columns, 150 mm × 4.6 mm; mobile phase, 40% acetonitrile/60% 25 mM sodium phosphate buffer, pH 7.0; flow rate, 1.5 mL/min; temperature: 23 °C. (B) Columns, 150 mm × 4.6 mm; mobile phase, 80% methanol/20% aqueous trifluoroacetic acid, pH 2.0; flow rate, 1.0 mL/min; temperature, 23 °C.

5.4. Hybrid organic–inorganic particles

Column packings based on sol–gel technology provide still another alternative for effective reversed-phase separations. This approach has been taken in an attempt to minimize some of the potential disadvantages of column packings based on silica particles (deleterious solute interaction with active silanol groups, dissolution at high pH, etc.). The first use of this technology for HPLC column packings was reported by Unger et al. [50], and a patent for preparing these particles was issued [51]. This technology largely stayed dormant until a group at Waters Associates developed a series of columns based on hybrid-organic chemistry in the late 1990's [53]. In hybrids, silica and organosilanes are combined to yield materials with properties that are somewhat intermediate between those of pure organic and pure inorganic substances.

These particles produce columns with good mechanical strength, efficiency, and good peak shape for basic compounds. One column type is based on material bonded with a trifunctional C18 silane. Another packing is made by modifying the surface of these particles with a monofunctional silane containing an embedded polar carbamate group. The advantage of these materials in producing good peaks shapes for basic compounds is shown in Fig. 15. It is claimed that

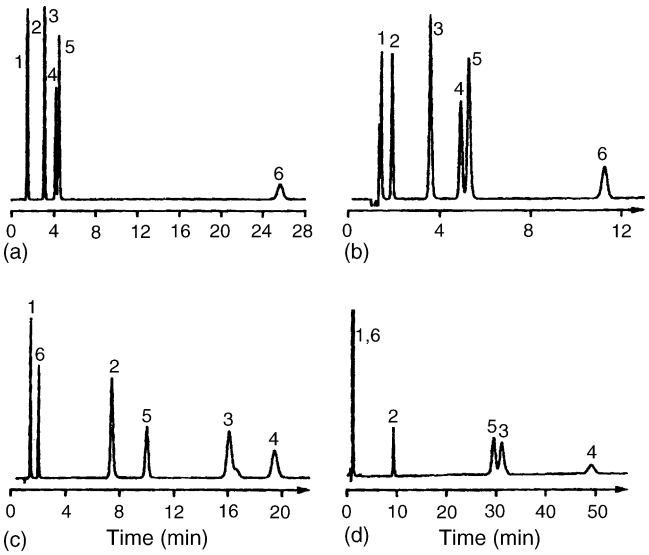

Fig. 15. Separations with hybrid organic–inorganic column packing with varying pH. (a) pH 2.5; (b) pH 5.0;(c) pH 8.0; (d) pH 10.6. Column, 150 mm × 3.9 mm, 5-μm XTerra® RP18; mobile phase, 65% 20 mM buffer/35% acetonitrile; UV detector, 210 nm for pH 2.5, 5.0, 8.0 and 230 nm for pH 10.6; flow rate, 1.0 mL/min; injection volume, 5 μL; peaks: (1) acetaminophen, (2) lidocaine, (3) doxepin, (4) imipramine, (5) nortriptyline, (6) ibuprofen. Reprinted with permission of *LC-GC* from [53].

the better peak shape for basic and other polar compounds is due to a significant reduced concentration of residual silanol groups on the particle surface. Note in Fig. 16, however, the XTerra® hybrid-organic particles show reduced stability with low pH mobile phases compared to a conventional C18 column (Inertsil® ODS-3) and a column with the sterically protected silane (StableBond®-C18). As with the study for the data of Fig. 8, these columns were continuously purged with the low pH mobile phase and the loss of stationary phase measured by periodically measuring the retention of a neutral hydrophobic test solute. On the other hand, columns of hybrid-organic and bidentate-C18 particles both demonstrate good stability and resistance to high pH mobile phases compared to a conventional C18 column, as shown in Fig. 17. These special stationary phases illustrate the high degree of sophistication and performance obtained in modern packings for reversed-phase HPLC.

