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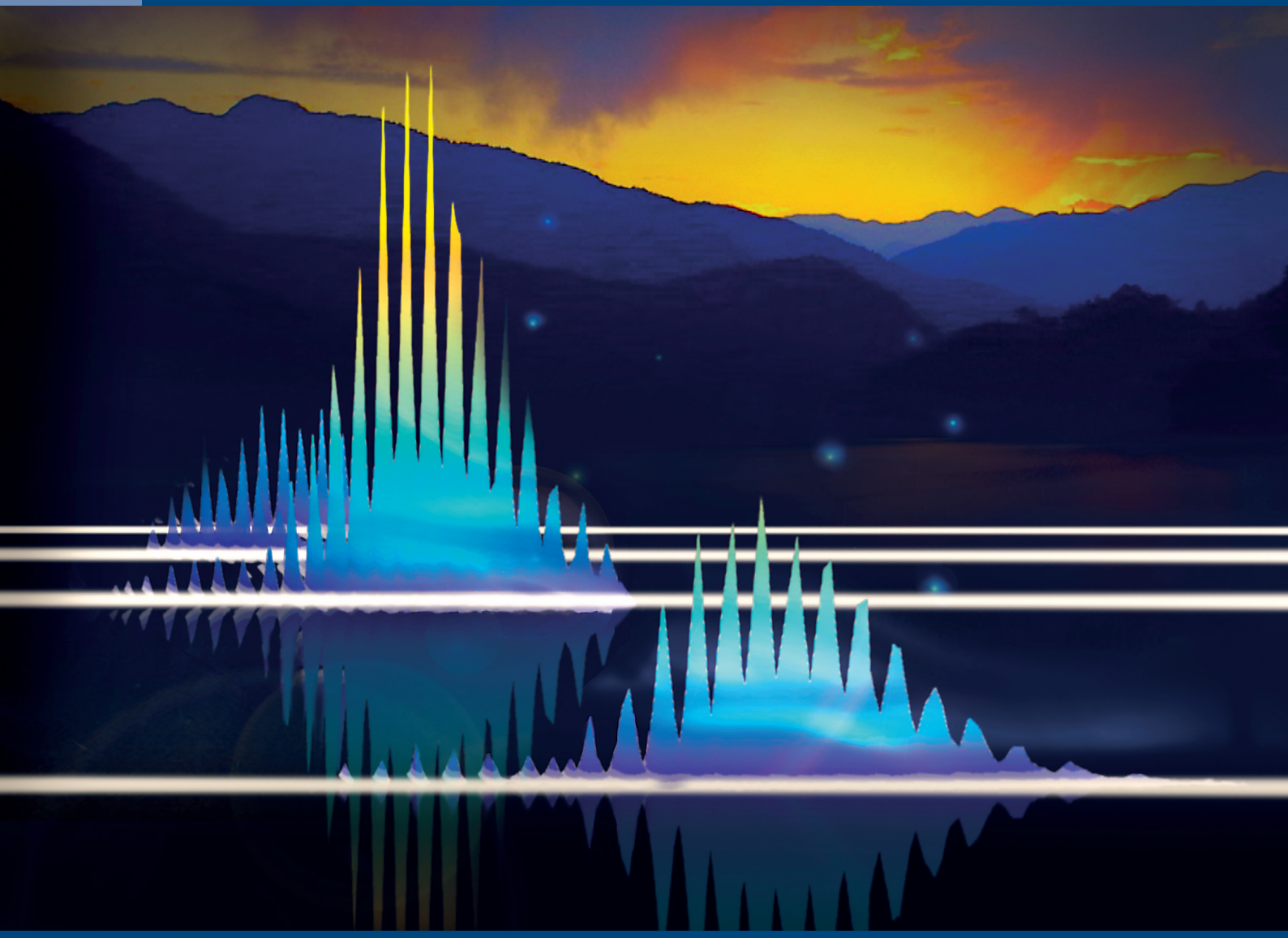

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REVIEW ARTICLE

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Mobile phase effects on the retention on polar columns with special attention to the dual hydrophilic interaction–reversed-phase liquid chromatography mechanism, a review

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Hydrophilic interaction liquid chromatography on polar columns in aqueous–organic mobile phases has become increasingly popular for the separation of many biologically important compounds in chemical, environmental, food, toxicological, and other samples. In spite of many new applications appearing in literature, the retention mechanism is still controversial. This review addresses recent progress in understanding of the retention models in hydrophilic interaction liquid chromatography. The main attention is focused on the role of water, both adsorbed by the column and contained in the bulk mobile phase. Further, the theoretical retention models in the isocratic and gradient elution modes are discussed. The dual hydrophilic interaction liquid chromatography reversed-phase retention mechanism on polar columns is treated in detail, especially with respect to the practical use in one- and two-dimensional liquid chromatography separations.

KEYWORDS

dual retention mechanism, gradient elution, hydrophilic interaction liquid chromatography, two-dimensional separation, water adsorption

1 | INTRODUCTION

RP systems, used as standard in contemporary HPLC, generally employ non-polar alkyl-silica bonded stationary phases. The mobile phase is usually a mixture of one or more organic solvents with water or an aqueous buffer. Lipophilic compounds generally show greater affinity to non-polar stationary phases in water-rich mobile phases than polar solutes in RP LC systems. Small strongly polar solutes such as carbohydrates or biopolymers may elute close to the column hold-up volume, so that their separation from one another and from polar matrix interferences may be difficult to accomplish, even in highly aqueous mobile phases [1]. On the other hand, the stationary phases in normal-phase (NP) chromatography are polar and-–opposite to RP HPLC-–the retention increases with increasing polarity of analytes. Less polar mobile phases enhance the retention. In non-aqueous mobile phases traditionally used in conventional NP (adsorption) chromatography, the retention results from the competition between the analytes and the organic mobile phase for localized polar adsorption centers on the adsorbent surface [2]. However, strongly polar compounds are often excessively retained in non-aqueous NP systems or are poorly soluble in non-polar or in weakly polar organic solvents. Often, their separation on polar stationary phases improves by adding water to the mobile phase [3]. Water accumulates close to

Abbreviations: ANP, aqueous normal phase; BEH, bridged ethylene hybrid; BIGDMA, bisphenol A glycerolate dimethacrylate; ERLIC, electrostatic repulsion-hydrophilic interaction liquid chromatography mode; LSER, linear solvation energy relationship; LSS, linear solvent strength model; MEDSA, *N*,*N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl)ammonium betaine; NP, normal phase; NPC, normal-phase adsorption chromatography

Conflict of interest: The authors declare no potential conflict of interest.

the polar adsorbent surface, where it forms a diffuse adsorbed layer. Alpert introduced the term HILIC for this separation mode [4,5]. Essentially, HILIC systems employ a "normal phase column" in combination with an aqueous–organic mobile phase, typical for RP systems. The HILIC technique provides appropriate retention and resolution for many polar compounds, often with better separation efficiency in comparison to the RP chromatography [6]. The diffusion coefficients of compounds in less viscous organic-rich mobile phases under HILIC conditions are approximately twice those under RP conditions, leading to improved separation efficiency (lower height equivalent of a theoretical plate) [7,8]. Another reason for the increasing popularity of HILIC is its excellent suitability for coupling to MS (LC–MS).

Growing interest in HILIC separations is demonstrated in several recently published review articles. An excellent overview of the progress in the development of polar stationary phases until 2006 was published by Hemström and Irgum [9]; more recent advances are included in reviews by Jandera [10,11] and by Buszewski and Noga [12]. Effects of the operating conditions on HILIC separations [13], especially on the separation efficiency [14] were reviewed, as well as the HILIC method development [15], coupling HILIC with MS and MS/MS systems [16,17] and implementation of HILIC systems in 2D separation modes [18]. HILIC applications are most frequent in biological [19,20], pharmaceutical [21], and metabolite [22,23] analysis. An excellent book on HILIC has appeared recently, covering various aspects of the technique: separation mechanism, stationary phases, method development, and applications [24].

2 | STATIONARY PHASES IN HILIC SYSTEMS

2.1 | A short column survey

Usually, 100–150 mm long, 4.6 mm id columns packed with 3–5 μm particles provide satisfactory HILIC separations; smaller diameter columns are preferred for HILIC coupled on-line with MS. With increasing column length and flow rate and with decreasing column particle size, the number of separated compounds (the peak capacity) increases, with maximum operation pressure as the limiting factor. Some HILIC columns may require lower flow rates for optimum performance, in comparison to the RP separations. Like in RP HPLC, the efficiency and column backpressure increase with decreasing particle size; the organic polymer columns usually show higher heights equivalent to theoretical plate in comparison to the silica-based columns [6].

