Separation efficiencies in hydrophilic interaction chromatography

Tohru Ikegami *, Kouki Tomomatsu, Hirotaka Takubo, Kanta Horie1, Nobuo Tanaka

Department of Biomolecular Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan

Available online 3 February 2008

Abstract

Hydrophilic interaction chromatography (HILIC) is important for the separation of highly polar substances including biologically active compounds, such as pharmaceutical drugs, neurotransmitters, nucleosides, nucleotides, amino acids, peptides, proteins, oligosaccharides, carbohydrates, etc. In the HILIC mode separation, aqueous organic solvents are used as mobile phases on more polar stationary phases that consist of bare silica, and silica phases modified with amino, amide, zwitterionic functional group, polyols including saccharides and other polar groups. This review discusses the column efficiency of HILIC materials in relation to solute and stationary phase structures, as well as comparisons between particle-packed and monolithic columns. In addition, a literature review consisting of 2006–2007 data is included, as a follow up to the excellent review by Hemström and Irgum.

© 2008 Elsevier B.V. All rights reserved.

Keywords: HILIC; Separation efficiency; Bare silica; Amino-silica; Amide-silica; Poly(succinimide)-silica; Sulfoalkylbetaine-silica; Diol-silica; CD-silica; Polymer monolithic columns; Silica monolithic column

Contents

1. Introduction .................................................. 475
2. Types of stationary phases for hydrophilic interaction chromatography .................................................. 475
3. Separation efficiencies of particle-packed hydrophilic interaction chromatography mode columns .................. 476
   3.1. Columns packed with bare silica .......... 476
   3.2. Columns packed with amino-modified silica ................................................. 479
   3.3. Columns packed with amide-modified silica ................................................. 483
   3.4. Columns packed with poly(succinimide)-modified silica .................. 485
       3.4.1. PolyHydroxyethyl A .......................................................... 486
       3.4.2. PolyGlycoplex .............................................................. 487
       3.4.3. PolyCAT A and poly(Sulfoethyl A) ................................................. 487
   3.5. Columns packed with sulfoalkylbetaaine-modified silica .......... 488
   3.6. Columns packed with cyano- and diol-modified silica ......................... 490
   3.7. Columns packed with cyclodextrin-modified silica .......................... 491
   3.8. Columns packed with other functionality-modified silica, and polymer particles ................................................. 493
4. Separation efficiencies of monolithic hydrophilic interaction chromatography mode columns .................. 493
   4.1. Polymer monolithic columns ..................... 493
   4.2. Silica monolith columns ............................................. 496
5. Conclusion .................................................... 499
Acknowledgements ........................................... 501
References ..................................................... 501

* Corresponding author. Tel.: +81 75 724 7801; fax: +81 75 724 7710.
E-mail address: ikegami@kit.ac.jp (T. Ikegami).

1 Present address: Eisai Co. Ltd., Japan.

0021-9673/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2008.01.075
1. Introduction

Recently, literature and research on hydrophilic interaction chromatography (HILIC) have been increasing drastically, along with various stationary phases developed for HILIC. HILIC is a kind of normal-phase liquid chromatography (NPLC), and has attracted the attention of researchers that study the separation of polar compounds in a wide variety of scientific fields. In HILIC mode, a mixture of water and organic modifiers in most cases acetoniitrile (MeCN) is employed with a polar stationary phase. Structural variations in HILIC type stationary phases are wider than those found in reversed-phase applications. In addition to classical bare silica andaminopropyl-bonded silica, silica gels modified with many polar functionalities such as amide, diol, cyano, derivatives of poly(succinimide), sulfoalkyl-betaine, cyclodextrin are applicable to HILIC mode separation. Polymer-based stationary phases such as sulfonated polymers, and diol-containing polymers can also be used. HILIC can be an alternative to reversed-phase chromatographic separation for polar compounds using isocratic and gradient elutions. Both particle-packed columns and monolithic columns have been used in HILIC. HILIC is suitable for electrospray ionization (ESI)-mass spectrometry (MS), because of the compatibility of the aqueous organic mobile phase to ESI-MS, which is a very powerful tool to detect and identify a wide range of polar compounds.

There are many examples of HILIC applications for the analysis of small polar molecules including biomarkers, nucleosides, nucleotides, carbohydrates, amino acids, peptides and proteins, that contribute to the pharmaceutical chemistry, agricultural and food chemistry, medicinal chemistry, proteomics, metabolomics, and glycomics. However, it seems that only limited attention has been paid to the separation efficiencies in HILIC. This review provides a perspective of HILIC type stationary phases, and discusses their separation efficiencies and retention tendencies.

First, various stationary phases for HILIC are introduced, and separation efficiencies of columns packed with particles, and suitable analytes for each stationary phase are discussed. Then, the same discussion is applied to monolithic columns to reveal the differences in these types of support for stationary phases.

2. Types of stationary phases for hydrophilic interaction chromatography

Although the acronym HILIC was already first suggested by Alpert in 1990 [1], the number of publications regarding HILIC has increased substantially since 2003, as outlined in the well-constructed review by Hemström and Irgum [2]. NPLC has been used widely to separate various compounds from non-polar compounds to highly polar compounds after chromatography was first introduced as a method of separation science [3,4]. NPLC consists of polar stationary phases, mainly bare silica or alumina, and less polar mobile phases formed by single organic solvents or mixed organic solvents, and has been the chromatographic mainstream for about 70 years [5]. The chemical modification of silica gels using chemical reactions began in the 1960s [6]. Reactions between alkylsilyl chlorides and silanol groups (Si–OH) is the most common method, and was reported in 1970 [7].

The first example of polar functional groups seems to be published in 1967 by Horvath et al., where glass beads have coated with polyethylene imine [8]. At the early stage of the development of stationary phase, the preparation of packing materials containing polar functional groups such as amino, sulfonic acid, carboxylic acid, ketone, and nitrile, for LC were reported by Halasz et al. in 1973 [9]. The authors employed chemical reactions between Si–Cl bonds in silica (prepared by chlorination of Si–OH bonds using thionyl chloride, SOCl₂) and various amines that possessed the above-mentioned functional groups. These products were useful as polar bonded phases, with intermediate polarities between those of reversed-phases and those of bare silica gels. In the same year, Scott and Grushka reported the preparation of a “polar bonded” phase, in which silica particles were modified first with 1-trimethoxysilyl-2-(4-chloromethylphenyl)ethane, and second with oligomers of glycin [10]. A similar stationary phase was reported by the same group to show that the height equivalent of theoretical plate, H of the column was 0.4–18 nm for a linear velocity at 3.7 mm/s [11].

The first generation of HILIC mode separation started in 1975. Linden et al. separated carbohydrates by an amino-silica phase, Bondapak (Waters, Milford, MA, USA) in a mixture of MeCN and water (75:25) [12]. Generally, amino-silica phases are prepared by reacting trialkoxysilanes, RSi(OR)₃, where R and R’ stand for aminoalkyl and alkyl groups, respectively, on silica and they can form polymeric phases with a certain thickness of the stationary phase to incorporate water. Before the introduction of HILIC separation, ion-exchange resin was used to separate carbohydrates. The formation of a water-enriched layer on the surface of highly polar stationary phases was reported in 1967 [13]. The existence of a water-enriched layer on the polar stationary phases under aqueous conditions, and a partitioning equilibrium of analytes between a mobile phase and the “wet” stationary phase, are a basic, and an accepted mechanism for HILIC [1,2]. Alpert suggested this mechanism referring to research by Orth and Engelhardt [14], and the partitioning mechanism has been recently substantiated [2].

The next generation of stationary phases for HILIC used diol- and amide-silica. The diol silica column has been used mainly for the separation of proteins [15,16]. According to the Tosoh, producer of TSKgel Amide-80, this amide-silica column has been available since at least 1985 [17]. This particular phase is described as “consisting of non-ionic carbamoyl groups that are chemically bonded to the silica gel”, but this phase is mainly called as an amide bonded silica. The aforementioned phase was used for the multidimensional mapping of oligosaccharides with an octadecyl silica (ODS) phase for twodimensional mapping, and with ODS and diethylaminoethyl (DEAE) phases for three-dimensional mapping [18–21]. After the application of this phase to the separation of peptides by Yoshida [22], the amide-silica phase became widely used in HILIC.
Stationary phases reported by Alpert contained polymeric structures of poly(succinimide) derivatives [1]. These columns are manufactured by PolyLC (Columbia, MD, USA), and due to differences in functional groups, different phases such as PolyGlycoplex, PolySulfoethyl aspartamide, and PolyHydroxyethyl aspartamide are available [23]. These columns have been widely used to separate various highly polar compounds, and recently, columns packed with 3 μm particles have been introduced to provide better separation [24].

Recently, a HILIC phase prepared by graft polymerization to incorporate 3-sulfopropyl(dimethylalkylammonium) inner salts, i.e. sulfoalkylbetaine functional groups onto silica and polymer particles has released. Sometime, this phase is called as a zwittrionic phase. These types of columns are available from SeQuant (Ume, Sweden), as ZIC-HILIC columns [25]. Originally, this phase was prepared for cation-exchange chromatography, and earlier reports described the separations of inorganic salts and proteins in fully aqueous mobile phases [26–28]. This phase is widely accepted by many researchers, and used often in more recent publications.

There are several stationary phases for HILIC mode separation in addition to the above-mentioned phases, and they all possess highly polar functionalities, such as cyano, hydroxy, diol, carbohydrates and so on, that attract water molecules from the mobile phase to form water-enriched layers. These layers seem to be important and facilitate partitioning equilibria between stationary and mobile phases.

In almost all published articles on HILIC mode separations, the elution order of analytes differs from their separation by RPLC, and analyte retention times are described. However, information on column separation efficiencies is still limited. One of the reasons could be the use of gradient elutions in their studies.

We would like to discuss the separation efficiencies of various HILIC phases in this review. Simple applications covered by two HILIC reviews [2,29] are omitted, and only literature published between 2006 and 2007 were added to this review. Therefore, articles containing isocratic separations were mainly surveyed; however, HILIC studies used in applications including proteomics, metabolomics, glycomics, medicinal science, agricultural and food chemistry reported within 2006–2007 are briefly mentioned. Comparisons of separation efficiencies between particle-packed and monolithic columns are discussed herein. Since the separation efficiency of each peak was estimated manually from the peak width at half height, considerable errors can be included in the calculation of separation efficiencies, N, and the column dead time, t₀ was calculated by an estimation of column porosity, ε = 0.65 if there is no peak of the t₀ marker. Plate heights depend on a variety of factors of dispersion parameters corresponding to each part of HPLC systems, also on linear velocity of the eluent. Though data employed herein might be not under control of extra-column effects (extra-column volumes at injection, detection, and tubing, and sample matrix), the chromatograms can be regarded as not the worst results.

3. Separation efficiencies of particle-packed hydrophilic interaction chromatography mode columns

Stationary phases for reversed-phase separation are modified with silanes that possess alkyl chains, between C4 and C30, and mainly C18. Not only “pure” alkyl groups, but also partially polar alkyl groups are employed to increase the retention of polar compounds. This approach does not seem to be so effective for the separation of highly polar compounds. Compared to stationary phases for RPLC, columns for HILIC separation have a wider variety of functional groups and are described as follows. All polar functionalities including anionic and cationic moieties would be able to form the water-rich regions on the surface of packing materials, and they are useful for HILIC separation. However, due to the wide variety of functionalities of stationary phases and target analytes, i.e. polar compounds, the knowledge on the applicability seems to be still limited. In this chapter, the fundamental characterization of columns such as plate number, N, and theoretical plate height, H, of stationary phases for HILIC is discussed with some emphasis on retention factors.

