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Review Article

The HILIC Equilibration Bottleneck: A Comprehensive Review of Kinetic Mechanisms, Column Hysteresis, and Mitigation Strategies

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ABSTRACT

Hydrophilic interaction liquid chromatography (HILIC) has become the separation mode of choice for polar and ionizable analytes that are poorly retained under reversed-phase conditions, yet its widespread adoption continues to be constrained by one persistent operational obstacle: the slowness with which a HILIC column re-establishes a stable partitioning equilibrium after any change in mobile-phase composition. This equilibration bottleneck arises because retention in HILIC is governed not by a simple, rapidly reversible adsorption event at the silica surface but by the gradual build-up, re-organisation, and depletion of a semi-immobilised water-rich layer that is themselves in slow exchange with the bulk eluent. The present review draws together the kinetic, thermodynamic, and instrumental evidence that has accumulated on this subject and organises it into a single mechanistic narrative. We examine how the water-rich layer forms and how its thickness depends on stationary-phase chemistry, pore architecture, and mobile-phase water content; we describe the kinetic steps -- convective transport, intraparticle diffusion, and interfacial water/buffer exchange -- that together set the time constant for equilibration; and we discuss the closely related but conceptually distinct phenomenon of column hysteresis, in which a column's retention behaviour depends on the direction and history of prior mobile-phase changes rather than on the current composition alone. Practical factors that modulate the severity of the bottleneck -- temperature, buffer identity and concentration, particle and pore size, gradient slope, and flow rate -- are reviewed with reference to their underlying kinetic origin. Finally, the review consolidates the mitigation strategies reported in the literature, ranging from extended isocratic pre-conditioning and elevated-flow re-equilibration to buffer engineering, temperature control, and the adoption of columns engineered for thinner or more kinetically labile water layers, and it considers how method-level and software-level compensation can be combined with these physicochemical approaches to deliver reproducible HILIC separations in routine and regulated analytical settings.

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INTRODUCTION

Hydrophilic interaction liquid chromatography occupies a distinctive place among modern separation modes. It was named and mechanistically framed by Alpert in 1990 as a complementary alternative to reversed-phase liquid chromatography (RPLC) for peptides, nucleic acids, and other strongly polar solutes that elute too close to the void volume under conventional C18 conditions [1]. In the decades since, HILIC has moved from a specialist technique into a mainstream tool for pharmaceutical impurity profiling, metabolomics, glycan mapping, and bioanalysis, largely because its high-organic mobile phases pair favourably with electrospray ionisation and permit the use of long, narrow-bore columns at low back pressure [2].

Despite these advantages, HILIC has earned a reputation among practising chromatographers for being comparatively difficult to render fully reproducible, particularly when methods are transferred between laboratories, instruments, or column batches. The dominant cause of this irreproducibility is not, in most cases, a flaw in the underlying separation chemistry but a failure to allow the column sufficient time -- expressed in column volumes -- to reach a stable state after the mobile phase composition has been changed, either at start-up, after a gradient run, or after a period of storage. This phenomenon, referred to throughout this review as the equilibration bottleneck, is the central subject of the discussion that follows.

The purpose of this review is threefold. First, we consolidate the mechanistic understanding of why HILIC columns equilibrate slowly, tracing the argument from the molecular structure of the stationary-phase water layer through to the kinetic steps that limit its re-formation. Second, we describe column hysteresis as a related but distinct manifestation of the same underlying slow kinetics, in which the equilibrium state reached by a column depends on the pathway by which it was approached. Third, we bring together the practical countermeasures that have been proposed and validated in the literature, with an emphasis on strategies that are compatible with regulated method validation, including those governed by ICH Q2(R1) principles for robustness and precision.

The scope of the review is deliberately focused on the equilibration process itself rather than on HILIC selectivity or retention prediction in general, both of which have been extensively reviewed elsewhere. Where selectivity and retention mechanism are discussed, it is specifically to establish why they give rise to slow, and sometimes path-dependent, equilibration behaviour, rather than as an independent treatment of HILIC method development. Readers seeking a broader treatment of HILIC selectivity, column chemistry, or applications outside the equilibration context are directed to the general reviews cited in Section 2.