Working at very high pressures increases the speed of separation in UHPLC, however secondary effects on HILIC separations were reported. For example, the HILIC retention of sugars in HILIC decreases at high pressures, probably due to changes in the hydration of strongly polar analytes as they move from the bulk highly organic mobile phase into the water-rich layer at the surface of the polar adsorbent [25].

Highly purified "sil-gel" spherical silica particles (type B silica) formed by the aggregation of silica sols in the air, stable to at least pH 9 are still most frequently used column materials in HILIC chromatography, either bare or modified by chemical bonding of polar ligands, mainly diol-, amide-, amine-, ion-exchange, zwitterionic, cyclodextrin, poly-(2-hydroxyethyl apartamide)-, polysuccinimide and other. HILIC stationary phases with various chemistries provide different retention times and separation selectivity for neutral, acidic, and basic compounds. Bare silica and diol stationary phases provide similar selectivities. Cyanopropyl columns show different selectivity for basic compounds. Aminopropyl stationary phases prefer acidic compounds, due to ion-exchange effects [26]. Retention of selected probes indicates specific interactions: Relative retention of cytosine versus uracil is a probe of "hydrophilicity" (polarity) of the HILIC phases, relative retention of adenosine versus adenine is a probe of the stationary phase hydrogen bonding activity. The relative retention of benzyltrimethylammonium versus cytosine characterizes the cation exchange/anion exchange character of the column [27,28].

Ikegami et al. [14] compared the separation properties of some types of particle-packed and monolithic polar columns (bare silica, amino-silica, amide-silica, diol-silica, cyano-silica, polysuccinimide-silica, sulfobetaine-silica, triazol-silica, and cyclodextrin-silica) frequently used for HILIC separations of carboxylic and amino acids, peptides, amines, amides and substituted urea derivatives, nucleic bases and nucleosides, poly-alcohols, oligosaccharides and carbohydrates. Guo and Gaiki [29] compared the retention of representative carboxylic acids, nucleosides, and nucleotides on bare silica and polar silica-bonded amide, amino, aspartamide, and sulfobetaine stationary phases commonly used in the HILIC mode. Silica gel showed the least retention, but the highest selectivity differences with respect to other polar bonded stationary phases. In another study, bare silica provided best separation of ten model peptides, followed by the zwitterionic ZIC-HILIC stationary phase [30].

Bonded amide, diol, and cyanophenyl polar ligands differ in HILIC selectivity from one another and from un-derivatized bridged ethylene hybrid (BEH) organic silica gel 1.7 μm particles. A HILIC generic screening protocol for the selection of the most suitable column for fast HILIC separations, is based on the chromatographic selectivity correlations of 28 polar test probes at weakly acidic (pH = 3) and weakly basic (pH = 9) conditions [31].

Schuster and Lindner [32] applied the linear solvation energy relationship (LSER) model using 68 test compounds comprising small neutral, basic, acidic, zwitterionic, and

amphoteric molecules with a broad range of solvatochromic parameters to characterize 22 silica-based polar stationary phases into basic, neutral, or acidic classes at pH = 3. The "acidic" modified columns exhibit only weak hydrogen bond acceptor properties and are less capable of achieving an even distribution of diverse analytes along the available retention window, in contrast to "basic" (bonded amino) and "neutral" columns. Of the "neutral" column class, amide and zwitterionic columns show a broader retention window for a wide range of analytes in comparison to diol-columns [33], and therefore are preferred as the first choice for HILIC separations [34]. The results of these studies confirmed major effects of hydrogen bonding and ionic interactions on the retention.

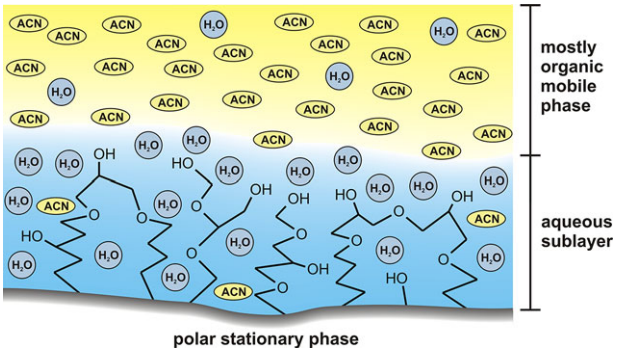

FIGURE 1 Schematic representation of a diffuse water layer at the surface of a polar stationary phase in a highly organic environment

2.2 | Water uptake from aqueous–organic mobile phases

In organic solvent normal-phase adsorption chromatography (NPC), the retention results from the competition between the solute and the polar solvent for the localized adsorption sites on the surface of a polar adsorbent, usually bare silica gel. In hydro-organic mobile phases both polar and hydrophobic adsorbent groups may cause preferential sorption of either acetonitrile or water, depending on the mobile phase composition [35]. The adsorbed liquid, especially water, changes the properties of the stationary phase, and—-in fact—-may become a part of it. In his original model of HILIC chromatography, Alpert [5] considered a partition-driven retention mechanism in which the analytes are distributed between the stagnant water-rich layer adsorbed onto the polar sorbent on one hand and the water-poor bulk mobile phase on the other, without any contribution of the sorbent backbone.

The solid stationary phase is not just an inert support for the adsorbed water layer in mobile phases containing 2–40% water in organic solvent (usually acetonitrile). There is an essential difference between the classical adsorption organic NPC and HILIC. In the conventional organic solvent NPC, a compact water layer forms on the adsorbent surface by taking up the traces of water present in non-polar or weakly polar organic solvents. Water is miscible at any proportion with acetonitrile, acetone, methanol or other polar organic solvents. In the water layer adsorbed from aqueous–organic mobile phases, the concentration of water progressively decreases from the polar surface towards the bulk organic-rich mobile phase outside (and, possibly even partly inside) the pores of the stationary phase. Hence, the adsorbed water layer is diffuse and lacks sharp boundaries (Fig. 1). Hydrogen-bonding, ionic and other interactions with bonded polar functional groups, ionized silanol groups of bare silica, or residual silanols on the silica-based polar bonded phases, may contribute to the analyte retention by adsorption effects.

Water uptake occurs even on non-polar surfaces; siloxane groups may adsorb water at very low organic solvent concentrations [36]. The amount of water adsorbed on polar columns plays important role in HILIC.

Polar stationary phases used in the contemporary practice of HILIC separations show large variability in the amount of adsorbed water in aqueous–organic mobile phases, depending on the column type. The water molecules close to the silica surface are almost immobilized by the hydrogen bonds to the silanol or other polar groups. NMR studies of bare silica and of silica with bonded zwitterionic sulfobetaine groups suggest that three types of water can be distinguished inside the 6–10 nm pores: 1) free water molecules, 2) "freezable" bound water, and 3) water bound within the polymeric stationary phase network that does not freeze at the regular water freezing temperature [37]. This may be the reason why separations are often irreproducible or fail in mobile phases containing <2% water in acetonitrile.

The Langmuir model, Eq. (1), enables measuring the adsorbed amount of water over a varying composition of the mobile phase [38]:

$$q_{\mathrm{i}} = \frac{\mathrm{a} \cdot \mathrm{c_m}}{1 + c_{\mathrm{m}} \cdot \frac{a}{q_{\mathrm{s}}}} \tag{1}$$

In Langmuir isotherms, q_i is the adsorbed water concentration, c_m is the water concentration in the bulk mobile phase, and a is the distribution constant of water in the pores of the stationary phase at very low c_m. q_s is the saturation capacity for water adsorption at the plateau of the isotherm. The experimental parameters of the Langmuir equation can be determined by the frontal analysis technique, where a sample solution is pumped through the column and the breakthrough volume of the sample from the column is usually monitored on-line in the column effluent using a non-specific (refractive index or low-wavelength UV) detector [39]. Later, Dinh et al. employed Karl–Fischer titration for the determination of equilibrium water concentration on "HILIC" adsorbents in contact with aqueous–organic mobile phases [40]. The direct Karl–Fischer titration of water has the advantage of being unaffected by possible interferences, when using a non-specific

detector for measuring breakthrough volumes on the frontal analysis plots. Approximately at the same time, Soukup et al. applied direct off-line coulometric Karl–Fischer titration of small collected column effluent fractions to measure the water breakthrough volumes, V_B, at varying concentrations of water in the aqueous-acetonitrile column feed (from 0% to column saturation) for the determination of water isotherms on various polar columns [41–43].

