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Review

Hydrophilic interaction chromatography

Separation of polar compounds on polar stationary phases with partly aqueous eluents is by no means a new separation mode in LC. The first HPLC applications were published more than 30 years ago, and were for a long time mostly confined to carbohydrate analysis. In the early 1990s new phases started to emerge, and the practice was given a name, hydrophilic interaction chromatography (HILIC). Although the use of this separation mode has been relatively limited, we have seen a sudden increase in popularity over the last few years, promoted by the need to analyze polar compounds in increasingly complex mixtures. Another reason for the increase in popularity is the widespread use of MS coupled to LC. The partly aqueous eluents high in ACN with a limited need of adding salt is almost ideal for ESI. The applications now encompass most categories of polar compounds, charged as well as uncharged, although HILIC is particularly well suited for solutes lacking charge where coulombic interactions cannot be used to mediate retention. The review attempts to summarize the ongoing discussion on the separation mechanism and gives an overview of the stationary phases used and the applications addressed with this separation mode in LC.

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1 Introduction

LC was discovered [1–3] by observation of colored compounds being separated on polar sorbents such as cellulose fibers or inorganic oxides, and eluted with nonpolar organic solvents. During the first century or so of its existence, this was the “normal” mode of operating a liquid chromatographic setup. When bonded phase columns started to appear in the 1970s, it was soon realized that a system of opposite polarity, *i.e.*, with a hydrophobic stationary phase and aqueous solutions of water-miscible organic solvents offered substantial advantages. The main drawbacks of normal phase HPLC on naked inorganic oxides were slow equilibration and a site heterogeneity that resulted in nonlinear isotherms, which in turn means peaks that are tailing/fronting and shift their retention times with concentration of the injected substance. Another factor that promoted the popularity of

“reversed” phase (RP) HPLC was its suitability for a large fraction of the bioanalytical solutes that were of interest. Pharmaceutical development has since long involved a screening of the water/octanol partitioning [4], which is related to the transport, absorption, and distribution of chemicals in biological systems, and hence the pharmacodynamic properties [5]. It turns out that most useful drugs have traditionally been found in a polarity interval that allows them to be well separated from most naturally occurring substances in blood plasma on an octadecyl-bonded phase, using as eluents aqueous buffers with a water-miscible solvent. The tremendous popularity of RP-HPLC left naked inorganic oxides behind, and solution phase chemistries such as micellar chromatography [6] were developed to allow separation of excessively retained compounds on regular RPs with eluents of suitable composition.

A problem that has been the focus of much attention is how to create retention in RP-HPLC for compounds with no or very low inherent distribution toward packings with conventional RP functionality. In those cases where some retention is seen, it usually calls for eluents with very low admixtures of organic solvents. Inadequate phase wetting and expulsion of eluent from the pore space often accompanies such efforts [7] and highly aqueous eluents are therefore often connected with nonrepro-

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Abbreviations: AEPD, 2-amino-2-ethyl-1,3-propanediol; HILIC, hydrophilic interaction chromatography; NP, normal phase; NPC, normal phase chromatography; PSP, paralytic shellfish poisoning; S-DVB, styrene-copoly-divinylbenzene; TEAP, triethylammonium phosphate

ducible retention times and low separation efficiencies. There have been numerous attempts to remedy this lack of retention, mainly by polar embedded or polar end-capped groups which decrease the risk of C_{18} phases to become dewetted. However these stationary phases provide lower retention than the standard C_{18} phases [8] and the approach is also unsuitable for compounds with low aqueous solubility. Really polar compounds will not be retained even on these more polar stationary phases.

Lack of retention for highly hydrophilic compounds is to a large extent caused by solvophilic factors, where polar functional groups have an ability to enter favorable dipolar bonds with the solvent, i.e., become solvated. Since nonpolar stationary phases cannot offer similar bonding, the solutes stay in solution and are thus eluted in the void volume. The functional moieties that convey this property to highly polar compounds are either charged groups or groups capable of entering strong dipolar or hydrogen bonds.

In those cases where the lack of retention is due to one or more charged functional groups, retention is easily accomplished by coulombic interaction mechanisms. Ion-exchange chromatography is an obvious choice, and can be used for practically all charged solutes, from small inorganic ions to proteins and other biological macromolecules. An alternative to ion exchange is ion pairing, which allows the use of RP columns that are less expensive and often have a better separation efficiency compared to dedicated ion exchangers. The increased retention is mediated by an ion pairing agent, which is an ionic compound of charge opposite to the solute, capable of entering into an ion pairing equilibrium. Ion pairing is predominantly used in cases where the dissociation/protonation equilibrium cannot be effectively used to transform the solute into a noncharged form within the applicable pH range of the column material being used. Retention can be seen as either a partitioning of uncharged ion pairs onto the hydrophobic stationary phase or a dynamic coating of the stationary phase with the ion pairing reagent leading to an ion-exchange-type retention mechanism; for a recent review see, e.g., Garcia [9]. Ion pairing reagents typically added to the mobile phase are, e.g., TFA or sodium heptane sulfonate, but these ion pairing agents have been shown to significantly reduce the signal intensity in MS with ESI [10].

Among the most problematic solutes in chromatography are compounds that are highly hydrophilic and uncharged. They lack the coulombic means of interactions, and the polar functional groups usually cause a low volatility and/or render the solutes too unstable to allow separation in GC. One way that has traditionally been used to increase the retention of this compound class is by converting one or more of the polar functional

groups into hydrophobic groups by chemical reactions, a technique referred to as derivatization [11]. In addition to increased retention, derivatization may also give other beneficial effects such as improved detectability. It has thus been widely used when UV and fluorescence detectors were mainstream and a goal was to introduce a property to the solute that differed from matrix components to enable detection in a "window" where few underivatized compounds gave signals. A striking example is formaldehyde, which in aqueous solution predominantly exists as its highly hydrophilic hydrate dihydroxymethane. The reaction with 2,4-phenyl hydrazine in acid solution converts formaldehyde quantitatively into the nonvolatile hydrazone, which has convenient optical absorption properties and lends itself to detection by MS [12]. Derivatization usually works well when a single and highly reactive group is being derivatized in a reaction known to yield a single product quantitatively under mild reaction conditions. For solutes containing multiple polar functional groups, the reactions frequently yield side products and are often not quantitative. Another drawback of derivatization as a means of increasing retention is that the main retention determinant will be the attached hydrophobic group. Closely related compounds therefore tend to be less well separated after derivatization. Derivatization is also time-consuming and increases the uncertainty in quantitative analysis.

Conceptually, the most rational way to address very hydrophilic and uncharged compounds in HPLC is by a "reversed RP", i.e., a separation mode where a polar stationary phase attracts and is enriched by the more polar part of the eluent and act as the retentive element, while the eluent at the same time offers reasonable solvent properties to allow a fast and linear distribution between the two phases. Hydrophilic interaction chromatography (HILIC) is such a technique, where analyte retention is believed to be caused by partitioning of the analyte between a water-enriched layer of stagnant eluent on a hydrophilic stationary phase and a relatively hydrophobic bulk eluent, with the main components usually being 5–40% water in ACN [13]. The use of water as the strongly eluting solvent gives HILIC a number of advantages over conventional normal phase chromatography (NPC); eluent preparation is less complicated since the need of total control over the solvent water content is omitted. Normal phase (NP) eluents are also nonpolar (often based on hexane) and polar analytes usually have a low solubility in these eluents. The interfacing with electrospray MS is also a problem with NP, since ionization is not easily achieved in totally organic, nonpolar eluents. The elution order in HILIC is more or less the opposite of that seen in RP separations [13], which means that HILIC works best for solutes that are problematic in RP. This

orthogonality [14] also houses one of the inherent advantages of HILIC, namely the possibility of creating coupled separations. When HILIC is used in conjunction with RP SPE, the strongly eluting solvent used to desorb the analytes from the SPE device is a weakly eluting solvent for HILIC or *vice versa*. Direct injection of the SPE eluate onto the separation column of opposite polarity is therefore often possible, which saves time and prevents sample losses [15]. This approach can however only be used for solutes that show retention on both RP and HILIC-type stationary phases, and an RP-SPE/HILIC scheme is obviously among the preferred combinations when the aim is to trap and separate polar compounds. The high ACN content also gives HILIC two additional advantages; high sensitivity in ESI-MS [16–18], and faster separations due to the lower viscosity of HILIC eluents compared to standard RP eluents [19]. Considering its growing importance and relative maturity, there have been surprisingly few reviews dedicated to HILIC. Strege [20] devoted parts of his 1999 review on natural products and modern high-throughput screening to HILIC. Naidong [17] reviewed the use of underivatized silica in HILIC mode combined with MS/MS for bioanalysis, Xie *et al.* [21] recently reviewed the use of polar phases in CEC, and Yoshida [22] has summarized the field of peptide separations by HILIC.

2 History

The acronym HILIC was suggested by Alpert in 1990 [13] to describe a chromatographic technique where the analytes interact with a hydrophilic stationary phase and are eluted with a relatively hydrophobic binary eluent in which water is the stronger eluting member. This separation technique had by then been used since 1975 in HPLC for the analysis of sugar and oligosaccharides [23, 24]. These early separations had a limited applicability since they could be run only under isocratic conditions due to detection by refractive index. HILIC mode separation had also been used for the determination of cocaine in serum [25]. It was first put forward by Rabel *et al.* [26] that this new separation mode was a variant of NPC. Yet, as early as 1952 Samuelson and Sjöström [27] described the separation of monosaccharides on an Amberlite IRA-400 ion-exchange column by means of stepped gradient elution starting at 99.5% ethanol, going toward pure water. That hydrophilic stationary phases like ion-exchange resins contained a water-enriched layer on the surface was first described by Gregor *et al.* [28], and that this stagnant water layer could be related to the uptake of nonelectrolytes by ion-exchange resins was suggested by Rückert and Samuelson [29] already in 1954. They also showed that the distribution (K_d) of glucose between 88% ethanol and the ion-exchange resin followed the lyotropic series, both for the immobilized ion and the counter ion. A later

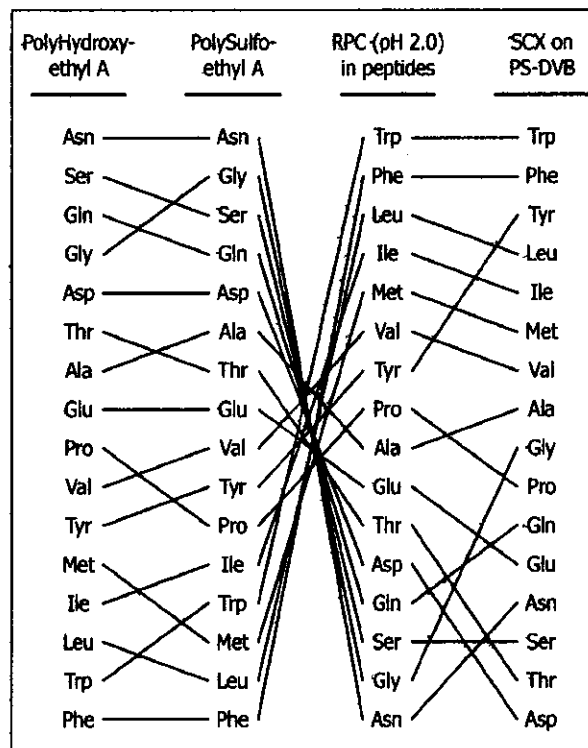


Figure 1. Relative order of elution of amino acids from various columns. Tie lines connect the same amino acid. Polyhydroxyethyl A and Polysulfoethyl A: Columns, 200 × 4.6 mm; mobile phase (isocratic), 10 mM TEAP (pH 2.8) containing 80% v/v CH₃CN. RP-HPLC: Linear AB gradient from water to CH₃CN (0.1% TFA in each). SCX: Step gradient of increasing salt and pH. Adapted from Alpert, 1990 [13] with permission.

paper by Havlicek and Samuelson [30] shows very good separations of oligosaccharides on regular gel-type ion-exchange resins.

HILIC has been steadily gaining interest and in the last few years it has emerged as a viable option to RP-HPLC for many applications dealing with polar and hydrophilic analytes. HILIC is a variant of NPC but utilizes water in a water-miscible organic solvent (most often ACN, but in some cases acetone or methanol) as the eluent in conjunction with a hydrophilic stationary phase. This means that the retention order in HILIC should be roughly the opposite of the order analytes are eluted from an RP column. Reversal of the retention order has been seen on C₁₈ columns at elevated levels of organic modifier [31, 32], on naked silica [32], and on a “hydrophilic polystyrene/DVB” stationary phase [33]. Alpert [13] also reported an almost complete reversal of retention order for amino acids as compared to RP (Fig. 1).

3 Mechanism

The distinction between HILIC and NPC is somewhat debated but the definition proposed by Alpert [13] seems

to be gaining acceptance, i.e., the term HILIC should be used (a) if the strongly eluting solvent is water and (b) the retention mechanism is by partitioning. Whereas retention in conventional NPC is predominantly governed by surface adsorption phenomena, Alpert suggested that the retention mechanism for HILIC was a partitioning between the bulk eluent and a water-rich layer, partially immobilized on the stationary phase. His arguments for suggesting a partitioning mechanism were (a) that carbohydrate separations and water retention experiments by Verhaar and Kuster [34], Orth and Engelhardt [35], and Nikolov and Reilly [36] carried out on underivatized silica and silicas derivatized with various amines had all been shown to involve a partitioning mechanism, since the stationary phases retain a semi-immobilized layer of water-enriched mobile phase; (b) a similarity with the extension of Albertsson's [37] aqueous two-phase partition systems to chromatography by Müller and Kutemier [38–40], with respect to (i) increase of retention time with size for an oligothymidylic [pd(T)_{12–30}] homologous series; (ii) retention for oligo(nucleic acids) following an opposite pattern compared to anion exchange; and (iii) extremely high sensitivity of the retention to eluent ionic strength. Let us now examine these fundamentals a little closer, but before we embark on this journey we should briefly review the basic equations describing pure partitioning and adsorptive interactions: RP chromatography has its roots in liquid–liquid chromatography and retention is considered to be (ideally) controlled by partitioning only. The relationship that is established for partitioning in RP separations (approximated to be valid over a narrow range of elution strengths) is

$$\log k' = \log k'_w - S\varphi \quad (1)$$

where k'_w is the capacity factor for the weaker eluent component (water) only as mobile phase, φ is the volume fraction (concentration) of the stronger member of a binary mobile phase mixture, and S is the slope of $\log k'$ versus φ when fitted to a linear regression model [41].

Well-proven models exist for describing the retention in NPC as function of a binary mobile phase composition. The first model for adsorption chromatography was proposed by Snyder [42], and has later been merged with a parallel model of Soczewinski and Wachtmeister [43, 44] into the Snyder-Soczewinski model [45, 46], where elution is depicted as taking place by a displacement exchange between solutes and solvent on the stationary phase surface. Contrasting the Snyder-Soczewinski model is the Scott-Kucera model [47], which has been disputed in the literature. The models are both quite complicated and difficult to employ. The NPC models are furthermore developed for classical solid-LC, where adsorption (direct surface interactions in the Snyder-Soczewinski model, or interactions with an absorbed solvent

layer in the Scott-Kucera model) occurs, mainly for non-polar to nonamphoteric polar solvents to which ethers, esters, and ACN belong. Water is (alongside alcohols, carboxylic acid, and phenols) part of the amphoteric polar group, where water is an extreme and is used in HILIC at concentrations which are way outside the bounds of the classical liquid–solid chromatography retention models.

For conventional NP chromatographic systems, where retention is based on surface *adsorption*, the relationship between the retention and the mole fraction X_B of the stronger solvent B in the eluent should adhere to the following expression [46, 48]:

$$\log k' = \log k'_B - \frac{A_s}{n_B} \log N_B \quad (2)$$

where k'_B is the solute retention factor with pure B as eluent, A_s and n_B are the cross-sectional areas occupied by the solute molecule on the surface and the B molecules, respectively, and N_B is the mole fraction of the stronger member B in the eluent. Plots made of $\log k'$ versus the linear and logarithmical function of the water contents in the eluents used in HILIC separations should thus give an indication on whether partitioning or adsorption is the dominating retention mechanism.

The immobilized water argument is unquestionably valid in view of the references cited by Alpert [13], i.e., there exists a water-enriched layer (most probably a compositional and structural gradient) in the immediate vicinity of a polar stationary phase in contact with an aqueous solvent mix, manifest in retention of water on naked and amino-functionalized silicas [34–36]. However, if we review these references more carefully, we find that Verhaar and Kuster [34] never made any experiments to actually assess the mechanism. Instead they referred to Samuelson [49], who pioneered separations of sugars on relatively weakly crosslinked gel-type ion-exchange materials. One has to differentiate between low crosslinked ion exchangers, which are highly charged and hydrophilic xerogels that swell considerably and uniformly in water, and silica, which is an impervious sintered aerogel that can retain water on the surface only. There is little doubt that a partitioning mechanism is prevalent in Samuelson's separations, but because of the radically different nature of these materials, the partitioning mechanism cannot be directly extrapolated to polar porous silica sorbents.

Orth and Engelhardt [35] did a series of experiments to elucidate the mechanism, among them studies on the ability of amino-, ethylenediamino-, and diethylenetriamino-silicas to retain water after switching from water/ACN to dichloromethane. This was coupled with retention studies for a number of mono-, di-, and trisacchar-

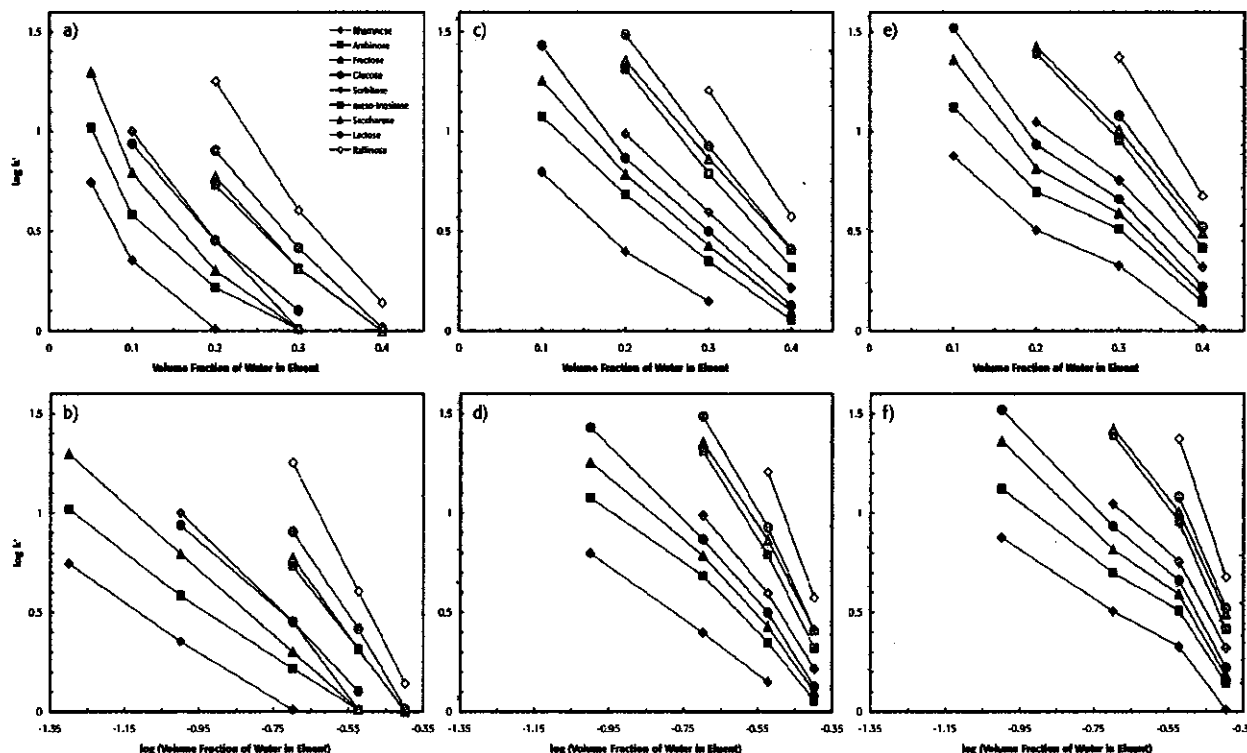


Figure 2. Dependence of $\log K$ on the water contents in ACN/water eluents on LiChrosorb Si 100 reacted with (a + b) amino-, (c + d) diamino-, and (e + f) triaminosilane, all plotted on the same scale. The upper plots (a, c, and e) were made from data extracted from Fig. 1 of Orth and Engelhardt [35], whereas the lower curves (b, d, and f) contain the same data plotted against the logarithm of the water fraction in the eluent.

ides on the same three phases. They concluded that, as is expected in a partitioning system, the retention decreased as the difference in polarity between the two phases was decreased, and that the opposite would be expected if a hydrophobic retention mechanism was responsible for the retention. Further, they argue that the retention increased with the number of amine/imine groups in the bonded moiety, which in turn was accompanied by an increasing amount of stationary eluents in the pores. They also noted that the mechanism appeared to be constant for all three phases and therefore excluded selective interaction, since the K' ratios did not change among the phases (K' for all probes were 2.97 ± 0.3 times higher on the diamine phase than on the amine phase, whereas the K' ratios between the triamine and amine phases were 3.4 ± 0.3). This was correlated to the amount of water trapped on the stationary phases (0.13, 0.23, and 0.25 g/g for the mono-, di-, and triamine phases, respectively). These observations could be explained only if the different amine ligands (that are of different basicity) were not involved in the separation directly, only indirectly through selective enrichment of water in the trapped mobile phase. A retention pattern that followed the polarity of the sugars (expressed by the number of hydroxyl groups) was finally taken as a circumstantial evidence for a partitioning mechanism.