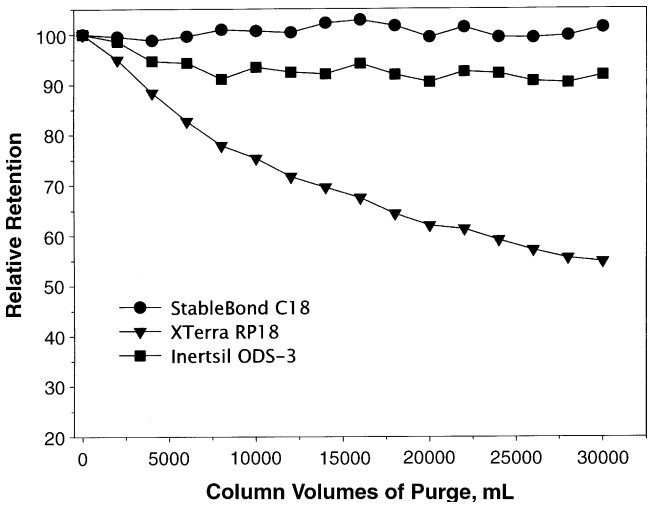

Fig. 16. Column stability at low pH. Columns as shown; purge solvent, 50% methanol/50% 1% aqueous trifluoroacetic acid, pH 1.3; flow rate, 3.0 mL/min; column temperature, 80 °C. Data courtesy of Murdoch University.

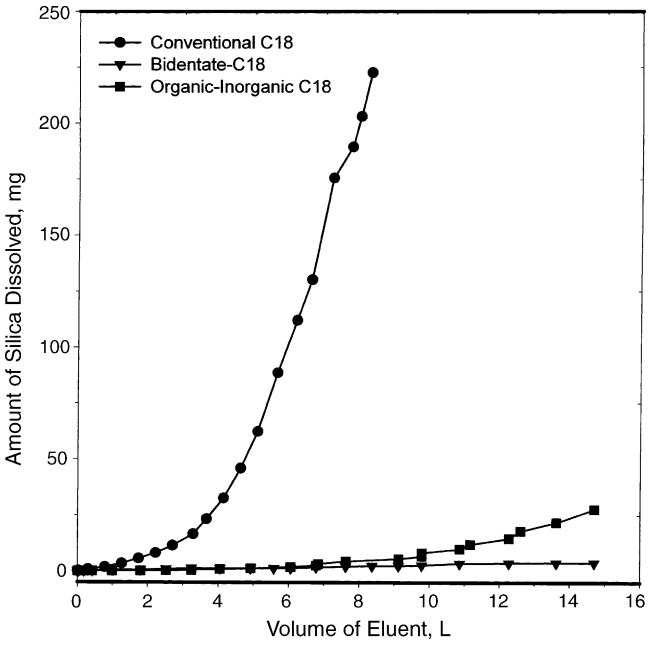

Fig. 17. Dissolution of silica supports at high pH. Columns: 150 mm × 4.6 mm conventional C18 (Agilent Technologies Zorbax® Rx-C18); 150 × 4.6 mm bidentate-C18 (Agilent Technologies Zorbax® Extend®-C18); hybrid organic–inorganic C18 (Waters Associates XTerra®-MS); mobile phase eluent, 50% methanol/50% 0.10 M sodium carbonate-bicarbonate buffer, pH 10.0; flow rate, 1.0 mL/min; temperature, 25 °C. Data from [59].