The water breakthrough volume, V_B, depends on the fraction of water, q_{ex}, contained in the inner pore volume in excess over the bulk mobile phase water concentration, c_m Eq. (2):

$$q_{ex} = \frac{(V_B - V_M) \cdot c_m}{V_i} \quad (2)$$

V_M is the total column hold-up volume, including the inner pore volume, $V_i = \varepsilon_i \bullet V_M$, and the outer (inter-particle) volume, $V_0 = \varepsilon_0 \bullet V_M$; ε_i and ε_0 are the inner pore and the inter-particle porosities, respectively. Unfortunately, the boundaries of the adsorbed water diffusion layer are difficult to determine and depend on the composition of the bulk mobile phase. Hence, the adsorbed water, q_{ex}, may be conveniently characterized in terms of the excess volume of water in the inner pore volume, Eq. (2), calculated from the difference between the total volume of mobile phase in the column, V_M, and the interparticle volume, V_0: $V_i = V_M - V_0$.

V_M is usually determined as the elution volume of a non-retained, non-excluded standard compound. Uracil or thiourea often used as the column hold-up volume, V_M, markers in RPLC, are quite strongly retained on polar columns under HILIC (NP) conditions [44]. Benzene and toluene are non-retained markers of column hold-up volume in acetonitrile-rich mobile phases [45]. However, their elution times on silica columns slightly increase as the water content in the mobile phase grows from 0 to 30%, probably because the water amount adsorbed close to the polar adsorbent surface, into which the markers cannot penetrate, depends on the water concentration in the bulk mobile phase [46]. Water adsorption isotherms on non-modified and monomeric functionalized silica phases show monolayer formation followed by multilayer adsorption, whereas water uptake on polymeric functionalized silica stationary phases may lead to the formation of hydrogels [39]. The water uptake correlates with the retention factors of neutral analytes, supporting the idea of coexistence of adsorption and partitioning of neutral solutes in the water concentration regime normally encountered in the HILIC mode [40].

A more accurate pycnometric method was devised by McCalley and Neue [46]. The column is alternately filled with water and methanol. After each flushing with a new solvent, the column ends are capped, the excess solvent is wiped off, and the column is weighed. V_0 can be determined as the elution volume of a high-molecular standard, such as polystyrene with $M_R = 2\,000\,000$.

The amount of water adsorbed on polar columns plays important role in HILIC. It strongly differs for the individual types of polar columns. Nineteen, fully porous, core–shell silica gel and silica-bonded columns with various polarity ligands (octadecyl, cholesterol, phenyl, nitrile, pentafluorophenylpropyl, diol, zwitterionic sulfobetaine, and phosphorylcholine) bonded on silica, hybrid organic-silica, and hydrosilated silica stationary phases all adsorb water from aqueous acetonitrile. The Langmuir model, Eq. (1), satisfactorily describes the water adsorption isotherms. However, there are large differences between the adsorption capacities of the individual stationary phases. Figure 2 shows six isotherm examples determined by frontal analysis with direct Karl–Fischer titration in column effluent fractions [43]. Non-polar long alkyl chain (YMC Carotenoid) and hydrosilated (Cogent Silica C, Cogent C18 Bidentate) columns show steep isotherms with low plateau water concentrations (low water uptake). The water adsorption isotherms on polar columns (YMC Triart Diol HILIC, ZIC HILIC, and TSK gel amide 80) show at least one order of magnitude higher water saturation concentrations, which often are not achieved even in mobile phases containing 20% water in acetonitrile [43].

Figure 3A shows saturation capacities of water on nineteen columns with different polarities, fully porous, core–shell silica gel and silica-bonded phases determined by frontal analysis and direct Karl Fischer titration in the column effluent fractions [43]. TSKgel amide, ZIC HILIC, ZIC cHILIC, and TSKgel NH_2 columns have high saturation capacities, corresponding to approximately 45% v/v water in the bulk mobile phase, which is consistent with their high affinity to water reported elsewhere [40]. Columns with hydroxyl and diol ligands such as the Ascentis Express OH column show relatively high saturation capacity, 35.4% v/v water, whereas the Xbridge HILIC, Atlantis HILIC, Ascentis Express ES-CN, and Ascentis Express F5 columns have lower saturation capacities, <9% v/v. At full column saturation, the excess adsorbed water, V_{ex}, fills up to 45.3% of the pore volume of normal silica-based columns, but only 2.6–5.5% of the pore volume of hydrosilated silica columns. The low affinity to water of the hydrosilated silica materials, results in the saturation capacities as low as 0.2–0.4% water in the inner pore volume, which are achieved in mobile phases containing 3–6% v/v water (for the silica hydride or bonded C18 Bidentate columns), in agreement with the Pesek aqueous normal phase (ANP) chromatography model. At increased temperature, the water saturation capacities of hydrosilated silica materials increase, but the adsorption isotherms become less steep [47].

Figure 3B shows the water uptake in terms of the number of "hypothetical" monomolecular water layer equivalents, N_w, at full saturation capacity of the 19 columns [43]. The number

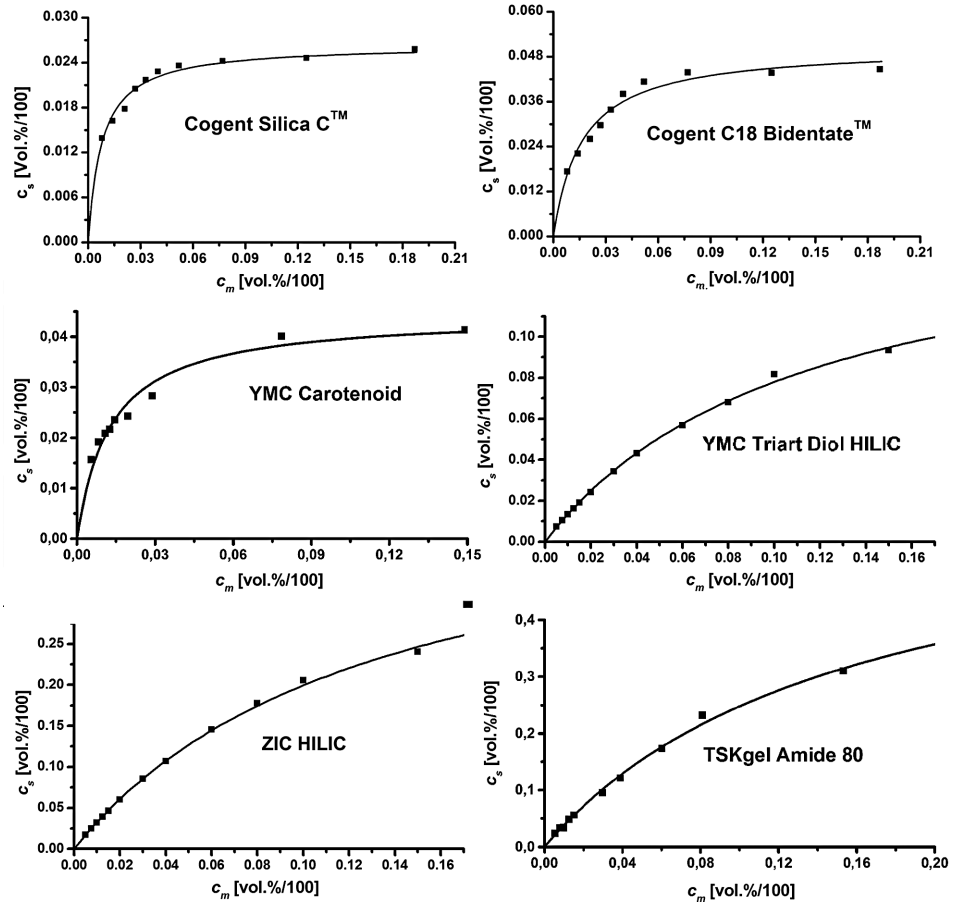

FIGURE 2 The Langmuir isotherms of water adsorbed on two hydrosilated (Cogent Silica, Cogent Bidentate), a C-30 (YMC carotenoid), a diol (YMC Triart Diol HILIC), a zwitterionic sulfobetaine (ZIC HILIC) and an amide (TSkgel Amide 80) silica based stationary phases. c_s – the volume fraction of the excess water contained in the pores of the stationary phase; c_m – the volume fraction of water in the mobile phase in equilibrium with the stationary phase. Based on the data of [43]

of adsorbed water layer equivalents generally agrees with the order of the column sorption capacities in Fig. 3A, with some exceptions from the rule. On strongly polar stationary phases, several water layer equivalents are captured from the mobile phase. Less than one monomolecular water layer equivalent (full horizontal line) was adsorbed on the silica hydride-based stationary phases and on moderately polar core–shell columns (Ascentis Express F5 and Ascentis Express CN) at the column saturation capacity. A sub-monomolecular layer of adsorbed water does not provide enough space for sample partition. Rather, competition between the adsorbed water and polar solutes, based on an NP adsorption mechanism [48], is more realistic. Hence, a low number of the adsorbed monomolecular equivalents, $N_W < 1$ may distinguish between the ANP and traditional HILIC systems [43]. Columns with bonded hydroxyl and diol ligands show stronger water adsorption in comparison to bare silica. The silica-bonded zwitterionic ZIC cHILIC stationary phase has very strong affinity to water, corresponding to more than nine water layer equivalents adsorbed at the saturation capacity.