Although their data unequivocally demonstrate that water is strongly retained on the surface of all three phases and increases with the length of the oligoethyleneimine chain, and that retention is a function of increasing polarity and size of the sugars, they never made an attempt to plot those data according to Eqs. (1), (2), to assess whether the mechanism actually was due to partitioning or of an adsorptive nature. A reassessment of their data is therefore shown in Fig. 2.

A few things can be noted from Fig. 2. First, the retention curves on the aminopropyl phase, which are all curved when plotted as a lin-log function (Fig. 2a), all turn out in the corresponding log-log plots (Fig. 2b) as straight lines with an average r^2 of 0.9981. The slopes of the lines range from -1.22 to -1.74 for the monosaccharides, -2.05 to -2.42 for the polyols, -2.58 to -2.95 for the disaccharides, and -3.69 for the trisaccharide. The good fit is consistent with an adsorptive process and the increase in slopes indicate that more interacting points are engaged as the number of available polar sites increase in the solutes. There is incidentally also a good fit between $\log K$ and the slopes in Fig. 2b at 20% water, the only concentration where all solutes were tested; $y = -2.09x - 0.9913$; $r^2 = 0.9732$. The di- and triamine silicas (Fig. 2c–f) are less conclusive when reassessed in the

log–log plots. At up to 30% water, the adsorption model seems to fit better, but between 30 and 40% water something appears to happen in the experiments, most evident from the discontinuous retention patterns on the triamine phase (Fig. 2e and f). This is also the water concentration range where the stationary phases start to become saturated and the enrichment levels off on underivatized and aminopropyl silicas, according to Verhaar and Kuster [34] and Nikolov and Reilly [36]. This finding could very well be taken as an indication of liquid–liquid partitioning. Although interesting, the paper of Orth and Engelhardt [35] does not provide conclusive evidence for a partitioning mechanism, since all the circumstantial evidence put forward basically support polar interactions that increase in strength with increasing number of polar groups on the solutes and on the stationary phases. It does not elucidate of which kind these interactions are. It is interesting to note that Verhaar and Kuster [34] saw a strong catalytic effect from aminopropyl silica on the mutarotation rate of sugars when used as a catalyst in stirred bulk experiments, and concluded that the amine groups were responsible for the high reaction rate. These results are hard to explain without the possibility of direct contact between the amino groups and the sugars, i.e., in the absence of surface interactions.

Nikolov and Reilly [36] base their argumentation for a partitioning interaction between the carbohydrates and stagnant water molecules on retention data taken from four different sources, which were correlated with calculated hydration numbers, those also from four different sources. Their data are visualized as a plot of retention times relative to sucrose versus carbohydrate hydration number in Fig. 3. Part of the spread in the plot can most probably be explained by data being gathered from eight different sources, compounded by molecular modeling data dating from 1958, 1972, 1978, and 1981. While it confirms that retention increases as a function of size and hydrophilicity of the sugars, the data presented in this plot can hardly be taken as an unequivocal evidence for partitioning as a retention mechanism.

The pertinent work of Müller [39] involves surface-initiated graft polymerization of acrylamide onto wide pore (1000 or 4000 Å) diol silicas, resulting in layers with estimated thicknesses of 140 and 460 Å, respectively. To this hydrophilic polymer graft is equilibrated the dextran-rich lower layer of a dextran/PEG aqueous two-phase polymeric system, which acts as the actual retentive medium for separation of DNA restriction fragments. The setup is in effect a liquid–liquid separation system where separations are mainly controlled by minute changes in charge between the two phases, accomplished by a salt gradient during elution to force the highly charged DNA polymers to move between the

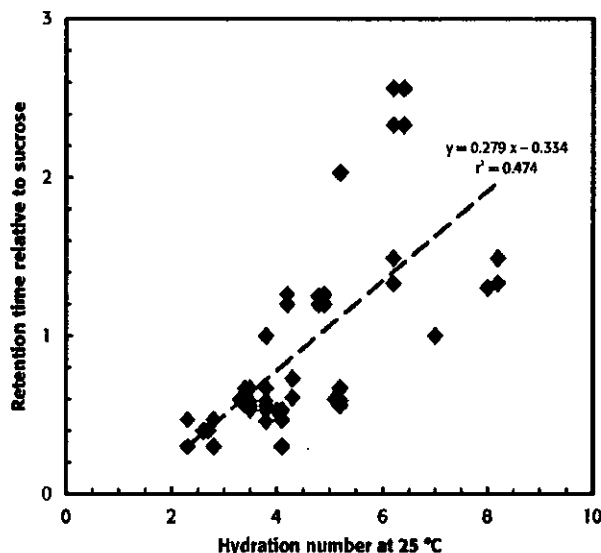


Figure 3. Retention as a function of calculated hydration numbers at 25°C for a series of mono-, di-, and trisaccharides on an amino-bonded silica in 80% aqueous ACN plotted from Table III in Nikolov and Reilly [36]. Dashed line is a linear regression with the equation and correlation coefficient, r^2 .

phases. This scheme is so immensely different from aqueous NPC that it cannot be used to infer a partition mechanism in HILIC.

Alpert [13] concluded cautiously that the phenomena responsible for partitioning between the two phases were obscure and might “involve some kind of dipole–dipole interactions.” He also suggested that a great deal of studies would be necessary to clarify the mechanism. Yet these caveats seem to have been overlooked in later works citing Alpert, as most authors seem to rely on the assumption he made, i.e., that retention is due to a partitioning mechanism. Let us therefore look at data from a few recent papers.

Guo and Gaiki have examined the effect of column temperature on retention and applied the van't Hoff equation, where linear and exothermic relationships indicated that there were no strong specific interactions between the solutes and the stationary phases. This can be taken as a relatively strong support of a partitioning mechanism rather than surface adsorption [50, 51]. They also concluded that the increase in retention time with salt concentration gave indirect support to partitioning as the primary separation mechanism.

Some researchers show what is claimed to be linear relationships between $\log k'$ and % of water over extended ranges of water admixtures. Guo and Huang [50] saw linearity from 10 to 35% water in ACN for 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS) and 2-amino-2-ethyl-1,3-propanediol (AEPD) on aminopropyl silica, and Valette *et al.* [52] concluded a similar linearity for three different

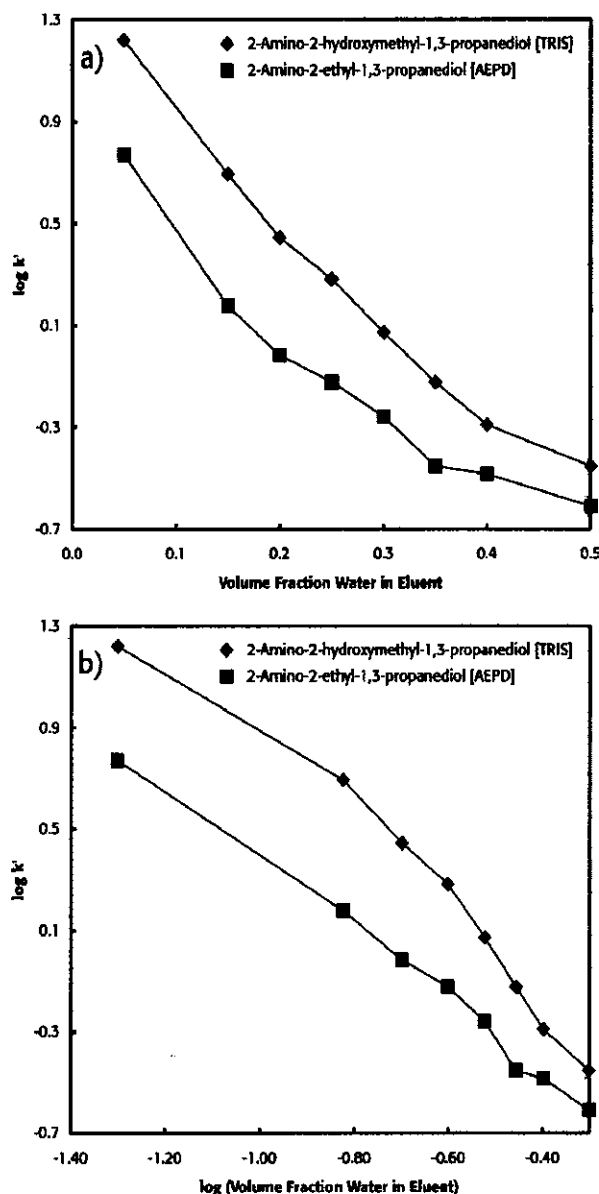


Figure 4. Linear (a) and logarithmic (b) plots of $\log K'$ vs. volume fraction of water in an ACN/water eluent on a Zorbax NH_2 column at 25°C. Plotted from the numerical data used to produce Fig. 3 in Guo and Huang [50].

tetracyclines, also on an aminopropyl silica. Reassessed, the data from Fig. 4 by Guo and Huang [50] (Fig. 4) show equally good (or bad) fits when $\log K'$ for TRIS is plotted against the linear and logarithmic volume fraction of water in the eluent ($r^2 = 0.968$ and 0.958 , respectively), whereas the plots for AEPD reveal a worse fit for the log–lin (Fig. 4a) than for the log–log (Fig. 4b) plot ($r^2 = 0.912$ vs. 0.992 ; the deviations in the lowest K' range may be due to difficulties of determining very low retention factors with great exactness).

TRIS, a small and *extremely* hydrophilic compound, fits partitioning better than surface adsorption but adheres

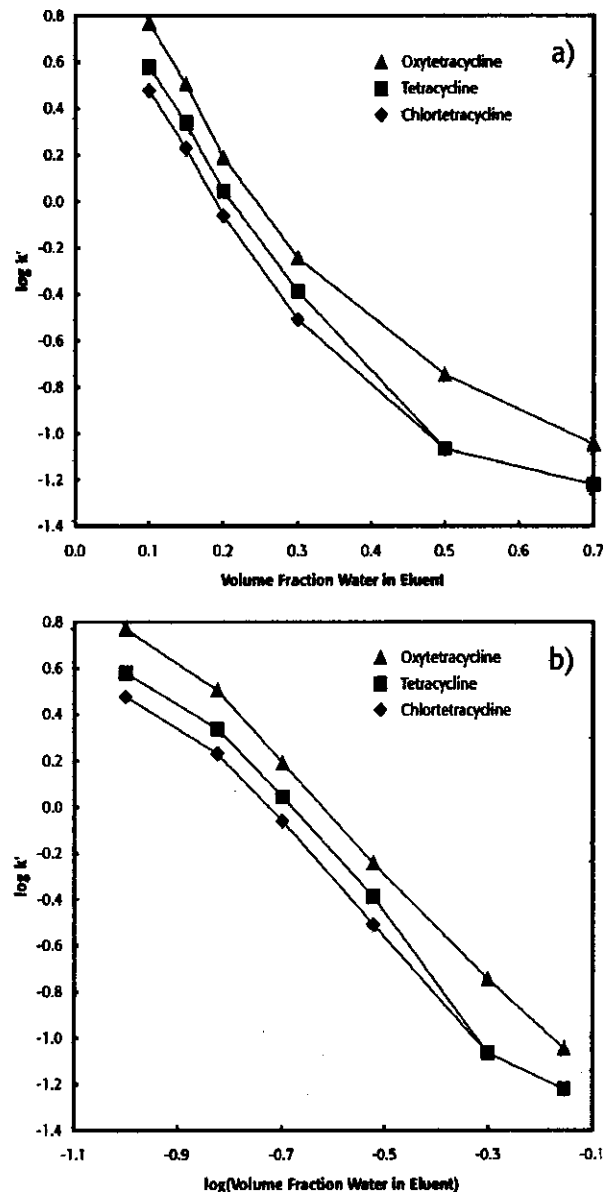


Figure 5. Linear (a) and logarithmic (b) plots of $\log K'$ vs. volume fraction of water in an ACN/water eluent containing 5 mM citrate buffer, pH 3.5 on a ThermoHypersil APS2 column. Plotted from the numerical data used to produce Fig. 3 in Valette *et al.* [52].

well to neither model, while the somewhat less hydrophilic AEPD (an ethyl group is substituted for one of the hydroxymethyl groups) seems to match the surface interaction model well. If two so similar compounds deviate so significantly from each other under otherwise identical conditions, it will be hard to draw any firm and general conclusions on whether the retention mechanism is purely partitioning or adsorption. It should be noted that these experiments were done in unbuffered ACN/water mixtures, which means that electrostatic interactions, most likely repulsion of the amine solutes from the amino phases, would be present at maximum strength.

If plots are made from the data of Valette *et al.* (Fig. 3 in [52]), we see a similar difficulty of drawing a firm conclusion on the retention mechanism. The lin-log plot (Fig. 5a) is largely linear in the range from 10 to 30% water, whereas the log-log plot (Fig. 5b) is linear from 15 to 50% (in the case of oxytetracycline to 70%) water. Again, it could be pointed out that it is difficult to make exact determinations of k' at the shortest retention times measured (lowest k' is 0.06).

The interpretation is complicated by electrostatic repulsion between the tetracycline solutes and the aminopropyl silica, both being positive at the eluent pH tested (3.5 and 5.0), and strong interaction between the citrate buffer used and the aminopropyl column. The general trend seems to be that when plotting $\log k'$ versus the fraction of water in the eluents the plots appear linear at intermediate levels [51] but curve upwards at both the lower and higher end of the ranges tested, i.e., the retention curves are U-shaped. Such U-shaped curves of $\log k'$ versus % of organic modifier were seen by Hearn [31] and Bij *et al.* [32] on both naked silica and octadecyl silica phases in RP eluents covering a very wide range of eluent strengths. The conclusion was that the deviations from linearity at high water content were due to hydrophobic interactions and at low water content due to "silanophilic" interactions. It should be pointed out that even though the term hydrogen bonding is used, Scott and Traiman [53] postulated that there will be no direct interaction between the surface silanols and the analyte since there will always be a tightly hydrogen-bonded monolayer of water associated with the silica surface that will not be removed by solvents.

In a recent paper by Guo and Gaiki [51], the retention times for acetylsalicylic acid and cytosine were plotted against the percentage of ACN in the eluent for four columns of different nature. A linear relationship would provide evidence for a partitioning mechanism, augmented by the observation that at extreme ACN concentration (95 % in the work of Guo and Gaiki) a deviation is evident toward excessive retention. When the data in the three papers cited above are visualized as $\log k'$ against the linear and logarithmic ratio of water (the eluting member) of the eluent, curvatures are evident in most of the plots. However, in general the data adhere better to a log-log plot, as is seen when $\log k'$ for cytosine on four different columns from Guo and Gaiki [51] are plotted as a linear or logarithmic function of the stronger eluent concentration (Fig. 6). What is often concluded to be deviations from a partition-based hydrophilic interaction separation mechanism in the extremes of the water admixture range could be equally well explained by a retention mechanism based at least partly on adsorptive interactions that would follow the empirical formula derived for ion-exchange sorbents [54].

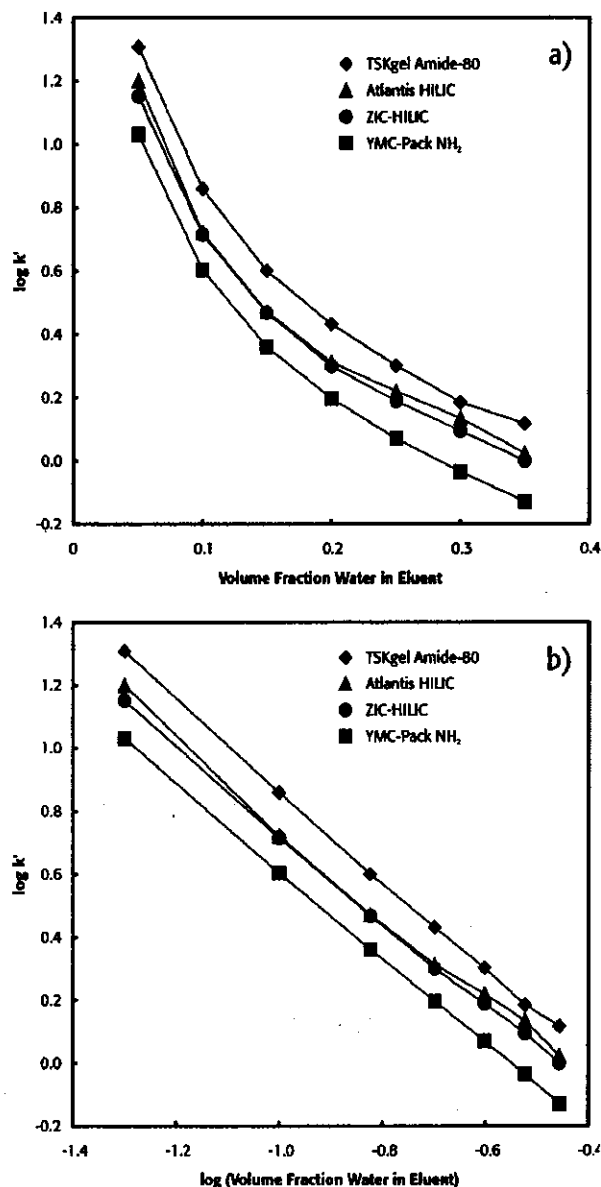


Figure 6. Plots of (a) $\log K$ vs. ϕ and (b) $\log K$ vs. $\log \phi$ for cytosine on four different silica-based columns, prepared from the numerical data used to produce Fig. 3 in Guo and Gaiki [51].

The straight lines obtained in the log-log plots for cytosine in Fig. 6b are striking, as they were obtained on four markedly different silica-based columns; underivatized, amide, aminopropyl, and a sulfoalkylbetaine zwitterionic material where functional moieties reside on grafted organic polymer chains instead of on short spacers as in the other materials. In comparison to the aminopropyl and amide-bonded phases, the polymeric zwitterionic material thus has a relatively thick interactive layer, and sulfoalkylbetaine polymers are soluble in water and known to retain water strongly. Consequently, it should be a near ideal phase for liquid-liquid partitioning. Particularly strong advocates of the Snyder-Soczewinski

model (cf. p. 399 in [46]) also predict that under NPC conditions where water or another “AB solvent” is part of the eluent, formation of a multilayer enriched in the strong solvent is likely. Yet the retention curves on all these phases are best represented by the original Snyder–Soczewinski adsorption model. The conclusion of Guo and Gaiki [51], based on the k' dependence, an increase in retention with increasing salt concentration in the eluent, and not least the van't Hoff plots, was still that the retention in HILIC is not mediated by strong stationary phase interactions, but follows the partition retention model suggested by Alpert.

As these four materials were all silica-based, it may be argued that residual silanol groups are responsible for the deviation from retention according to the partition model. Chambers and Fritz [55] evaluated a porous (80–100 Å) styrene-*copoly*-divinylbenzene (S-DVB) strong cation exchange (SCX) material (55% crosslinked) with varying degree of sulfonation in HILIC-type separation of several neutral polar compounds. They were able to find a fitting of $\log k'$ for ethylene glycol, propylene glycol, glycerol, D-fructose, and dextrose obtained with 5–30% water in ACN as eluents to the following expression

$$\log k' = A\phi_{\text{H}_2\text{O}}^2 + B\phi_{\text{H}_2\text{O}} + C \quad (3)$$

and a regression coefficient of 0.999 was obtained. Although not referenced, this expression matches the empirical function by Schoenmakers *et al.* [56] for retention by partitioning in RP. However, if the same data (taken from Table 5 in [55]) are plotted as $\log k'$ versus $\log \phi_{\text{H}_2\text{O}}$ and $\phi_{\text{H}_2\text{O}}$, they produce the curves shown in Fig. 7.