6. Stationary phase selectivity

Reversed-phase column packings with aliphatic C18 or C8 stationary phases allow adequate separations of many compounds of interest. Recently, however, there has been a proliferation of stationary phases with other functionalities for use in reversed-phase chromatography [54]. These functionally diverse stationary phases provide a different separation selectivity than traditional C18 or C8 stationary phases and are especially useful when the chromatographer is restricted to using a particular mobile phase such as in LC–MS studies. This approach is illustrated in Fig. 18 for the separation of a herbicide mixture using CN, phenyl and C8 bonded stationary phases. Varying separation selectivity by optimizing the mobile phase is the most powerful approach for optimizing separation resolution [10]. However, varying selectivity by changing the stationary phase also is an effective alternative, and this approach is being used more and more frequently [55].

The approach of changing separation selectivity with the stationary phase is especially useful in rapid, automatic method development systems for obtaining separations in combinatorial chemistry and related studies [56]. Here, columns with different stationary phases are rapidly scouted for desired reversed-phase separation selectivity using automatic column switching with a single mobile phase. Current approaches often use available stationary phases in somewhat a random manner in an effort to find a suitable separation. Typically, the stationary phases used are C18, C8, phenyl, cyano, and perhaps other functionalities. Different commercial C18 columns also often show different separation selectivities, usually based on differences in silica supports or stationary phase chemistry. Therefore, it is not uncommon to see C18 columns from different manufacturers used in this searching routine.

There is a more systematic approach that could be taken, providing columns with the required stationary phases are available. The opportunity is to expand this column optimization and switching concept by providing a range of columns with stationary phase functionalities that encompass the entire range of potential chemical selectivity for band spacing changes. Present available columns with stationary phase functionalities do not have this total capability, and other stationary phase functionalities are needed.

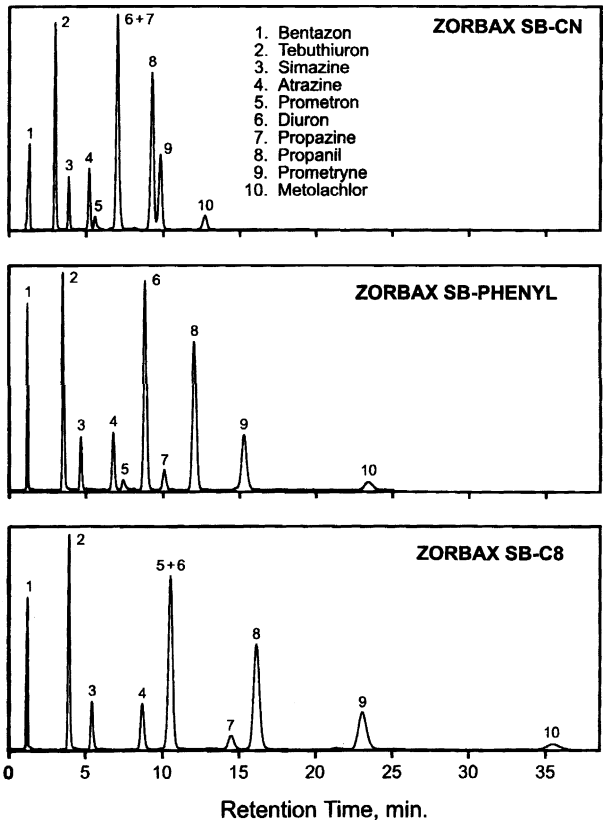

Fig. 18. Comparison of column stationary phase selectivity. Columns, 150 mm × 4.6 mm Zorbax® SB-C8, SB-Phenyl, SB-CN; mobile phase, 35% acetonitrile/65% water; flow rate, 1.0 mL/min; temperature, 22 °C; detector. Reprinted with permission of John Wiley and Sons from [57].