3 | MOBILE PHASE EFFECTS AND SEPARATION MECHANISM

HILIC of polar compounds on polar stationary phases employs aqueous–organic mobile phases, usually containing 60–95% organic solvent (acetonitrile) in water or an aqueous buffer (often volatile enough, such as ammonium formate or ammonium acetate, compatible with MS detection and on-line identification). The separation mechanism on polar columns in aqueous–organic mobile phases is complex, as suggest different separation selectivities for various polar compounds, which indicates that the solute adsorption on polar functionalities of the solid phase surface may play important role in the retention [10].

3.1 | Retention LC models in aqueous–organic mobile phases

Snyder developed the displacement model of retention in adsorption chromatography in the early 1960s [49]. This

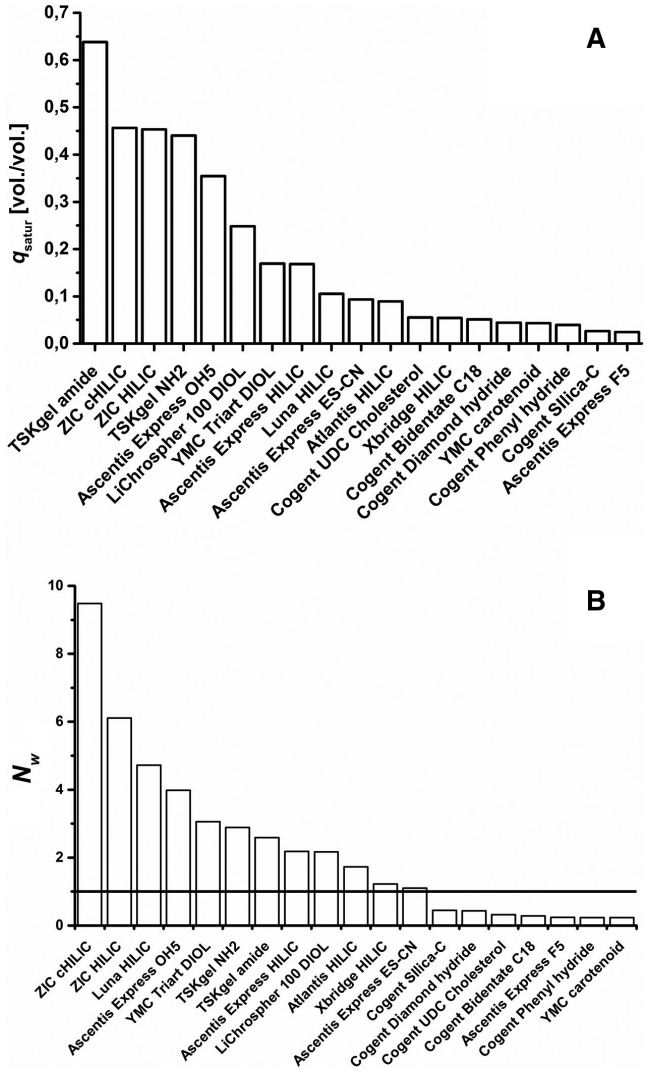

FIGURE 3 (A) Comparison of the excess water saturation capacities, q_{satur}, on nineteen polar columns. (B) Comparison of the equivalent number of adsorbed monomolecular water layers, N_w, inside the pores at the full saturation capacity of the columns. Based on the data of [43]

model assumes that the retention results from the competition between the molecules of the solute and of the strong solvent for the adsorption sites. The adsorbent surface is covered with an adsorbed layer of solvent molecules and the adsorption of a solute molecule is accompanied by the desorption of one or more solvent molecules from the adsorbent surface, depending on the specific adsorbent surface, A_s, adsorbent activity, α', and solvent strength of the mobile phase, ε.

$$\log k'_{ab} = \log k'_{a} + \alpha' \cdot A_s \left(\varepsilon_a - \varepsilon_{ab}\right) \tag{3}$$

The solvent strength ε_{ab} of a mixed binary phase comprised of a stronger (more polar) solvent B (solvent strength ε_b) and a less polar solvent A (solvent strength ε_a), controls the retention factor k_{ab} of the analyte in the adsorption LC systems:

$$\varepsilon_{ab} = \varepsilon_a + \frac{\log\left[x_b\left(10^{\alpha' \cdot n_b(\varepsilon_b - \varepsilon_a)} + 1 - x_b\right)\right]}{\alpha' \cdot n_b} \tag{4}$$

With some simplification, Eqs. (3) and (4) lead to the simple Snyder–Soczewinski model equation, Eq. (5) describing the retention factor, k, as a function of the concentration of the stronger (more polar) solvent, φ, in binary mobile phases comprised of two solvents of different polarities [50].

$$k = k_0 \cdot \varphi^{-m} \tag{5}$$

Combining Eqs. (3) and (4) of the basic Snyder adsorption model, we obtain Eq. (6):

$$\log k'_{ab} = \log k'_{a} - \frac{A_s}{n_b} \cdot \log\left[x_b\left(10^{\alpha' \cdot n_b(\varepsilon_b - \varepsilon_a)} - 1\right) + 1\right] \tag{6}$$

Eq. (6) can be formally re-written as Eq. (8):

$$k' = (b + a \cdot \varphi)^{-m'} \tag{7}$$

with:

$$b = \left(k'_a\right)^{-\left(\frac{n_b}{A_s}\right)},\ a = \left(k'_a\right)^{-\left(\frac{n_b}{A_s}\right)} \cdot \left(10^{\alpha' \cdot n_b(\varepsilon_b - \varepsilon_a)} - 1\right),\quad m' = \frac{A_S}{n_b} \tag{8}$$

The parameters a, b, m, and m' in Eqs. (5) and (7) are experimental constants depending on the solute and on the chromatographic system. For φ = 0, $b = 1/(k'_a)^{1/m'}$, where k'_a is the solute retention factor in pure weak solvent). For compounds very strongly retained in the non-polar solvent, k'_a is very high and the parameter b is very small. By neglecting b, Eq. (7) simplifies to the Snyder–Soczewinski Eq. (5).

Equation (5) characterizes the Snyder–Soczewinski model of retention in organic normal phase adsorption LC. The parameter m is the stoichiometric coefficient characterizing the number of molecules of the strong solvent necessary to displace one adsorbed molecule of the analyte. In Eq. (5), the concentration of the strong solvent can be expressed in volume fractions, φ [51].

RP systems employing non-polar or moderately polar columns (usually silica gel with bonded alkylsiloxane ligands) and aqueous–organic mobile phases represent the standard in contemporary HPLC. The surface of the alkyl bonded stationary phases does not contain localized adsorption centers (except for some more or less shielded residual silanol groups), but a layer of the adsorbed solvent on the non-polar bonded ligands, where either non-localized adsorption or partition control the retention. In the linear solvent strength model (LSS) of RP LC introduced by Snyder et al., log$K$

decreases proportionally to the increasing volume fraction φ of the strong eluent (organic solvent) Eq. (9) [52].

$$\log k = \log K_D + \log \frac{V_S}{V_M} = a - m \cdot \varphi \quad (9)$$

K_D is the distribution constant characterizing the solute non-specific surface adsorption or the partition between the surface layer of the adsorbed solvent and the mobile phase. V_S and V_M are the volumes of the stationary and the mobile phase in the column, respectively.

Equation (5) have been often used to characterize the retention in the adsorption systems and Eq. (9) in the partition systems [53]. Both Eq. (5) and (9) were applied to the systems with immiscible mobile phases, such as in NP organic adsorption chromatography or RP LC with long-chain chemically bonded alkylsilica materials [52]. The application of the theoretical models relies on the knowledge of the volumes of both the stationary and mobile phases in the column, which are not easy to exactly define in HILIC systems, where the amount of adsorbed water changes with the composition of the bulk aqueous–organic mobile phase.

Several more complex equations were introduced to describe the effects of the mobile phase on the retention. Generally, the three-parameter model equations provide better fit to the experimental data than the two-parameter models. That is why some authors formally extended Eq. (5) and Eq. (9) by adding second-order φ^2 terms, which however often lack physical meaning [54–56].

In the HILIC mode, the retention on polar columns increases at decreasing concentration of water in aqueous/organic mobile phases. Acetonitrile shows the best retention and separation selectivity among all water-soluble organic solvents in HILIC (ANP) chromatography, because methanol and other lower alcohols provide significant hydrogen bonding interactions and compete with water for the solvation of the polar adsorbent surface, decreasing thus the amount of adsorbed water layer on the polar stationary phases [31].