The topmost line (dextrose) in Fig. 7b has an exceptionally good fit ($r^2 = 0.9999$) and the regression coefficient decreases slightly as we move toward lower retention (0.9997, 0.9992, 0.9967, and 0.9883 for D-fructose, glycerol, ethylene glycol, and propylene glycol, respectively). Corresponding r^2 for the log–lin plots in Fig. 7a range from 0.972 to 0.876. Thus, the Snyder–Soczewinski model [45, 46] fits these data remarkably well and appears to be the more valid model also for this markedly different material run under HILIC conditions. Since these separations were carried out on a sulfonated organic matrix and not on a silica-based material, we cannot ascribe this good fit to a surface adsorption phenomenon on silica. Instead it appears that retention in LC relies on strong interactions (ionic, ion-dipole, and hydrogen bonding), in which the elution is mediated by a stoichiometric substitution that follows the Snyder–Soczewinski model. This is also in agreement with the commonly accepted model for retention models for ionic interactions [57]. Retention based on weaker interactions (such as in RP) that do not require a strict stoichiometric exchange of eluent components follows the partition model. Plots based on data in the original HILIC paper by

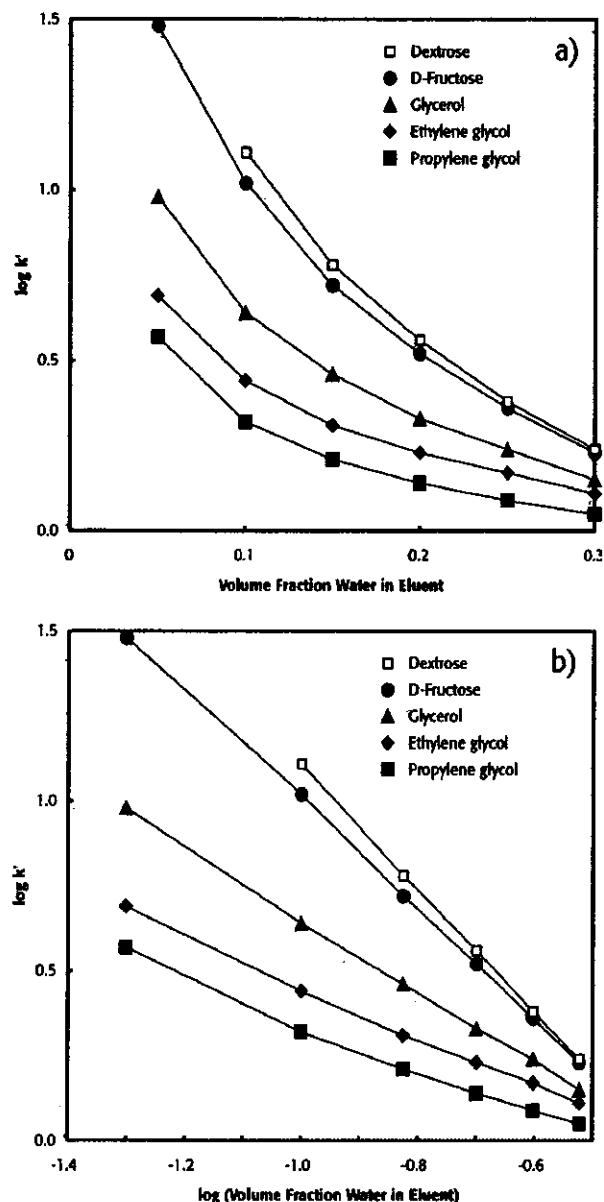


Figure 7. Linear (a) and logarithmic (b) plots of $\log K'$ vs. $\phi_{\text{H}_2\text{O}}$ in an ACN/water eluent for sugars and glycols separated on a sulfonated (2.63 mmol/g) porous S-DVB material in the Ca^{2+} form. Plotted using data from Table 5 in Chambers and Fritz [55].

Alpert are simply not possible since many of the compounds are plotted with negative k' (Fig. 5 in [13]).

An adsorptive retention model was also concluded for peptides in HILIC in a recent review by Yoshida [22]. On an amide silica, Yoshida found that when $\log k'$ for a series of peptides covering a large residue number range (obtained in gradient runs) was plotted against the logarithm of the water content, the data points invariably fell on straight lines [54] (Fig. 8). The conclusion was that the elution pattern is analogous to nonaqueous NPC, and would indicate a retention mechanism more similar to

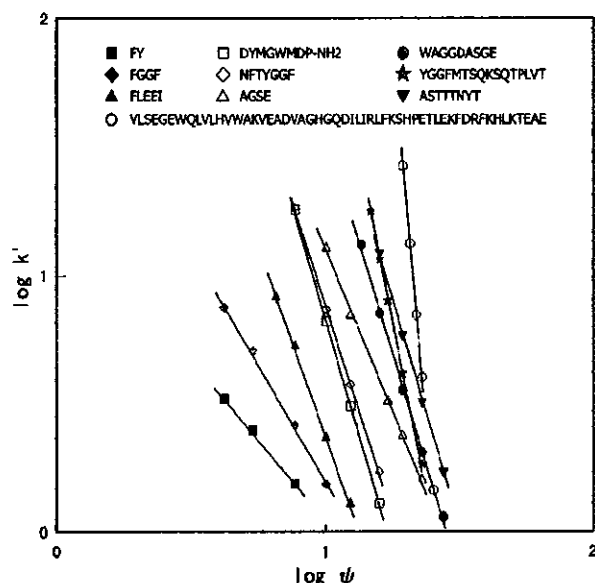


Figure 8. Plots of the retention factor, $k'(\Psi)$, of peptides on the volume fraction of water (Ψ) in ACN/water eluents obtained by isocratic elution. Adapted (legend integrated in figure) from Yoshida [54] with permission.

the surface adsorption established for NP separations. Yoshida [22] also suggested hydrogen bonding as the principal interaction mode in HILIC.

An intermediate theory was put forward by Berthod *et al.* [58] who suggest a retention mechanism that is a combination of partitioning and hydrogen bonding. They plotted the DP of a number of oligosaccharides *versus* $\log k'$ and the linearity of the plot was compared to the RP separation of a homologous series. They also found that the slopes of these plots decrease as the water content in the mobile phase increases and that this decrease in $\log k'$ *versus* % of water is nonlinear. When the logarithm of $\Delta G_s^0/RT$ ($\log k' = \text{constant} \cdot \Delta G_s^0$) is plotted against % of water, the experimental data fall more or less on straight lines with the same slope for all oligosaccharides. They also used these relationships to extrapolate to hypothetical retention times in 100% ACN, and although extrapolation to a retention time of 600 million years is a bit outside the experimental domain, the conclusion that hydrogen bonding must play a major part in the retention at extremely low water content is logical. More important is probably the conclusion that the retention mechanism for sugars on CD-bonded phases in HILIC mode is due to both partitioning and hydrogen bonding, and that discrimination between these two contributions is impossible because the actual phase ratio is dependent on the amount of water absorbed in the interfacial hydrated layer.

The use of other eluents than ACN and water has been examined, but methanol as the strongly eluting solvent gave wider peaks and only minor changes in selectivity

[52, 59]. The use of alcohols as the weakly eluting solvent has also been attempted and this might be useful if the analyte is not soluble in ACN. When Li and Huang [60] compared the separation of epirubicin from three of its analogs with methanol, isopropanol, THF, and ACN as weakly eluting solvents with 10% aqueous buffer, methanol gave no retention. Isopropanol gave some retention but was unable to separate the structural isomers from each other. THF should, based on its solvent strength ($\epsilon^0 = 0.48$), result in longer elution times than ACN ($\epsilon^0 = 0.50$) but the strongest retained peak (doxorubicin) had a retention time roughly four times as long in ACN as in THF, and the retention order was also somewhat different. This is explained by THF being a better hydrogen bond acceptor than ACN, a difference that affects the apparent pK_a of the analytes giving the analytes higher pK_a in ACN than in THF. Later in the paper, it is shown that an increase in pH gives a similar change of retention order, but the retention time was only slightly affected. Thus it seems equally rational to assume that the hydrogen bond acceptor capabilities of THF makes it disrupt the immobilized water layer of the stationary phase (*i.e.*, increase the eluting strength compared to what might be assumed by considering the ϵ^0 value in RP).

At present all manufacturers of HILIC column recommend the use of buffered eluents in HILIC in order to reduce the electrostatic interactions between charged analytes and deprotonated silanol groups of the stationary phase. These interactions are a major factor when separating charged molecules on all forms of silica columns; for example, basic analytes can be separated by an ion-exchange mechanism on pure silica. It has been shown that columns prepared from silica of higher purity provide lower retention and less selectivity than columns made from silica containing metal impurities [61, 62]. This is explained [61] as a mixed mode phenomenon caused by the higher acidity of silanols "activated" by the proximity to a metal impurity, mainly aluminum and iron. It should be pointed out that these experiments were made using eluents with relatively high water contents, making RP or direct metal ion interactions likely as the secondary separation mode. However, these findings should translate to HILIC separations as well. Electrostatic repulsion of acidic or negatively charged species can lead to complete expulsion of such analytes from the pore space of silica-based separation materials. Ammonium salts of formate or acetate are mostly recommended due to their high solubility in eluents with high ACN content, a buffering range suitable for most HILIC applications, and are also compatible with MS detection due to their volatility.

Worth mentioning is that the retention properties of large peptides in both HILIC and RP-HPLC can be understood by the contact region concept [63]. Using mixed-

mode HILIC/cation-exchange chromatography (HILIC/CEC), Mant and coworkers [64, 65] investigated the contact region concept by separating amphipathic α -helical peptides, where a substitution in the hydrophilic face of the peptide gave a large effect on retention in HILIC but no effect in RP-HPLC, whereas a substitution in the hydrophobic face gave no effect in HILIC but a large effect in RP-HPLC. Unfortunately no conclusions about the HILIC separation mechanism can be drawn from these results since both hydrogen bonding and partitioning will give these results. Regardless of whether the retention mechanism in HILIC is by partitioning, adsorption, or any mix of the two, mixed mode separations play an important role – in particular for solutes that have, or can be converted into charged form under the conditions of the separation [66]. This has been systematically investigated by Mant and coworkers [64, 65, 67–71], using native and modified peptides as model solutes. It should in this connection also be mentioned that Euerby *et al.* [72] concluded that both alkyl- and phenyl perfluorophases exhibit high retentivity for bases with mobile phases containing high organic modifier and low buffer concentration and that this retentivity is controlled by an HILIC mechanism. They also showed that fluorinated phenyl (PFP) phases were more retentive than fluorinated alkyl chain phases for basic analytes. This retention behavior for the PFP phase has been also reported by Bell and Jones [73].

4 Separation materials for HILIC

Although the number of commercially available columns designed specially for HILIC is growing, there is still not a substantial variety in phase compositions. A summary of phases that have been used for dedicated HILIC separations is found in Table 1. To facilitate an understanding of the underlying concepts, stationary phases will be consistently referred to by their chemistry and not by brand names. For commercial sources consult Table 1, where crossreferences to composition and applications are found for most of the works cited. It is natural to start the treatise on naked inorganic stationary phases, notably unmodified silica, since separations in the early days of “high aqueous NP” chromatography that can be considered as the foundation of HILIC were all based on underivatized [36] or aminopropyl-bonded silica [23, 24, 34, 36].

4.1 Underivatized silica

A large fraction of the recently published works are still using conventional nonmodified (naked) silicas like Beta-sil [18, 74–78], Hypersil [79, 80], and Kromasil [60, 81]. Atlantis HILIC is a silica claimed to be specially made for HILIC, and has been used in several recent publications [16, 82–86]. Other recent additions to the chromatogra-

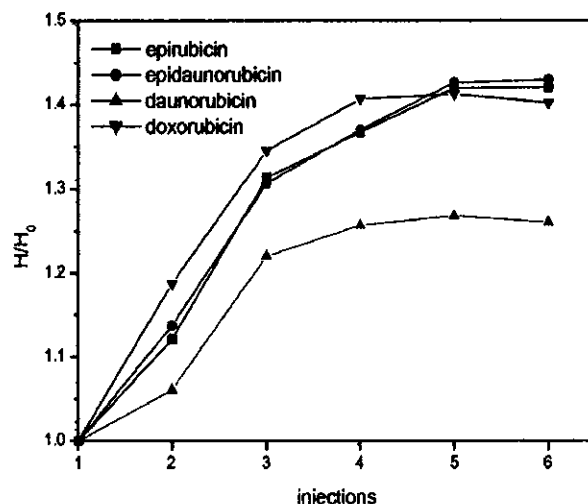


Figure 9. Plots of peak height divided by the peak height for the first injection (H/H_0) for six consecutive injections. Kromasil KR100-5SIL (5 μ m); mobile phase: 90:10 v/v ACN/sodium formate buffer (20 mM, pH 6.5). Reproduced from Li and Huang [60] with permission.

phers toolbox such as Alltima HILIC have not yet gained popularity in the scientific literature.

A review on the use of naked silica in bioanalytical HPLC/MS/MS was published a couple of years ago by Naidong [17]. Underivatized silica in HILIC mode has been found to provide consistent sensitivity improvements over RP of up to 120 times (typically two to ten times). A common notion has been that underivatized silica is impossible to use in bioanalytical separations and other applications where polar solutes present in complex matrices are to be separated. This skepticism has its roots in conventional NPC, where fully organic eluents were used and the surface effects were not efficiently shielded by a high concentration of a polar additive. Water is among the most powerful deactivating agents and when a significant fraction of water (10% water corresponds to 5.5 mol/L) is added to the eluent, bare silica seem to be sufficiently deactivated to allow reproducible results over an extended number of injections. However, severe irreversible adsorption has also been observed on bare silica in HILIC mode. Li and Huang [60], *e.g.*, noted that it took six injections to get a stable signal when epirubicin and its analogs were separated in a 90% ACN/10% 20 mM sodium formate buffer, pH 6.5 (Fig. 9).

Overloading is more evident for separation materials that rely on a mechanism that is at least partly based on surface adsorption rather than partitioning and can be a problem in sample preparation by HILIC and other schemes where complex samples are loaded at above normal analytical concentrations. Certain solutes seem to be more affected than others. The effect of overloading a silica column in HILIC mode was shown by Li and Huang

Table 1. Separation columns designated specifically for HILIC mode with published applications

Brand name	Manufacturer	Support	Functionality ^{a)}	Applications
Atlantis®	Waters	Silica, 100 Å	Underivatized	Polar basic compounds [261]; acetylcholine and choline by HILIC/ESI-MS [262]; levosulpiride [82] and tiapride [83] in human plasma by HILIC/MS/MS; enhanced ESI-MS sensitivity for polar analytes [16]; allantoin in human biological samples by HILIC/ESI-MS [84]; dencichine in Panax medicinal plant species by HILIC/MS/MS [85]; glycine betaine [86]
Betasil®	Thermo Hypersil	Silica, 100 Å	Underivatized	Fluconazole [74], paroxetine [75], cetirizine [77], and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol [76] in human plasma by HILIC/MS/MS; decreasing the sensitivity loss for basic compounds by TFA mobile phases in HILIC/ESI-MS/MS [18]; allantoin in human biological samples by HILIC/ESI-MS [84]
Chromolith®	Merck	Silica (monolith)	Underivatized	Mono- and divalent cations by HILIC/ELSD [263]; lowering the sensitivity loss for basic compounds by TFA mobile phases in HILIC/ESI-MS/MS [18]
Hypersil®	Thermo Hypersil	Silica, 120 Å	Underivatized	Atenolol [79] and nicotinic acid/metabolites in human plasma by HILIC/MS/MS [80]
Kromasil®	EKA Chemicals	Silica, 100 Å	Underivatized	Isolation and purification of epirubicin [81]; parameters affecting the HILIC separation of epirubicin and its analogs [60]
Spheri-5 Silica	Brownlee (Alltech)	Silica, 80 Å	Underivatized	Acyclovir in pregnant rat plasma and tissues by HILIC/ESI-MS [139]
Cogent Type C Silica™	Microsolv	Silica, 100 Å	Silica hydride	See manufacturer's application notes.
Alltima™ Cyano	Grace Alltech	Silica	3-Cyanopropyl	Piperazine in pharmaceuticals by HILIC-ELSD [117]
Inertsil® Diol	GL Sciences	Silica	2,3-Dihydroxypropyl	Amino acids, glycine, sucrose, and urea [110]
Lichrospher® Diol 100	Merck	Silica	2,3-Dihydroxypropyl	Acetylcholine and choline in microdialysis samples by HILIC/MS/MS [111]
ProteinPak™ 60	Waters	Silica (discontinued)	2,3-Dihydroxypropyl	Glucose polymers [264]; glucooligosaccharides and polysaccharide hydrolysates [265]
Silasorb Diol	Chemapol	Silica	2,3-Dihydroxypropyl	Cleanup of poly(amidoamine) dendrimers prior to MALDI-TOF MS [109]
YMC-pack® Diol-120-NP	YMC	Silica	2,3-Dihydroxypropyl	Validation of an HILIC method for an undisclosed drug substance and its likewise secret metabolites [14]
Amide Silica	(Unspecified)	Silica	Amide	N-linked oligosaccharides [100, 102]
TSKgel Amide-80	Tosoh Bioscience	Silica	Amide	Peptides [54, 233, 266]; pyridylaminated sialo sugar chains [219]; quaternary ammonium compounds [133]; small polar compounds in food analysis by HILIC/ESI-MS [155]; hydroxybenzoate saxitoxin analogs in dinoflagellates [160]; chitine fragments [198]; cyanobacterial toxins by HILIC/MS [164]; 1-deoxynojirimycin in mulberry leaves [150]; polar compounds contributing to umami taste and mouth-drying oral sensation of morel mushrooms [156]; elucidation of yeast sulfur metabolism [253]; PSP toxins [166, 165, 167]; monosaccharides [227, 267]; identification of a bitter inhibitor [157]
GlycoSep N	ProZyme	Silica	Amide	Oligosaccharides [224]; separating glycan mixtures and analyzing oligosaccharide profiles [221]; 2-aminoacridone derivatized complex glycans released from hen ovalbumin [223]; 2-aminoacridone-derivatized carbohydrates in urine [199]; fluorescence derivatized glycans [225]; O-glycan characterization in nutritional supplements [222]
Nucleodex® β-OH	Macherey-Nagel	Silica	β-CD	Phosphorylated carbohydrates [209]
Cyclobond™ I 2000	ASTEC	Silica	β-CD	Underivatized oligosaccharides [58, 197]
Perhydroxyl-CB[6]uril silica	Custom synthesis	Silica	Perhydroxyl-cucurbit[6]uril	Narceine, berberine, ephedrine, atropine, brucine, and nicotine [128]

Table 1. Continued ...

Brand name	Manufacturer	Support	Functionality ^{a)}	Applications
PolyHydroxyethyl A	PolyLC	Silica	Poly(2-hydroxyethyl aspartamide)	Peptides, nucleic acids, and other polar compounds [13]; peptides [67]; tyrosine protein-kinase specificity [234]; desalting of electroeluted proteins [241]; glycopeptides [268]; site-specific glycosylation microheterogeneity in recombinant human interferon- γ [269]; urea, allantoin and lysine pyroglutamate in cosmetic samples [134]; glucosinolates in vegetables [201]; folates in human plasma by HILIC/MS/MS [137]; citrulline in dried blood spots [138]; SPE enrichment of avoparcin from kidney samples [254]; ionic compounds by pressurized flow CEC [270]; sucrose in a plasma-derived process solution [271]; glycoalkaloids in transgenic potatoes [104]; glycopeptide microheterogeneity [272]; peptides by HILIC-CEC [273]
PolyGlycoplex	PolyLC	Silica	Poly(succinimide)	<i>N</i> -(<i>p</i> -nitrobenzyloxy)aminoalditol derivatized oligosaccharides [274]; complex carbohydrates [88]
μ Bondapak™ Carbohydrate	Waters	Irregular silica (discontinued)	3-Aminopropyl [WAX]	Reducing carbohydrates (in assessing losses due to Schiff's base formation) [107]
Luna® Amino	Phenomenex	Silica, 100 Å	3-Aminopropyl [WAX]	Dichloroacetic acid in drinking water [158] and in rat blood and tissues [159] by HILIC/MS/MS
Hypersil® APS2	Thermo Hypersil	Silica, 120 Å	3-Aminopropyl [WAX]	Tetracycline antibiotics [52]
Spherisorb® NH ₂	Waters	Silica, 80 Å	3-Aminopropyl [WAX]	Peptides [145]
Zorbax® NH ₂	Agilent	Silica, 70 Å	3-Aminopropyl [WAX]	Tris(hydroxymethyl)methylammonio ion [50]; DNA methyltransferase inhibitor zebularine and three metabolites in murine plasma [143]
Micropellicular AP-Silica	Custom synthesis	Silica (micropellicular)	3-Aminopropyl [WAX]	Fructo-oligosaccharides [275]
YMC-Pack® Polyamine II	YMC	Silica	Polyamine [WAX]	Atosiban and other oxytocin analogs [145]
Amino-bonded Zirconia	Custom synthesis	Zirconia	Mono-, di- and triamine bonded by a propyl spacer [WAX]	Carbohydrates by pulsed amperometric detection [98]
SynChropak CM300	Eichrom (Eprogen)	Silica, 300 Å	Carboxymethyl [WCX]	Acetylated core histones [243], ageing-related histone modifications [249, 248]
PolyCAT A	PolyLC	Silica	Poly(aspartic acid) [WCX]	Phosphorylated H1 histones [244]; microheterogeneity of mammalian H1(0) histone [245]; deamidation of H1 histones [246]; mammalian histone H1 and avian histone H5 [247]; allelic and nonallelic variants of histone H1 [240]; sequence variations in human histone H1.2 and H1.4 subtypes [250]
PolySulfoethyl A	PolyLC	Silica	Poly(2-sulfoethyl aspartamide) [SCX]	Peptides [67, 68, 276]; amphipathic α -helical peptides [65]; cyclic peptides [64]; purification of synthetic serine side-chain acetylated peptides from closely related impurities [69]; amphipathic α -helical peptides with L- and D-amino acid substitutions in the hydrophilic face [70]; basic compounds [135]; hydrophilicity/hydrophobicity of amino acid side-chains in amphipathic α -helices [71]; basic pharmaceuticals in human serum [277] and peptides [278, 273] by CEC

Table 1. Continued ...