3.1. Columns packed with bare silica

Bare silica is a classical phase in NPLC, and that is still widely used in recent studies of HILIC, and especially with HILIC-MS systems. Naidong reviewed the separation–detection of bioanalytical samples by HILIC-MS/MS [29]. The mobile phase in HILIC, mainly a mixture of MeCN and aqueous buffer, is suitable and compatible for electrospray ionization source that are interfaced to MS systems. Bare silica columns employed for HILIC separations include, Betasil (Thermo Scientific, Waltham, MA, USA) [30], Hypersil (Thermo Scientific) [31], Inertsil (GL Science, Tokyo, Japan) [32], Kromasil (EKA Chemicals, Göteborg, Sweden) [33], Supelcosil LC-Si (Sigma–Aldrich, St. Louis, MO, USA) [34], Allitima (Alltech, Nicholasville, KY, USA) [35], Spheri5 Silica (Alltech) [36], and Supersphere Si (Trentec, Gerlingen, Germany) [37]. There are many publications on the separations using Atlantis (Waters) [38–46]. At the time of the review by Naidong, the particle size for all these columns was 5 μm [29], but a 4 μm particle-packed column [37], and 3 μm particle-packed columns were used in recent studies [38,41–46]. Recently, an Acquity column packed with 1.7 μm particles was released from Waters for HILIC mode ultrahigh-performance liquid chromatography (UPLC) separations [47,48]. This column was applied to a LC–MS system as a complementary separation method for RPLC–MS and a gas chromatography–time-of-flight-MS (GC–TOF-MS) to comprehensively analyze metabolomes in human urine for the diagnosis of kidney cancer [49]. MS systems and nuclear magnetic resonance (NMR) spectroscopy can be useful detectors for HILIC mode separation. For HILIC-NMR/MS systems, water and formic acid were changed to D₂O and DCO₂D, but CH₃CN was used as an organic modifier [37].

Almost all the literature on HILIC using bare silica columns employs gradient elutions, and it is difficult to evaluate their separation efficiencies. Monolithic silica columns, Chromolith-
Si (Merck, Darmstadt, Germany) were used in a few publications [50] and the use of monolithic silica capillary columns in HILIC was recently reported [51,52]. Monolithic silica columns are discussed in Section 4.2.

Bare silica columns were used to separate small polar compounds, especially pharmaceuticals, and examples of separations of carbohydrates, peptides or proteins using HILIC are limited. HILIC-MS/MS systems were employed for the analysis of atenolol [31], acyclovir [36], propofol and its glucuronide derivatives [43], gabapentin and metformin [41], glutathione and its derivatives [39,44], iminocitadine triacetate [38], zanamivir [45], levofloxacin [42], carvedilol [46], omeprazole and its derivatives [30], and spectinomycin [35], while UV–Vis detectors were also used for the analysis of epirubicin and its analogues [33], carnosine, anserine, balenine, creatine, and creatinine in meat [40], pseudoephedrine, diphenhydramine, and dextromethorphan [34].

The retentivity and the separation efficiency of a bare silica, Atlantis HILIC Silica was compared with three HILIC columns, an amino-silica, YMS-Pack NH2, an amide-silica, TSKgel Amide-80, and a sulfoalkylbetaine-modified silica, ZIC-HILIC, using five aromatic carboxylic acids and five nucleic bases and nucleosides [53] as shown in Fig. 1. Each column was 250 mm × 4.6 mm I.D., 5 μm. The column dead time, t₀ for the flow rate 1.5 mL/min, was estimated to be 1.8 min assuming a porosity of 0.65. For the analytes employed, k and H were compared for four different stationary phases as shown in Fig. 2. The Atlantis HILIC Silica phase showed the smallest retention for the 10 test samples, and the highest separation efficiency around N=25 000 (H = 10 μm).

As shown by Olsen, three bare silica columns from different manufacturers, Zorbax SIL, Nucleosil, and YMC silica provided different retentivities, efficiencies, and peak shapes for nucleosides [54]. As shown in Fig. 3, peak tailing was observed in
the case of Nucleosil and Zorbax SIL for pyrimidines, while no significant tailing was found for purines.

This kind of difference is also known for RPLC columns [55,56], and recently reviewed by Neue recently [57], but there is limited detailed research on HILIC columns.

Compounds 1–4 (Fig. 4) were not separated on the Kromasil KR100-5SIL phase (250 mm × 4.6 mm I.D., 5 μm) and were eluted as a non-retained peak in MeOH, but partially separated in THF, and completely separated in MeCN as shown in Fig. 5A. The separation efficiency in MeCN was estimated as \( N = 10000, H = 25 \mu m \). Among three different buffer solutions of pH 5.0, 4.2, and 2.9, the most acidic conditions afforded the best separation efficiencies of \( N = 10000–12000, H = 21–25 \mu m \), while \( N = 6000–9000, H = 28–42 \mu m \) was obtained at pH 5.0 [33].

Naidong compared the separation efficiency of a HILIC column, Betasil Silica with those of two RPLC columns, Inertsil ODS2 C18 and Chromolith SpeedROD RP-18e by the backpressure-flow rate plot and the Van Deemter plot as shown in Fig. 6 [29]. The Betasil column showed an intermediate efficiency between the Inertsil and the Chromolith RP columns. The optimal flow rate for the Betasil column was 1–2 mL/min, (linear velocity was estimated as \( u = 1.6–3.2 \text{ mm/s} \)) and the plate height was around 20 μm for fluconazole (\( k = \text{ca.} 2 \)).

Phenyl-β-d-glucuronide (1), and glucuroconjugated metabolites (propofol-glucuronide (2), 1-quinol-glucuronide (3), and 4-quinol-glucuronide (4)) in Fig. 7 were analyzed on an Atlantis HILIC column (150 mm × 2.1 mm I.D., 3 μm) with an Atlantis HILIC guard cartridge (10 mm × 2.1 mm I.D.) in a mixture of MeCN/water/100 mM NH4OAc buffer (pH 5) (87/1/12, v/v/v) at 25 °C and at a flow rate of 200 μL/min [43]. The efficiency was estimated as \( N = 3500–4500, H = 33–43 \mu m \), and these values were apparently worse than expected separation efficiency by RP columns packed with 3 μm particles. The existence of isopropyl group in 2–4 (Fig. 7) led to a decrease in retention.
of these glucuronides, compared to phenyl-β-D-glucuronide (1) which showed a typical retention tendency in HILIC.

The user of this type of column must keep in mind that bare silica phases sometimes undergo severe irreversible adsorption of analytes on the columns, as reported by Li and Huang: epirubicin was absorbed on the column under neutral or weakly acidic conditions [33].

### 3.2. Columns packed with amino-modified silica

Many column manufactures supply 3-aminopropyl bonded silica phases for NPLC and HILIC modes. For example, columns used for HILIC are Bondpak AX (Waters) [12], μ-Bondapak Carbohydrate (Waters) [58], Spherisorb NH2 (Waters) [59], YMC-Pack NH2 (YMC, Kyoto, Japan) [53,59], Luna Amino (Phenomenex, Torrance, CA, USA) [60], Hypersil APS2 (Thermo Science) [61,62], Zorbax NH2 (Agilent, Santa Clara, CA, USA) [63], apHera NH2 (ASTEC, Whippany, NJ, USA)) [64], Alltima Amino (Alltech) [65], PALPAK Type N (Takara Bio, Otsu, Japan) [66], and Micropellicular [67]. YMC Pack Polyamine II (YMC) contains polyamine moieties on silica surfaces [59]. Separation targets of amino-silica phases are carbohydrates [12,58,62,67,68], amino acids [64], peptides [59], carboxylic acids and nucleosides [49,50,60], tetracycline antibiotics [61], oxytocin analogues [59], and 2-amino-2-ethyl-1,3-propanediol (AEPD) and tromethamine [63].

By comparing the retentivities of the four columns in Fig. 1, [53], a 250 mm amino column, YMC-Pack NH2 gives in the range of 5000–17 000 (i.e., \(H = 15–50 \mu m\)) for benzoic acid derivatives, nucleic bases, and nucleosides peaks with \(k\) values.
Fig. 5. Effect of organic modifier on the separation of epirubicin and its analogues. (A) Column: Kromasil KR100-5SIL (250 mm × 4.6 mm I.D., 5 μm); mobile phase: sodium formate buffer (20 mM, pH 2.9) modified with various organic solvents (10:90, v/v). (B) Comparison of the chromatographic separation of epirubicin and its analogues at different buffer pH as shown in the Figure. Compounds: (1) epidaunorubicin; (2) daunorubicin; (3) epirubicin; (4) doxorubicin.

The retention tendency of the amino column is characteristic; for aromatic carboxylic acids, retention factors, $k$ are larger than in the other three columns, and the separation efficiency for these compounds was around $N = 5000$, which was less efficient than the other three columns as shown in Fig. 2. On the other hand, the amino column shows a similar separation efficiency to the ZIC-HILIC column for nucleic acids and nucleosides. By studying on the relationship between $k$ and the MeCN concentration in the mobile phase, the amino column was found to have significantly larger retention for salicylic acid even in 65% MeCN, while the $k$ value decreased drastically in 65% MeCN on other columns as shown in Fig. 8. For cytosine, the same study showed that the amino column has a similar tendency to other columns. By the Van’t Hoff plot for acetylsalicylic acid and salicylic acid, only the amino column showed positive retention enthalpy.

The retention time of salicylic acid on the amino phase decreases when a mobile phase with a lower MeCN concentration is used, or an increased salt concentration in the mobile phase is employed. These findings suggest that the hydrophilic partition interaction and the ion-exchange interaction exist between analytes and the amino stationary phase for analytes possessing acidic functionalities. For the carboxylic acids, the amino-silica column could generate only low efficiencies.
of $N = \text{ca.} 5000$, $H = 50 \mu m$. In addition, salt concentration and buffer pH effects indicated that the amino-silica phase was quite sensitive to these conditions in the case of analytes that possessed $pK_a$ values in the pH range of the mobile phase employed.

Although amino-silica phases are useful separation media in NPLC and HILIC, the reactive nature of amino functional groups can cause several severe problems from an analytical point of view. For example, the irreversible adsorption of analytes on the phase is often discussed: with aldehyde compounds, the primary amino group in the amino-silica phase forms a Schiff base, which results in the adsorption of carbonyl compounds, and simultaneously changes the functionality of the stationary phase. Thus, it is easy to understand why amino-silica phases were not chosen to separate carbohydrates in more recent publications, though nobody ever produced any evidence that this occurs under HILIC conditions with reducing sugars.

Oyler et al., employed amino-silica columns to separate cyclic peptides, atosiban diastereomers. HILIC on a Spherisorb NH2 (250 mm × 4.6 mm I.D., 5 μm) separated ten atosiban diastereomers with a good separation efficiency, $N = 7000–11000$, $H = 23–35 \mu m$ except for one diastereomer at a flow rate of 0.5 mL/min [59]. However, under a faster flow rate, 1.2 mL/min, resolution of these compounds turned into worse, apparently. Separation of the same peptides on a YMC Polyamine II column resulted in worse separation of the analytes compared to the Sperisorb NH2 column [59]. Among amino-silica phases, the Zorbax NH2 column was tested over a period of several weeks, to take eleven separate runs with 1800 column volumes of the mobile phase. After that, theoretical plate numbers for each peak were greater than 94% of their original values. Moreover, retention factors and selectivities were hardly affected [54]. A very slow equilibrium when substituting a citrate buffer by an acetate buffer on the amino-phase was also reported. It took several hundred column volumes of mobile phase to stabilize the baseline monitoring [61]. Each brand of amino-silica columns showed significantly different retention factors and selectivities for certain compounds. Comparisons of separation characteristics for three different amino-silica columns are shown in Fig. 9 [54].

Contrary to the findings of Olsen, the self-decomposition of the stationary phase by amino groups in aqueous eluents was shown, [69,70] and peak shape deterioration using HILIC were also demonstrated [71]. Due to the self-decomposition reaction, the use of amino-phase in HILIC-MS causes higher backgrounds with ion suppression for certain analytes, and these phenomena are not conductive to the sensitive detection of analytes [71]. When analyzing ginsenosides by LC–MS, these analytes were detected as [M+Na]+ or [M+138]+ adducts [72]. The latter cation was caused by the addition of [NH$_3$CH$_2$HSi(OH)$_3$]$^+$, which was caused by the stationary phase. There are several reports on amino-phases based on different silica supports. Zirconia particles immobilized with amino functionalities were used in HILIC to separate carbohydrates under a gradient elution. Six analytes containing $N$-acetyl-chitooligosaccharides and maltoligosaccharides were completely separated [73].

Guo and Huang analyzed 2-amino-2-ethyl-1,3-propanediol (AEPD) and tromethamine using three amino-silica columns (150 mm × 4.6 mm I.D., 5 μm). The separation efficiency of the system for these amines in aqueous MeOH was estimated as 2800 ($H = 54 \mu m, k = 0.2$) and 3600 ($H = 42 \mu m, k = 0.3$), while those in aqueous MeCN was 3700 ($H = 41 \mu m, k = 1.2$) and 6100 ($H = 25 \mu m, k = 2.5$). The separation by amino-silica phases, Zorbax NH2, YMC-Pack NH2, and Nucleosil NH2 resulted in significantly different chromatograms, although $k$ values of the three chromatograms were almost the same. Slightly leading peaks were obtained by YMC-Pack NH2 and Nucleosil NH2, while Zorbax NH2 gave good shaped peaks with the highest separation efficiency of $N = 4600$ and 7300 for AEPD and tromethamine [63].