The effect of the volume fraction of water, φ_{H2O}, in the mobile phase on the solute retention factor, $k = ((V_R/V_M) - 1)$, can be described by simple two-parameter equations:

$$\log k = a_1 - m_{HILIC} \cdot \log \varphi_{H2O} \quad (10)$$

$$\log k = a' - m_{HILIC} \cdot \varphi_{H2O} \quad (11)$$

φ_{H2O} is the volume fraction of water, "a_1" is the (extrapolated) logarithm of the retention factor of the solute in pure water, and "a'" is the logarithm of the retention factor in the pure organic solvent. The parameter "m_{HILIC}" characterizes the rate of decreasing retention (k) with increasing content of water in the mobile phase. Equation (10), or Eq. (11), have been applied to HILIC separations in a limited range of low water concentrations. In fact, some experimental HILIC systems show better data fit for either of the two equations [10,11]. On the other hand, both Eq (5), and Eq. (9) fail to describe satisfactorily the retention of some acidic, basic, and neutral polar compounds under HILIC conditions [26]. Theoretically, a second organic solvent can substitute water in the mobile phase. Usually, the retention in "non-aqueous HILIC chromatography" (e.g., methanol/acetonitrile) is weaker than in the conventional HILIC separations [42,57].

Equation (12) obtained by combining Eqs. (10) and (11) was claimed to provide an excellent fit to the HILIC retention data, better than polynomial empirical equations [58]:

$$\ln k = a + b \cdot \ln \varphi_{H_2O} - c \cdot \varphi_{H_2O}. \quad (12)$$

The ratio of the parameters b and c of Eq. (12) was tentatively employed to estimate the importance of the relative contributions of partitioning and surface adsorption mechanism [59]. Equation (12) provides improved fit to the experimental HILIC data of oligosaccharides with respect to the two-parameter model equations (Eqs. (10) and (11)) [60]. In spite of different conclusions of some studies, it is highly probable that the adsorption and the partition retention mechanisms actually coexist in HILIC. The proportion of the mechanisms obviously depends on the solute, the hydration and the charge of the stationary phase polar functional groups and on the eluting conditions [10,11]. The application of Eq. (12) to the experimental systems suggests that the transition from a partitioning to a surface adsorption mechanism for neutral compounds occurs at >75–80% acetonitrile on diol, silica, and amino columns. The differences between the columns probably depend on the different degree of hydration of the stationary phases.

Often, electrostatic interactions, either attractive (ion exchange) or repulsive (ion exclusion or ion repulsion in the electrostatic repulsion-hydrophilic interaction liquid chromatography (ERLIC) mode, participate in the retention mechanism of ionized compounds, especially on SAX or WAX columns. The addition of salts, weak acids, bases, or ion-pairing reagent additives to the mobile phase usually significantly improves the separation in the mixed HILIC/ion-exchange mode [10,12]. Adjusting the pH and the salt (buffer) concentration may significantly improve the retention selectivity, peak profile, and separation, however with very different selectivity effects for acids and bases [61]. On the bare silica columns, acids show much stronger retention in mobile phases containing TFA, in comparison to ammonium formate buffers, where on the contrary, bases are more strongly retained [62].

At increased concentrations of acetonitrile, both basic and acidic peptides can be resolved in a single run. On either SAX or WAX columns, the HILIC and electrostatic repulsion retention mechanisms superimpose in the ERLIC mode

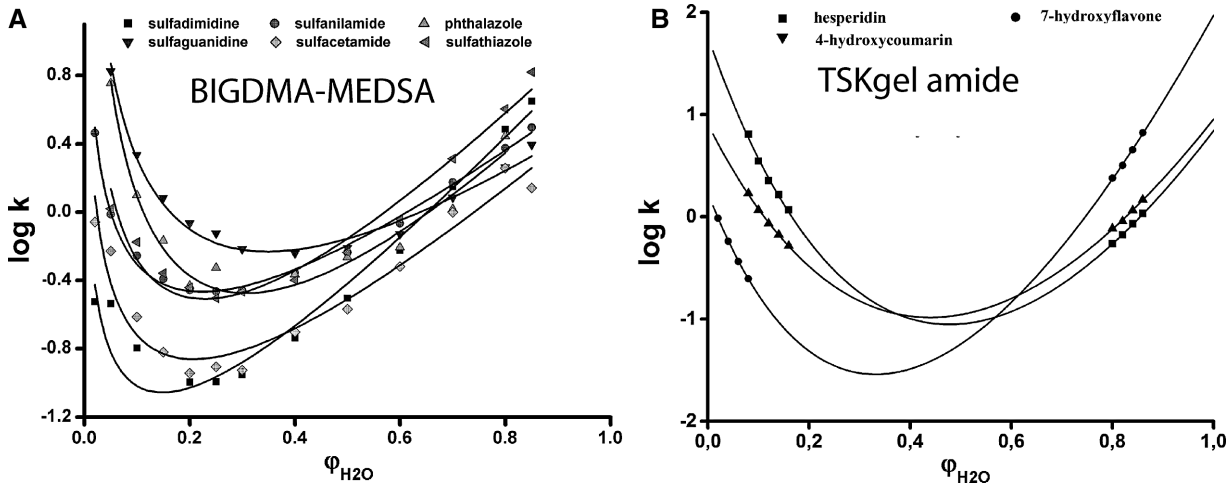

FIGURE 4 Effect of the volume fraction of aqueous buffer, φ_{H2O}, in aqueous–organic mobile phase on the retention factors, k, of sulfonamides on a monolithic column BIGDMA-MEDSA (**A**) and of flavonoids on a carbamoyl bonded silica column TSKgel Amide-80 (**B**). Points are the experimental data and lines the best fit plots of Eq. (16). Based on the data of [95]

at a low pH, which allows independently adjusting the HILIC and the ion-exchange selectivities in highly organic mobile phases [63,64].

The effects of the adsorbed water on the retention of aromatic carboxylic acids, flavonoids, benzoic acid derivatives, nucleic bases, and nucleosides in aqueous/acetonitrile mobile phases over the full composition range are illustrated by the examples in Fig. 4 for an organic polymer monolith zwitterionic column and a silica-bonded amide column. The graphs of the retention factors versus the volume fraction of water in the mobile phase show "U-profile" characteristic for dual hydrophilic interaction–RP retention mechanism. The minimum on the graph marking the changing retention mechanism depends on the amount of adsorbed water [65].

3.2 | Gradient elution in HILIC systems

The main objective of gradient elution is improving single-run separations of samples containing analytes largely differing in retention. Increasing the concentration of the stronger component (solvent) during gradient elution increases the retention of early eluting compounds, while simultaneously speeds up the elution of strongly retained solutes. In RP gradient systems, the proportion of a polar organic solvent in aqueous–organic mobile phase increases. On the contrary, in HILIC the retention of polar compounds decreases by increasing the water proportion in the mobile phase during the gradient run.

Prediction of the retention times in gradient HPLC is important for compound identification, for setting the peak integration time windows, and in fundamental studies of retention [66]. Different gradient models related to isocratic retention models (Eqs. (5), (7), (9), etc.) characterize in different ways the effects of the gradient program on the retention. For linear gradients the volume fraction of the strong solvent B increases proportionally to the volume of the mobile phase passed through the column, V: $\varphi = A + B \bullet V$. The effects of the gradient ramp (B) and gradient range (φ_G–A) on the elution volumes, $V_{R(g)}$, can be predicted from the parameters of the isocratic model equations, Eq. (9) for the LSS model yields gradient Eq. (13) [52,67,68]:

$$V_{\mathrm{R(g)}} = \frac{1}{m \cdot B} \log \left[2.31 m B \cdot \left(V_{\mathrm{M}} \cdot 10^{a - m \cdot A} - V_{\mathrm{D}} \right) + 1 \right] + V_{\mathrm{M}} + V_{\mathrm{D}} \quad (13)$$

The Snyder–Soczewinski isocratic Eq. (5) provides the gradient equation, Eq. (14) [69]:

$$V_{\mathrm{R(g)}} = \frac{1}{B} \left[(m+1)\, B \left(k_0 \cdot V_{\mathrm{M}} - V_{\mathrm{D}} \cdot A^m \right) + A^{(m+1)} \right]^{\frac{1}{m+1}} - \frac{A}{B} + V_{\mathrm{M}} + V_{\mathrm{D}} \quad (14)$$

The three-parameter isocratic Eq. (7) turns into the gradient equation, Eq. (15) [70]:

$$V_{\mathrm{R(g)}} = \frac{1}{a \cdot B} \left\{ \left(m' + 1 \right) b \cdot B \left[V_{\mathrm{M}} - V_{\mathrm{D}} (b + a \cdot A)^{\mathrm{m}} \right] + (b + A \cdot a)^{m'+1} \right\}^{\frac{1}{m'+1}} - \frac{b + A \cdot b}{b \cdot B} + V_{\mathrm{M}} + V_{\mathrm{D}} \quad (15)$$

V_M is the column hold-up volume, A and B are the gradient parameters, a, b, k_0, m, and m' are the best-fit regression constants of Eq. (9), (5), and (7), respectively. V_D is the instrumental dwell volume (i.e., the volume of the gradient mixer and of the connecting tubing between the mixer and the column inlet) containing the starting mobile phase in which less retained compounds may move some distance under isocratic conditions, before the front of the gradient program.