The review is organised as follows. Section 2 revisits the retention mechanisms proposed for HILIC. Section 3 focuses specifically on the water-rich layer. Section 4 develops the kinetic argument for the equilibration bottleneck. Section 5 introduces semi-empirical kinetic models of the equilibration process. Section 6 addresses column hysteresis. Section 7 places HILIC equilibration behaviour in comparative perspective against RPLC. Section 8 surveys the experimental variables that modulate equilibration behaviour. Section 9 reviews the analytical evidence base. Section 10 presents mitigation strategies. Section 11 discusses representative applications where the bottleneck has practical consequences. Section 12

offers a forward-looking perspective. Section 13 discusses statistical and system-suitability considerations, and Section 14 concludes.

2. Overview of the HILIC Retention Mechanism

HILIC employs a polar stationary phase -- typically underivatised silica, or silica bonded with amide, diol, amino, zwitterionic, or other hydrophilic functional groups -- in combination with a mobile phase that is predominantly organic (commonly acetonitrile) but that always contains a minimum proportion of water, usually in the range of two to forty percent [5,7]. This arrangement is often described, somewhat loosely, as the mechanistic mirror image of RPLC: whereas RPLC retains hydrophobic solutes by partitioning them into a non-polar bonded layer, HILIC is understood to retain polar solutes by allowing them to partition into, or adsorb onto, a polar, water-enriched region associated with the stationary-phase surface.

The retention mechanism, however, is now recognised to be considerably more complex than a single partitioning equilibrium. Contemporary treatments describe HILIC retention as arising from a superposition of at least three contributing effects: (i) liquid-liquid partitioning of the solute between the bulk, organic-rich mobile phase and a water-enriched liquid layer associated with the stationary-phase surface; (ii) direct adsorption of the solute onto exposed silanol or other polar functional groups at the silica surface; and (iii) electrostatic or ion-exchange interactions between charged or ionisable solutes and residual charged sites on the stationary phase [4,8,15]. The relative weight of these three contributions varies with stationary-phase chemistry, solute structure, and mobile-phase pH and ionic strength, which is one reason that HILIC selectivity can differ so markedly between columns that are nominally similar.

Molecular dynamics simulations have added considerable resolution to this picture. Simulations of acetonitrile-water mixtures confined within a silica nanopore show that water is not merely enriched near the surface but forms a structured, several-molecule-thick region in which water activity, density, and mobility differ substantially from the bulk mobile phase [6,10]. Because solute partitioning is governed by the properties of this near-surface region rather than by the nominal bulk-phase composition, the practical retention behaviour of a HILIC column is inseparable from the physical state of this water-rich layer -- and, critically, from how quickly that layer can adjust when conditions change.

An additional layer of complexity arises from mobile-phase pH and buffer composition, which govern the ionisation state of both residual silanol groups on the stationary phase and any acidic or basic functional groups on the solute. Because pH measured directly in a predominantly organic solvent (the so-called solvent-specific, or "ss", pH) can differ substantially from the value measured in the aqueous buffer prior to mixing (the conventional, or "ww", pH), the effective electrostatic environment experienced by a solute at the stationary-phase surface is not always straightforwardly predictable from the nominal aqueous buffer pH alone. This complicates both retention prediction and, as discussed later, the kinetics of re-establishing a stable ionic environment after a compositional change, since the true, in-column pH and ionic strength are themselves dependent on the same slow water and buffer redistribution processes that govern the water-rich layer.

3. The Water-Rich Layer: Formation and Structure

The concept of a water-rich layer associated with the stationary phase is central to virtually every modern account of HILIC retention. McCalley and Neue used frontal analysis and related chromatographic measurements to estimate the thickness of this layer directly, finding that even nominally "bare" silica phases retain a water-enriched region whose extent depends on the water content of the mobile phase and on the specific surface area of the packing [3]. Later nuclear magnetic resonance studies of neat silica and zwitterionic phases confirmed that a fraction of the adsorbed water behaves as a distinct, less mobile population relative to bulk water, consistent with the existence of a genuinely different physicochemical environment near the surface.

Subsequent work systematically compared water-uptake behaviour across a panel of commercially available HILIC phases. Dinh, Jonsson, and Irgum developed methods to quantify the water-absorbing capacity of twelve different stationary phases and demonstrated that the amount of water retained at the surface varies severalfold between phase chemistries, correlating with the polarity and hydrogen-bonding capacity of the bonded functional group [9]. Amide- and zwitterionic-bonded phases, for example, typically present a thinner, more tightly bound water layer than bare silica, whereas certain highly hydrophilic bonded phases can sequester disproportionately large amounts of water relative to their surface area.