Amino-silica phases prepared from 3 μm particles were employed for the separation of tetracycline antibiotics (Figs. 10 and 11). For the most retained solute, oxytetracycline, in 85% MeCN, $N = 3800$ ($H = 13 \mu m$) was obtained on an amino-column, ThermoHyperSil APS2 (50 mm × 4.6 mm I.D.) with a same efficiency for a short-time retention factor stability test [61]. After 5 weeks of daily use, theoretical plate numbers were re-evaluated to show they were greater than 90% of the initial separation efficiency without change in retention factors [61].
Fig. 9. The separation of 2-amino-2-ethyl-1,3-propanediol (AEPD) and tromethamine. (A) Column: Zorbax NH2, 150 mm × 4.6 mm I.D., 5 μm; Mobile phase: (A) MeCN/water (80/20), (B) MeOH/water (80/20) and (C) MeOH/water (90/10); Column temperature: 25 °C; flow rate: 1 mL/min; samples: (1) AEPD, (2) tromethamine; Injection volume: 50 μL. (B) Columns: (A) Zorbax NH2, (B) YMC-Pack NH2, (C) Nucleosil NH2 column; mobile phase: MeCN/water (80/20, v/v). Other conditions are the same to (A) [63].

A HILIC-ESI-MS/MS using an amino-silica column, Aste-capHera NH2 (150 mm × 4.6 mm I.D., 5 μm) was employed to analyze methionine, threonine, and taurine with a 60% MeOH as the mobile phase. A separation efficiency of $N = 1500–2600$ ($H = 58–100$ μm) was generated at a flow rate of 0.6 mL/min ($u = 3$ mm/s) with a split ratio of 1/6 [64]. In a capillary electrochromatography, CEC mode, a polymer monolithic amino column (280 mm × 100 μm I.D.) was employed to separate bile acids and their conjugates [74] and saccharides [68] using an ESI-Ion trap (IT)-MS as a detector. The separation efficiency is very high compared to the above-mentioned separations, and is discussed later, in Section 4.1.
3.3. Columns packed with amide-modified silica

HILIC type columns packed with amide-bonded silica particles are one of the most popular separation media. Among these, TSKgel Amide-80 from Tosoh (Tokyo, Japan) is widely used for the separation of monosaccharides [75], peptides [22,76], 2-pyridylamino (PA)-sugar [77], ammonium [78], paralytic shellfish poison (PSP)-toxin [79], and amino acids [80]. A TSKgel Amide-80 (250 mm × 2.0 mm I.D., 5 μm) was applied to a metabolomics study of pumpkin phloem exudates with a HILIC-ESI MS system [81].

A column, GlycoSep N from ProZyme (San Leandro, CA, USA) was also employed to separate carbohydrates including oligosaccharide [82], 2-aminoacridone (AA) derivatized carbohydrates [83], and derivatized glycan [84]. Due to the chemical stability, i.e. low reactivity of the amide moiety, this type of columns was less sensitive to the pH of the mobile phase, and the irreversible absorption of analytes to the column was limited [85]. The Amide-80 column was used for the separation of peptides by Yoshida [22,85], and two-dimensional or three-dimensional mapping of oligosaccharides [18–20], however, descriptions in these reports were limited to the retention times of peptides or retention factors of oligosaccharides, and unfortunately, the separation efficiency of the column was not mentioned.

Yanagida et al. developed a method to separate oligomeric proanthocyanidins, polyphenolic catechins contained in an apple...
using a HILIC-MS system. The logarithm of the octanol–water partition coefficient, log \( P \) values for standard samples of polyphenolic catechins (Fig. 12) were plotted against each degree of polymerization \( D_p \), and the logarithm of retention factors, log \( k \) on a TSKgel Amide-80 column as shown in Fig. 13 [86]. It was apparent that compounds with larger \( D_p \) values simultaneously resulted in the higher hydrophilic nature of polyphenols, and larger log \( k \) values. Numbers of hydroxyl groups in analytes were plotted against log \( k \) on amide-silica as shown in Fig. 14 to show there was a linear relationship between the two parameters. Still there are few examples of the structure–retention relationship studies using HILIC.

The complete separation of tetramers and pentamers of proanthocyanidins would have been difficult under the conditions in the above-mentioned isocratic separation, because of the significantly broadened peak of the tetramer and the pentamer. The separation efficiency of peaks 4a and 5a in Fig. 15 were estimated as to be \( N = 1000 \) for both, with \( k = 7 \) (4a) and 17 (5a), respectively [86].

Simultaneous analyses of mono- and oligosaccharides by HPLC using a TSKgel Amide-80 column with a post-column fluorescence derivatization showed detection limits of \( \beta \)-glucose and maltohexaose as 1.78 and 2.59 pmol [87]. Karlsson et al. demonstrated the separation of native monosaccharides on a TSKgel Amide-80 column, followed by ELS detection, with detection limits of 0.3–0.5 \( \mu \)g for each saccharide [88]. N-Acetylgalactosaminic acid (Neu5Ac) and glucuronic acid (GlcA) peaks were completely separated from other analytes, and their separation efficiency was estimated to be \( N = ca. 2000 \) (\( H = 125 \mu \)m) at 40 \( ^\circ \)C, and \( N = 3000 \) (\( H = 83 \mu \)m) for Neu5Ac, and \( N = 2400 \) (\( H = 104 \mu \)m) for GlcA at 60 \( ^\circ \)C, respectively as shown in Fig. 15.

Wuhrer et al. prepared a capillary column packed with amide silica (100 mm \( \times \) 75 \( \mu \)m I.D., 5 \( \mu \)m) to evaluate a nano-LC-ESI-MS system for the analysis of underivatized oligosaccharides. Due to the lower degree of sample dilution of the capillary column, very high sensitivity of the saccharides at low-femtomole level was achieved [89]. A similar column was also employed for the analysis of glycosilated proteins [90].

The TSKgel application reported that an isocratic separation by a TSKgel Amide-80 (250 mm \( \times \) 4.6 mm I.D., 5 \( \mu \)m) using a MeCN–water (75:25) mixture gave 80 000 theoretical plates per meter for mannitol at room temperature, at the optimal flow rate, between 0.15 and 0.3 mL/min using a reflective index (RI) detector [17]. By a rough estimation, a 25 cm column can provide \( N = 20 000 \) with \( H = 12.5 \mu \)m. Under a practical flow rate of 1.0 mL/min (\( u = 1.5 \) mm/s), the \( H \) value reached around 20 \( \mu \)m, and thus \( N = 12 000 \). The temperature effect on the separation efficiency of the Amide-80 column was also studied. At 80 \( ^\circ \)C, the separation efficiency against a flow rate for a non-reducing carbohydrate, mannitol showed small differences in the range from 0.2 to 2.0 mL/min (\( u = 0.30–3.0 \) mm/s), while in the case of a reducing carbohydrate, \( \beta \)-glucose, the separation efficiency was significantly lost under practical separation conditions (flow rate at 1.0 mL/min) as shown in Fig. 17A.

Very recently, Tosoh released a new column, TSKgel Amide-80 packed with 3 \( \mu \)m particles [91]. By comparing \( H–u \) curves...
Fig. 16. Separation of monosaccharides by a TSKgel Amide-80 columns. Column: TSKgel Amide-80, (250 mm \( \times \) 4.6 mm I.D., 5 µm); mobile phase: MeCN–ammonium formate buffer (concentration is shown in the Figures) (82:18); compounds: (Fuc) L-fucose, (Man) D-mannose, (Gal) D-galactose, (GlcNAc) N-acetyl-D-glucosamine, (Neu5Ac) N-acetylneuraminic acid, (GlcA) D-glucuronic acid [88].

between 3 and 5 µm particle-packed columns as shown in Fig. 17B, the new column provides better separation efficiencies even under fast separation conditions. The minimum \( H \) reaches 7 µm at around \( u = 2.7 \) cm/min, i.e. 0.45 mm/s. At \( u = 1.0 \) mm/s, \( H \) was estimated at around 8 µm.

A mannitol peak obtained using a TSKgel Amide-80 column (150 mm \( \times \) 4.6 mm I.D., 3 µm) in MeCN–water (75:25) gave a value of \( N = 18 000 \) within 7 min. The separation efficiency was comparable to a conventional column (250 mm \( \times \) 4.6 mm I.D., 5 µm), but the 5 µm particle packed column gave a similar separation efficiency around 10 min as shown in Fig. 18. Mannitol in pharmaceutical formulations was analyzed using a TSKgel Amide-80 column (250 mm \( \times \) 4.6 mm I.D., 5 µm), with an ELS detection [75]. By a chromatogram obtained in a mixture of MeCN and 0.1% CF₃CO₂H in water (75:25) with a flow rate of 1.0 mL/min, the separation efficiency for mannitol was estimated as \( N = 13 000 \) (\( k = 2.5 \)). The plate height was calculated as 19 µm, thus the separation efficiency of the system was comparable to that exhibited in Fig. 16.

The retentivity of Amide-80 was compared with other HILIC columns, as shown in Fig. 1 [53]. The 250 mm Amide-80 column gave \( N \) in the range of 15 000–20 000 (i.e., \( H = 12–16 \) µm) for \( k \) values below 5. For peaks with \( k = 4–5 \), the separation efficiency slightly worsened when compared to less retained analytes. On the Amide-80 column, acids were less retained in 85% MeCN, except for 3,4-dihydroxyphenylacetic acid, which was strongly retained giving a split peak with significant tailing. Compared with the other three columns, the amide column showed smaller decreases in separation efficiencies by the increasing of retention factors as shown in Fig. 2.

3.4. Columns packed with poly(succinimide)-modified silica

Alpert reported new types of silica-based stationary phases in 1983 by further modifying aminopropyl silica phases [92]. The treatment of an aminopropyl silica phase with a poly(succinimide) resulted in the formation of covalent bonds to anchor polymers. This stationary phase, poly(succinimide) silica can be a platform for several HILIC stationary phases. Hydrolysis or aminolysis reactions of the remaining succinimide rings using hydroxide, 2-aminoethanol, and 2-aminoethylsulfonic acid leads to the preparation of poly(aspartic acid), poly(2-hydroxyethyl)aspartamide, poly(2-sulfoethyl)aspartamide bonded silica phases as shown in Fig. 19 [1].

These phases are available from PolyLC as PolyCAT A, PolyHydroxethyl A, PolyGlycoplex, and PolySulfoethyl A, respectively. Although these stationary phases are applied in a wide variety of fields, their separation efficiencies have not been
Fig. 17. The Van Deemter plots on TSKgel Amide-80 columns. (A) Column: TSKgel Amide-80, (250 mm × 4.6 mm I.D., 5 μm); mobile phase: MeCN/water = 80/20; temperature: 80 °C; detection: RI; sample: A, α-glucose, B, mannitol [14]. (B) Columns: (♦) TSKgel Amide-80, (150 mm × 4.6 mm I.D., 3 μm), (▲) TSKgel Amide-80, (250 mm × 4.6 mm I.D., 5 μm); mobile phase: MeCN/water = 75/25; temperature: 40 °C; detection: RI; Flow rate: 1.0 mL/min; injection volume: 10 μL; sample: mannitol [91].

Fig. 18. Comparison of chromatograms for sugar alcohols on TSKgel Amide-80 3 μm and 5 μm columns. A: TSKgel Amide-80, (150 mm × 4.6 mm I.D., 3 μm); B: TSKgel Amide-80, (250 mm × 4.6 mm I.D., 5 μm); Chromatographic conditions are the same to Fig. 17B except for the column temperature was 25 °C. Compounds: (1) ethylene glycol; (2) glycerin; (3) erythritol; (4) xylitol; (5) mannitol; (6) inositol [91].

Fig. 19. Preparation scheme of poly(succinimide)-based stationary phases.

Fig. 19. Preparation scheme of poly(succinimide)-based stationary phases.

discussed in detail. In this chapter, examples of application using each phase are introduced first, and then separation efficiencies are discussed.