The three-parameter model equation, Eq. (15), in classical organic solvent adsorption chromatography provided errors of the prediction of the retention times < 3% for gradients starting from at least 3% propanol in hexane [71,72]. Recently, Neue and Kuss proposed another empirical gradient elution three-parameter model [55].

Two- or more-parameter gradient models allow direct calculations of the gradient retention data from the parameters measured under isocratic, or gradient conditions. Optimizing of gradient elution by numerical calculation is possible using gradient models that do not allow direct analytical solution. However, with some multi-parameter model fitting equations, the physical meaning of the parameters is unclear, and improved fit may be due just to including the experimental data subject to large errors, such as very low $k < 0.1$–0.5.

A common practice of the optimization of gradient elution in RP LC employs running two gradients to obtain the input data for the optimization of the final gradient, using the LSS model (Eq. (13) or its modifications) to calculate simulated gradient separations at various separation conditions. A commercial optimization software DryLab calculates and compares hundreds of simulated gradient chromatograms for various combinations of the gradient range and steepness covering by a dense net the retention space, to select the combination of conditions providing optimum resolution of the target sample compounds [73]. The approach may include additional separation parameters such as temperature, pH, or two strong solvents for optimization in a three-dimensional retention space [74]. Computerized optimization based on two initial "scouting" gradients has important practical advantage of fast acquisition of the input gradient data, in comparison to the significantly longer time necessary for the acquisition of the isocratic retention times over a broad enough composition range of the isocratic mobile phases. On the other hand, the agreement between the experiment and the gradient retention data predicted from the isocratic model parameters may provide a proof of the validity of the underlying theoretical model. "Scouting" gradient data also allow rapid determination of the parameters of the isocratic retention equations such as Eq. (7) or Eq. (9) and adjusting optimum isocratic conditions [75].

Equation (13) is frequently used for data prediction in RP gradient LC [76]. It has been occasionally employed for predicting the retention volumes in the HILIC mode with linear gradients of water in acetonitrile (with φH2O is used instead of φorg). A disadvantage of the HILIC gradients is a relatively long time of equilibration needed to achieve reproducible data. Further, the amount of the adsorbed water changes during the water gradient, which may affect the accuracy of the predicted gradient retention times from the isocratic retention data [41]. However, satisfactory prediction of gradient retention data of metabolites by numerical calculations based on the Linear Elution Strength model was reported on an UHPLC BEH amide column [77]. In an earlier study, we found significant errors of prediction for water gradients on hydrosilated silica columns [41], which show very low water adsorption (less than a monomolecular layer) [43].

The experimental HILIC gradient retention volumes of nucleobases and nucleosides agree with the values predicted from Eq. (13) on the TSKgel Amide-80 and YMC Triart Diol columns adsorbing significant water amounts (Fig. 2). For six linear water gradients differing in steepness (gradients 96–88, 88–76, and 88–80% acetonitrile in 15, 20, and 25 min), the average differences between the predicted and experimental values are in between 0.55–1.97%. This is much better agreement than the earlier results with the hydrosilated columns [41], and is deemed satisfactory for predicting the gradient data in the HILIC systems. The accuracy of the HILIC gradient prediction improves for columns showing high water adsorption. Probably, increasing the water volume fraction in the bulk mobile phase during HILIC gradient elution affects less the retention on the columns with high water adsorption capacities, which usually contain significant amounts of adsorbed water already at the start of the HILIC gradient elution. Hence, the relatively low increase in the water adsorbed during a HILIC gradient increases the polarity of these columns less significantly in comparison to the hydrosilated columns showing low (sub-monomolecular) water adsorption [43,48].

4 | DUAL RETENTION MODE HILIC–RP MECHANISM ON A SINGLE COLUMN

4.1 | The effect of a broad range of aqueous–organic mobile phase composition on the retention of polar compounds

The LSER model suggests that the retention on polar columns in the HILIC mode is controlled mainly by polar (proton-donor/acceptor, dipole-dipole, etc.) interactions, depending on the column type.

Many polar columns contain non-polar structural moieties, which are subject to solvophobic interactions with less polar samples, giving rise to dual HILIC-RP mode retention mechanism. Here, we distinguish between the dual mechanism and the mixed-mode retention mechanism (presenting a cocktail of interactions largely independent of the mobile phase composition such as solvophobic, polar, attractive, or repulsive electrostatic effects) [63]. On the other hand, the dual retention mechanism strongly depends on the composition of the aqueous–organic mobile phase. At high concentrations of acetonitrile, HILIC mechanism controls the retention. At gradually increasing water concentration, the retention decreases, until the analytes are very weakly retained and elute

close to the column hold-up volume. When further increasing water concentration, some compounds may become again retained due to increasing solvophobic interactions of non-polar structural moieties with the column, showing a typical RP behavior in aqueous-rich mixed mobile phase. In the presence of dual retention mechanism, the graphs of the retention factors, k, of the analytes versus the volume fraction of water, φ_{H2O}, show characteristic U-shape profiles over a broad range of binary aqueous–organic mobile phase composition, with the retention minimum corresponding to the transition from the HILIC to the RP mechanism (see the examples in Fig. 4). The U-profile retention plots can be often described by a four-parameter (Eq. (16)) over a broad composition range of aqueous–organic mobile phases (at $\varphi_{H2O} > 0.02$) [78]:

$$\log k = a + m_{\mathrm{RP}} \cdot \varphi_{\mathrm{H2O}} - m_{\mathrm{HILIC}} \cdot \log\left(1 + b \cdot \varphi_{\mathrm{H2O}}\right) \quad (16)$$

The parameter m_{RP} characterizes the effect of the increasing concentration of water in the mobile phase on the retention, due to the RP mechanism in water-rich mobile phases, whereas the parameter m_{HILIC} is a measure of the water contribution to the decrease of retention in highly organic mobile phases (HILIC range). The constant a depends on the solute and on the type of the organic solvent. Finally, b is the correction term accounting for the retention in mobile phases with very low concentrations of water [79].

The "U-turn transition" volume fraction of water, φ_{min}, where the RP mechanism begins to dominate over the HILIC interactions, can be calculated using Eq. (17) [78].

$$\varphi_{\mathrm{min}} = 0.434 \cdot m_{\mathrm{HILIC}}/m_{\mathrm{RP}} - 1/b \quad (17)$$

4.2 | Chemically bonded silica gel columns

Bare silica usually does not provide dual HILIC-RP behavior, which was however observed on various polar silica-bonded columns, with aminopropyl [80], cyanopropyl, diol, PEG, amide [65], cyclodextrin [81], zwitterionic [82], and mixed mode ion-exchanger [83] bonded ligands. Neutral and acidic small polar compounds are more strongly retained at the HILIC than at the RP conditions.

The U-shaped retention plots were observed, e.g. for beta-blockers atenolol and propranolol [26], for phenolic acids and flavonoids [78,79] on various polar silica bonded columns. Some examples of the plots of the retention factors of six sulfonamides and three flavonoids versus the volume fraction of the aqueous acetate buffer, φ_{H2O} (10 mM NH_4Ac, 0.1% HCOOH), in the aqueous–organic mobile phase, are shown in Fig. 4. The HILIC mechanism controls the retention in the low aqueous buffer concentration range (< 20–50%), depending on the sample and the stationary phase. In the mobile phases with >60% of aqueous component, the RP mechanism controls the retention.

The sugar groups in the molecules of glycoside flavonoids significantly increase the retention in the HILIC mode with respect to their parent aglycons (e.g., flavone, hydroxyflavone, or biochanin A), which show higher retention in the RP mode. Eq. (16) (lines) provides very good description of the experimental retention data (points) with the coefficients of determination $D^2 = 99.04$.

Silica-bonded zwitterionic stationary phases, which contain both positive and negatively charged moieties, often show dual mode HILIC–RP retention mechanism and U-shape retention plots, depending on the mobile phase composition [84]. This behavior is probably correlated with a strong water adsorption on the zwitterionic moieties [40]. The mixed-mode anion–cation exchange HILIC materials improve the separation selectivity for small molecule drugs by combining ionic interactions with polar hydrophilic interactions.