Table 2
Stationary phase selectivity

S. no.	Stationary phase functionality	Chemical interactivity with solute
1	Amino (tert.)	Basic interactions
2	C18 or C8	Hydrophobic, dispersion forces
3	CN (cyano)	Largely dipolar
4	Phenyl	π–π Interactions, dispersive
5	Amide	Basic and dipolar interactions
6	Ether	Largely basic, some H-bonding interactions
7	Nitro	Strongly dipolar
8	Diol	H-bonding, basic-acidic possibilities
9	Fluoroalcohol	Acidic interactions

Selection of optimum stationary phases for selectivity changes can be based on chemical interaction insights using the solvent selectivity triangle concept that has long been used to vary separation selectivity by changes in mobile phase constituents [57]. With the solvent selectivity triangle approach as a rough guide, several specific stationary phases likely would round out the entire reversed-phase chemical-interaction possibilities for greatest selectivity changes. Examples of these stationary phases and their interactive possibilities are given in Table 2.

Based on the solvent selectivity triangle, widest selectivity changes (coupled with experimental practicality) could involve the use of phases 1, 2, 3 (or 5), 7, 8, and 9 from Table 2. Fig. 19 shows a system of several stationary phase functional groups that results in an interactive separation selectivity (inner triangle) that encompasses a very wide range of chemical interactive possibilities. When coupled with a hydrophobic

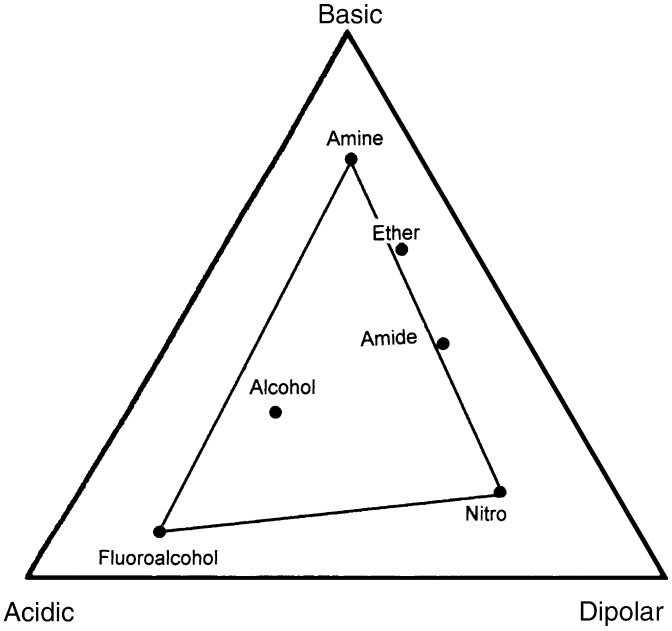

Fig. 19. Stationary phase selectivity triangle.

phase like C18 for dispersive interactions, the capability for band spacing changes with stationary phase changes is very large indeed. For practical applications, columns with these different stationary phases should exhibit *k* retention values within a factor of about two of each other in order to use a single isocratic mobile phase. For difficult separations, it would also be useful to consider connecting two columns with different stationary phases to obtain intermediate selectivity [55].

It should be noted that an accurate system for predicting the selectivity of reversed-phase columns has been described by Snyder et al. [58]. This approach gives great promise of allowing the rapid selection of appropriate column types to produce the desired selectivity for a particular separation. In addition, this approach allows the prediction of a different column that will produce a closely similar separation to the column currently in use, essentially guaranteeing that a column for a needed separation will always be available.

Using column selectivity as a means of obtaining the desired separation is especially attractive in situations where appropriate separating conditions must be rapidly obtained, such as in rapid LC–MS identification studies. Column selectivity often is required to gain adequate separations when the mobile phase cannot be altered and mobile phase selectivity changes are not possible. Finally, some detectors restrict the type of mobile phases that can be used; separation selectivity must be approached using different column functionalities since the mobile phase again cannot be varied.