3.4.1. PolyHydroxyethyl A

Separations with phosphorylated and non-phosphorylated amino acids, dipetptides, derivatized maltooligoglycosides, and oligothymidylic acid were shown by Alpert [1]. Peptides [59] and glycopeptides by HPLC [93,94] and by CEC [95], carbohydrate derivatives [96], and ionic compounds by CEC mode [97] were separated using this stationary phase. This phase can also be used for solid phase extraction media [98].

Tolstikov and Fiehn compared the separation ability of TSKgel Amide-80 and PolyHydroxyethyl A columns using a mixture of amino acids and carbohydrates (standard compounds) in HILIC-MS mode [99]. As shown in Fig. 20, peak widths by the PolyHydroxyethyl A column are wider than those by the TSKgel Amide-80 column, although the elution order of standard compounds is quite different for the two columns.

Tolstikov et al. used a PolyHydroxyethyl A column (150 mm × 0.6 mm I.D., 3 μm) to analyze plant leaf extracts. In the mass chromatogram, metabolites were eluted in the order of polar lipids, flavonoids, glucosinolates, saccharides, and amino acids [81]. For the separation of dipeptide standards by a PolyHydroxyethyl A column (200 mm × 4.6 mm I.D., 3 μm) with a mixture of MeCN–triethylammonium phosphate (TEAP) buffer (10 mM, pH 2.8) (8:2), and a flow rate of 2 mL/min, provided
Fig. 20. HILIC/MS base peak chromatograms of a mixture of standards. Columns: (A) PolyHydroxyethyl A, 150 mm × 1.0 mm I.D., 5 μm; (B) TSKgel Amide-80, 250 mm × 2.0 mm I.D., 5 μm; mobile phases: A, MeCN, B, 6.5 mM ammonium acetate (pH 5.5, adjusted by acetic acid); gradient: 15–35% B from 10 to 60 min for (A); 15–55% B from 10 to 60 min for (B); compounds: (1) N-acetyl-d-glucosamine; (2) sucrose; (3) d-(+)-raffinose; (4) l-methionine; (5) N-methyl-1-deoxynojirimycin; (6) l-alanyl-l-alanine; (7) 1,4-dideoxy-1,4-imino-d-arabinitol; (8) uridine-5-diphosphoglucose; (9) stachyose; (10) glucosaminic acid; (11) 2-amino-2-deoxy-d-glucose; (12) maltoheptaose [99].

separation efficiencies \(N = 2300–2900\) \((H = 69–87\) μm) for peaks of \(k = 6–25\) [1]. With higher concentrations or higher pHs of buffer solutions, separation efficiencies, retention factors, and selectivities decreased resulting in poorer resolutions.

Oyler et al., employed a PolyHydroxyethyl A columns (200 mm × 4.6 mm I.D., 5 μm) to separate atosiban diastereomers with a fair separation efficiency, \(N = 5000–6000, H = 33–37\) μm except for one diastereomer \((N = 1700, H = 119\) μm). Among ten diastereomers, 3 peptides were co-eluted with other peptides, and resolution with the PolyHydroxyethyl A columns was worse compared to that with an amino-silica column [59].

Under completely aqueous conditions, a PolyHydroxyethyl A column (100 mm × 4.6 mm I.D., 3 μm) with a TEAP buffer \((10\) mM, pH 2.8), and a flow rate of 0.5 mL/min, provided separation efficiencies of \(N = 4500–5000\) \((H = 20–22\) μm) for peaks of \(k = 5–7\) [1]. The use of smaller particles results in higher separation efficiencies in this HILIC column. However, the separation efficiency by this phase is lower than conventional RPLC columns packed with 3 μm or 5 μm particles.

3.4.2. PolyGlycoplex

The separation of oligosaccharides using a PolyGlycoplex column was reported by Alpert et al. [23]. The same column was employed to separate \(N\)-(p-nitrobenzyloxy)aminoalditol derivatized oligosaccharides [100].

The HILIC separation of small carbohydrates containing monosaccharides, \(N\)-acetylneuraminic acids, and sialyl sugars by a PolyGlycoplex column \((200\) mm × 4.6 mm I.D., 5 μm) in MeCN10 mM TEAP (pH 4.4) (80:20) provided \(N = 5000\) for peaks of \(k = 10, 23,\) and 26. Plate heights were calculated to be \(57–66\) μm at a flow rate of \(1.0\) mL/min \((u = 1.6\) mm/s) as shown in Fig. 21 [23]. Fig. 22 is an example of the HILIC mode separation of \(p\)-nitrobenzylxoy (PNB) derivatives of xylglucans (XG) on a PolyGlycoplex column \((200\) mm × 9.4 mm I.D., 5 μm) in 70% MeCN. For peaks of XG7, XG8, and XG9, separation efficiencies were estimated as \(N = 9500\) \((k = 4.5), 10000\) \((k = 6.3),\) and 9000 \((k = 8.4),\) respectively from Fig. 22. Plate heights were calculated as \(20–22\) μm at flow rate of \(1.0\) mL/min \((u = 0.37\) mm/s) [23].

3.4.3. PolyCAT A and poly(Sulfoethyl A)

The polyCAT A column was used to separate histones under a gradient elutions, and separation efficiencies were not estimated [101]. The ring opening reaction of succinimide residues on silica gel by 2-aminoethylsulfonic acid lead to a poly(2-sulfoethyl aspartamide) phase, which possessed strong cation-exchange properties. The aforementioned phase was employed to sep-

Fig. 21. Chromatogram of small carbohydrates. Column: PolyGlycoplex, 200 mm × 4.6 mm I.D., 5 μm; mobile phase: MeCN–10 mM TEAP (pH 4.4) buffer (80:20, v/v); flow rate: 1.0 mL/min; detection: UV, \(1 = 275\) nm; Compounds: (A) monosaccharides; (B) 2,3-didehydro-2,6-anhydro-N-acetylneuraminic acid; (C), (D) N-acetylneuraminic acid; (E) N-glycolylneuraminic acid; (F) sialyl(2→3)lactose; (F) sialyl(2→6)-N-acetyllactosamine; (G) sialyl(2→6)lactose; (H) disialyllactose [23].
arate peptides using HPLC [102] and CEC [95], and purify peptides [103], basic compounds [104], and basic pharmaceuticals [105]. In a fully aqueous mobile phase, the phase was used as a cation-exchange media, as reported by Gilar et al. [106]. Chromatograms by Alpert provided limited examples of an isocratic separation of amino acids by the use of this phase [1].

For peaks in Fig. 23 until 8 min (k<6), the separation efficiencies were calculated as N = ca. 4000, H = 50 μm. The separation efficiency at the last peak was estimated as k = 12.5, N = 3000, H = 67 μm. This separation was done at 2.0 mL/min (u = 3.1 mm/s) flow rate, and judging from the above-mentioned example with the PolyGlycoplex column, the flow rate may have been too fast to provide optimal separation efficiency. The same column was employed to separate cyclopeptides using mixtures of MeCN-15 mM TEAP buffer (pH 2.8) in the following ratios; 95:5, 90:10, 75:25, or 60:40 at a 1 mL/min flow rate. In 60 and 75% MeCN, the separation efficiency was poor, but in 90%, N = 9000 for a peak of k = 3 was generated. In 95% MeCN, retention factor became larger, but the separation efficiency decreased to N = 5000 for a peak of k = 3.3, and N = 3000 for a peak of k = 9.1. Therefore, this kind of phase should be used for analytes with small k values (from 2 to 5) to provide optimal separation efficiencies under isocratic conditions [1].

Column bleeding with poly(Sulfoethyl A) was reported by Mihailova et al. [107]. The authors found several interfering peaks with m/z ratio of 526, 568, 659, 712 and 714 in a two-dimensional LC–MS/MS study. Peaks were regarded as the products from column bleeding of the poly(Sulfoethyl A) column used as a strong cation-exchange media. When a new column was tested, levels of interfering peaks were negligible. The authors concluded that the lifetime of the column is around 1 month, before significant column bleeding becomes problematic.

3.5. Columns packed with sulfoalkylbetaine-modified silica

Irgum et al. introduced sulfoalkylbetaine zwitterionic functionality, given as CH₂CH₂N(CH₃)₂⁺CH₂CH₂CH₂SO₃⁻ to polymer supports to prepare ion-exchange materials for the separation of inorganic compounds [26,27] and proteins [28]. This phase can separate anion and cation species simultaneously due to its zwitterionic structure. Recently, similar functionality was immobilized on silica surfaces, and this type of column, the ZIC-HILIC, was released from SeQuant. The ZIC-HILIC column is one of the most popular HILIC columns, and is widely used to analyze aminoglycosides [108] including neomycin [109], morphine and its derivatives [110], small and polar compounds [111], metabolomes [112,113], glucosinolates [114], and PSP-toxins [115]. This phase can also be used for solid phase extraction or trapping media of N-glycosylated peptides [116] and Glycosylphosphatidylinositol (GPI)-anchored peptides [117]. Schettgen et al. employed a ZIC HILIC column (100 mm × 4.6 mm I.D., 3.5 μm) in an isotope-dilution HILIC-MS system, using [²H₄]lysine (lysine-d₄) as an internal standard [118]. Under their experimental conditions, retention times for lysine and lysine-d₄ were the same, and no deuterium effect was observed on the retention times. Phytosiderophores and their metal complexes in plants were analyzed by a ZIC HILIC-ESI-MS system. Complexes of mugineic acid (N-(3-(3-carboxypropylamino)-2-hydroxy-3-carboxypropyl)azetidine-2-carboxylic acid) and its derivatives with Fe²⁺, Fe³⁺, Ni²⁺, Zn²⁺, and Cu²⁺ were separated under a gradient elution, whereas metal complexes eluted earlier than their corresponding metal free ligands [119].
Takegawa et al. have reported the separation of N-glycopeptides [120], PA-N-glycans and PA-N-glycopeptides [121] using HILIC-ESI-MS systems with the ZIC HILIC phase. To optimize and evaluate ZIC-HILIC LC–MS or two-dimensional LC, ZIC-HILIC-RP-LC–MS systems, Heck and co-workers selected a set of peptides from digests of bovine serum albumin, α- and β-casein, as a standard sample [122]. They found that peptides possessing at least two acidic residues showed longer retention times at pH 8, while peptides containing more than two basic residues or lacking acidic residues showed longer retention times at pH 3.

The orthogonality of the ZIC HILIC phase against RP phase was also studied. The authors concluded that the separation using ZIC-HILIC at pH 6.8 was better than at pH 3 for a comprehensive analysis of peptides, although the orthogonality with RP was highest at pH 3.

A novel zwitterionic HILIC stationary phase immobilized with phosphorylcholine type polymers was reported by Jiang et al. [123]. A zwitterionic phase, KS-PolyMPC was prepared by multi-step chemical modifications as shown in Fig. 24. A column packed with this phase showed different elution orders for three peptides from a RP phase derived from the same silica support as shown in Fig. 25. Apparently, the separation efficiency was worse in the HILIC mode (top) than the RP mode (bottom).

The separation efficiency of the KS-PolyMPC was estimated from the peak width at a half height for the chromatogram in 60% MeCN-buffer in Fig. 26 as \( N = 6000 \) for three peptides with \( k < 1.8 \), \( N = 4000 \) for two peptides with \( k < 4.0 \), and \( N = 1500 \), and bradykinin with \( k = 7.5 \), respectively. Since the column dimension was 150 mm × 4.6 mm I.D., 5 μm, the plate height was estimated as \( H = 25–100 \) μm. The mobile phase contained a smaller fraction of MeCN compared to the usual HILIC mode, and the elution order was different from that predicted by the hydrophilicity. The authors supposed that the separation of peptides was mainly controlled by ion-exchange interactions [123].

By comparing four HILIC type columns, on their retentions and the separation efficiencies, separation efficiencies in the range of \( N = 12,000–22,000 \), i.e. \( H = 11–21 \) μm were obtained by using a ZIC-HILIC column (250 mm × 4.6 mm I.D., 5 μm) for carboxylic acids, nucleic bases, and nucleosides [53]. The retention enthalpy value of salicylic acid estimated from the Van’t Hoff plots indicated that the ZIC-HILIC phase had somewhat specific interactions for the separation as shown in Fig. 8. However, no ion-exchange effect was observed for benzoic acid derivatives on the ZIC-HILIC phase under the experimental conditions.

Further assessments of the separation efficiencies of the ZIC-HILIC column were carried out, according to the application data by the column manufacturer, SeQuant. For the separation of nucleic bases by a ZIC-HILIC column (150 mm × 2.1 mm I.D., 5 μm), separation efficiencies were estimated as 13,000 for adenine \( (k = 1.1) \), 10,000 for guanine \( (k = 1.7) \), 12,000 for cytosine

---

Fig. 24. Synthetic scheme of the zwitterionic stationary phase KS-polyMPC [123].