Some mixed-mode silica-based HILIC/IEX stationary phases provide separations of polar and ionic solutes under HILIC conditions in the organic solvent-rich mobile phases, and separations of the less-polar compounds under the RP conditions, in more aqueous mobile phases. Depending on the solute character, the columns may show attractive or repulsive electrostatic interactions increasing the separation selectivity of acidic, cationic, polar, and moderately polar compounds such as peptides varying in hydrophobicity/hydrophilicity. In the HILIC mode, the RP/WAX phases differ from the typical HILIC stationary phases, such as TSKGel Amide-80, ZIC-HILIC, or polysulfoethyl A, to which they offer complementary application possibilities [85].

U-shape retention plots due to dual HILIC-RP retention mechanism were observed on many less common silica-bonded stationary phases. For example, aqueous extracts of *Hedyotis Diffusae* herba grass were separated on a beta-cyclodextrin bonded phase at the RP conditions into fractions, which were re-analyzed on-line on the same column in the HILIC mode [81]. A phosphate ester bonded silica provides dual retention mechanism due to polar interactions with phosphate groups and solvophobic interactions with short alkyl chains, which were used for dual mode HILIC-RP separations of phenols, nucleosides, and nucleic bases [86]. A short peptide Boc-Phe-Allo-Phe-OH grafted onto porous silica provides separation of small polar and non-polar drugs in the HILIC-RP modes due to multiple interactions with the carbonyl and phenyl groups. In comparison to the octadecyl- and phenyl-bonded silica bonded phases, the column shows enhanced selectivity for planar molecules in the HILIC mode [87].

A mixed-mode glutamine silica-bonded stationary phase containing an amino alcohol group and two different amide groups, one a polar head and the other one embedded into aromatic phenyl ring, shows retention of polar, moderately polar, and non-polar analytes [88]. The column shows similar properties to zwitterionic stationary phases and provides a dual

retention mechanism. Six nucleotides separated in 10 min in the HILIC mode with phosphate buffer (pH 3.25); phytohormones and phenolic compounds were separated under the RP conditions [89].

4.3 | Hydrosilated silica columns

The ordinary silica gel (B-type) is transformed to the C-type "hydride silica", by modifying up to 95% of the original surface silanols (Si–O–H) to non-polar silicon hydride Si–H groups. The silica hydride material is less polar and shows less attraction for water [48] and improved pH stability in comparison to the sil-gel columns; hence the authors suggested denoting the retention mechanism on hydrosilated silica as "ANP chromatography" rather than HILIC. However, HILIC separations essentially are conducted in NP systems. Water adsorbed on hydrosilated materials at the point of column saturation fills only 2.6–5.5% of the pore volume, which corresponds to approximately half a monomolecular adsorbed water layer [41,43]. Hence, a low water saturation capacity (less than a monomolecular water equivalent (Fig. 3B)) may be used for distinguishing the ANP from the HILIC systems. According to recent studies, the water uptake on the hydrosilated surface could probably be explained by the effects of the electrical interfacial double layer at the adsorbent surface, characterized by the zeta potential, rather than by the adsorption on residual silanol groups [90,91].

Hydrosilated silica with bonded low-polarity ligands such as C18, cholesterol [92], perfluorinated [93], undecanoic acid [94], etc., show dual retention mechanism in aqueous NP (HILIC in buffered mobile phases containing >50–70% acetonitrile, or RP in more aqueous mobile phases). For example, the undecanoic acid silica hydride stationary phase shows increased separation selectivity for mono-, di-, and triphosphate nucleotides with respect to the unmodified silica hydride column [94].

4.4 | Organic polymer monolithic columns

Like silica-based zwitterionic stationary phases, the zwitterionic polymethacrylate monolithic columns show dual HILIC and RP mechanisms with orthogonal selectivities in the HILIC and the RP ranges and provide excellent separations of flavonoids and phenolic acids (and other polar analytes) on a single column in the two retention modes. The recently introduced zwitterionic capillary column prepared by copolymerization of the *N*,*N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl)ammonium betaine as a zwitterionic functional monomer and bisphenol A glycerolate dimethacrylate as a crosslinking monomer (BIGDMA-MEDSA) shows a dual mechanism, HILIC at high concentrations of acetonitrile and RP in the water-rich mobile phases [95,96].

5 | HILIC MODE IN 2D LC SEPARATION SYSTEMS

The number of peaks that can be separated in a single HPLC run can hardly exceed one hundred. However, in clinical, pharmaceutical, food, natural and environmental analysis, molecular biology, and elsewhere, we encounter samples, that may contain huge numbers of compounds, in concentrations possibly spanning over ten orders of magnitude. The peak capacity, i.e. the maximum number of peaks that can be separated in a sample, can be significantly increased by combining two or more different separation mechanisms in multi-dimensional separation systems [97–101].

The 2D peak capacity can theoretically reach the product of the peak capacities in the individual separation systems, where the retention in the first dimension is not correlated with the retention in the second dimension (orthogonal systems). In practice, most 2D systems show a lower number of resolved peaks due to more or less significant retention correlation in the two dimensions [102]. An on-line comprehensive LC × LC system is most efficiently used for samples requiring peak capacities up to 600. The off-line or stop-flow systems provide higher peak capacities, at the cost of long separation times. The effect of the stopped flow on the band broadening is negligible. Coupled HILIC and RP separation systems offer two completely different retention mechanisms and a very high degree of orthogonality in comparison to other 2D LC systems [18]. Hence, 2D separation systems combining HILIC and RP modes allow significant increasing of the number of compounds resolved in complex samples [99] and favorably compare with other LC 2D coupled system in terms of peak capacities, analysis times, and peak production rates [103]. The expected increase in the number of really separated compounds (the practical peak capacity) that can be achieved in various off-line and on-line multi-dimensional HILIC-RP coupled setups and the price to be paid for it in the terms of the analysis time and sample dilution (i.e., decreased sensitivity) have been reviewed in detail [99].

Generally, the peak capacity is higher in the gradient mode than at isocratic conditions. Hence, the number of resolved peaks increases when simultaneous gradients are used in the first dimension (HILIC) and in the second dimension (RP) of a comprehensive on-line 2D setup [104]. Optimization of the gradient range and of the gradient profile, especially in the second dimension, can significantly increase the practical 2D peak capacity [105,106].

The off-line approach using two (or more) separate columns is very simple, does not necessitate any special instrumentation, and allows independent optimization of the HILIC and RP separation systems; the fractions collected from the first, HILIC column, can be pre-treated by evaporation to dryness before injection onto a C18 column in the second dimension [107]. Off-line coupling of HILIC and

RP-LC systems provide a powerful tool for separation of procyanidins [108] and flavonoids [109]. Another recent example reports an off-line RP-HILIC method coupled with MS for impurity profiling of infusion solutions [110]. Unfortunately, the off-line procedures are labor- and time-demanding.

On-line combinations of HILC and RP HPLC are subject to incompatibility problems of the mobile phases in the HILIC and RP modes. Highly concentrated organic solvents used in HILIC separations provide weak retention in the RP systems, whereas the mobile phases rich in water used in the RP HPLC are often too strong HILIC eluents. The mobile phase from the first (HILIC) dimension used to transfer sample fractions to the second (RP) dimension, may significantly decrease the retention and cause asymmetric or even split peaks, with detrimental effects on the separation [111].

The mobile phase incompatibility problems in 2D HILIC-RP systems can be improved in several ways [103]:

1. Transferring only small-volume fractions on-line from the first dimension to the second dimension column (for example, 2 μL onto a 50 × 3 mm id C18 column) often minimizes the sample solvent effects in the second dimension, however sometimes with possible negative impacts on the sensitivity of 2D separations [99].
2. The mobile phase strength in the acetonitrile-rich fractions transferred from the HILIC column can be modulated by diluting with water before introducing the fractions into the second dimension (RP), using a make-up flow mediated by an additional auxiliary pump [112]. A more sophisticated approach relies on trapping the fractions from the first column on small auxiliary column(s) [113].

For example, 2D HILIC × RP separation of phenolic compounds in green tea was reported using a diol HILIC column in the first dimension and a gradient of water and methanol in acetonitrile with acetic acid additive. 50 μL fractions were collected with 1 min frequency and evaporated under nitrogen to 2 μL before introduction onto a C18 column in the second dimension [103].