Brand name	Manufacturer	Support	Functionality ^{a)}	Applications
ZIC-HILIC® and ZIC-pHILIC®	SeQuant	Silica (ZIC-HILIC); Porous polymer (ZICpHILIC)	Polymeric sulfo- alkylbetaine zwitterionic [SAX+SCX, 1:1]	Neomycin by HILIC/MS/MS [141]; trapping of N-glycosylated peptides [255]; aminoglycosides in serum [142]; morphine, morphine-3-glucuronide and morphine-6-glucuronide in microdialysis and plasma samples [154]; metabolic fingerprinting of rat urine by LC/MS [148, 149]; polar and hydrophilic compounds [279]; homocysteine, methylmalonic acid and succinic acid [136]; selective SPE enrichment of GPI-anchored peptides extracted from in-gel trypsin digested porcine renal membrane dipeptidase for structural analysis by MALDI-QTOF-MS/MS [257]
Hydrolyzed GMA-co-EDMA	Custom synthesis	Methacrylic copolymer	2,3-Dihydroxy- propyl	CDs, dextrans, PVPs in HILIC mode, and polyoxyethylenes and nonionic surfactants in NP mode [131]
Asahipak® NH ₂	Shodex	Poly(vinyl alcohol) gel	Amine [WAX]	Mono-, di-, and maltooligosaccharides by pulsed amperometric detection [280]; oligosaccharides [96]; 5-fluorouracil in plasma and tissues by HILIC/APCI-MS [95]
GlycoSep C	ProZyme	"Polymeric"	DEAE [WAX]	Fluorescently labeled oligosaccharides from glycoproteins [220]
Astec apHera™	ASTEC	PVA copolymer	Polyamine [WAX]	Taurine and methionine in carbohydrate-rich matrices by HILIC-MS/MS [97]
Acrylamide CEC phase	Custom synthesis	4% Crosslinked polyacrylamide	Dodecyl chains and sulfonic acid	Urea herbicides and carbamate insecticides [281]
Excelpak CHA-P44	Yokogawa (Agilent)	Styrene/divinyl- benzene	Sulfonic acid [SCX, Zn ²⁺ form]	Mono-, di- and maltooligosaccharides by pulsed amperometric detection [280]
Sulfonated S-DVB	Custom synthesis	Styrene/divinyl- benzene	Sulfonic acid [SCX]	Glycols and monosaccharides [55]
GlycoSep H	ProZyme	(Discontinued)	N/A	Separation of glycan mixtures and analyzing oligosaccharide profiles [221]

^{a)} All phases are covalently bonded, unless otherwise noted. [SAX, SCX, WAX, and WCX] denotes Strong and Weak Anion- and Cation-exchange functions, respectively. Links to manufacturers: Agilent, www.agilent.com; ASTEC, www.astecusa.com; Brownlee, N/A; Chemapol, www.chemapol.cz; Eichrom, www.eichrom.com; EKA Chemicals, www.kromasil.com; GL Sciences, www.inertsil.com; Grace Alltech, www.alltechweb.com; Macherey-Nagel, www.macherey-nagel.com; Merck, www.merck.de; Microsolv, www.microsolvtech.com; Phenomenex, www.phenomenex.com; PolyLC, N/A; ProZyme, www.prozyme.com; SeQuant, www.sequant.com; Shodex, www.shodex.com; Thermo Electron, www.thermo.com; Tosoh Bioscience, www.tosohbioscience.com; Waters, www.waters.com; YMC, www.ymc.co.jp/en/index_e.html; Yokogawa Electrical Works, N/A; N/A = no website found.

[60] (Fig. 10), and for an adsorptive process one would normally expect a Langmuirian-type isotherm, *i.e.*, one that is concave upward or "levels off." However, the peak shapes from the modestly overloaded injections were instead indicative of an *anti*Langmuirian isotherm, characterized by reluctant initial solute sorption that facilitates further sorption of solute from solution as the concentrations increases. These isotherms are unusual in LC and may hint at a multilayer absorption process [87], at least in this particular case.

One of the properties that make underivatized silica inherently attractive in LC-MS(/MS) is the absence of ligands that may detach and show up as spurious peaks in the mass spectra. Recent bonded phase materials for HILIC seem to have addressed this issue, and various functionalized silicas are today starting to become as common as naked silica in HILIC-MS applications.

Although water will shield the surface, it will still not prevent the dissociation of silanols and the cation exchange properties that accompany this dissociation. In fact, the presence of water will promote formation of free silanols and also make the dissociation more facile. The negatively charged surface will attract cationic solutes, but also cause a decreased retention of negatively charged polar compounds, since these are electrostatically repelled from the surface. Addition of electrolyte (preferentially a buffer) is therefore mandatory to control the mixed mode separations that the dissociated silanols give rise to, in particular with basic solutes. Applications have been published for a range of very polar natural compounds and pharmaceuticals, of which a representative subset is shown in Fig. 20.

The purity of silica has been used as a sales argument, and the rationale lays in the induction effect that some

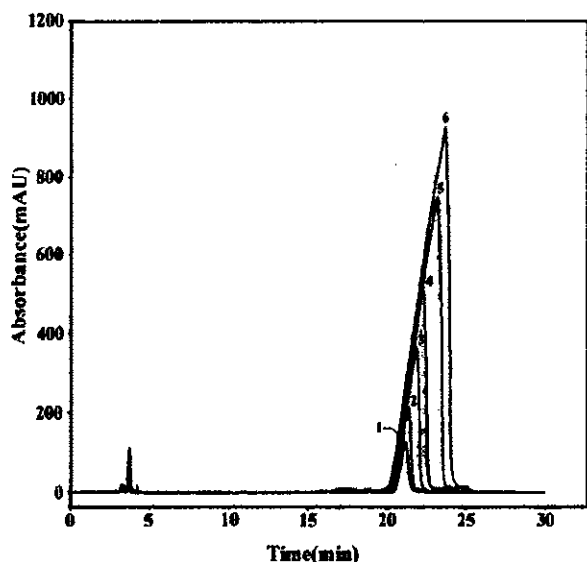


Figure 10. Chromatograms of epirubicin of increasing sample sizes on a 250 mm \times 4.6 mm Kromasil KR100-5SIL (100 Å, 5 μ m, specific surface area 330 m²/g). Mobile phase: 90% ACN/10% 20 mM sodium formate buffer, pH 2.9. Injected sample: (1) 0.8 μ g; (2) 4.1 μ g; (3) 8.2 μ g; (4) 16.4 μ g; (5) 32.2 μ g; (6) 49 μ g. Reproduced from Li and Huang [60] with permission.

metal ions have on the acidity of the silanol group, and as can be seen in Fig. 11 there are significant differences between silica columns from different manufacturers [59]. This might be due to different purity of the silica used in the preparation of the stationary phase [61] but might also arise from differences in procedures used in the column preparation. The peak shape problems seem to have been resolved on silicas designed especially for HILIC.

4.2 Aminopropyl silica

Aminopropyl-bonded silica was the first bonded stationary phase to be routinely used for carbohydrate separations in HILIC mode. Its advantage over underivatized silica is an increased rate of mutarotation, which prevents formation of double peaks due to anomer resolution. Naked silica was also introduced in these first works on silica-based HILIC separation, but a small amount of ammonia must usually be added to accomplish the mutarotation [88]. Most column manufacturers supply aminopropyl columns for either HILIC or NPC applications. Aminopropyl- and naked silica are more reactive than alkyl phases so irreversible adsorption is more problematic for these columns than for RP-HPLC columns [59].

Aminopropyl silica also shows extended equilibration times with certain buffer ions, compared to materials derived from polymeric matrices. For instance, Valette *et al.* [52] noted that it took several hundred column

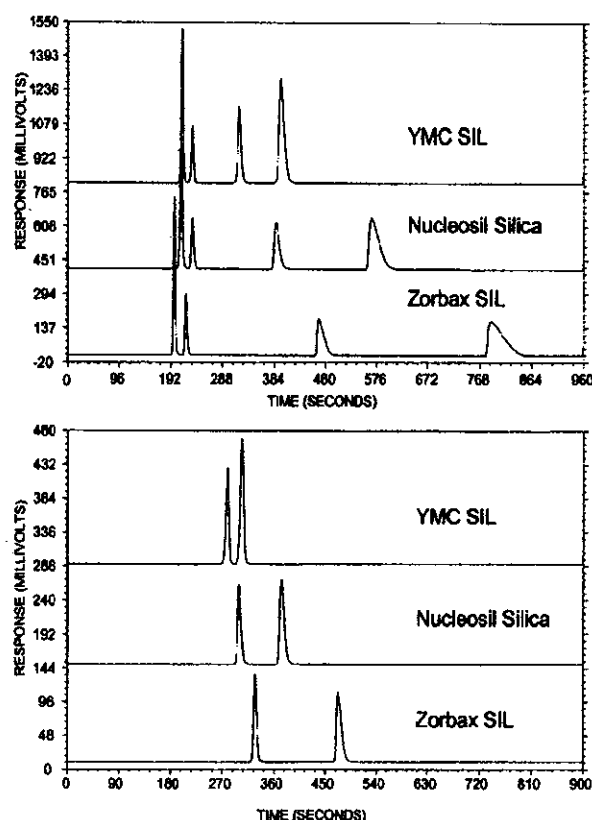


Figure 11. Pyrimidines (upper) and purines (lower) on silica columns using 5 mM phosphoric acid in water/ACN (25:75) for pyrimidines, and (30:70) for purines as mobile phase. UV detection at 275 nm. Pyrimidines, ~0.05 mg/mL (in order of elution): 5-fluorouracil, uracil, 5-fluorocytosine, cytosine; purines, ~0.02 mg/mL (in order of elution): acyclovir, guanine. Reproduced from Olsen [59] with permission.

volumes to reach a stable baseline when changing from citrate to acetate buffer at the 100 mM level. Aminopropyl silicas of different brands also show large differences in HILIC performance [50, 59] (Fig. 12), and care should be taken when selecting a column not intended primarily for HILIC.

A serious concern with aminopropyl silica is the limited stability in aqueous eluents [89, 90] that leads to fast release of ligands with accompanying peak shape deterioration under HILIC conditions [91]. This instability seems to be related to a catalytic selfpredatory action of the primary amino group on the ligand and other highly hydrophilic-bonded phase silicas appear to be far more stable toward phase stripping [91]. Among silicas, the "Type C" (hydride) silica produced by the hydrosilylation procedure of Pesek occupies a special position [92]. It could arguably qualify as a naked silica, since the majority of the surface silanols have been replaced with silicon hydride (Si–H) groups. Octadecyl RP materials prepared from Type C silica show a unique selectivity for many polar compounds in high organic eluents (Fig. 13) [93].

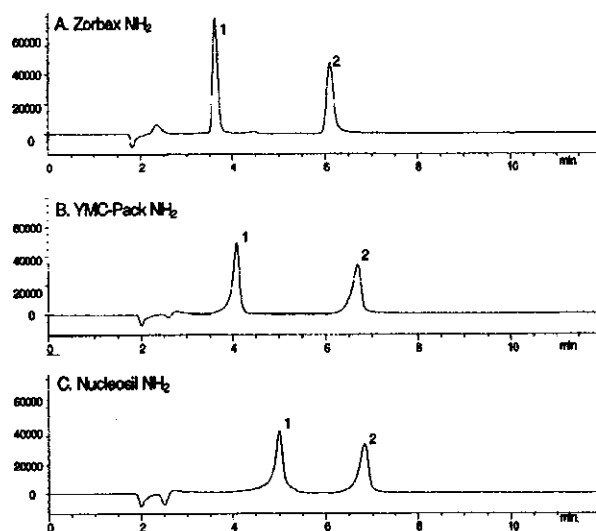


Figure 12. Chromatograms for the separation of 2-amino-2-ethyl-1,3-propanediol (Peak 1, AEPD) and TRIS (Peak 2, TRIS) on three amino columns at 25°C. Mobile phase: ACN/water (80:20 v/v) at 1 mL/min. Injected: 50 μ L AEPD and TRIS (~1 mg/mL) each in the mobile phase. Reproduced from Guo and Huang [50] with permission.

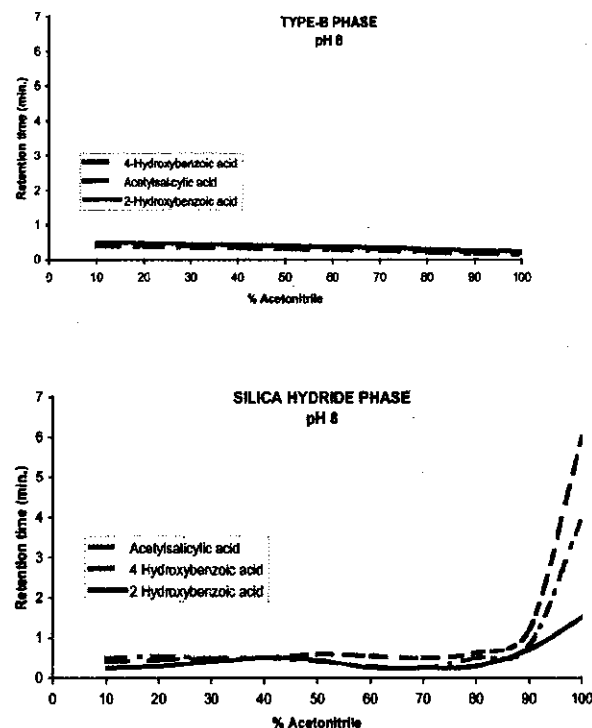


Figure 13. Retention time as a function of ACN percentage in the mobile phase for three acids at pH 8 on C_{18} -bonded phases prepared from conventional Type B silica and hydride (Type C) silica. Reproduced from Pesek and Matyska [93] with permission.

In a recent paper, Matyska *et al.* [94] evaluated several procedures for preparing amino-bonded phases based on functionalization of Type C silica, and compared these phases to naked Type C silica with and without endcap-

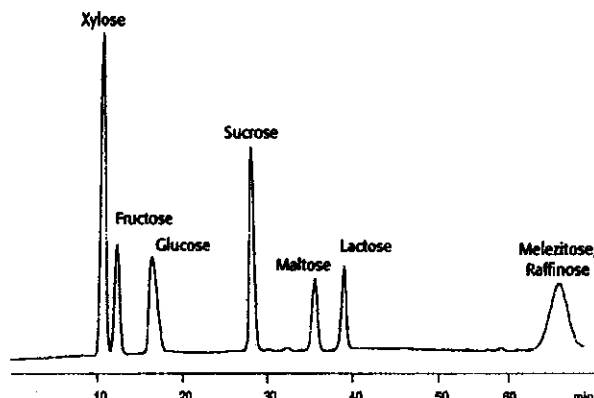


Figure 14. Sugars separated on an amino phase prepared by cleavage of *N*-(3-butyryl) phthalimide bonded to a Type C silica by Pt catalysis. Isocratic elution with water/ACN (15:85). Adapted (legend added in figure) from Matyska *et al.* [94]. Reproduced with permission.

ping of residual silanols for separation of underivatized mono- and oligosaccharides in HILIC mode. Retention was seen on the non-endcapped naked Type C silica, but it was lower than for naked Type B silica and disappeared when the Type C silica was endcapped. It was therefore concluded that the retention seen on the non-endcapped Type C silica was due to residual silanols and that Type C silica does not provide retention of sugars in HILIC mode. Among the amination schemes, the best results were seen for the phase prepared with *N*-(3-butyryl) phthalimide followed by hydrazinolysis (Fig. 14). No stability study was included, but bonded phases prepared from Type C silica are attached through a Si–C bond and phases with other ligands are known to have a stability superior to phases prepared by conventional silylation schemes [93].

4.3 Nonsilica-based amino packings

Amino packings based on organic polymers have much better stability compared to aminopropyl silica [95]. They can be used under acidic and basic conditions ((a) <http://www.shodex.com/english/dc030237.html>; (b) <http://www.shodex.com/english/dc030238.html>; (c) http://www.astecusa.com/l_chromatography/amino.htm. All as of April 30, 2006.) and are applicable in HILIC-type separations, e.g., for “size fractionation” in the structural analysis of oligosaccharides [96], and for the analysis of fluorouracil [95], and taurine and methionine [97]. Micropellicular zirconia particles modified by several different aminosilylation protocols have also been used in HILIC with good results [98].

4.4 Amide silica

Yoshida [99] has examined HILIC mode separations of peptides on an amide-bonded silica, a hydrophilic phase containing a carbamoyl (amide) group on a short alipha-

tic spacer [51, 99]. The amide group is less reactive than the amine and lacks its basicity. Retention on these columns is thus less sensitive to eluent pH and less prone to irreversible chemisorption, and the absence of primary amino groups precludes Schiff's base formation with reducing sugars and other carbonyl compounds. Amide silica shows good recovery and stability even after 500 injections [99], and was used in 2-D mapping of oligosaccharides by HILIC nearly 20 years ago [100–102].

4.5 Poly(succinimide)-bonded silica and its derivatives

Several polar silica-based bonded phases are based on the covalent reaction of poly(succinimide) with an aminopropyl functionalized silica. The primary reaction produces a polyamide/imide surface layer bonded by multi-point attachment, whereby parts of the poly(succinimide) rings are opened to form amide bonds with the aminopropyl moieties. Only a fraction of the succinimide rings are engaged in this bonding and the remaining rings remain intact and susceptible to reaction with nucleophiles, which can be used to produce a variety of functional silicas. Among these are poly(aspartic acid) weak cation exchangers formed by alkaline hydrolysis, poly(2-sulfoethyl aspartamide) strong cation exchangers by aminolysis with taurine, and poly(2-hydroxyethyl aspartamide) silica from reaction with ethanolamine. The latter is the stationary phase that was synthesized by Alpert [13] especially for use in HILIC (Fig. 15). Various poly(succinimide)-based phases have been used in numerous works with HILIC and mixed mode separations in the 1990s (see Table 1), but the dedicated poly(2-hydroxyethyl aspartamide) HILIC phase seems to have lost some of its momentum, which might be due to a somewhat lower efficiency compared to more recent dedicated HILIC stationary phases [103], a limited long-time stability [104], or column bleeding, as recently reported for a poly(succinimide)-based phase [105].

4.6 Diol silica

Diol silica is the bonded phase that most closely resembles naked silica in overall polarity [66, 106]. It is prepared by reaction of silica with an alkoxy-activated glycidypropylsilane, followed by acid-catalyzed ring-opening hydrolysis of the oxirane group to form a siloxane-linked 2,3-dihydroxypropyl ligand. Diol phases were among the first bonded silicas to be developed, and one of the purposes was to address the troublesome adsorption properties of naked silica. The high polarity and hydrogen bonding properties of diol columns, along with a relative absence of dissociable moieties, should be nearly ideal for HILIC applications where an intended mixed mode interaction encompassing ionic interac-

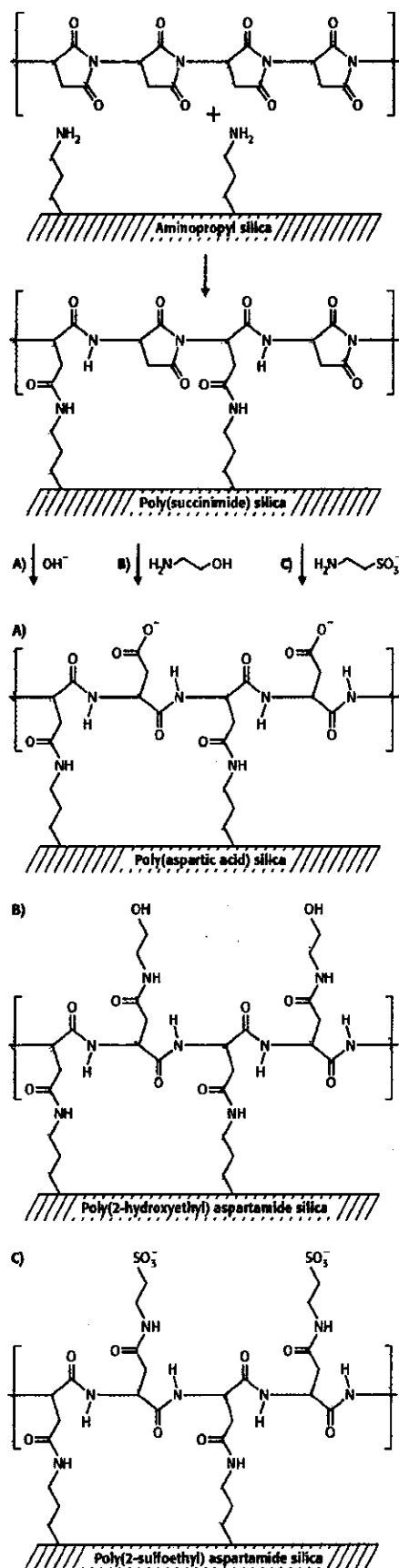


Figure 15. Silica-based phases based on activation with poly(succinimide) and subsequent hydrolysis/aminolysis.

tions with silanol groups is not desired. Yet surprisingly few applications of diol silica have been published.