Fig. 25. Chromatograms from the separation of three peptides mixture. Columns: (A) KS-polyMPC, 150 mm × 4.6 mm I.D., 5 μm, (B) Kromasil C18, 150 mm × 4.6 mm I.D., 5 μm; Mobile phases: (A) MeCN/10 mM ammonium acetate buffer (pH 7) (60:40, v/v) (B) MeCN/10 mM ammonium acetate buffer (pH 7) (5:95, v/v); flow rate: 1 mL/min; detection: UV, λ = 214 nm [123].

Fig. 26. Chromatogram from the separation of six peptides mixture on the KS-polyMPC zwitterionic column. Columns: KS-polyMPC, 150 mm × 4.6 mm I.D., 5 μm, mobile phases: MeCN/10 mM ammonium acetate buffer (pH 6) (60:40, v/v); flow rate: 1 mL/min; detection: UV, λ = 214 nm [123].
(k = 3.2), respectively [25]. Thus the plate height was in the range of H = 12–15 μm. The separation of dehydroascorbic acid and ascorbic acid by a ZIC-HILIC column (150 mm × 4.6 mm I.D., 5 μm) resulted in N = 9000–10 000, H = ca. 15 μm as mentioned above. However, the separation of morphine and its glucuronides by a ZIC-HILIC column (50 mm × 4.6 mm I.D., 5 μm) resulted in poorer separation efficiencies of N = ca. 1500 and H = 33 μm for the two glucuronides (k = 2.8 and 4.1), and N = 3400 for mor-

phine (k = 6.1). A significant band broadening was observed when separating oligopeptides by a ZIC-HILIC column (100 mm × 4.6 mm I.D., 5 μm). For neurotensin (k = 0.7) and Gly-His-Lys (k = 5.5), N = 3000, H = 33 μm was obtained, but for bradykinin (k = 2.3), N = 1800, H = 55 μm was obtained. The Van Deemter plot on a ZIC-HILIC column (150 mm × 4.6 mm I.D.) revealed that the optimal flow rate was relatively low, less than 0.5 mL/min (u = 0.83 mm/s). The ZIC-HILIC shows good stability and low column bleeding among 19 columns tested by ELSD, suggesting that the column is suitable for high-sensitivity LC–MS analyses [25].

3.6. Columns packed with cyano- and diol-modified silica

A diol bonded phase has been prepared by immobilizing silanol groups using glycidoxypropyltrimethoxysilane, to provide a hydrophilic and neutral stationary phase bonding, for a good recovery of proteins [15]. According to a column character-

ization using a quantitative structure retention relationship (QSRR) [124,125], the overall polarity of the diol-bonded phase is close to the bare silica phase, although the diol-bonded phase showed slightly stronger retention for test compounds under a supercritical fluid chromatographic conditions with a mixture of CO2 and MeOH (90:10) as a mobile phase [126]. The log k values on the diol column (a product of Princeton Chromatography, Cranbury, NJ, USA) for the test samples were similar to those of a bare silica phase (Kromasil), while those of a cyano column (a product of Princeton Chromatography) showed different patterns with smaller k values than silica and amino-silica phases.

Diol-bonded phases possess hydrophilic surfaces, and silanol groups can be partially blocked by a silylating agent to avoid the irreversible absorption of polar analytes onto the stationary phase. On a diol type column, LiChrosorb-DIOL (250 mm × 4.6 mm I.D., 10 μm, Merck), provided N = 5300, H = 47 μm for sucrose (k = 2.3) and N = 3400, H = 74 μm for lactose (k = 3.4) in 75% MeCN as estimated from a chromatogram [58]. In the same report, an amino-silica phase (LiChrosorb-NH2, 250 mm × 4.6 mm I.D., 10 μm, Merck) also separated the same samples under the same mobile phase to give N = 3200, H = 78 μm for galactose (k = 1.8), N = 5600, H = 45 μm for sucrose (k = 2.8) and N = 4700, H = 53 μm for lactose (k = 4.1). In the diol phase, galactose and lactose peaks were broad in 85% MeCN, due to anomerization [58]. Non-reducing saccharides, such as sucrose, had no such effect. This situation was improved by adding ethyldiisopropylamine (EDIPA) to the mobile phase or by the separation at a higher temperature: the N value for a lactose peak in 85% MeCN at 30 ºC was 600 with k = 6.0, while it changed into N = 1300, k = 3.4 in 85% MeCN–0.1% EDIPA at 40 ºC. The k value for sucrose at 40 ºC was less than 2/3 of that at 30 ºC, but the N value of each sucrose peak was constant at 1600.

A mixed mode HILIC column, Acclaim Mixed Mode HILIC-1 was released from Dionex. The stationary phase contained alkyl chains which possessed 1,2-diol functionality, and could be used in RP and HILIC modes with wider application range than conventional diol columns [127].

The log k–log k plot between a cyano-silica phase and a bare silica showed that a significant number of analytes possessed different selectivities between the two columns [126]. The application of the cyano-silica to the HILIC mode is still limited. Determination of piperadine in pharmaceutical drug substances by a cyano-silica, Alltima Cyano (Grace Alltech) [128]. The separation efficiency of the cyano silica, Alltima Cyano (250 mm × 4.6 mm I.D.) in MeCN–water-0.15% nitric acid (95:4:85:0.15) as a mobile phase, with an ELSD detection for piperadine, piperadine citrate, and estropipate (piperadine salt of estrone sulfate) was estimated as N = 13 000, and H = 19 μm [128].

A cyano-silica column, TSKgel CN-80T did not retain peptides in MeCN–water with a gradient elution, while the peptides were strongly adsorbed onto an amino-silica phase, and TSKgel Amino-80 provided sufficient recovery [129]. A chromatogram by Padaraskas et al. provided one of the limited example of a cyano-silica in HILIC [130]. For the separation of a denaturant in alcohol formulations, a cyano-silica column (Separón SGX, TESSEK, Prague, Czech Republic) possessed larger retention for Bitterx (denatonium benzoate), crystal violet, and methylene blue than bare silica and amino-silica columns. In a mixture of MeCN–5mM NaTFA (90:10, v/v), the cyano-silica column provided N = 17 000 for peaks around k = 4.5, and N = 9500 for peaks at k = 10.3, respectively. However, the final optimization of the separation conditions seemed to be carried out using a bare silica column [130]. The separation efficiency of a diol type column, Inertsil Diol (150 mm × 4.6 mm I.D., 5.1 μm, GL Science) was tested using urea, sucrose, and glycine. In a mobile phase of 90% MeCN, the k value of glycine was around 25, while in 80% MeCN, it decreased into 4. The effect of temperature on ln k and on the plate height, H for the three analytes was examined to show that the ln k values decreased linearly as the tempera-

ture rose, while the H value did not change linearly as shown in Fig. 27 [131].

At elevated temperatures, an extra-column effect became greater than a lower temperature in terms of separation efficiency losses for less retained solute, while the decrease of the resistance of mass transfer at higher temperatures resulted in the decrease of plate height for a long retained analyte, glycine. For sucrose, the column provided H = ca. 18 μm at a 0.35 mL/min flow rate at 30 or 40 ºC. A conventional HPLC column packed with 5 μm particles, was categorized as slow in terms of separation, and at 1.0 mL/min, the H increased to 30 μm. The column effectively separated amino acids in HILIC, although the optimal flow rate was somewhat slow, at 0.3–0.5 mL/min. The orthogonal separation characteristics of the diol phase against RPLC were examined for pharmaceuticals and impurities (exact structures were not shown, but they contained –NH2, –OH,
further in Section 4.1. This issue is discussed similarly modified silica column is connected as a pre-column acidic conditions, and the lifetime of the phase is extended if the studied not only in analytical (chromatographic) chemistry, but interesting structure and molecular functions, CDs are widely been studied in molecular recognition chemistry. Due to their formation of inclusion compounds within their cavities has hydrophobic cavities due to their unique toroid structures, and of 

\[ \text{HCONH}_2, \text{ or } \text{OCONH} \] functionalities. A diol column (YMC-Pack, 250 mm \times 4.6 mm I.D., 5 \mu m) gave \( N = 24,000 \) for a less retained compound \( (k < 1) \) and \( N = 20,000 \) for a retained compound \( (k > 4) \). A direct comparison of the separation efficiency of the diol phase and RPLC was difficult, since the separation in RPLC mode was carried out with a linear gradient mode \[132,133\]. A sensitive HILIC LC-ESI–MS–MS system was developed to analyze choline and acetylcholine in microdialysis samples. A 125 mm \times 4 mm I.D., 5 \mu m, Merck) was employed for a gradient elution. The separation of both target analytes was good \[133\]. The importance of the sample solvent was discussed: when samples were dissolved in 90% MeCN, all analytes were concentrated as narrow bands, while those dissolved in water showed band broadening. A similar separation was reported using a longer column of the same packing material in gradient elutions using a mixture of HCOOH in H2O–MeCN, and a gradient elution that started with hexane, ethyl acetate, MeCN, and 0.1% HCOOH in H2O \[134\].

Diol-bonded silica releases its silylation reagent slowly under acidic conditions, and the lifetime of the phase is extended if the similarly modified silica column is connected as a pre-column \[135\].

In a CEC mode, a polymer monolithic cyano column (270 mm \times 100 \mu m I.D.) was employed to separate saccharides \[68\] using ESI-IT-MS as a detector. This issue is discussed further in Section 4.1.

### 3.7. Columns packed with cyclodextrin-modified silica

Cyclodextrins (CD) are cyclic oligosaccharides that consist of \( 1 \)–4 linkages of glucose units. CDs possess a relatively hydrophobic cavities due to their unique toroid structures, and the formation of inclusion compounds within their cavities has been studied in molecular recognition chemistry. Due to their interesting structure and molecular functions, CDs are widely studied not only in analytical (chromatographic) chemistry, but also in organic chemistry, pharmaceutical science, enzymology, biochemical sciences, food and cosmetics sciences. Molecular interactions that form inclusion complexes can be used as driving forces in the separation chemistry. The well-known CD are \( \alpha, \beta, \gamma \)-CD, in which, six, seven, and eight glucose units are linked together with 174, 262, and 427 Å³ cavity volumes, respectively \[136\]. Among these, \( \beta \)-CD is the most studied and widely used in the field of separation science. The CD-bonded silica phase has been used for chiral separations for many years \[137–139\], and CD-bonded monolithic silica columns of a conventional size \[140\] and a capillary size \[141\] are also known.

A Cyclobond I 2000 (\( \beta \)-CD bonded phase) column (ASTEC) was used to separate native oligosaccharides including arabinosides, celluloside, cyclodextrins, isomaltoside, maltotides, mannosides, and xylosides that possessed degrees of polymerization \( (D_p) \) from 1 to 8, using 70% MeCN as a mobile phase, to estimate chromatographic parameters, \( k, R_s, \) and \( N \) \[142\]. Throughout the measurements, resolution, a \( R_s \) of around 2.5 was obtained to separate oligomers differing by one saccharide unit. The relationship between \( k \) and \( D_p \) is interesting: generally, higher \( D_p \) yields larger \( k \) values for all saccharides, however, oligosaccharides with the same \( D_p \) with different carbohydrate units sometimes lead to quite different \( k \) values. One of the reason of the selectivities will be understood by that the columns function in the HILIC mode through interaction with the hydrophilic aqueous layer on the exterior of the CD molecule. The selectivity, alpha, for one unit of saccharides did not seem to be constant. Larger molecules gave slightly smaller alpha values, and this tendency was different from \( \alpha \) (CH\(_2\)) in the RPLC mode \[55\]. The separation efficiency was in the range of \( N = 2300–7800 \), and usually around 4000 for a 25 cm column, \( H \) = ca. 60 \mu m. These values were quite low value for a modern HPLC system, though they could have been better at very slow linear velocities. To separate \( \alpha-, \beta-, \) and \( \gamma-CDs \) at a 1.0 mL/min of flow rate, the \( N \) value was 3000, but it became \( N = 7000 \) at a 0.05 mL/min of flow rate \[142\].
Separation efficiencies were estimated for the separation of oligosaccharides up to tetramers, and sugar alcohols by Cyclobond III (γ-CD bonded phase) and Cyclobond I columns (25 cm in length) as \( N = 4000 - 13,000 \) (\( H = 19 - 63 \mu m \)) for oligosaccharides \((k = 2.5 - 9.5)\), and \( N = 5000 - 13,000 \) (\( H = 19 - 50 \mu m \)) for sugar alcohols, even at a 1.5–2.0 mL/min of flow rate of the aqueous MeCN mobile phase [143]. The Van Deemter plot for fructose, sucrose, and lactose on a 25 cm β-CD column (Cyclobond I) showed that the optimal linear velocity for these three saccharides was different as shown in Fig. 28. Relatively fast separation, \( u = 2 \text{ mm/s} \) was preferred for long retained analytes such as sucrose \((k = 2.71)\), and lactose \((k = 3.84)\), while \( u = 0.4 \text{ mm/s} \) was the optimum for fructose \((k = 1.25)\). The same study on a 25 cm α-CD column (Cyclobond III) also showed the same tendency, but with better efficiencies. The CD-bonded columns are stable and reproducible compared to amino-silica phases. After 3000 injections of standard samples, the old column provided comparable selectivities and efficiencies to the new column.