On-line 2D separations can employ either serial or parallel column setups. Serial column coupling does not require complex instrumentation, but allows only a moderate increase in peak capacity, due to the additivity of the contributions of the individual columns, in contrast to the comprehensive on-line 2D LC × LC providing multiplicative effects on peak capacity and considerably higher peak production rate (i.e., the number of resolved compounds in a pre-set separation time). A set of serially coupled columns containing different stationary phases behaves like a new column with a modified selectivity, which may enable separations of compounds with widely differing properties [114]. An octadecylsilica column serially coupled with a HILIC column in the second dimension, with a gradient of up to 80% acetonitrile in water, allowed the separation of a broad range of pharmaceuticals in a single run [115]. A serial combination of a ZIC HILIC sulfobetaine column and an amide column with a HILIC gradient run from 95 to 35% aqueous buffer in acetonitrile allowed simultaneous separations of polar and non-polar metabolites in a mouse serum sample [116]. The tandem coupling of a C18 column with a zwitterionic column was employed for separation of polar and non-polar phenolic compounds in wine, with a single gradient of simultaneously increasing concentration of acetonitrile and a decreasing salt concentration [117].

A 2D capillary LC method, employing a 100 mm × 150 μm id C18 RP column serially coupled with a 250 mm × 200 μm id (polyhydroxyethyl aspartamide) HILIC column resolved eleven quaternary ammonium compounds in brain extracts, including acylcarnitines of low polarity. To overcome the mobile phase incompatibility problems, the two columns were connected by a T-piece allowing addition of a make-up flow of 95% acetonitrile to the effluent from the RP column before the transfer to the HILIC column [118].

An interesting solution to the problem of different elution strengths needed to elute polar and non-polar compounds, employed a column-switching setup where the sample was first injected onto two serially coupled HILIC 100 mm × 2.1 mm BEH columns. A plug of the early eluting weakly polar compounds is caught on a trapping RP column, where the analytes are stored until the complete separation of polar compounds on the HILIC columns. Then the configuration of the switching valves changes to redirect the weakly polar compounds onto a phenyl-hexyl RP column, for elution with increasing acetonitrile concentration gradient in 0.02% aqueous formic acid [119].

A crucial point affecting the separation time in comprehensive 2D LC is the performance of the column used in the second dimension, which should allow highly efficient fast chromatographic separations. For this purpose, UHPLC with a short column packed with sub-2 μm particles is used at a very high operating pressure [120]. On-line connection of a capillary HILIC column in the first dimension and an ultrahigh performance RP-LC in the second dimension, coupled with MS, was used for high-resolution separation and detailed characterization of anthocyanins and related pigments in berries and aged red wine [122].

Because of a low flow resistance, core–shell columns or monolithic columns can be used in the second dimension with conventional liquid chromatographic instrumentation [121], at higher flow rates in comparison to particle-packed columns operated at the same operating pressure. Very good band symmetry and retention time repeatability in the gradient separations of phenolic compounds and flavonoids could be achieved in the optimized comprehensive HILIC × RP setup with a 0.5 mm id monolithic sulfobetaine HILIC capillary column coupled with various 2.5–5 cm long, 3 mm id monolithic and core–shell C18 columns. A flow rate of a few microliters

per min is used on the capillary column in the first dimension; the short C18 columns were operated at flow rates of 3–5 mL/min in the second dimension [111].

Polar columns showing a dual RP-HILIC mechanism allow a combined 2D RP × RP and HILIC × RP setup to be used. In the first dimension, the RP mode in a highly aqueous mobile phase alternates with the HILIC mode in a mobile phase with a high acetonitrile concentration. In the second dimension, the RP mode is used.

A zwitterionic polymethacrylate sulfobetaine capillary column prepared by the copolymerization of *N*,*N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl)ammonium betaine as the functional monomer and bisphenol A glycerolate dimethacrylate as the cross-linking monomer (BIGDMA-MEDSA) shows a dual HILIC/RP mechanism at high concentrations of acetonitrile and RP behavior in the water-rich mobile phases [95]. The BIGDMA-MEDSA column in the first dimension coupled on-line with a short monolithic or core–shell C18 column was used for combined alternating HILIC × RP and RP × RP comprehensive 2D separations of polyphenolic compounds. During the HILIC × RP period, a gradient of decreasing acetonitrile concentration was used for the separation in the first dimension. At the end of the gradient, the polymeric monolithic micro-column was equilibrated with a highly aqueous mobile phase and was ready for the second sample injection in the RP × RP period. This time a gradient of increasing concentration of acetonitrile was used in the first dimension. Figure 5 presents 2D chromatograms of flavones and related polyphenolic compounds, acquired with a single first-dimension BIGDMA-MEDSA capillary column in two experiments with consecutive injections of the sample, the first one into a decreasing and the second into an increasing acetonitrile gradient [123]. The automated dual LC × LC approach allows obtaining three-dimensional data in a relatively short time.

6 | CONCLUDING REMARKS

A number of new types of polar stationary phases appeared during the past years, based either on silica gel, other inorganic supports, or on organic polymers. Large differences in retention and separation selectivity of various polar solutes, depending on the chemistry of stationary phases, indicate that the retention is controlled by a sample-dependent mix of non-polar and selective polar (hydrogen bonding, dipole–dipole, ion-exchange, and ion repulsion (ERLIC)) interactions participates in separation.

The column types used in HILIC strongly differ in the adsorption capacity for water. The columns based on hydrosilated silica show less than a monomolecular water layer capacity, which suggests that the adsorption process (i.e., aqueous NP LC) controls the retention rather than the partition between two liquid phases.

On the other hand, silica-bonded polar columns such as DIOL, amide, some organic polymer monolithic columns, and especially zwitterionic columns show strong affinity to water, providing adsorption saturation capacity up to 8–10 equivalents of monomolecular layers. The amount of adsorbed water affects the retention behavior in the HILIC mode [65], such as the column parameters of the LSER model, characterizing the separation system response to the molecular structure descriptors of solutes, the size of the molecules, the hydrogen bonding basicity, the hydrogen bonding acidity, the polarity,

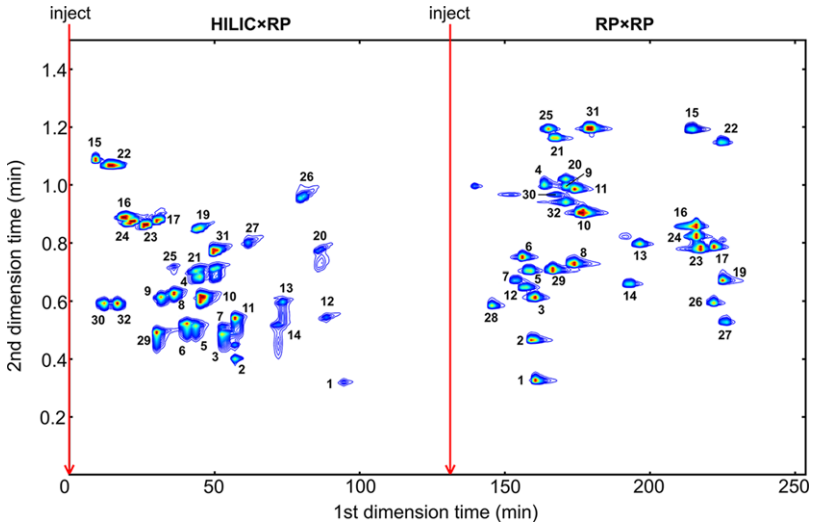

FIGURE 5 Dual comprehensive 2D chromatogram of polyphenolic and flavonoid compounds recorded in subsequent HILIC mode (left, gradient of decreasing acetonitrile concentration) and RP mode (right, gradient of increasing acetonitrile concentration) on a single monolithic capillary zwitterionic polymethacrylate BIGMA-MEDSA, 160 × 0.53 mm id, column in the first dimension coupled on-line with a Chromolith HighResolution, 50 × 4.6 mm id, column in the second dimension. Based on the data of [123]

polarizability, and the ionic properties. Further, the accuracy of prediction of gradient elution data increases for columns with large water saturation capacities.

Many polar columns show dual retention mechanism, HILIC in the mobile phases with low concentration of water, and RP in the organic solvent-rich mobile phases. The dual retention mechanism columns can be used for separations with differences in selectivity depending on the proper adjusting of the aqueous–organic mobile phase. The mobile phase composition marking the transition from the HILIC to the RP separation mode on a single column correlates with the water saturation capacity [11].

We can expect future development of tailor-made HILIC stationary phases intended for specific types of samples. Another almost unexplored promising field is better utilization of mobile phases for adjusting the retention mechanism (HILIC-RP) on a single column to extend separation selectivity by alternating use of different separation conditions. These possibilities can probably be used in future 2D combinations implementing the HILIC mode in off-line, serial, stop-and-go, and comprehensive setups, to improve peak capacities for complex samples containing hundreds of compounds.

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