The practical consequence of these findings is that the water-rich layer is not a static structural feature of the column but a dynamic reservoir whose size and composition must re-equilibrate with the bulk mobile phase whenever that mobile phase changes. Because the total amount of water held in this layer can represent a substantial fraction of the water introduced through several column volumes of mobile phase, any perturbation to the aqueous content of the eluent forces a comparatively large mass of water to migrate into or out of the porous particle structure before a new steady state is reached. This mass-transfer requirement, rather than any single molecular interaction, is the physical root of the equilibration bottleneck discussed in the next section.

Comparable behaviour has been reported for aqueous normal-phase separations on silica, where water adsorption from acetonitrile-water mixtures follows a nonlinear isotherm that is itself sensitive to water activity in the bulk phase [11]. This nonlinearity means that the amount of additional water needed to re-establish the layer is not simply proportional to the change in bulk-phase water content, further complicating attempts to predict equilibration time from first principles.

A further practical implication concerns column-to-column and batch-to-batch reproducibility. Because the thickness and behaviour of the water-rich layer depend sensitively on details of the underlying silica -- residual metal content, surface silanol density, pore-size distribution, and the uniformity of any bonded-phase coverage -- two columns nominally of the same product line can, in principle, present measurably different equilibration requirements if manufactured in different batches or if subjected to different histories of use and storage prior to installation. This underlines the practical value of empirically verifying equilibration behaviour for a specific column-buffer combination during method development, rather than relying solely on generic vendor or literature recommendations, particularly for methods intended for long-term regulated use.

4. Kinetic Mechanisms Underlying the Equilibration Bottleneck

4.1 Water-layer adsorption and desorption kinetics. When the water content of the mobile

phase entering a HILIC column is changed -- for example, by starting a new isocratic run at a different composition, or by returning to initial conditions after a gradient -- the water-rich surface layer must adjust to the new equilibrium thickness dictated by the adsorption isotherm described above. Because this adjustment requires net transport of water molecules into or out of the near-surface region, and because the surface region is itself only slowly exchanging with the pore fluid, the process cannot occur instantaneously. Reported equilibration times for HILIC columns are typically two to four times longer than those required for an equivalent change on a reversed-phase column operating under comparable conditions.

4.2 Diffusion limitations within the porous particle. A second, additive kinetic constraint arises from ordinary intraparticle diffusion. Because the water-rich layer exists throughout the internal pore structure of a porous silica particle, not merely at the outer particle surface, water and buffer species must diffuse through a tortuous, partially water-saturated pore network to reach or leave the interior of each particle. McCalley's systematic study of column equilibration time demonstrated that full equilibration is achieved more rapidly on columns with larger pore diameters and at elevated column temperature, both of which act to increase the effective diffusion coefficient of water within the pore space and thereby shorten the diffusional path time [16].

4.3 Ion-exchange and buffer re-equilibration kinetics. For basic, acidic, or zwitterionic stationary phases, and for many analytes bearing ionisable functional groups, a further kinetic process is superimposed on water-layer equilibration: the re-establishment of an appropriate distribution of buffer ions, including any residual silanol counter-ions, across the stationary-phase surface. Because buffer salts are themselves concentrated within the water-rich layer, changes in buffer identity or concentration in the bulk mobile phase must propagate into this layer through the same diffusion-limited pathway that governs pure water transport, and the presence of specific ion-exchange sites can introduce additional, slower equilibration steps, particularly for phases bearing residual acidic silanols or bonded amino/ammonium groups.

4.4 Molecular-level evidence from simulation. Molecular dynamics studies of confined acetonitrile-water mixtures provide a microscopic rationale for these observations. Such simulations show that water molecules within a silica nanopore exhibit markedly reduced translational mobility relative to bulk solvent, particularly within the one to two molecular layers closest to the silica surface [6,10]. Because the rate-limiting step for macroscopic equilibration is transport through this low-mobility region, even small increases in the thickness of the immobilised layer -- driven, for instance, by higher surface silanol density or smaller pore diameter -- can produce disproportionately large increases in the observed equilibration time. This nonlinear relationship between structural parameters and kinetic behaviour helps explain why nominally similar HILIC columns from different manufacturers, or even different batches of the same product, can display substantially different equilibration requirements.

4.5 A composite kinetic picture. Taken together, these three processes -- surface-layer adsorption/desorption, intraparticle diffusion, and buffer/ion re-equilibration -- act in series rather than in parallel, and the overall equilibration time observed at the column outlet reflects whichever step is slowest under the prevailing conditions. In practice, this means that the equilibration

bottleneck is not a single, fixed property of a HILIC column but a composite kinetic phenomenon whose magnitude depends on the interaction of stationary-phase chemistry, mobile-phase composition, column geometry, and temperature, all of which are considered in more detail in Section 6.