Brons and Olieman [107] evaluated a diol silica in parallel with several silicas with primary amino groups, and a silica with dimethylamino-bonded groups. Losses of galactose and lactose were severe on brand new primary amino columns and decreased as the columns were used. The dimethylamino silica showed a low retention ($k' \sim 1.5$ in 90:10 ACN/water) and could not resolve sucrose and lactose. The diol silica showed no irreversible retention of the reducing sugars tested. The absence of amino groups did, however, give double peaks due to slow mutarotation, and addition of 0.1% diisopropylethylamine (a base with very low nucleophilicity) to the eluent resolved this issue. Herbreteau *et al.* [108] tested several bonded phase columns for the separation of carbohydrates and polyols and concluded that the diol phase was best suited because reducing sugars did not form Schiff's bases as they do on amino columns. More recently, Peterson *et al.* [109] used a diol silica alongside an RP separation to purify the first generation (G1) in a step-by-step synthesis and purification of poly(amidoamine) PAMAM dendrimers. Tanaka *et al.* [110] used urea, sucrose, and several amino acids to demonstrate the utility of a new diol column in HILIC mode. The need for buffering was seen and in a series of experiments TFA was added at concentrations ranging from 0.001 to 0.1% to a 90:10 ACN/water eluent. This significantly affected the retention of glycine, while urea and sucrose remained at the same elution times. An ion exchange effect was thus evident that can be attributed to residual silanol groups. After an eluent with 0.1% TFA had been used, the column required 10 h of washing to restore the previous separation performance. Uutela *et al.* [111] based the development of a validated method for acetylcholine and choline in salt-containing microdialysis samples with electrospray MS/MS on a diol silica, and found that the analytes eluted without coeluting endogenous compounds suppressing the ionization. Wang *et al.* [14] found a diol silica useful when developing a validated method for four (undisclosed) polar pharmaceuticals. There has also been an attempt to integrate "classic" NP and HILIC to cover the entire polarity spectrum from hexane to water [112] on a diol column.

4.7 Cyclodextrin (CD)-based columns

Cyclodextrin (CD) phases are silica-based packings with a cyclic oligosaccharide composed of five or more 1→4 linked α -D-glucopyranoside units as the bonded phase. Naturally occurring CDs start from six glucose units and the nomenclature for the most common members of the series is α -, β -, and γ -CD for the six-, seven-, and eight-membered rings. The topological configuration of these molecules resembles toroids, with the wide and the nar-

row entrances exposing secondary and primary hydroxyl groups to the solvent, respectively. Although not hydrophobic, the toroid interior is considerably less hydrophilic than the exterior, and can thus host hydrophobic molecules. Because the cavities are formed from optically active sugars, the CDs also have chiral recognition properties and are consequently used as chiral selectors in many areas of separation science [113]. The high density of exposed hydroxyl groups is what makes the CD phases attractive in HILIC. CDs were introduced as ligands in aqueous NPC for the separation of sugar alcohols and mono-, di-, and oligosaccharides as early as 1989 [114], and it is obviously an intriguing phase when the desire is to accomplish chiral separations in HILIC mode [115, 116]. In fact, Risley and Strege [115] showed that HILIC is a promising separation mode for polar chiral compounds, as the enantiomers of a compound that separated well in an aqueous ACN eluent in HILIC mode failed to resolve under more conventional, nonaqueous NP conditions on CD or teicoplanin functionalized silicas.

4.8 Cyanopropyl silica

Cyanopropyl silica also emanates from the early days of aqueous NPC, but only a few applications in HILIC-like separations are found in recent times [117]. Although commonly referred to as a polar phase, the absence of hydrogen bond donor capabilities [106] causes a low NP retention on cyanopropyl silica, and it also finds uses in the RP mode. When Yoshida and Okada [118] compared several packing materials for the separation of peptides, the cyanopropyl phase failed to give any retention. Cyanopropyl silica therefore seems impractical since HILIC is already run at elevated ACN concentrations. Another practical aspect that may have precluded the use of cyano phases in HILIC is the mechanical instability that these phases suffer when operated with solvents of intermediate polarity. This instability is caused by a decrease in the interparticle forces that maintain the integrity of the bed in either nonpolar or highly polar solvents [66].

4.9 Sulfonated S-DVB

Sulfonated S-DVB can be used for HILIC separations if the degree of sulfonation is sufficiently high, and sulfonated S-DVB gels were actually among the first materials in which aqueous NPC was demonstrated [27, 29, 30]. Chambers and Fritz [55] brought this concept to HPLC by surface sulfonating porous S-DVB resins. With an eluent consisting of 5% water in 95% ACN, the separation of ethylene glycol, propylene glycol, and glycerol was found to be strongly dependent both on the degree of sulfonation and on the counter-ion bound to the sulfonic acid groups on the stationary phase. Increasing the sulfonation gave a nearly linear increase in retention and the Ca^{2+} form gave much higher retention than the H^+ form.

4.10 Sulfoalkylbetaine silica

A recent addition to the palette of HILIC phases is based on a grafted polymeric layer with sulfoalkylbetaine zwitterionic moieties of 3-sulfopropyltrimethylammonio inner salt as functional groups on a wide-pore silica. The concurrent presence of a quaternary ammonium and a sulfonic acid group in a 1:1 ratio on the same pendant moiety bestows a zero net charge, but sulfoalkylbetaine zwitterions are still potent osmolytes with a strong ability of binding water to surfaces [119]. The way in

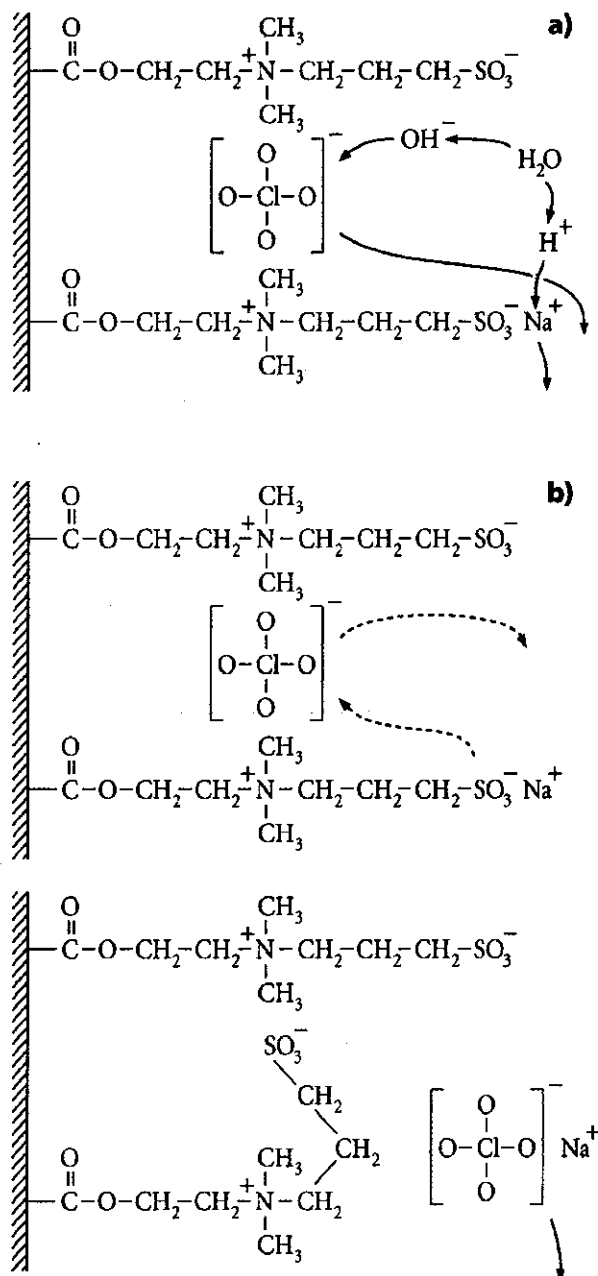


Figure 16. Schematically depicted elution processes for ionic solutes on a sulfoalkylbetaine zwitterionic material mediated (a) by water splitting, and (b) by zwitterion self-association. Reproduced from Viklund *et al.* [126] with permission.

which these phases interact with water is very special, since water splitting is part of the scheme (Fig. 16). The hydrogen bonding pattern of water associated with sulfoalkylbetaine zwitterionic polymers in solutions is known to be minimally perturbed and water associated with such zwitterions at interfaces exists more or less as bulk liquid water [120–122]. These materials were initially developed for the separation of inorganic salts, small ionic compounds, and also proteins in low salt fully aqueous eluents [123–127], but have currently found their primary use in HILIC due to a combination of the water-retaining properties of the (zwitter)ionic environment, and a low surface charge that does not promote strong ion exchange interactions. Its selectivity follows the general pattern in HILIC with increased retention as a function of solute polarity, but is largely free from the silanophilic and pronounced ionic retention properties of underivatized silica and packings containing amino groups. It has a slight electrostatic contribution from the sulfonic group, presumably because it is the distal charged moiety in the zwitterionic functional group (Fig. 16). This low negative excess charge is essentially independent of pH and can be modulated up and down by the addition of chaotropic/cosmotropic salts or divalent cations at millimolar concentrations [124–127]. In a recent study by Guo and Gaiki [51], a silica-based sulfoalkylbetaine zwitterionic phase was compared to a bare silica, an aminopropyl silica, and an amide silica, and the retention on the sulfoalkylbetaine zwitterionic phase was found to be least affected by changes in pH among the columns compared. They also noted that the sulfoalkylbetaine material was different from the other commercially available HILIC phases tested [51].

4.11 Dedicated synthesis

Recently a rather special HILIC phase was described by Liu *et al.* [128]. The base material is silica, and the ligand perhydroxyl-cucurbit[6]uril (Fig. 17), derived from cucurbit[6]uril (a macrocyclic cavitant made up from six glycoluril units in a cyclic arrangement) by persulfate perhydroxylation. Prior to perhydroxylation, the cavities are hydrophobic and have found uses in various schemes based on host–guest chemistries [129, 130]. The perhydroxylated ligand phase could separate a number of alkaloids, albeit not with illustre efficiency (Fig. 18). A study of its selectivity in comparison with conventional phases was not included.

An all-polymeric diol phase has been devised by Svec and coworkers [131, 132], based on copolymerization of glycidyl methacrylate and ethylene dimethacrylate with subsequent hydrolysis of the oxirane group. Most of the applications shown were run in NP mode using hexane and THF as eluents, producing well resolved chromatograms.

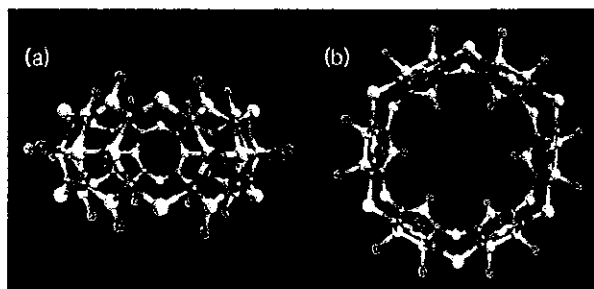


Figure 17. X-ray crystal structure of perhydroxyl-cucurbit[6]uril; (a) side view and (b) top view. Reproduced from Jon *et al.* [130] with permission.

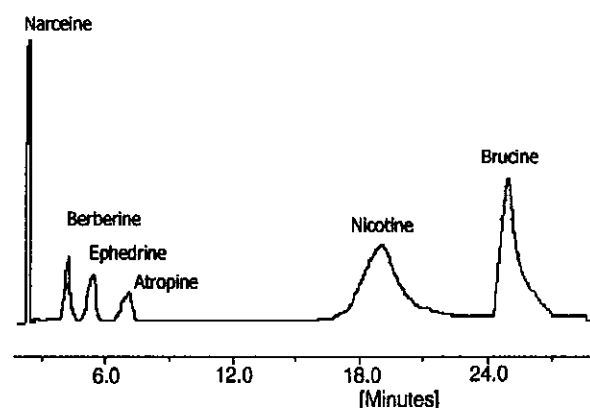


Figure 18. Separation of alkaloids on a silica-based phase with perhydroxyl-cucurbit[6]uril as functional groups, using 5 mM phosphate buffer/CH₃CN, pH 3.48 (20:80 v/v) at a flow rate of 1 mL/min. Adapted (legend added) from Liu *et al.* [128] with permission.

grams of polar oligomers, but these diol phases should be equally suitable for HILIC.

5 Applications

Although “NP” chromatography with aqueous eluents has been known for quite a long time, it has taken a surprisingly long induction period for HILIC applications to take off outside the field of carbohydrate analysis. Of all publications found in an ISI Web of Science search for (“hydrophilic” or “HILIC”) AND “chromatogr*” in title or abstract, cleaned for nonrelevant entries and reviewed, two-thirds have been published since the year 2000, and one-fourth was published in 2005 alone (Fig. 19).

5.1 Small molecules

HILIC excels with small and very polar analytes, and in particular basic analytes where ion pairing has been necessary to obtain retention in RP. Only a brief summary of the analytes can be given here and Fig. 20 shows the structures of some of the small and basic molecules separated by aqueous NPC. Li *et al.* [79] developed a validated method for atenolol based on an underivatized

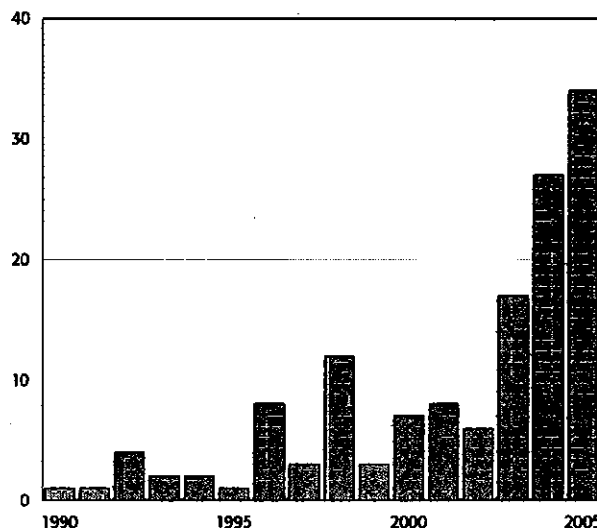


Figure 19. Number of publications containing separations with HILIC as one of the separation principles from ISI Web of Science with search term HILIC OR (“hydrophilic interaction” AND chromatogr*).

silica. Grumbach *et al.* [16] also used naked silica to separate a number of highly polar compounds, among them choline and acetylcholine. Glycine betaine is a similarly polar substance, also separated on silica in a study of stress-induced induction of choline oxidase production in transgenic rice [86]. Guo [133] compared aminopropyl and amide silicas and found quaternary ammonium compounds to have a considerably higher retention on the amide column, and that the effect of addition of ammonium acetate to the eluent at different concentrations had an opposite effect on retention time for the two columns. Among the few applications of cyanopropyl silica is the determination of piperazine in drug substances [117]. Dallet *et al.* [134] used a poly(2-hydroxyethyl aspartamide) silica with UV detection to determine urea, allantoin, and lysine pyroglutamate in cosmetic samples, and urea, sucrose, and glycine were used as test solutes when Tanaka *et al.* [110] evaluated a newly developed diol silica in HILIC mode. Optimum flow rate was found to be one-third of that of an RP separation on a column of comparable dimensions, and it was argued that the stationary phase mass transfer slows down the separation process [110]. A range of basic pharmaceuticals were used as test probes by McKeown *et al.* [61] when they assessed the effect of silica purity on the retention in HILIC (see above), and indapamide, methatone, celiprolol, atenolol, timolol, and isoprenaline were separated in a mixed cation exchange/HILIC mode by pressurized CEC mode on poly(2-sulfoethyl aspartamide) silica [135]. Separation of homocysteine, methylmalonic acid, and succinic acid in clinical diagnostics was accomplished in 3 min by Appelblad and Abrahamsson [136] on a sulfoalkylbetaine zwitterionic column with ESI-MS detection. An underiva-

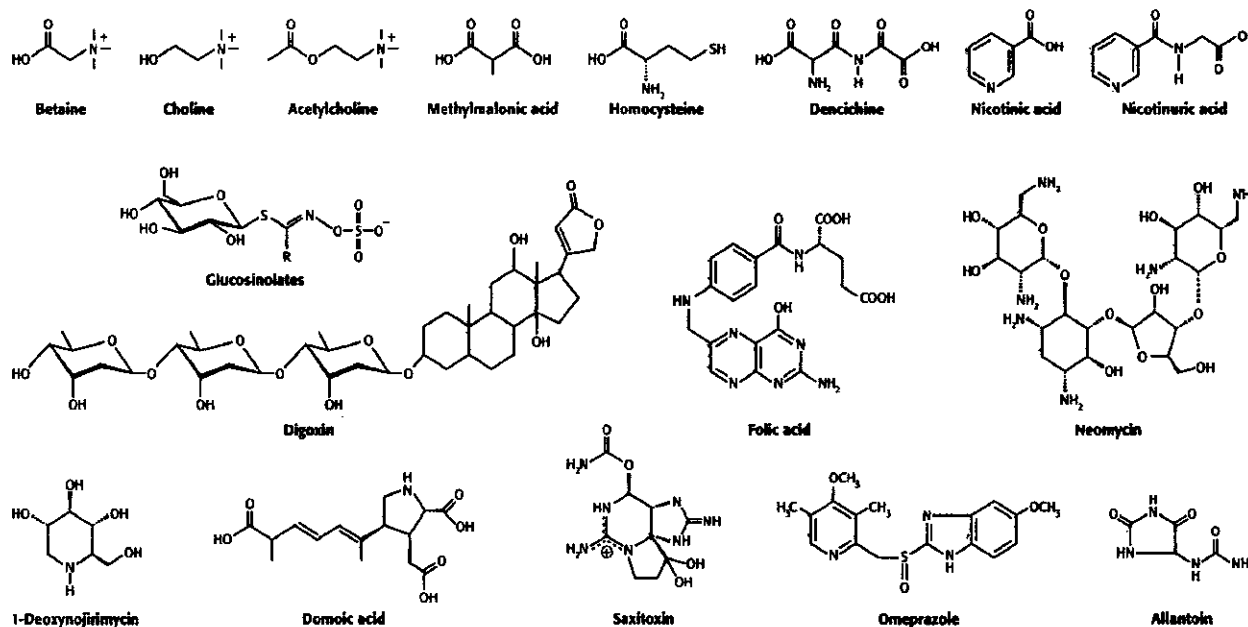


Figure 20. Some representative polar and basic compounds separated by HILIC. See Table 1 and the text for a listing and discussion of the separation of the individual compounds.

tized silica was used for the determination of allantoin in human biological samples with ESI-MS detection [84].

5.1.1 Bioanalysis and pharmacokinetics

HILIC also has a role in modern pharmaceutical development, where potent drug substances are analyzed at very low concentration levels in bioanalytical applications. Many modern drugs are highly polar compounds and RP separations which have been commonplace in bioanalysis cannot adequately address many of these. A recent review by Naidong [17] summarizes the use of MS/MS with underivatized silica columns in bioanalysis.

Among the more interesting applications is the determination of folates (folic acid, tetrahydrofolate, 5'-methyltetrahydrofolate, and 5'-formyltetrahydrofolate) in human plasma [137], a separation that was tested on both RP and several polar columns prior to selecting a poly(2-hydroxyethyl aspartamide) silica for the final method development (Fig. 21). On all the four RP columns tested, folate peaks were tailing and coeluting species suppressed the ionization, and an SPE cleanup was required to reduce interferences from plasma components. Retention times on a cyanopropyl column were very long with broad and tailing peaks, and an attempt to add ammonium acetate or ammonium formate suppressed the ionization. No peaks were observed on an aminopropyl column.