The Cyclobond I column was applied to a LC-ESI–MS system to analyze oligosaccharides up to 11 glucose units. The limits of detection were as low as 50 pg for test analytes [144].

The Cyclobond I column was employed for the HILIC mode separation of polar test compounds as shown in Fig. 29, and amino acids such as aspartic acid, proline, leucine, methionine, and norvaline. The cyclodextrin-bonded column showed greater retention than that of TSKgel Amide-80 phase for several analytes [145]. For a compound A, the chiral separation was realized under the HILIC conditions.

A β-CD-bonded phase, Nucleodex beta OH (Macherey-Nagel, Düren, Germany) was used for the separation of phosphorylated carbohydrates [146]. Novel silica-based stationary phases modified with β-CD, glucose and maltose using a “click chemistry” developed by Lei and Liang [147], were examined to separate nucleosides, saccharides, and sugar alcohols under HILIC conditions [148].

Judging from a chromatogram obtained under an isocratic conditions, the Click Maltose column (150 mm × 4.6 mm I.D.) provided \( N = 8000 - 10,000 \) (\( H = 15 - 19 \mu m \)) for uracil \((k = 1.0)\), adenosine \((k = 2.6)\), guanosine \((k = 5.3)\), in MeCN–10 mM NH₄OAc buffer (85:15), respectively. The Click β-CD column separated 11 carbohydrate derivatives under a gradient mode separation. Further detailed characterization of these stationary phases is required to examine their potentials as a new stationary phases.
3.8. Columns packed with other functionality-modified silica, and polymer particles

Various silica-based phases modified with different functionalities from above-mentioned groups, are discussed in this category. The temperature responsive nature of poly(N-isopropylacrylamide), PNIPAAm was incorporated to silica-based phases by Kanazawa et al. The column packed with PNIPAAm modified silica particles showed RP characteristics over the lower critical solution temperature (LCST), while it exhibited a hydrophilic nature at 5 °C [149]. PNIPAAm hydrogel-modified silica phase was also reported. Starting from amino-silica, immobilization of a radical initiator, 4,4′-azobis(4-cyanovaleric acid), and on-silica polymerization of N-isopropylacrylamide with N,N′-methylenbis(acrylamide) as a crosslinking agent resulted in the preparation of the hydrogel on silica surface [150]. The aforementioned phase showed a hydrophilic nature under 5 °C, although a fully aqueous mobile phase was employed in these studies. These phases have the potential to be used in HILIC, but their separation efficiencies seem poor at lower temperatures.

A chiral stationary phase, Chirobiotic T (ASTEC), modified with a macromycyclic antibiotic, teicoplanin was employed for chiral separations under HILIC conditions [145]. With a 25 cm column, $N = 7000–17000 (H = 15–36 \mu m)$ was achieved. Liu et al. reported [151] the synthesis and chromatographic characterization of a silica-based phase functionalized by perhydroxycucurbit[6]uril, a macrocyclic compound with a cavity [152]. The cavity of the cucurbit[6]uril was similar to that of α-cyclodextrin [153]. In a mixture of MeCN and phosphate buffer (5 mM, pH 3.48) (80:20, v/v), a good separation of alkaloids was carried out on a column packed with the cucurbit[6]uril-silica (150 mm × 4.6 mm I.D., 5–7 μm). For less retained solutes such as narceine and berberine, $N = 2000–4000 (H = 38–75 \mu m)$ was obtained, while, for nicotine, significant broadening of the peak, $N = 600 (H = 250 \mu m)$ was observed. Interestingly, for brucine, $N = 8000$ was provided though it had larger retention than nicotine.

In 2007, a silica-based column, the Cosmosil HILIC phase was released from Nacalai Tesque (Kyoto, Japan). The stationary phase contains a 1,2,4-triazol group (the immobilization method of the polar heterocyclic compound to the silica surface is not shown), and possesses unique retentivity due to the basic nature of the triazol [154]. By a Cosmosil HILIC column (250 mm × 4.6 mm I.D., 5 μm), water soluble vitamins, carboxylic acids, amino acids, and polar pharmaceuticals were separated with high efficiency; for analytes of $k = 0.5–6.5$, $N = 12500–22000$, $H = 11–20 \mu m$ and a flow rate of 1.0 mL/min were obtained. Application data for 154 compounds are available. For the separation of phosphorylated glucose, amino acids (histidine and O-phospho-L-serine) and peptides, the separation efficiency was worse ($H = 30–45 \mu m$) than with other examples. The column can also be used as a weak anion-exchange stationary phase by changing mobile phases.

A cellulose-based column was used to separate $N$-linked oligosaccharides from hydrazinolysates of glycoproteins using a mixture of 1-butanol–ethanol–water (4:1:1, v/v) or a mixture of ethanol–water (1:1, v/v) [155].

Polymer-based stationary phases with amino groups are also known. A column, Phenomenex AshaipakNH250D was applied for the separation of 5-fluorouracil in plasma and tissue using a HILIC-APCI-MS system [156]. These types of phases were employed for the separation of PA-oligosaccharides [157], or taurine and methionine in matrices rich in carbohydrates [64]. According to a chromatogram of the separation of taurine, threonine, and methionine on an ASTEC apherNAH2 under isocratic conditions with 60% MeOH, the separation efficiency by a column (150 mm × 4.6 mm I.D., 5 μm) was estimated as $N = 1500–2600 (H = 58–100 \mu m)$.

Porous, spherical polystyrene–divinylbenzene particles functionalized with neutral hydrophilic groups, Rogel-P (not mentioned in detail) were invented by Yang and Verzele. A column packed with particles, Rogel-P, was evaluated in 40% MeCN. The efficiency of 50 000 plates/meter was obtained for phenol [158]. Generally, columns packed with polymer-based particle for the HILIC mode generate poorer separation efficiencies than those of silica-based columns, and the use of polymer-based materials should be limited under separations using strong acidic or basic conditions.

4. Separation efficiencies of monolithic hydrophilic interaction chromatography mode columns

Monolithic materials, one-piece structure consisting of skeletons and through-pores have recently attracted the attentions of chromatographers recently. First, organic polymer monolithic columns [159,160], then silica monolithic columns [161,162] were reported. Monolithic materials possess several features as supports for chromatographic stationary phases such as higher mechanical stabilities in comparison to particle-packed columns, large through-pores which results in higher permeabilities or lower backpressure than particle-packed columns, small-sized skeletons to allow fast mass transfer within the stationary phase, high porosities in monolithic columns lead to low phase ratios, i.e. lower sample capacities and smaller retentivities than particle-packed columns. These findings were reviewed repeatedly over the years [163–185], and judging from these reviews, organic polymer monolithic columns have been mainly used in CEC and for the separation of biological molecules (biopolymers), while monolithic silica columns have been employed for both of HPLC and CEC mainly of small molecules.

4.1. Polymer monolithic columns

Investigations on the use of organic polymer monolithic columns is still limited, and there are only a few reports on the subject even in 2007. Xie et al., prepared porous hydrophilic monoliths using acrylamide and N,N-methylenebis(acrylamide) as starting materials, however, they did not report on their chromatographic characteristics [186]. Viklund and Irgum prepared a polymer monolith column containing zwitterionic functionality of sulfalkylbetaine moiety in 2000 [187]. The column was obtained by a photo-induced copolymerization of trimethyl-
lolpropane trimethacrylate (TRIM) and N,N-dimethyl-N-methacryloyloxyethyl-N-(3-sulfopropyl)ammonium betaine (SPE) in a glass column (150 mm × 2.7 mm I.D.). This column has the potential for HILIC separation, but has been supplied for the cation-exchange mode separation of proteins. The SPE monomer was co-polymerized with ethylene dimethacrylate (EDMA) in a glass tube (150 mm × 2.7 mm I.D.) by photo-induced polymerization [28]. The column was evaluated in a fully aqueous mobile phase for the separation of inorganic salts and proteins.

A similar methacrylate-based monolithic column in capillary was reported by Smith et al., to provide \( N = 15 \times 100 \, \text{m}^{-1} \) at a linear velocity of 2 mm/s [188]. The column was prepared from SPE and EDMA in a 100 μm I.D. capillary. Under HILIC conditions, no evidence of swelling or shrinking of the column bed was observed. When the estimation of the column efficiency as above was adopted to two chromatograms in this report, it was found that bases and neutral compounds were separated with \( N = 1900–4500 \) (\( H = 66–158 \, \text{m} \)) on a column (300 mm × 100 μm I.D.), while \( N = 800–1300 \) (\( H = 230–375 \, \text{m} \)) was obtained for benzoic acid derivatives with the same column as shown in Fig. 30.

Polymer-based monolithic columns in capillaries were prepared by reacting between tris(2,3-epoxypropyl) isocyanurate (TEPIC) and 4-[(4-aminocyclohexyl)methyl]cyclohexylamine (BACM) or trans-1,2-cyclohexanediamine (CHD) in the presence of poly(ethylene glycol). This type of monolithic column apparently had a different morphology compared with previously reported polymer monolith [189]. In 90% MeCN, a TEPIC–BACM column (364 mm × 100 μm I.D.) separated nucleosides with efficiencies of \( N = 3200–27 \, 000 \) (\( H = 13–113 \, \text{m} \)) as shown in Fig. 31. Column efficiencies and permeabilities were quite high, and for benzene in MeCN/20 mM sodium phosphate buffer pH 7.060/40 (v/v), optimum plate height reached 5 μm within the linear velocity range of 1–2 mm/s.

Fig. 30. Chromatograms obtained by a poly(SPE-co-EDMA) monolithic column. Column: 285 mm × 100 μm I.D.; Detection: UV, \( \lambda = 214 \, \text{nm} \); flow rate: 800 nL/min; injection: 100 nL. (A) Mobile phase: MeCN–water (50 mM ammonium formate pH 3.0) (95:5, v/v); compounds: (1) toluene; (2) methacrylamide; (3) acrylamide; (4) thymine; (5) uracil; (6) adenine; (7) thiourea; (8) cytosine. (B) Mobile phase: MeCN–water (50 mM ammonium formate pH 3.0) (75:25, v/v); Compounds: (B) benzoic acid, (3-HB) 3-hydroxybenzoic acid, (2-HB) 2-hydroxybenzoic acid, (3.4-DHB) 3,4-dihydroxybenzoic acid, (3.5-DHB) 3.5-dihydroxybenzoic acid, (3.4-5-THB) 3,4,5-trihydroxybenzoic acid, (2.6-DHB) 2.6-dihydroxybenzoic acid [188].

Fig. 31. Chromatograms on a TEPIC–BACM monolithic column. Column: TEPIC–BACM, 36.4 cm × 100 μm I.D.; mobile phase: MeCN–water (90:10, v/v); detection: UV, \( \lambda = 210 \, \text{nm} \) (off column, 9 cm × 50 μm I.D.); pressure: 55 kg/cm²; flow rate: 0.5 mL/min (split method); temperature: 30 °C; compounds: adenine, thymine, uracil, cytosine, adenosine, guanosine, uridine, and cytidine [189].
Fig. 33. Normal-phase CEC/negative-ion ESI-MS analysis of the bile acids in Fig. 32. Column: polymer monolith, 30 cm × 100 μm I.D.; mobile phase: MeCN–water–240 mM ammonium formate buffer (pH 3) (60:35:5, v/v/v); field strength, 400 V/cm; injection, 6 kV, 5 s, compounds: a mixture of standards containing free bile acids, together with glycine and taurine conjugates (TLCA: 0.2 mg/mL, TDCA: 0.1 mg/mL, TCA: 0.05 mg/mL, GLCA: 0.5 mg/mL, GDCA: 0.2 mg/mL, GCA: 0.2 mg/mL, and CA: 0.1 mg/mL) [74].