7. Conclusions

These and other discussions show that current commercial stationary phases are rugged, efficient and repeatable for a wide variety of reversed-phase separations. Sophisticated stationary phases now are available for difficult separations with excellent peak shapes throughout the pH 1–12 range with appropriate mobile phases. Some examples from the author's and other laboratories attempt to present the rationale for development of certain stationary phases and provide a background for some of the advantages and limitations of these materials. Column stationary phase selectivity is a powerful alternative to changing the mobile phase to obtain desired separations. Expansion of available stationary phase functionalities will provide a wider range of column stationary phase selectivities for reversed-phase separations.

References

[1] J.F.K. Huber, J. Chromatogr. Sci. 7 (1969) 855.
[2] L.R. Snyder, J. Chromatogr. Sci. 7 (1969) 595.
[3] J.J. Kirkland, in: J.J. Kirkland (Ed.), Modern Practice of Liquid Chromatography, John Wiley and Sons, New York, 1971 (Chapter 5).
[4] L.R. Snyder, J.J. Kirkland, Introduction to Modern Liquid Chromatography, John Wiley and Sons, New York, 1974 (Chapter 8).
[5] W.R. Melander, C. Horvath, High Performance Liquid Chromatography, vol. 2, Academic Press, New York, 1980.
[6] C.J. Bossart, ISA Trans. 7 (1968) 283.
[7] W.A. Aue, C.R. Hastings, J. Chromatogr. 42 (1969) 319.
[8] I. Halasz, I. Sebestian, Angew. Chem., Intern. Ed. 8 (1969) 453.
[9] J.J. Kirkland, J.J. DeStefano, J. Chromatogr. Sci. 8 (1970) 309.
[10] L.R. Snyder, J.J. Kirkland, Introduction to Modern Liquid Chromatography, second ed., John Wiley and Sons, New York NY, 1979 (Chapter 7).
[11] I. Halasz, in: J.J. Kirkland (Ed.), Modern Practice of Liquid Chromatography, John Wiley and Sons, New York, NY, 1971 (Chapter 9).
[12] E.W. Abel, F.H. Pollard, P.C. Den, G. Neckless, J. Chromatogr. 22 (1966) 23.
[13] H.N.M. Stewart, S.G. Perry, J. Chromatogr. 37 (1968) 97.
[14] J. J. Kirkland, Paul C. Yates, U. S. Patent 3,722,181, 27 March 1973.
[15] J. J. Kirkland, U. S. Patent 3,505,785, 14 April 1970.
[16] J.J. Kirkland, Anal. Chem. 43 (1971) 36A.
[17] J.J. Kirkland, J.J. DeStefano, J. Chromatogr. Sci. 8 (1970) 309.
[18] D.C. Locke, J.T. Schmermund, B. Banner, Anal. Chem. 44 (1972) 90.
[19] O.E. Brust, I. Sebestian, I. Halasz, J. Chromatogr. 83 (1973) 15.
[20] K.K. Unger, N. Becker, P. Roumeliotis, J. Chromatogr. 125 (1976) 115.
[21] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, second ed., John Wiley and Sons, New York, 1997 (Chapter 5).
[22] J.J. Kirkland, J.W. Henderson, J. Chromatogr. Sci. 32 (1994) 473.
[23] M.J. Wirth, H.O. Fatunmbi, Anal. Chem. 65 (1993) 822.
[24] J.J. Kirkland, Chromatographia 8 (1975) 661.
[25] M. Przybyciel, R.E. Majors, LC-GC North Am. 20 (2002) 516.
[26] N. Nagae, T. Enami, S. Doski, LC-GC 20 (2002) 964.
[27] Z. Li, S.C. Rutan, S. Dong, Anal. Chem. 68 (1996) 124.
[28] J.J. Kirkland, J.L. Glajch, R.D. Farlee, Anal. Chem. 61 (1988) 2.
[29] J.J. Kirkland, J.D. Martosella, J.W. Henderson, C.H. Dilks Jr., J.B. Adams Jr., Am. Lab. 31 (1999) 22.
[30] K. Szabó, N.L. Ha, Ph. Schneider, P. Zeltner, E.Sz. Kováts, Helv. Chim. Acta 67 (1984) 2128.
[31] G. Schomburg, A. Deege, J. Köhler, U. Bien-Vogelsang, J. Chromatogr. 282 (1983) 27.
[32] R.V. Arenas, J.P. Foley, Am. Chromatogr. Lab. 26 (1994) 32CC.
[33] J. Li, P.W. Carr, Anal. Chem. 69 (1997) 2193.
[34] R.A. Henry, Am. Lab. 34 (2002) 18.
[35] J.L. Glajch, J.J. Kirkland, J. Köhler, J. Chromatogr. 384 (1987) 81.
[36] J.J. Kirkland, C.H. Dilks Jr., J.E. Henderson, LC-GC 11 (1993) 290.
[37] B.E. Boyes, J.J. Kirkland, Pept. Res. 6 (1993) 249.
[38] J.J. Kirkland, LC-GC 14 (1996) 486.
[39] B. Wheals, J. Chromatogr. 187 (1980) 65.
[40] R.K. Iler, The Chemistry of Silica, John Wiley and Sons, New York, 1979.
[41] J.J. Kirkland, M.A. Van Straten, H.A. Claessens, J. Chromatogr. A 691 (1995) 3.
[42] H.A. Claessens, M.A. Van Straten, J.J. Kirkland, J. Chromatogr. A 728 (1996) 259.
[43] J.J. Kirkland, J.W. Henderson, J.J. DeStefano, M.A. Van Straten, H.A. Claessens, J. Chromatogr. A 762 (1997) 97.
[44] J.J. Kirkland, M.A. Van Straten, H.A. Claessens, J. Chromatogr. A 797 (1998) 111.
[45] J.J. Kirkland, J.B. Adams Jr., M.A. Van Straten, H.A. Claessens, Anal. Chem. 70 (1998) 4344.
[46] J.J. Kirkland, J. Chromatogr. Sci. 34 (1996) 309.
[47] T.L. Ascah, B. Feibush, J. Chromatogr. 506 (1990) 357.
[48] J.E. O'Gara, B.A. Alden, T.H. Walter, J.S. Peterson, C.L. Niederlaender, U.D. Neue, Anal. Chem. 67 (1995) 3908.
[49] T. Czajkowska, M. Jaroniec, J. Chromatogr. A 762 (1997) 147.
[50] K.K. Unger, N. Becker, P. Roumeliotis, J. Chromatogr. 125 (1976) 115.