HILIC on a poly(2-hydroxyethyl aspartamide) column has also been used in combination with MS/MS for determination of citrulline in dried blood spots for graft monitoring in intestinal transplantation [138]. MS/MS was

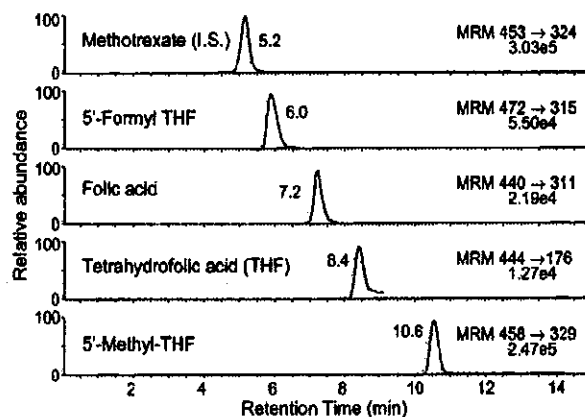


Figure 21. LC-MS-MS analysis of folate species (10 ng/mL) spiked into human plasma. HILIC was used with negative ion electrospray mass spectrometric detection. Reproduced from Garbis *et al.* [137] with permission.

likewise used for the detection in a method for omeprazole and 5-OH-omeprazole in human plasma with an underivatized silica [78]. Bare silica was also used for the determination of the antiviral agent acyclovir in plasma, amniotic fluid, and placental and fetal tissue from rat [139] by MS/MS detection. Determination of the bis-triazole antifungal agent fluconazole in human plasma has been done with liquid-liquid extraction into methyl-*t*-butyl ether (MTBE), evaporation and reconstitution with 0.05% TFA in CH₃CN, all performed in the 96-well plate format. Final determination with HILIC on a naked silica column provided increased sensitivity compared to a C₁₈ separation, and the silica column could be used for > 500

injections without column deterioration [74]. An underivatized silica column was also used in combination with MTBE extraction for analyzing the antidepressants paroxetine in human plasma with MS/MS for detection, with a 2.5-fold increase in sensitivity over a corresponding C_{18} method [75]. Underivatized silica has been used for determination of the antipsychotic drug levosulpiride in human plasma after alkaline treatment and liquid–liquid extraction with ethyl acetate [82]. The same authors also used a similar set-up with dichloromethane as the extractant for the related drug tiapride [83].

Isoniazid, an antituberculous prodrug, is also a compound where retention in RP is difficult to accomplish. HILIC was successful when used in combination with a Waters Oasis HLB extraction plate in the 96-well format [140]. The same work also showed a similar approach for the antihistamine cetirizine in plasma, this time using an MCX μ -Extrelute plate. Underivatized silica was used for both separations, and the columns were stable for >1200 injections. The method for cetirizine was revisited a year later, this time using a Strata-X SPE extraction plate [77]. A brief summary of sample preparation approaches in the 96-well format for HILIC was published by Naidong *et al.* [62].

An HILIC method was developed by Uutela *et al.* [111] based on a diol phase as an alternative to using ion pair or ion-exchange chromatography for the separation of acetylcholine and choline from cationic salts and endogenous compounds in microdialysis samples using MS detection. The sensitivity was five times better than previous methods. Underivatized silica has been used in the separation step for the determination of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, a nitrosamine metabolite specific to tobacco [76].

A validated method for fast separation of nicotinic acid from its metabolites nicotinamide and nicotinuric acid by HILIC-MS-MS was set up for a pharmacokinetic study by Hsieh and Chen [80] using a naked silica column. Total analysis time per sample was less than 2 min (Fig. 22).

5.1.2 Antibiotics and cytostatica

Most of the antibiotics are derived from microbial products exuded to protect the organisms against biological threats in the environment. Water solubility is obviously an advantage in a first line defense and most antibiotics in use are either native or derived hydrophilic substances. The tetracyclines comprise a relatively old group of antibiotics, where the precursor tetracycline is produced by *Streptomyces*. It was the first group of designed antibiotics, where synthetic modifications were used to produce new variants. Some of the more important members of this group of antibiotics are shown in Fig. 23. Valette *et al.* [52] studied the separation of three tetra-

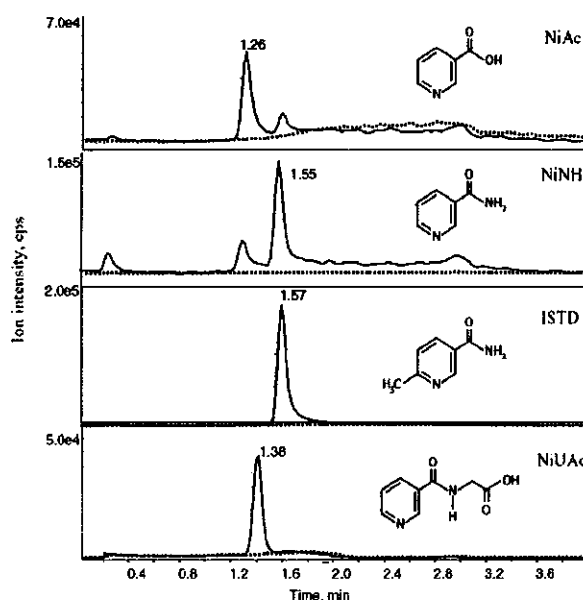


Figure 22. HILIC/APCI-MS/MS chromatograms for nicotinic acid (NiAc), nicotinamide (NiNH₂), and nicotinuric acid (NiUAc) with 6-methylnicotinic acid as internal standard (ISTD) from 5 mg/mL spiked standard dog plasma under gradient conditions. Adapted (structures added) from Hsieh and Chen [80] with permission.

cyclines on naked and aminopropyl silicas. The underivatized silica could not be used because of peak tailing, attributed to strong interactions of the protonated tertiary amino group of the tetracyclines with dissociated silanols. Better efficiency was obtained with an aminopropyl silica, but only when citrate buffer was used. As discussed above, attempts to substitute acetate for citrate buffer (pH 3.5) resulted in a four-fold loss of separation efficiency and drift toward longer retention times that lasted for several hundred column volumes. This was discussed in terms of metal complexation and masking of silanol groups by the amino groups of the derivatized stationary phase. A stable method could only be established using an ACN/citrate buffer eluent.

Using a silica-based sulfoalkylbetaine zwitterionic column in combination with an Oasis MCX cleanup step, Oertel *et al.* [141] developed a method for determination of neomycin in human plasma with ESI-MS detection. Compared to the single amino group of the tetracyclines just discussed, neomycin (Fig. 20) has no less than six primary amino groups, which makes it highly susceptible to silanophilic and ion exchange interactions of the kind discussed by Valette *et al.* [52]. Neomycin also has an inherently low sensitivity in ESI-MS, which calls for optimal eluent conditions with respect to organic contents and buffers. Using the zwitterionic column stable retention times could be obtained with a steep gradient (from 80 to 20% CH₃CN in 10 mM ammonium acetate in 30 s), providing conditions that were optimal for electrospray

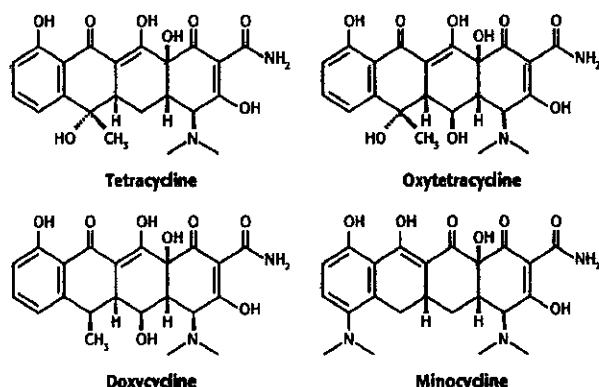


Figure 23. Some of the members of the tetracycline antibiotics family.

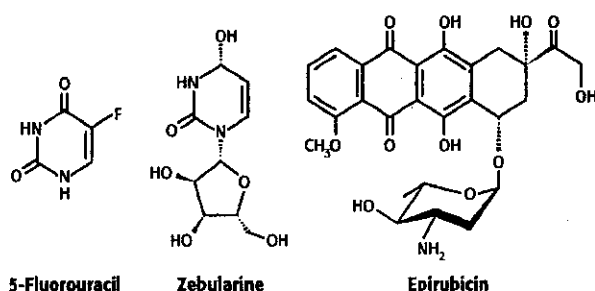


Figure 24. Structures of cancer drugs of different classes for which HILIC methods have been developed (see text).

MS/MS. The method was later extended to several other aminoglycoside antibiotics of similar structure (amikacin, gentamicin, kanamycin, neomycin, paromomycin, and tobramycin) in human serum for use in therapeutic drug monitoring at and below the therapeutic level [142].

Several cancer drugs are also highly hydrophilic compounds (Fig. 24) that are typically used in low doses because of their potency. A method for determining 5-fluorouracil, a pyrimidine analog antimetabolite pro-drug primarily used against colorectal cancer, was published by Pisano *et al.* [95]. After having evaluated a silica-based amino column, they found it to have a very short lifetime, causing significant shifts in retention time, a high background (Fig. 25), and also deposition of silica on the voltage needle and on the curtain plate of the MS. After switching to a poly(vinyl alcohol)-based amino column the retention time reproducibility was improved substantially and depositions in the mass spectrometer inlet were absent. A HILIC method was recently developed to monitor the pharmacokinetics of zebularine, a novel anticancer drug of the DNA methylation inhibitor type [143]. Radioactive detection was used to trace the ^{14}C marked substance and its major metabolites uridine, uracil, and dihydrouracil in murine plasma. Baseline separation was achieved on a 250 mm \times 4.6 mm aminopro-

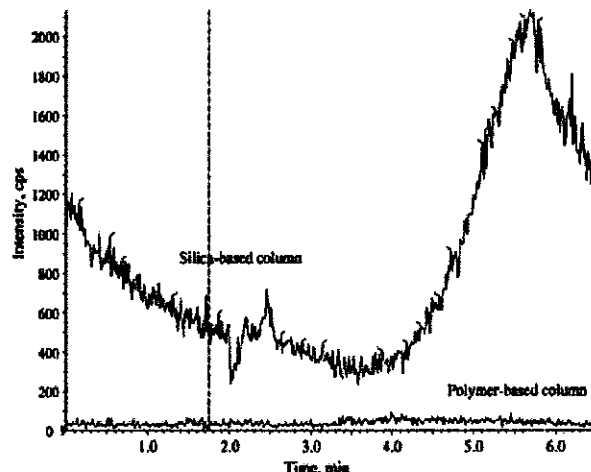


Figure 25. Multiple reaction monitoring traces of 5-fluorouracil after methanol injection on a silica-based and a polymer-based amino column. Reproduced from Pisano *et al.* [95] with permission.

pyl column, whereas an attempted RP separation failed. Epirubicin is another class of chemotherapy agents and belongs to the anthracycline drug family. The carbohydrate moiety along with polar substituents on the tetracyclic backbone makes it difficult to establish retention in RP. In the development of an HILIC method for this substance on a high purity naked silica [60], the choice of organic modifier was found to be particularly important, since it simultaneously affects many of the separation parameters; see Fig. 10 and the corresponding part of the mechanism section.

Only a handful of preparative HILIC applications seem to have been developed. One of these is the use of a poly(2-hydroxyethyl aspartamide) column for purification of peptides that were too hydrophilic for separation on C_{18} or SDVB columns in a study of copper-binding peptides that stimulate angiogenesis [144]. Another preparative separation by HILIC is by Huang and Li [81] and describes purification of epirubicin from crude solution under overload conditions. This separation is interesting to review in connection with the analytical separation just mentioned [60]. The preparative paper uses the same underivatized silica, but as opposed to the analytical method, the eluents used for preparative separation are exceptionally rich in water (95–100%) for an HILIC separation, and an RP retention pattern was observed on a high purity silica under these conditions. The peaks were triangular and tailing, and the center of gravity moved toward earlier retention times with higher loading, as would be expected for a Langmuirian isotherm (Fig. 26). A stepwise elution with an increase in ACN contents from 5 to 40% was used to rapidly elute the more strongly retained components. This retention behavior was attributed to the use of a high purity silica and is rational in view of the "U-shaped" retention curves that

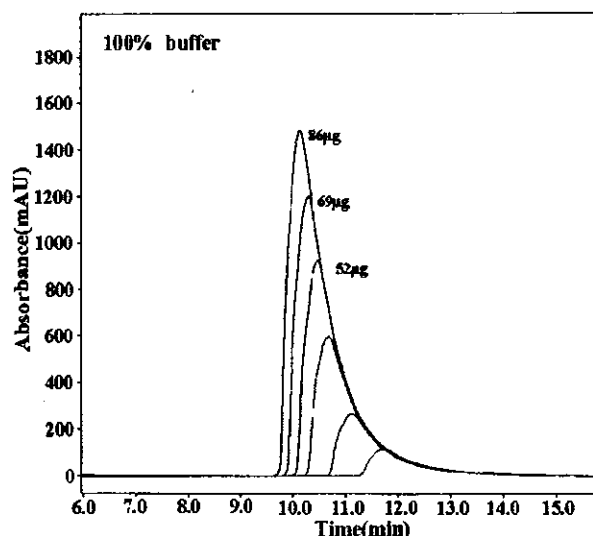


Figure 26. Peak shape of epirubicin at the sample size increases on a Kromasil silica under overload conditions. Fully aqueous mobile phase: Sodium formate buffer (20 mM, pH 2.9) pumped at 1.0 mL/min. UV detection at 280 nm. From Huang and Li [81], reproduced with permission.

are frequently seen for highly amphiphilic compounds when eluted over a wide range of eluent strengths, as discussed above. It is worth noting that useful separations of the same compound in analytical and preparative applications could be accomplished on the same packing using such widely different conditions.

5.1.3 Pharmaceutical development and drug discovery

Oyler *et al.* [145] introduced peptide separations by HILIC to MS by separating an oxytocin analog, atosiban, and nine diastereomers on aminopropyl silica, polyamine silica, and a poly(2-hydroxyethyl aspartamide) with ESI-MS detection. On the aminopropyl column, baseline separation of all ten solutes was accomplished but high salt concentration (250 mM sodium perchlorate) was needed to get acceptable peak shape and was thus not compatible with ESI-MS detection. The high amount of salt needed can probably be explained by both the analyte and the column being positively charged. The retention was also very sensitive to water contents in the eluent. In contrast, the polyamine column, which has predominantly secondary and tertiary amine groups, gave a good peak shape with only 5 mM ammonium acetate. However, the selectivity was different and fewer peaks were separated. Also the poly(2-hydroxyethyl aspartamide) silica failed to resolve all solutes. Besides, the solutes selected had retention in RP, and they concluded that HILIC could be a complement but failed to come up with a true HILIC-ESI-MS method. This feat was accomplished by Strege [146], who tested seven different columns (a poly(2-hydroxyethyl aspartamide) silica, an amide silica,

two amino phases, two cyano phases, and a CD phase) with ACN/water gradients, either with or without low concentrations of volatile buffers added. The test solutes were selected to represent acidic and basic compounds of interest in drug development from natural products, and none of these compounds showed retention in RP with a mobile phase containing only 2% ACN in ammonium acetate, pH 5.5. Without buffering, the amide, cyano, and CD phases showed a typical HILIC behavior, manifest in a substantial retention when 25% water was used in the aqueous ACN eluent. The retention virtually disappeared when the water level was increased to 40%. None of the solutes were eluted from the amino columns under these conditions. When increasing amounts of ammonium acetate was added to the eluent at relatively low concentrations, the retention times first responded significantly, then leveled off and the retention times became more stable. A concentration of 6.5 mM was found to be a good compromise between retention time stability and ionization suppression. A separation of hydrophilic compounds in a fermentation extract showed around 70 resolved peaks after an RP-SPE cleanup step [146]. Strege *et al.* [147] later developed the HILIC-MS approach to drug discovery through a mixed-mode anion/cation exchange/HILIC (ACE-HILIC) approach by coupling two oppositely charged ion-exchange columns in series and eluting in HILIC mode for drug discovery of natural products.

Lately, the use of HILIC as a complement to RP separations in metabolic fingerprinting has been performed [148]. Rat urine samples were separated by both HILIC on a polymeric sulfoalkylbetaine zwitterionic silica and by RP with ESI-MS detection, and the resulting data were subjected to multivariate data analysis by partial least squares discriminant analysis (PLS-DA) to provide a metabolic fingerprint [149]. It was concluded that metabolite detection was enhanced by combining the two chromatographic techniques, and that many potential biomarkers detected in the fraction containing the highly polar compounds would normally have been discarded in the wash when using RP-HPLC.

Fishing for bioactive molecules is a branch of drug discovery where HILIC is potentially useful, since it has the ability to retain highly hydrophilic and uncharged compounds, which behave essentially as stealth when chromatographic techniques are used in attempts to isolate the active substances. Kimura *et al.* [150] determined 1-deoxynojirimycin (Fig. 20) in mulberry leaves with good retention on an amide silica, when a reasonable retention could not be achieved on RP, ligand exchange and aminopropyl columns. Extraction from mulberry leaves was improved over conventional water extraction by using ACN/6.5 mM ammonium acetate, pH 5.5 (50:50) as extraction medium with sonication. The crude extract

was filtered and injected directly into the HILIC system, where the eluent had the same components at a 81:19 ratio. In spite of injecting the sample in a stronger eluent, the peak for 1-deoxynojirimycin was well defined and baseline separated from the other components.

Another highly hydrophilic plant constituent that has recently been analyzed by HILIC is dencichine (β -N-oxalyl-L- α , β -diaminopropionic acid), a hemostatic agent present in traditional Chinese medicinal herbs, but also a compound known as a neurotoxic agent from *Lathyrus sativus* (grass pea) seed. Koh *et al.* [85] developed a validated method for rapid determination of this compound based on an underivatized silica with MS/MS detection. With three dissociable groups (pK_a values 1.95, 2.95, and 9.25) dencichine will be zwitterionic in the pH range of most usable buffer systems. Attempts to use C_{18} directly were futile, and ion pairing with 0.1% TFA also failed to establish retention. Ion exchange was written off because of incompatibility with MS detection. In short, this is a perfect candidate for HILIC, and retention was seen once a bare silica column was tested. However, with 60% aqueous ACN the peak was initially very broad and it was necessary to increase the ammonium formate concentration in the aqueous part of the eluent to 200 mM in order to arrive at an acceptable peak shape.

Several different HILIC phases also played an important role in the isolation of an NCX_{IF} , a low abundance low molecular weight compound of hitherto unknown structure that acts as an inhibitor of the cardiac Na–Ca exchanger. What is known is that NCX_{IF} is a small, polar, and uncharged compound that is poorly retained even on polar-embedded RP columns. HILIC on an amide silica column was used alongside gel filtration, cation exchange, and C_{30} RP separation a few years ago [151], and recently the same authors revisited the problem [152] with a set of new columns of which two were HILIC phases (poly(succinimide) and poly(2-hydroxyethyl aspartamide) silica) and one a β -cyclodextran silica operated in NP mode in their continued efforts to purify the elusive substance for identification. The HILIC and NP separations were found imperative for the overall purification scheme, but provided a much lower yield of the NCX_{IF} activity (30–60%) compared to the RP columns.

5.1.4 Drugs of abuse

HILIC separations have also been applied to drugs of abuse. A method for cocaine and seven of its metabolites in human body fluid and tissue was developed, based on a liquid extraction step followed by separation on an underivatized silica with a gradient from 80 to 56% ACN in 2 mM ammonium acetate [153]. A sulfoalkylbetaine zwitterionic silica column was used in combination with a C_{18} precolumn for on-line desalting to establish a validated method for morphine and its 3- and 6-glucuro-

nides in plasma and microdialysis samples [154]. An isocratic eluent composed of 70% ACN and 30% 5 mM ammonium acetate gave separation of the gluconurides, which is necessary in MS detection since they share the same precursor and product ions. A good long-term stability was obtained and the analytical column could be used for 3000 injections.

5.2 Agricultural and food chemistry

Considering its suitability to separation of hydrophilic substances it is surprising that only a few publications dealing with food and drink analysis make use of HILIC. Using an amide-bonded silica, Schlichtherle-Cerny *et al.* [155] separated and identified a number of amino acids, glutamyl di- and tri-peptides, and their glycoconjugates (Amadori compounds) by analyzing what eluted in the void of a traditional RP separation of the low molecular fraction in a wheat gluten hydrolysate (Fig. 27). When the same procedure was applied to a parmesan cheese extract the HILIC-ESI-MS trace contained more than 25 unique substances, more or less well separated, including arginine, lysine, glutamic acid, and a number of polar di-peptides. Using a somewhat similar approach, ultrafiltration, gel permeation, and RP-SPE followed by HILIC separation on an amide-bonded silica, Rotzoll *et al.* [156] isolated a new glycoside, (S)-morelid from morel mushrooms.

Poly(2-hydroxyethyl aspartamide) HILIC and RP columns have been used for the quantification of the two major steroidal glycoalkaloids in potatoes, α -chaconine and α -solanine. Zywicki *et al.* [104] concluded that both RP and HILIC methods proved to be precise and accurate enough to be used. However, the RP method was more rugged than the HILIC method for maintaining the analyte peak shape, as the poly(2-hydroxyethyl aspartamide) column lost its peak shape after 100 injections. Some of the superiority may be due to a step gradient being used in the RP method as opposed to a slow gradient in the HILIC method, and a better performance of the RP method may also be explained by the RP method being used for potato skin only which contain higher levels of glycoalkaloids than potato flesh, which were the samples used to evaluate the HILIC method.