Fig. 34. Electrochromatogram of a mixture of mono- and disaccharides. Column: polymer monolithic cyano column, 27 cm × 100 μm I.D.; mobile phase: MeCN–water–240 mM ammonium formate buffer (pH 3) (76:23:1, v/v/v); field strength, 500 V/cm; injection, 4 kV, 10 s; compounds: each sugar (0.8 mg/mL); mass range: 100–600 Da [68].

A long column (150.5 cm) produced very high separation efficiencies of $N = 140\ 000–210\ 000\ (H = 7–11\ \mu m)$ for alkylbenzenes, but no such result has been reported for polar compounds [189].

Fig. 35. Electrochromatographic separation of anomeric maltooligosaccharides from Dextrin 20 (degree of polymerization, DP) 1–9). Column: polymer monolithic amino column, 28 cm × 100 μm I.D.; mobile phase: MeCN–water–240 mM ammonium formate buffer (pH 3) (70:29:1, v/v/v); field strength: 500 V/cm; injection: 1 kV, 10 s; compounds: dextrin sample, 4 mg/mL; Mass range: 150–1300 Da [68].

Fig. 36. Relationship between log $t_R$ and DP of linear maltooligomers. Experimental conditions were the same as shown in Fig. 37, except that the mobile phase was MeCN–water–240 mM ammonium formate buffer (pH 3) (60:39:1, v/v/v) [68].

Que and Novotny reported on the preparation of polymer monolithic columns containing amino functionalities, [2-(acryloyloxy)ethyl]trimethylammonium methyl sulfate (2-AETMA) and 3-amino-1-propyl vinyl ether (APVE) in a capillary. The column (300 mm × 100 μm I.D.) was applied to an analysis of bile acids (Fig. 32) by a CEC-ESI-MS system to generate very high separation efficiencies, 610 000 plates/m using 60% MeCN as a mobile phase as shown in Fig. 33 [74].

Que and Novotny prepared a cyano-phase polymer monolith for CEC mode also using 2-cyanoethyl acrylate as one component of the column. This column and the above-mentioned amino column were employed in the analysis of neutral mono- and oligosaccharides, and sugar alcohols in a CEC-ESI-MS system [68]. The mono- and oligosaccharides were well separated on the cyano phase as shown in Fig. 34, in a mixture of MeCN–water–NH$_4$OCHO buffer (76:23:1, v/v/v). At a higher concentration of MeCN, MeCN–water–NH$_4$OCHO buffer (95:4:1, v/v/v), anomers of fucose, N-acetylglucosamine, and glucose were separated completely.

Sugar alcohols, fucitol, ribitol, xylitol, N-acetylglucosaminitol, glucitol, and mannitol were also separated using the
cyano column in MeCN–water–NH₄OCHO buffer (95:4:1, v/v/v). Using the amino column [74], maltooligosaccharides (degree of polymerization 1–9) were separated in MeCN–water–NH₄OCHO buffer (70:29:1, v/v/v) as shown in Fig. 35. The separation efficiency of the column was calculated as 200 000 plates/m, \( H = 5 \mu m \). Oligosaccharides were eluted in the order of their molecular size as shown in Fig. 36, where \( \log R \) was plotted against the degree of polymerization of maltooligosaccharides.

Even though they are used in CEC to separate polar compounds, probably due to their high backpressure drop under HPLC conditions, applications with polymer monolithic columns to HILIC are still limited. Without a few exceptional columns, it is difficult to generate higher separation efficiencies than the particle-packed HILIC columns in HPLC mode.

4.2. Silica monolith columns

In spite of extensive researches on monolithic silica columns, example of their use in the HILIC mode is still surprisingly limited. Merck released a monolithic bare silica column, Chromolith Performance Si, (100 mm × 4.6 mm I.D.)[190], but there is only one example of its use in a HILIC separation. Pack and Risley evaluated the above-mentioned Chromolith Si in the HILIC mode for inorganic ions such as lithium, sodium, potassium, chloride, and drugs like naproxen and warfarin in 80% MeCN with ELS detection[50]. For these substances, the efficiency was estimated as \( N = 1000–4800 \), for rapid separation with a flow rate of 2.0 mL/min. In 60% MeCN with a 1.0 mL flow rate, the retentions of ionic species was smaller, and the efficiencies were better \( N = 5000 \), than those in 80% MeCN. The column efficiency, however, is much lower compared to that of octadecylsilated monolithic silica column, Chromolith Performance C18, which generates \( H = 8 \mu m \) at around a 2 mm/s flow rate.

The HILIC mode separation using monolithic silica columns in capillaries were recently reported by Demesmay et al.[51,52]. Alkaloids were analyzed in CEC by a bare monolithic silica column (85 mm (effective length) × 75 \( \mu m \) I.D.) and in MeCN-1mM Tris buffer (pH 7.5) (95:5, v/v) with high separation efficiencies, \( N = 9300–17 500 \) as shown in Fig. 37 [51]. In the CEC mode, the plate height reached to 5 \( \mu m \) (Fig. 38).

Since Puy et al. used a small sized (85 mm (effective length) × 75 \( \mu m \) I.D.) monolithic silica capillary column, the total separation efficiency of the HPLC system was below 20 000 plates.

Another approach to prepare HILIC type monolithic silica columns utilizes polymerization of vinyl monomers having functional groups, such as amides, carboxylic acids, sulfonic acids, amines, and ammonium salts, to immobilize various functionalities onto silica surfaces modified with methacryloyl groups. In this procedure, monolithic silica capillary columns modified with 3-(methacryloxypropyl)trimethoxysilanes, or 3-(methacrylamidopropyl)trimethoxysilanes can be used as “universal platforms”. Into the column, a solution containing the above-mentioned monomer, and a radical initiator such as 2,2′-azobisisobutyronitrile or ammonium peroxydisulfate, were charged, and polymerization reaction was carried out. After washing out polymers that did not bind to the column surfaces, polymer-coated monolithic silica capillary columns were obtained [191–197]. Starting from the modification of

---

**Fig. 37.** Separation of xanthines in CEC on unmodified monolithic capillary. Column: W-SMC-2 silica monolithic column, 39 cm × 75 \( \mu m \) I.D. (effective length: 8.5 cm); mobile phase: MeCN–Tris 1 mM (pH 7.5) (95:5, v/v); field strength: \( V = 30 kV \); detection: UV, \( \lambda = 214 \) nm; Compounds: (1) naphthalene; (2) caffeine; (3) theophylline; (4) 1,7-dimethylxanthine; (5) b-hydroxyethyltheophylline; (6) dyphylline [51].

**Fig. 38.** The Van Deemter plots by the monolithic silica columns with or without the hydrothermal treatment. Symbols: not tailored, (□) in CEC and (△) in nano-LC; 1 h tailored, (■) in CEC and (▲) in-LC; column: SMC-2: not tailored, 46.6 cm × 75 \( \mu m \) I.D.; mobile phase: MeCN–water (95:5, v/v); Solute: dyphylline (\( k = 0.75 \)); detection: UV, \( \lambda = 214 \) nm; 1 h tailored: \( L = 14 \) cm × 75 \( \mu m \) I.D. (effective length: 8.5 cm); mobile phase: MeCN–Tris 0.1 mM (pH 7.5) (98:2, v/v); solute: dyphylline (\( k = 0.3 \)); detection: UV, \( \lambda = 214 \) nm[52].
silica using 3-methacryloxypropyltrimethoxysilane (MOP) or 3-methacrylamidopropyltrimethoxysilane (MAS), various vinyl monomers such as acrylamide (AA) [191,194], acryl acid (AA) [192,193], octadecyl methacrylate (OM) [196,197], 3-diethylamino-2-hydroxypropyl methacrylate (DAHMA), 2-(triethylammonium)methyl methacrylate chloride (DMAEA-Q), p-styrenesulfonic acid sodium salt (pSSA), and 2-acrylamide-2-methylpropanesulfonic acid (AMPS) [195] were used. These polymer-coated monolithic columns were characterized as HILIC phases for AA and AAm, RP phases for OM, cation-exchange phases for pSSA and AMPS, and anion-exchange phases for DAHMA and DMAEA-Q, respectively as shown in Fig. 39.

This method for the stationary phase preparation has several advantages over the preparation of particle-packed columns as follows: (1) the MOP or MAS modified monolithic silica column can be used as universal platforms, and various stationary phases can be prepared by changing only the monomers; (2) there is no need to prepare silanes possessing highly reactive functional groups; (3) a good permeability and column efficiency can be maintained after the polymerization; (4) it is free from column packing; (5) the amount of bonded phase can be controlled by the polymerization conditions; (6) the phase ratio can be increased compared to the silica modification using chloroorganosilanes.

In earlier stages of this research, a monolithic silica column coated with polyacrylamide (PAAm) generated $H = 1.6 \mu m$ for uridine in 90% MeCN (the bare silica showed $H = 1.0 \mu m$) as shown in Fig. 40 [191]. After the silica support was improved to increase the phase ratio [198], the PAAm type column could generate $H = 7 \mu m$ for an optimal flow rate of 1 mm/s, as shown in Fig. 41 [194].

Fig. 39. Synthetic scheme of the polymer-coated monolithic silica columns.

![Fig. 39](image)

Fig. 40. Chromatograms of nucleic bases and nucleosides separations on a poly(acrylamide)-coated monolithic silica column, PAMS-(100). Column: PAMS-(100)-1 (38 cm x 100 \mu m I.D.); mobile phase: MeCN–water (90:10, v/v); temperature: ambient; detection: UV, $\lambda = 260$ nm. Linear velocity: $u = 1.2 \text{mm/s}$ at $\Delta P = 2.1 \text{MPa}$; compounds: (1) thymine; (2) uracil; (3) adenine; (4) uridine; (5) adenosine; (6) cytosine; (7) guanine; (8) cytidine; (9) guanosine [191].

Fig. 41. The Van Deemter plot with a column backpressure plot for the a poly(acrylamide)-coated monolithic silica column, 200T-PAAm. Column: 200T-PAAm, 285 mm x 200 \mu m I.D.; mobile phase: MeCN–13 mM ammonium acetate buffer (pH 4.7) (80:20, v/v); temperature: 30 °C; detection: UV, $\lambda = 210$ nm; solute: guanosine ($k = 3.8$); sample volume: 1.0 \mu l injection (split), column: ZIC-HILIC, 150 mm x 4.6 mm I.D., 5 \mu m; mobile phase: MeCN–buffer (80:20, v/v); temperature: 25 °C; solute: cytosine ($k = 1.3$) [194].

Fig. 42. Kinetic plot of $\log(t_0/N^2)$ vs. $\log N$ with the assumed maximum pressure of 20 MPa for the (♦) MS-200T-PAAm, (●) the ZIC-HILIC, (□) the TSKgel Amide-80, 5 \mu m, and (△) the TSKgel Amide-80, 3 \mu m columns. Chromatographic conditions for TSKgel Amide-80 columns are as follows: Column: TSKgel Amide-80, 250 mm x 4.6 mm I.D., 5 \mu m and 150 mm x 4.6 mm I.D., 3 \mu m; mobile phase: MeCN–water (75:25, v/v); temperature: 40 °C; detection: RI; Solute: mannitol ($k = 2.5$); sample volume: 10 \mu l injection. For other chromatographic conditions, see Fig. 43 [194].
By kinetic analyses in which \( \log(t_0/N^2) \) values were plotted against \( \log N \) values under a limiting of pressure of 20 MPa, the PAAm modified column was compared with a ZIC-HILIC column, and two TSKgel Amide-80 columns packed with 3 \( \mu \)m and 5 \( \mu \)m particles to find that the monolithic type column could generate the same separation efficiency as particle-packed columns, with three time faster separation times than the columns packed with 5 \( \mu \)m particles, as shown in Fig. 42 [194]. This column was operated with a fast linear velocity, \( u = 7.6 \) mm/s without showing a severe loss in the separation efficiency as shown in Fig. 43. The above-mentioned column provides an example of the separation of oligosaccharides in HILIC and detection using a LC-ESI–MS [194].