[51] K. Unger, J. Schick-Kalb, U. S. Patent 4,017,528, 12 April 1977.
[52] J.J. Kirkland, J.W. Henderson, J.D. Martosella, B.A. Bidlingmeyer, J. Vasta-Russell, J.B. Adams Jr., LC-GC 17 (1999) 634.
[53] Y-F. Cheng, T.H. Walter, Z. Lu, P. Iraneta, B.A. Alden, C. Gendreau, U.D. Neue, J.M. Grassi, J.L. Camody, J.E. O'Gara, R.P. Fisk, LC-GC 18 (2000) 1162.
[54] R.E. Majors, LC-GC 18 (2000) 586.
[55] J.J. Kirkland, B.E. Boyes, J.J. DeStefano, Am. Lab. 26 (1994) 36.
[56] D. Wu, M. Berna, G. Maier, J.J. Johnson, Pharm. Biomed. Anal. 16 (1997) 57.
[57] L.R. Snyder, J.J. Kirkland, Introduction to Modern Liquid Chromatography, second ed., John Wiley and Sons, NY, 1979 (Chapter 6).
[58] L.R. Snyder, J.W. Dolan, P.W. Carr, J. Chromatogr. A 1060 (2004) 77.
[59] H.A. Claessens, Trends Anal. Chem. 20 (2001) 563.