Semipreparative separation on an amide silica played an important role in a highly orthogonal fractionation scheme used in identifying 1-carboxymethyl-5-hydroxy-2-hydroxymethylpyridinium inner salt as a potential bitter-suppressing candidate in a combinatorial library of pyridinium betaine type taste modulatory compounds [157]. Taurine and methionine have been quantified in carbohydrate rich energy drinks without other sample preparation than dilution and the method has been validated in terms of accuracy, specificity, precision, linearity, detection limits, and stability, using threonine as

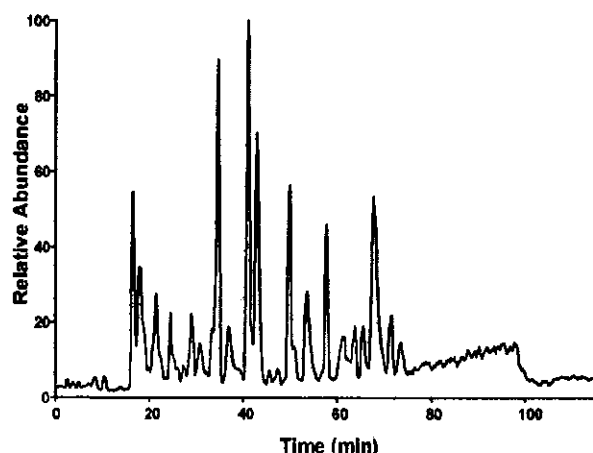


Figure 27. HILIC separation of a polar hydrolysate fraction of wheat gluten, unretained by RP-HPLC, with ESI-MS detection in the positive ionization mode. Eluent: ammonium acetate buffer, pH 5.5/ACN gradient with 13–40% water in 90 min. Reproduced from Schlichtherle-Cerny *et al.* [155] with permission.

internal standard [97]. So far, the only application of HILIC to drinking water is for the quantitation of dichloroacetic acid [158], a method that was subsequently extended to rat blood and tissues [159]. The authors tested a number of state-of-the-art RP columns but were unable to come to terms with the peak shifting and distortion due to differing salt concentrations (mainly Na^+) in the tapwater samples. The final method utilized an aminopropyl silica in ion-exchange/HILIC-MS-MS mode with ACN/40 mM ammonium formate (60:40), a buffer concentration that resulted in the best peak shape with minimal ion suppression. The method was insensitive to sample salt content and there was no need of the derivatization step used in most previous methods [158].

5.3 Toxins

Many naturally occurring toxins are highly hydrophilic compounds [160–163]. Particularly well suited for HILIC separations are marine toxins associated with paralytic shellfish poisoning (PSP). When Negri *et al.* [160] isolated three novel hydroxybenzoate saxitoxin analogs from *Gymnodinium catenatum*, HILIC separation on amide silica was a vital part of the preparative and analytical separation toolkit. Amide silica was also used by Dell'Aversano *et al.* [164], who separated and quantified a large number of cyanobacterial toxins including various analogs of saxitoxin, cylindrospermopsin, and anatoxin-a in algal samples with selected reaction monitoring MS detection. The method is robust and analyses can be done without sample cleanup or preconcentration. It was later extended to domoic acid [165] and a wider range of PSP toxins [166], and recently applied along with other methods for studies of the toxin profile of *Mytilus galloprovincialis* [167].

5.4 Synthetic polymers

Size exclusion or gel permeation chromatography are standard techniques for separation of synthetic high polymers and oligomers. Another, less well-known separation technique is precipitation–redissolution, or gradient polymer elution chromatography [168], which is based on narrowing of the solubility range in relation to the solvent properties as the molecular weight of a polymer increases, according to the Flory-Huggins solution theory [169]. HILIC-like conditions were used by Svec *et al.* [131] to separate poly(vinyl pyrrolidone) of molecular weights up to 360 kDa on an all-polymeric diol phase, using a gradient of 0–50% water in acetone. Contrary to common behavior in partition chromatography, peaks were sharper as the molecular weight increased, which hints at a precipitation/redissolution mechanism. Dextran oligomers with 3–12 glucose units were also baseline separated using a gradient of 10–40% water in acetone. When the eluent was extended to 60% water, dextrans with MW of up to 147 kDa were eluted, and using a weaker gradient (8–20% water), α -, β -, and γ -CD were baseline separated. HILIC on diol silica has also been used in a low pressure format in the initial stages of purification of poly(amidoamine) PAMAM dendrimers [109].

5.5 Carbohydrates

The scope of carbohydrate analysis is very wide, ranging from analysis of sugars [170, 171] and nucleosides [172] in the most diverse samples, characterization of low and high molecular weight polysaccharides and their derivatives in the food [173] and cellulose industries [174], synthetic glycopolymers [175], to charting of differences in complex glycan structures of low abundance glycoproteins (“glycoproteomics” or “glycomics”) [176, 177].

Common to carbohydrates is that they possess a set of properties that has made chromatographic separation difficult. They are rich in hydroxyls and other polar functional groups, lack the thermal stability required for native analysis by GC, and are, as saturated carbohydrates, hard to detect by optical means with reasonable sensitivity. Separation trends have changed with the availability of new techniques from derivatization GC [178] to high-performance anion-exchange chromatography [179, 180] and early adoption of HILIC [23, 24, 181]. Today conventional chromatographic separations are facing serious competition from CEC [182, 183] and CE [184], due to the complexity of carbohydrates in biological systems. Detection techniques in liquid phase separations have also varied over time, from insensitive refractive index to pulsed amperometric detection [185] and evaporative light scattering [186]. Nowadays MS is the standard technique for detection in complex carbohydrate analysis [187]. A review on carbohydrate separa-

tions by HILIC was published as early as 1996 [181] and prior to that aqueous NPC on polar silica-based columns was already featured in several reviews on carbohydrate separation [188–190]. A comprehensive review of carbohydrate separation by HILIC is beyond the scope of this treatise. HILIC is well covered in numerous recent reviews on subareas of carbohydrate analysis, such as the separation of low molecular weight carbohydrates in food and beverages [173], sugars in traditional Chinese drugs [171] and marine samples [170], glycoalkaloids and their hydrolysis products in *Solanaceae* plants [191], glycosaminoglycans [192], glycoproteins [193], assessment of protein glycosylation [194], and the fluorescence derivatization for HPLC of glycoprotein carbohydrates [195]. Instead some focus will be given to novel ways of carrying out HILIC-type separations and novel sample cleanup procedures using the aqueous NP principle. A list of applications is found in Table 1.

5.5.1 Mono- and di- and oligosaccharides

A substantial fraction of carbohydrate separations by “aqueous NPC” still use aminopropyl silica, in spite of the sensitivity to hydrolysis and the Schiff's base formation on these materials [107]. These separations have been around for >30 years [23, 24], have been included in numerous reviews [173, 181, 189, 196], and will not be covered here. Armstrong and Jin [114] introduced CD-bonded phase columns to the separation of saccharides up to tetrasaccharides, deoxysaccharides, and sugar alcohols, using aqueous ACN or acetone eluents in isocratic or gradient modes. The α -CD column was found to be slightly more efficient than the β -CD column and the stability and reproducibility was far superior to amino-bonded columns. In spite of the α -CD column being found more efficient, the same author recently published a method for underivatized oligosaccharides and polysaccharide hydrolysis products on a β -CD-bonded phase with ESI-MS detection [197]. Oligosaccharides produced by enzymatic hydrolysis of chitin were characterized for anomer conformation and size, with an amide silica used for the separation [198]. The analysis of oligosaccharides in rat urine as a noninvasive evaluation of toxicity has also been performed on an amide silica, following a sample cleanup step with a graphitized carbon SPE step and labeling with 2-aminoacridone [199].

5.5.2 Glucosinolates

Glucosinolates are a group of water-soluble glucosides with a sulfonated oxime and a thio-linked glycone as characteristic polar features, and are differentiated by the side group R attached to the central carbon atom (Fig. 20). Bogdanov has prepared a list of 116 naturally occurring glucosinolates with structures on the web (Bogdanov, B., <http://ns.pmf.ukim.edu.mk/bbogdanov/glucosi->

[nolates/webG2.htm](http://ns.pmf.ukim.edu.mk/bbogdanov/glucosinolates/webG2.htm); as of April 24, 2006), all of which are highly hydrophilic or amphiphilic compounds with a permanent anionic charge due to the sulfonate group. The side chain is a strong retention determinant and glucosinolates can be separated with RP, although the retention is very low and calls for a gradient starting with a fully aqueous 0.1% TFA eluent to only 10% methanol over 30 min [200]. Previous methods have relied on ion pairing or desulfation and RP separation, but Troyer *et al.* [201] introduced a method based on poly(2-hydroxyethyl aspartamide) silica by which intact glucosinolates are separated by HILIC in isocratic mode. This HILIC method was later used for analytical separation of glucosinolates purified from plant sources by high-speed counter-current chromatography [202].

5.5.3 Saponins

Saponins is the collective name for a group of highly amphiphilic compounds that are found mainly in the skin of plants where they are part of the waxy protective coating, but also in their seeds and roots. The group comprises steroid glycosides, steroid alkaloids, or triterpenes. Many saponins are known to be more or less toxic and are then termed sapotoxins; an example is the cardiac glycoside digoxin extracted from *Digitalis*. Other saponins such as the ginsenosides of *Panax* roots are believed to be beneficial to health. Due to their pronounced amphiphilicity, saponins can be separated both by RP [203, 204] and HILIC. In HILIC mode [205], saponins can be separated with ACN–water eluents on wide-pore aminopropyl silica, and the pore size is important for the separation. Preparative HILIC on a 300 mm \times 20 mm aminopropyl silica column has been used in combination with an initial size exclusion (SEC) step and finally orthogonal C_4 and phenyl RP steps, for the isolation and identification of a series of cytotoxic ester saponins from *Archidendron ellipticum* [205, 206].

5.5.4 Phosphorylated carbohydrates

Due to their charge, phosphorylated carbohydrates are difficult to retain under RP conditions compatible with MS detection. Common separation modes are ion exchange [207] or ion pairing/micellar RP [208]. Feurle *et al.* [209] showed that a β -CD column could be used in a mixed HILIC/anion exchange mode for the separation of a variety of phosphorylated monosaccharides. The anion exchange properties were established by inclusion of ammonium ions from the buffer in the CD cavities. The method has since been used for the determination of glycerophosphoinositol in cell extracts [210].

5.5.5 Glycans

The use of RP, graphitized carbon, and NP (HILIC) with online MS or MS/MS detection in studies of protein glyco-

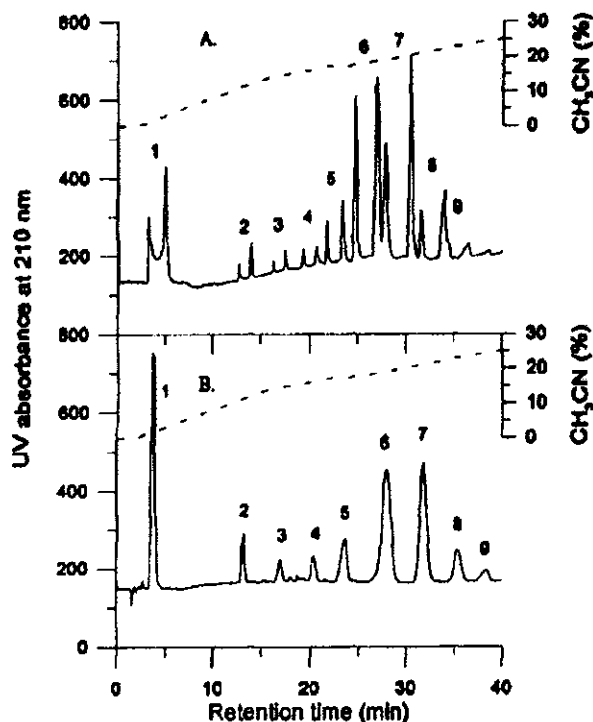


Figure 28. Separation of chito-oligosaccharides on a graphitized carbon column at 50°C, eluted with a gradient of ACN in water, (A) without, and (B) with 10 mM ammonia added to both eluent members. The legend numbers denote DP. Reproduced from Fan *et al.* [213] with permission.

sylation was recently well reviewed by Wührer *et al.* [194]. HILIC is part of an overall scheme, where derivatization for retention in RP and detection in UV, fluorescence, and improved fragmentation in MS ("tagging") plays an important role. Derivatized oligosaccharides and glycans can be separated by RP techniques and an advantage is claimed to be that laboratories equipped for proteomics by RP-LC/MS could implement these schemes for protein glycosylation studies with minimal effort and investments. Apart from permethylation [211], all common derivatization schemes target the reducing end of the cleaved oligosaccharide and the polar groups that promote retention in HILIC are therefore left largely intact. Graphitized carbon [212] occupies an intermediate position, as it provides retention also for underivatized oligosaccharides, with a retention mechanism that appears to be hydrophobic in nature [213]. Elution is accomplished by water alone or relatively low and increasing concentrations of methanol or ACN in aqueous buffers containing TFA [214] with ammonia sometimes added to catalyze the mutarotation between α and β anomers and the column operated at elevated temperature [213] (Fig. 28). It is amazing to see the same oligosaccharides eluted by HILIC in the same work, with a selectivity pattern that is essentially identical (Fig. 29). The ability to trap underivatized oligosaccharides from aqueous solutions makes graphitized carbon useful in desalting of oligosacchar-

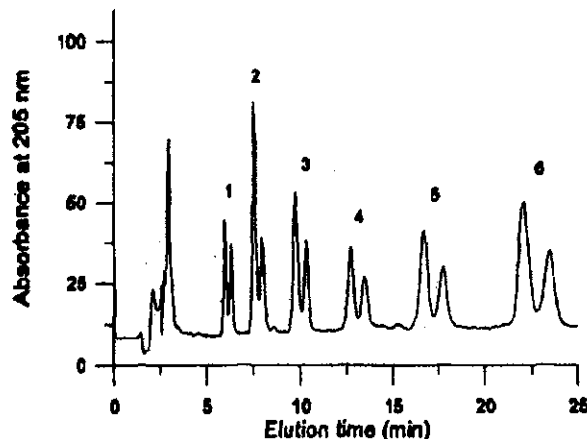


Figure 29. Separation of chito-oligosaccharides on an amide silica column, eluted isocratically with 32% water in CH_3CN . Legend numbers as in Fig. 28. Reproduced from Fan *et al.* [213] with permission.

ides from glycoproteins [215]. An alternative trapping technique uses aminopropyl silica in the HILIC mode [216]. Yet graphitized carbon has a substantial advantage over silica-based phases, as the hydrolytic stability allows the use of high pH and elevated temperature without column deterioration, and it is a worthy competitor of HILIC in protein glycosylation investigations [217, 218].

Common practice in mapping of glycans has been to release the glycans from glycoproteins with either a chemical (hydrazinolysis) step or by enzymatic means. The released glycans are thereafter derivatized at the reducing terminal with a fluorescent reagent and the derivatized glycan is subjected to a series of separation steps of which HILIC is often one. Derivatization reagents used are 2-aminopyridine [96, 100, 102, 219], 2-aminobenzamide [220–222], 2-aminoacridone [223, 224], or 3-(acetylamino)-6-aminoacridine [225], and separations in HILIC mode have almost invariably taken place on various brands of amide silica [100, 102, 219, 221–225]. These methods have been reviewed by Anumula [195, 226]. Standard separation methods for the analysis of complex carbohydrates include, apart from HILIC, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [226], and CE and microelectrophoresis [184].

Quite surprisingly, HILIC has not been used for separation of underivatized monosaccharides common in N-linked oligosaccharides from glycoproteins until last year, when Karlsson *et al.* [227] developed an isocratic method for separation of L-fucose, D-galactose, D-mannose, N-acetyl-D-glucosamine, N-acetylneuraminic acid, and D-glucuronic acid, based on an amide silica with evaporative light scattering detection.

5.5.6 Glycopeptides

Conventional HILIC mode separations of fluorescently labeled oligosaccharides was miniaturized for analysis by ESI-MS at the subfemtomole level by Wührer *et al.* [228]. The protocol was based on separation on an amide silica microcolumn and still involved derivatization with 2-aminobenzamide. However, as it was concluded that the sensitivity of fluorescence was conquered, subsequent works by the group of Wührer has disposed of the derivatization step which was intended to give RP retention and detectability by fluorescence for high sensitivity. Since HILIC is based on polar interactions, the retention generally increases with the length of the glycan chains, and the elution positions thus provide a rough indication of glycan size [229]. A further evolution of this concept is a miniaturized setup for characterization of glycopeptides, where the protein is digested by Pronase (a mixture of proteases from *Streptococcus griseus*) which produces short peptide fragments. The (glyco)peptides are then subjected to HILIC separation whereby retention is mainly brought about by polar interactions with the hydroxyl groups of the glycans. Separation of identical peptide fragments with different glycan structures is thereby possible, and in combination with MS/MS the method is applicable to rapid, high sensitive characterization of site-specific protein glycosylation (Fig. 30) [230]. It was recently also shown by Alvarez-Manilla *et al.* [231] that the increased size of glycosylated tryptic peptides enables the use of size exclusion chromatography as a pre-separation step for enrichment of glycopeptides prior to subsequent glycan release and characterization.

A procedure termed “hydrophilic affinity isolation,” deceptively similar to a one-step HILIC procedure, was used by Wada *et al.* [232] to selectively trap and enrich tryptic glycopeptides in glycoproteomics. The glycoprotein digest was applied to a microcentrifuge tube containing microcrystalline cellulose or Sepharose CL-4B, and a mixture of 1-butanol, ethanol, and water (4:1:1 v/v) was added. After 45 min of shaking followed by washing with the trapping solvent, the glycopeptides were released with ethanol/water (1:1 v/v) and the reconstituted sample subjected to RP-HPLC. The chromatogram in Fig. 31 shows an essentially clean baseline with two glycopeptide fragments. It is worth noting that this was not an SPE-type cleanup but a one step trap-and-elute procedure based on a slurry of the carbohydrate sorbents. Even better performance would probably be possible if the sorbent is used as a conventional SPE format, or as an online cleanup cartridge.

5.6 Amino acids and peptides

Following carbohydrates, peptide separations were among the first applications of HILIC and it has been

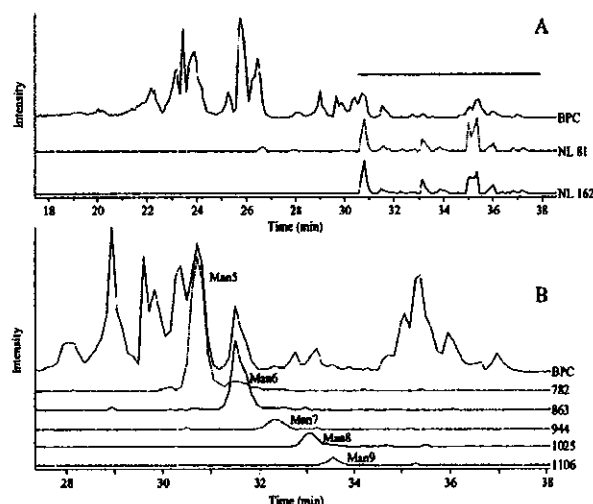


Figure 30. HILIC separation of a 5 ng aliquot of *N*-glycosylated peptides from an RNase B Pronase digest with ESI-MS with automatic MS/MS. Extracted ion chromatograms of double-protonated glycopeptide species with an N60LT62 peptide moiety are given in (B). BPC, base peak chromatogram. Reproduced from Wührer *et al.* with permission; see [230] for details.