A monolithic silica column coated with poly(acrylic acid) PAA, also generated \( H = 9–10 \) \( \mu \)m at a flow rate of 1.0 mm/s. When the separation impedance \( E \) was compared to that of a particle-packed column, the difference in column supports became clear. The particle-packed column lost its column efficiency at linear velocities higher than 1 mm/s, while the monolithic column showed no significant loss in its separation efficiency until \( u = 3 \) mm/s as shown in Fig. 44 [194].

Using the column in a LC-ESI–TOF MS system, 9 peptides were separated within 4 min. This type of stationary phase could be used for a weak cation-exchange phase and a HILIC phase to separate proteins, peptides, nucleosides, and oligosaccharides [192]. The above-mentioned cation-and anion-exchange station-
with polar polymers will provide more efficient and faster separation than particle-packed columns.

5. Conclusion

The characteristics of stationary phases for HILIC in terms of the separation efficiencies are summarized in Table 1. Due to the lack of separation examples in the isocratic mode in many cases, the table is not fully completed. HILIC separation targets were roughly separated into eight groups, such as (1) acids, (2) bases, (3) polar small compounds like amides or ureas, (4) Polyols (5) amino acids, (6) peptides, (7) nucleic bases and nucleosides, and (8) oligosaccharides and carbohydrates. Judging from the table, several sugges-

<table>
<thead>
<tr>
<th>Column</th>
<th>Bare silica</th>
<th>Amino-silica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acids</td>
<td>10 μm, 4 aromatic carboxylic acid, 85% MeCN–20 mM AB, UV [53]</td>
<td>44–60 μm, 4 aromatic carboxylic acid, 85% MeCN–20 mM AB, UV [53]</td>
</tr>
<tr>
<td>Bases</td>
<td>–</td>
<td>20–35 μm, 2 aminoalcohols, 80% MeCN, RI [63]</td>
</tr>
<tr>
<td>Amides, ureas</td>
<td>10 μm, salicylamide, 85% MeCN–20 mM AB, UV [53]</td>
<td>50 μm, salicylamide, 85% MeCN–20 mM AB, UV [53]</td>
</tr>
<tr>
<td>Polyols</td>
<td>21–25 μm, epirubicines, 90% MeCN, 20 mM FB (pH 2.9), UV [33]</td>
<td>13 μm, 3 μm column, tetracyclines, 85% MeCN, 1 mM CB (pH 5), UV [61]</td>
</tr>
<tr>
<td>Amino acids</td>
<td>–</td>
<td>58–100 μm, 3 amino acids, 60% MeOH, ESI-MS [64]</td>
</tr>
<tr>
<td>Peptides</td>
<td>21–25 μm, 3 flavonoids, 75% MeCN–30 mM AB, UV [25]</td>
<td>–</td>
</tr>
<tr>
<td>Nucleic bases and nucleosides</td>
<td>10–12 μm 6 compounds, 85% MeCN–20 mM AB, UV [53]</td>
<td>14–20 μm 6 compounds, 85% MeCN–20 mM AB, UV [53]</td>
</tr>
<tr>
<td>Oligosaccharides and carbohydrates</td>
<td>33–43 μm by a 3 μm column, gluconolactones, 87% MeCN–100 mM AB, [43]</td>
<td>43–180 μm by a 10 μm column, disaccharides, 85% MeCN [58]</td>
</tr>
</tbody>
</table>

| Remarks | Irreversible adsorption | Irreversible adsorption, Slow column equilibration, column bleeding |

<table>
<thead>
<tr>
<th>Column</th>
<th>Amide-silica</th>
<th>Poly(succinimide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acids</td>
<td>14 μm, 4 aromatic carboxylic acid, 85% MeCN–20 mM AB, UV [53]</td>
<td>–</td>
</tr>
<tr>
<td>Bases</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Amides, ureas</td>
<td>15 μm, salicylamide, 85% MeCN–20 mM AB, UV [53]</td>
<td>–</td>
</tr>
<tr>
<td>Polyols</td>
<td>250 μm, polyphenols, 84% MeCN, UV [86]</td>
<td>–</td>
</tr>
<tr>
<td>Amino acids</td>
<td>–</td>
<td>50–67 μm, amino acids, 80% MeCN–5 mM TB (pH 2.8), UV [1]</td>
</tr>
<tr>
<td>Peptides</td>
<td>Many examples in gradient elutions</td>
<td>33–37 μm, atosiban, 89% MeCN–10 mM TB, UV [59], 69–87 μm, dipptides, 80% MeCN–10 mM TB, UV [1]</td>
</tr>
<tr>
<td>Nucleic bases and nucleosides</td>
<td>13–16 μm 6 compounds, 85% MeCN–20 mM AB, UV [53]</td>
<td>–</td>
</tr>
<tr>
<td>Oligosaccharides and carbohydrates</td>
<td>7 μm by a 3 μm column, 13 μm by 5 μm column, mannitol, 75% MeCN [91]</td>
<td>57–66 μm, sialyl sugars, 80% MeCN–10 mM TB, 20–22 μm, xyloglucans, 70% MeCN–10 mM TB, PAD [23]</td>
</tr>
</tbody>
</table>

| Remarks | Good recovery of analytes | Column bleeding |

<table>
<thead>
<tr>
<th>Column</th>
<th>Sulfoalkylbetaaine</th>
<th>Diol/cyano</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acids</td>
<td>12–21 μm, 4 aromatic carboxylic acid, 85% MeCN–5 mM AB, UV [53]</td>
<td>–</td>
</tr>
<tr>
<td>Bases</td>
<td>15 μm, morphine, 70% MeCN–20 mM AB, UV [25]</td>
<td>19 μm, piperadine and its salts, Cyano, 95% MeCN, ELSD [128]</td>
</tr>
<tr>
<td>Amides, ureas</td>
<td>13 μm, salicylamide, 85% MeCN–20 mM AB, UV [53]</td>
<td>17 μm, methylene blue, Cyano, 90% MeCN–5 mM TFA, UV [130]</td>
</tr>
<tr>
<td>Polyols</td>
<td>26–35 μm, 3 flavonoids, 75% MeCN–30 mM AB, UV [25]</td>
<td>28–30 μm, urea, Diol, 80% MeCN, RI [58]</td>
</tr>
<tr>
<td>Amino acids</td>
<td>–</td>
<td>24–27 μm, glycine, Diol, 80% MeCN, RI [58]</td>
</tr>
<tr>
<td>Peptides</td>
<td>33 μm, neurotensin, 56 μm, bradykinin, 70% MeCN–50 mM AB, UV [25]</td>
<td>–</td>
</tr>
<tr>
<td>Nucleic bases and nucleosides</td>
<td>17–21 μm 6 compounds, 85% MeCN–20 mM AB, UV [53] 36–84 μm for 6 nucleotides, 70% MeCN–100 mM AB, UV [25]</td>
<td>18–30 μm, sucrose, Diol, 80% MeCN, RI [58] 47 μm, sucrose, 74 μm, lactose, by a 10 μm Diol column, 75% MeCN, RI [58]</td>
</tr>
<tr>
<td>Oligosaccharides and carbohydrates</td>
<td>33–38 μm, morphine glucuronides, 70% MeCN–20 mM AB, UV [25]</td>
<td>Column bleeding under acidic conditions</td>
</tr>
</tbody>
</table>

| Remarks | Low column bleeding, Relatively slow optimal flow rates | Column bleeding |
tions for the selection of HILIC columns are given, as follows.

(1) Classical HILIC columns such as bare-silica columns and amino-silica columns are limited by the choice of analytes, due to the strong adsorption phenomena of compounds with specific functionalities. (2) The polymer-coated silica columns based on poly(succinimide) have retentivities for a wide range of analytes, and the use of the amide silica column packed with 3 \( \mu \)m particles may be the choice for the efficient separation of highly polar compounds. (4) The sulfonated silica column can be applied to the separation of a wide range of analytes, but the optimal flow rate of the column is relatively lower than that of amide-silica columns, and not suitable for fast separations. The mixed mode separation of the HILIC and the ion-exchange LC will decrease the separation efficiency. (5) Columns packed with carbohydrate-modified silica particles were used mainly for the separation of oligosaccharides with a fair to good separation efficiencies, and they may make an interesting research subject for chiral separation using HILIC. (6) Cyanogenic and diol-silica columns can be employed in HILIC, but the basic research for the scope and limitation of target analytes is required. (7) Newly released column packed with triazol-bonded silica particles shows good separation efficiencies for a wide range of analytes. (8) Examples of polymer-based monolithic columns are still limited, and their separation efficiencies are not so high in the HILIC mode, though some polymer monoliths in capillaries can generate very high separation efficiencies in the CEC mode. (9) Only limited monolithic silica columns for HILIC are commercially available, and bare silica monolith columns show worse separation efficiencies compared to the RP mode. Monolithic silica column in capillaries can be modified with various polar functionalities to have separation efficiencies for a wide range of analytes. These columns generate better kinetic performance compared to the particle-packed columns.

Many researchers tend to use newly released smaller particle-packed columns, in many cases 3 \( \mu \)m particles except for bare silica columns packed with 1.7 \( \mu \)m particles. However, the basic characteristic of the separation efficiency of these columns is ignored. It is difficult to know differences in separation efficiencies between columns packed with 5 \( \mu \)m particles and those packed with 3 \( \mu \)m particles. There are no common test samples to evaluate separation efficiencies of HILIC columns. In addition, a survey of good \( t_0 \) markers is also required. Although the HILIC mode separation is said to be a good alternative to RPLC, the knowledge of the basic chemistry of HILIC is limited when compared to the RPLC. Generally lower performance of HILIC columns than RPLC columns is an interesting subject to study.

### Table 1 (Continued)

<table>
<thead>
<tr>
<th>Column</th>
<th>Cycloextrin</th>
<th>Triazol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acids</td>
<td>–</td>
<td>11 ( \mu )m, oxalic acid, 50% MeCN–10 mM PB (pH 7), UV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17 ( \mu )m, benzoic acid, 50% MeCN–10 mM AB, UV</td>
</tr>
<tr>
<td>Bases</td>
<td>–</td>
<td>33 ( \mu )m, benzamide, 90% MeCN–10 mM AB, UV</td>
</tr>
<tr>
<td>Amides, ureas</td>
<td>–</td>
<td>17 ( \mu )m, urea, 90% MeCN, UV 20 ( \mu )m, cyanuric acid, 50% MeCN–10 mM AB, UV</td>
</tr>
<tr>
<td>Polyols</td>
<td>13–24 ( \mu )m, norvaline, Chirobiotic T column, 50–80% MeCN–6.5 mM AB [pH 5.5] [145]</td>
<td>11 ( \mu )m, leucine, 13 ( \mu )m, valine, 14 ( \mu )m, isoleucine, 70% MeCN–10 mM AB, UV</td>
</tr>
<tr>
<td>Amino acids</td>
<td>–</td>
<td>14 ( \mu )m, L,2,6-hexanetriol, 90% MeCN, ELSD</td>
</tr>
<tr>
<td>Peptides</td>
<td>–</td>
<td>35 ( \mu )m, oxtocin, 70% MeCN–10 mM AB, UV</td>
</tr>
<tr>
<td>Nucleic bases and nucleosides</td>
<td>15–19 ( \mu )m, nucleosides, maltose column, 85% MeCN–10 mM AB, UV [148]</td>
<td>14 ( \mu )m, uridine, 90% MeCN–10 mM AB, UV</td>
</tr>
<tr>
<td>Oligosaccharides and carbohydrates</td>
<td>19–63 ( \mu )m, oligosaccharides, 78% MeCN, RI [143]</td>
<td>30 ( \mu )m, glucose, 60% MeCN–20 mM PB (pH 7), RI 18 ( \mu )m, glucose-1-phosphate, 30 ( \mu )m, glucose-6-phosphate, 60% MeCN–20 mM PB (pH 7), RI</td>
</tr>
<tr>
<td>Remarks</td>
<td>–</td>
<td>All data are available in [154]</td>
</tr>
</tbody>
</table>

Separation efficiencies, analytes, mobile phases, and detection were summarized. AB: ammonium acetate buffer; CB: citrate buffer; FB: sodium formate buffer; PB: sodium phosphate buffer; TB: triethylammonium phosphate buffer.
Investigations to elucidate the scope and limitation of each stationary phase for HILIC should be carried out to more efficiently utilize this attractive separation mode in liquid chromatography more efficiently.

Acknowledgements

We acknowledge the financial support from a Grant-in-Aid for Scientific Research funded by the Ministry of Education, Sports, Culture, Science and Technology, Nos. 17350036 and 19550088.

References