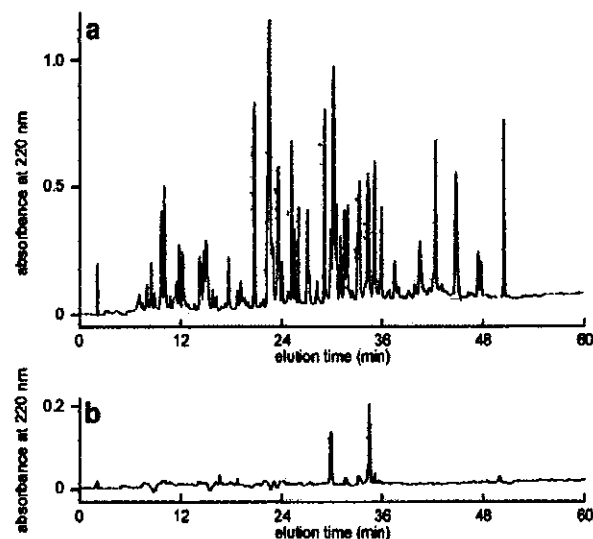


Figure 31. RP-HPLC of tryptic peptides from transferrin. (a) Total digest; (b) Sepharose-binding peptides. The Sepharose-binding fraction from the tryptic digest of carbamidomethylated human transferrin (200 µg) was recovered and analyzed. Reproduced from Wada *et al.* [232] with permission.

shown on practically all commonly used HILIC stationary phases [13, 67, 99, 145]. That HILIC has an inverse retention order relative to RP was shown by Alpert [13] for amino acids and for peptides by Mant and Hodges, using peptides specially designed for testing RP and ion-exchange columns [67]. Mant and Hodges were also the first to realize that cation exchange (CEX)-HILIC mixed mode separation can rival the efficiency of RP chromato-

graphy for certain peptides [68]. Using amphipathic α -helical and β -sheet peptides they also showed that a substitution in the hydrophilic face of the peptide gave a large effect on retention in HILIC but no effect in RP-HPLC, and that a substitution in the hydrophobic phase gave no effect in HILIC but a large effect in RP-HPLC [64, 65]. This proves that the retention characteristics of large peptides are governed by their contact region and not by the overall hydrophobicity/hydrophilicity in both RP and HILIC separations. The same strategy was also utilized for the purification of a 21-residue synthetic amphipathic α -helical peptide from serine side-chain acetylated impurities [69] and for determining the apparent hydrophilicity/hydrophobicity of different amino acid substitutions [71]. Temperature has also been shown to increase retention and improve separation of these CEX-HILIC separations of amphipathic peptides [70]. Separation of peptides on both silica and on packings with an intentional charge invariably has an electrostatic component, making such HILIC setups pronounced mixed mode separations [64, 65, 67, 68]. Yoshida [233] has contributed to the fundamental understanding of HILIC by developing a set of "hydrophilicity retention coefficients" from 121 peptides separated on an amide silica in ACN/water eluents with 0.1% TFA. The predicted retention times had a reasonably good fit ($r^2 = 0.94$) and the observed retention times were generally within 10–20% of those predicted. Boutin *et al.* [234] separated phosphorylated peptides from γ - ^{32}P -ATP and inorganic ^{32}P in tyrosine protein kinase assays by a poly(2-hydroxyethyl aspartamide) silica, which was used both for sample preparation by SPE and for HPLC in HILIC mode. Peptide separations by HILIC were recently reviewed by Yoshida [22].

Although not strictly an HILIC work, attention should be drawn to the paper of Hyun *et al.* [235], who studied the retention behavior and enantiomeric resolution of α - and β -amino acids on silica-based chiral stationary phases with sodium *N*-(*S*)-1-hydroxymethyl-3-methylbutyl)-*N*-undecylaminoacetate and sodium *N*-(*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate as bonded selectors in aqueous methanol. An increasing retention was seen for many of the amino acids in methanol concentrations in the range 30–50%, and the discussion encompasses interesting aspects of retention as a function of the lipophilic/hydrophilic balance of the amino acids.

5.7 Proteomics – coupled-column systems (2-D-HPLC)

Coupled-column HPLC systems (2-D-HPLC) have emerged as a response to the extreme sample complexity faced in the search for low abundance components in biological samples, most notably in proteomics, metabolomics, and glycomics. The key to success in 2-D-HPLC is *orthogonality*, i.e., the selectivities in the two separation dimensions

should be the results of pronounced different sets of retention mechanisms. Equally important is also that the eluate from the first dimension can be injected on the second dimension column without band-broadening and disturbances in the column equilibrium. Furthermore, 2-D-HPLC is nowadays used almost exclusively in combination with MS detection, and the eluate from the second dimension should therefore be compatible with ESI. This is a tough set of constraints and very few separation mode combinations are considered feasible. Because of its general applicability, good resolving power and familiarity, few would argue against RP being given an almost mandatory position as one of the separation dimensions for samples where RP retention can be accomplished.

The combination of SCX with RP [236–238] has become a common setup in proteomics, but it is applicable only to positively charged solutes, which most peptides fortunately are at low pH. Against this combination has been argued that SCX is not completely orthogonal to RP since retention in ion exchange is driven mainly by charge (which is a strong negative retention promotor in RP). The separation efficiency in SCX is inferior to RP and HILIC (cf. Fig. 32A, E, and F), and since retention in ion exchange is driven primarily by coulombic interactions, peptides tend to cluster as a function of their net charge as a majority of tryptic peptides carry either a +2 or +3 charge under the conditions typically used (cf. Fig. 33F) [237, 239]. For neutral solutes, RP must be combined with a different separation mode that does not rely on charge, and the options left are essentially HILIC, size exclusion, or RP at a radically different pH. Although SEC eluents are easily combined with RP and a good orthogonality is warranted by the separations relying on completely different principles, SEC suffers from limited resolution (Fig. 32D). The reason is that in the (ideal case of) absence of enthalpic interactions, all solutes must elute at k' that are negative in the common sense, i.e., in a "retention window" extending from the interstitial volume for a totally excluded solute to the sum of the interstitial volume and the eluent contained in the pore space for a totally nonexcluded solute [41].

The figures referred to in the preceding paragraph were taken from a recently published paper by Gilar *et al.* [239], who investigated several different separation mode combinations (among which HILIC in combination with RP at acidic pH in the second dimension was one) for their orthogonality in the resolution of tryptic digests with ESI-MS detection. In HILIC on an underivatized silica, they saw a slight correlation between peptide charge and retention, which was attributed to ionic interactions with charged silanols, and claimed that the separation resembled that on a strong cation exchanger (Fig. 33E and F). After employing a binning procedure to

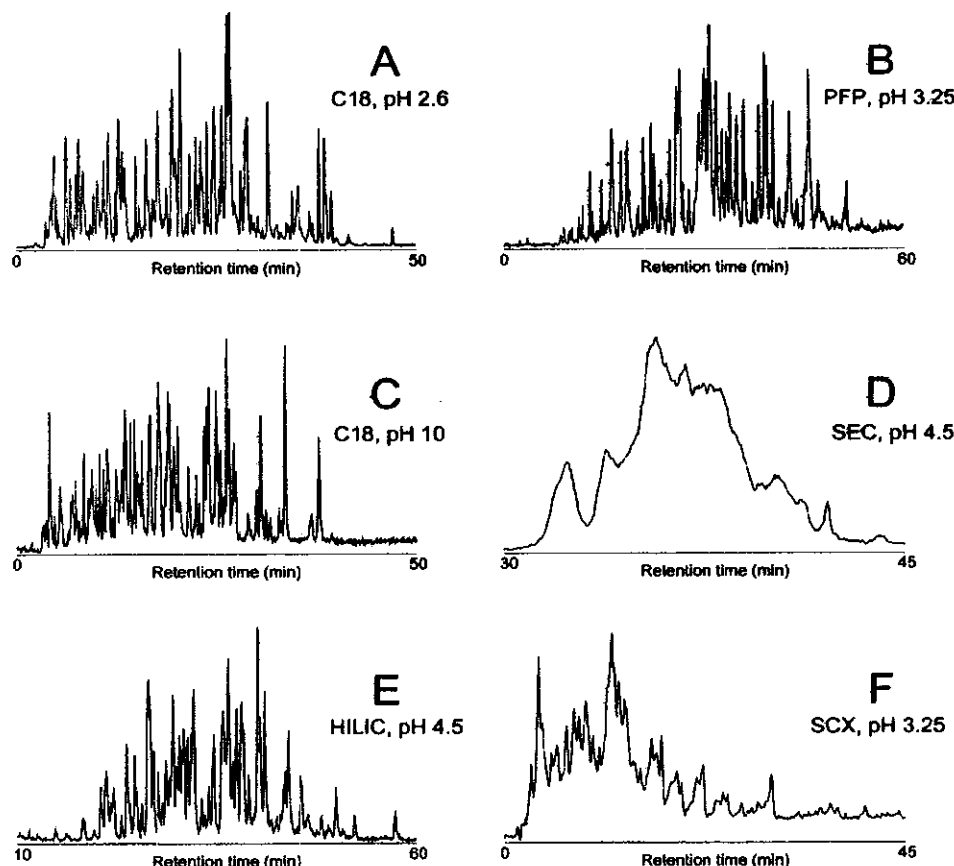


Figure 32. LC-MS chromatograms of a phosphorylase b tryptic digest (~100 peptides) in different LC separation modes. (A) C₁₈, pH 2.6; (B) perfluorophenyl RP; (C) C₁₈, pH 10; (D) SEC on a diol silica; (E) HILIC on underivatized silica; (F) SCX on poly(2-sulfoethyl aspartamide) silica. The C₁₈ separations are performed on packing materials of (A) and (E) 3 μ m, (C) 3.5 μ m particle size, all other on 5 μ m particle size columns (Gilar, M., personal communication, Waters Corporation, May 1, 2006). All columns were 150 mm \times 2.1 mm except SEC (three 250 mm \times 4.6 mm connected in series) and run at an eluent flow rate of 0.2 mL/min. Reproduced from Gilar *et al.* [239] with permission.

assess the utilization of the 2-D separation space, they found that the highest orthogonality (69% of theoretical full orthogonality) was seen with HILIC in the first dimension, whereas a 2-D combination with high pH RP in the first dimension had a corresponding score of 53%. The experimental procedure for the HILIC separation was not described, but it was carried out under conditions that would warrant a valid comparison (Gilar, M., personal communication, Waters Corporation, May 1, 2006). Based on a slightly higher column efficiency of high pH RP over HILIC in the first dimension, coupled with a shorter effective analysis time for HILIC, they arrived at the somewhat curious conclusion that the RP–RP combination with high and low pH in the first and second dimensions gives the best practical peak capacity. One of the arguments in favor of RP/RP was a presumed poor solubility of peptides in high organic (HILIC) solvents, yet the peak shapes were good and no precipitation was seen in the injected solutions [239]. It is worth noting that the first 10 min of the HILIC separation are missing in Fig. 32E. In a corresponding poster presenting this concept (Olivova, P., Gilar, M., Dorschel, C. A., Gebler,

J. C., Poster presented at ASMS 2005, Waters Library No.: 720001223EN. <http://www.waters.com/WatersDivision/SiteSearch/AppLibDetails.asp?LibNum=720001223EN>, as of April 23, 2006.), the HILIC chromatogram shows numerous peaks eluting in this retention time range. The second dimension RP mode separation employed a gradient from 0 to 45% CH₃CN in 22 mM formic acid, whereas the HILIC separation (in spite of charge contribution from the silanol groups of the underivatized silica) was performed with a gradient of 90–40% ACN in only 10 mM ammonium formate buffer, pH 4.5. In view of the favorable ionization properties of HILIC eluents in ESI-MS [16–18], it would have been particularly interesting to see the opposite combination (RP with HILIC in the second dimension) tested.

5.8 Proteins

Protein separations in HILIC are not abundant, probably because most integral proteins are well separated by common protein separation techniques using eluents without significant admixtures of organic solvents, but

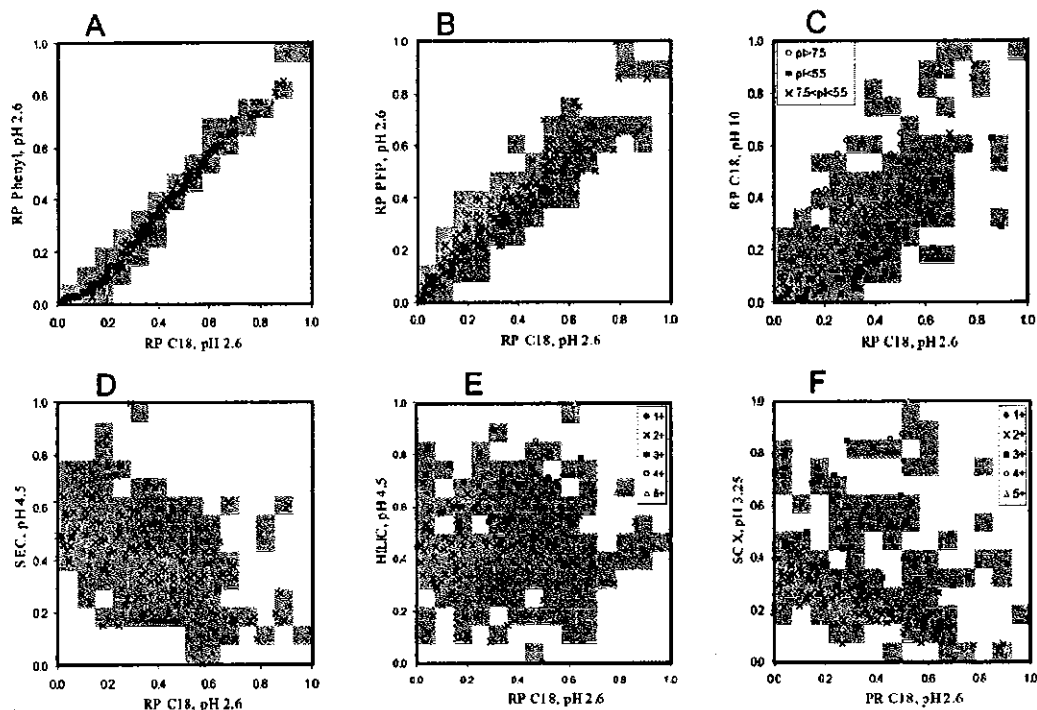


Figure 33. Normalized retention time plots for selected 2-D-LC systems with RP at pH 2.6 in the second dimension. First dimension separations were (A) RP on phenyl silica; (B) RP on perfluorophenyl silica; (C) RP on C₁₈ at pH 10; (D) SEC on diol silica; (E) HILIC on underivatized silica; (F) SCX on poly(2-sulfoethyl aspartamide) silica [239]. Numbers in the legends of plots E and F are the charges of peptides in the low pH RP separation. True orthogonality would exist if peaks were distributed totally evenly in the plots and no correlations were evident. The graphics is from a poster presented at ASMS 2005, and is used instead of the one appearing in the published paper [239] because it shows the binning concept (gray shaded squares) and has an additional color coding of the symbols. Reproduced from Olivova *et al.* (Olivova, P., Gilar, M., Dorschel, C. A., Gebler, J. C., Poster presented at ASMS 2005, Waters Library No.: 720001223EN. <http://www.waters.com/WatersDivision/SiteSearch/AppLibDetails.asp?LibNum=720001223EN>, as of April 23, 2006.) with permission.

also because of solubility issues [240]. A baseline separation of ovalbumin, cytochrome c, and BSA has been shown on a poly(2-hydroxyethyl aspartamide) silica, eluted with a gradient from 75% *n*-propanol in 50 mM formic acid to 50 mM formic acid only [241]. Using the packing material in the SPE format, a 30 kDa membrane protein extracted from an SDS-PAGE gel could be freed from staining agents and SDS and concentrated in HILIC mode. The protein recovery usually ranged between 50 and 65% for 30–45 kDa proteins. The authors had problems with single proteins producing multiple peaks, indicative of on-column denaturing [242].

Histones are the only integral proteins where HILIC has been regularly used. These proteins help to build the cellular chromatin by acting as a core, around which DNA is wound. There are several classes of histones and all are highly basic proteins. Lindner *et al.* [243] have developed mixed mode weak cation-exchange/HILIC methods for separation of acetylated core histones and phosphorylated H1 histones [244], and have used these separations for characterization of microheterogeneity [245], age-dependent deamidation [246–248] and trimethylation [248, 249], and sequence variations [250]. The separations

have been based on wide-pore silicas with either carboxymethyl [243, 248, 249] or poly(aspartic acid) [244–247, 250] functionalization using gradients of sodium perchlorate in 70% ACN at acidic pH. Mizzen [251] recently summarized the techniques for purification and analyses for variants and posttranslational modifications of histone H1, in which HILIC has a vital position.

5.9 Metabolomics

With the primary goal of metabolomic analysis being the unbiased relative quantification of all metabolites in a biological system, the expectations on separation science must be high [252]. HILIC has been recognized as an important tool in metabolomics, since metabolites are often very polar compounds. A frequently cited paper is by Tolstikov and Fiehn [103], who used HILIC combined with ESI-MS for metabolomic screening of oligosaccharides and sugar nucleotides in plants. Three different silica-based microbore columns with poly(2-hydroxyethyl aspartamide), amide, and aminopropyl functionalities were used. The aminopropyl column gave broad peaks and irreproducible retention times. Good separations and somewhat different separation patterns were

obtained on the two remaining columns, with amide silica concluded to be slightly better. Amino compounds were markedly more retained than analogs lacking an amino group, which is an advantage since it complements the low retention for amines in RP separations. An amide silica was also recently used in the profiling of sulfur metabolites (cysteine, homocysteine, methionine, cystathionine, γ -glutamylcysteine, S-Ado-Hcys, and glutathione) in yeast [253].

5.10 Sample preparation by HILIC SPE

One could argue that the use of HILIC as an intermediate separation step in a multidimensional protocol is a sample pretreatment, but only a few works have reported HILIC in regular sample preparation. Jenö *et al.* [241] used a poly(2-hydroxyethyl aspartamide) silica for removal of SDS and staining components from proteins extracted from SDS-PAGE gels. The organic part of the eluent (*n*-propanol) is rather unconventional for HILIC, but with a decreasing concentration from 75% in 50 mM formic acid to aqueous formic acid only, clean extracts for further handling were obtained. Curren and King [254] used a poly(2-hydroxyethyl aspartamide) silica in the SPE format for cleanup and enrichment of avoparcin, a macrocyclic glycopeptide antibiotic with a molecular weight of nearly 2 kDa. The sample was loaded as an aqueous 70% solution of ethanol with 6 mM triethylammonium phosphate (TEAP) and eluted by pure water. The reconstituted extract was separated on a column with the same kind of stationary phase but with smaller particle size and larger pores, using 47% aqueous ACN with 15 mM TEAP as eluent. In spite of two nonorthogonal techniques being used for cleanup and final separation, the peak from avoparcin extracted from swine kidneys was fully resolved, which may be attributable to the use of ethanol/water with an ion pairing reagent in the SPE step and ACN/water in the final HILIC separation. Yu *et al.* [216] used a 96-well microelution plate packed with aminopropyl silica for desalting of N-linked glycans enzymatically released from glycoproteins. Hagglund *et al.* [255] used a sulfoalkylbetaine zwitterionic HILIC phase packed in GELoader tips to trap sialylated glycopeptides which were not retained on RP sample preparation sorbents. Lindegårdh *et al.* [256] used the same zwitterionic material for trapping of amoxicillin from human plasma. The most recent application of sulfoalkylbetaine zwitterionic microcolumns in sample preparation is by Omaetxebarria *et al.* [257] and shows how GPI-anchored peptides can be selectively extracted from in-gel trypsin digested porcine renal membrane dipeptidase for structural analysis by MALDI-QTOF-MS/MS, using less than 1 pmol of tryptic digest (Fig. 34).

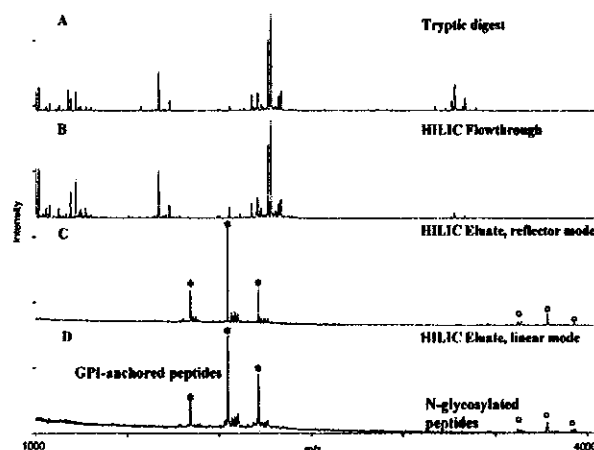


Figure 34. MALDI-TOF mass spectra of peptides from porcine and membrane dipeptidase (MDP) tryptic digest. (A) Peptides from the tryptic digest of MDP. (B) MDP tryptic digest from the void volume of the HILIC step. (C) HILIC-enriched fraction analyzed in MALDI reflector TOF-MS mode. (D) HILIC-enriched fraction analyzed in MALDI linear TOF-MS mode. In all cases, 0.5 μ L sample and 0.5 μ L of 2,5-dihydroxybenzoic acid (20 μ g/mL) in 70% ACN/0.1% TFA were mixed directly on the MALDI-MS target. Reproduced from Omaetxebarria *et al.* [257] with permission.

6 Summary

In conclusion, we believe that Alpert was correct when he claimed the acronym HILIC and defined it as an identifiable HPLC mode based on “the combination of hydrophilic stationary phases and hydrophobic, mostly organic, mobile phases.” HILIC is certainly a technique of its own, and is still probably best referred to as a liquid–liquid chromatography technique, where the actual stationary phase is the enriched layer of the more polar eluent (water) residing on the surface of the stationary phase. This “pure HILIC” separation mode appears, however, to be quite rare since most real HILIC separations are in essence multimodal – either due to coulombic interactions or due to hydrogen bonding – either to the stationary phase or to tightly bound water on the stationary phase at low water content in the eluent. It should be clear that retention in HILIC must involve more than just simple liquid/liquid partitioning between the bulk eluent and an adsorbed layer of water on the stationary phase. The elusive mechanism is due to the strong part of the eluent mixture being water, and although it is our most abundant and indispensable solvent, it never ceases to come up with new surprises [258–260]. In order to better understand the retention mechanisms of HILIC, the actual role and the structure of water at the surface of HILIC materials should therefore be further elucidated.

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7 References

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