5. Quantitative and Semi-Empirical Models of Equilibration Kinetics

Beyond the largely descriptive, mechanistic account developed in Section 4, several groups have attempted to place HILIC equilibration behaviour on a more quantitative footing, borrowing conceptual tools from classical chromatographic rate theory. In the simplest treatment, the approach of the water-rich layer to its new equilibrium thickness after a step change in mobile-phase composition is modelled as a first-order relaxation process, in which the deviation from the final equilibrium value decays exponentially with the number of column volumes passed. Such a model, while a simplification of the true multi-step kinetic sequence described in Section 4, captures the empirically observed two-phase behaviour reasonably well: a comparatively fast initial relaxation, corresponding to convective displacement of mobile-phase composition through the interparticle void volume, followed by a much slower, diffusion-limited approach to the true stationary state within the particle pores.

A useful, if approximate, analogy can be drawn with the Van Deemter treatment of band broadening, in which contributions from eddy diffusion, longitudinal diffusion, and resistance to mass transfer are summed to yield an overall plate-height expression. In the equilibration context, an analogous composite time constant can be envisaged as the sum of a convective transit time (set by the column dead volume and flow rate), an intraparticle diffusion time (scaling with the square of the effective diffusion path length and inversely with the diffusion coefficient of water or buffer ions within the pore fluid), and an interfacial exchange time associated with the adsorption/desorption step at the silica surface itself. Because the diffusion term scales with the square of particle or pore radius, it is typically the dominant contributor for smaller-pore, fully porous particles, which is consistent with the experimentally observed benefit of larger pore diameters described in Section 8.2.

Attempts to fit such composite models to experimental breakthrough curves have generally required at least two exponential terms -- a fast and a slow component -- to adequately describe the data, reinforcing the view that a single, lumped time constant is an oversimplification. Nonetheless, even this two-term description is of considerable practical value, since it allows a laboratory to estimate, from a small number of calibration experiments on a given column and buffer system, the number of column volumes required to reach a specified fraction (for example, 95 or 99 percent) of full equilibration, rather than relying solely on generic literature recommendations that may not transfer precisely between column batches or instrument configurations.

It should be emphasised that these semi-empirical models remain approximations, and that no fully predictive, first-principles model capable of forecasting equilibration time purely from stationary-phase specifications (surface area, pore size, bonded-phase density) and mobile-phase composition is yet available. Closing this gap is one of the more promising directions for future methodological work, as discussed further in Section 10.

Table 1. Representative column-volume (CV) recommendations for HILIC equilibration reported in the literature

Equilibration stage	Typical recommendation (column volumes)	Primary rationale
Initial start-up / after storage	20 - 50 CV	Full re-formation of the water-rich layer and buffer distribution across a previously dry or organic-stored column
Between gradient injections	8 - 20 CV	Repeatable partial equilibrium sufficient for acceptable retention-time precision
Isocratic method start-up	30 - 50 CV	Slow approach to a genuinely stationary water-layer thickness under constant composition
Elevated-flow conditioning	Same CV count, higher flow rate	Delivers required column volumes in reduced wall-clock time owing to low mobile-phase viscosity

6. Column Hysteresis: Origins and Manifestations

6.1 Definition and phenomenology. Column hysteresis, in the HILIC context, refers to the observation that the retention behaviour of a column exposed to a given mobile-phase composition can depend on the direction from which that composition was approached -- that is, whether the column was previously equilibrated at a higher or a lower water content -- rather than being a unique function of the current composition alone. This behaviour is distinct from simple slow equilibration, in which a column eventually reaches the same steady state regardless of history but merely takes time to do so; true hysteresis implies that two different histories can, for a period, produce two different retention states at nominally identical current conditions.

6.2 Mechanistic origin. Hysteresis in HILIC is generally attributed to structural or compositional metastability within the water-rich layer itself. Because the layer's thickness and degree of ordering depend on a slow adsorption/desorption equilibrium rather than an instantaneous partition, a column that has recently been exposed to a high-water mobile phase may retain a thicker, more extensively hydrated surface region than one that has been approached from a low-water, high-organic condition, even after both have nominally been "equilibrated" for the same number of column volumes at the target composition. Related metastable states have also been proposed to arise from slow reorganisation of buffer ions or residual silanol ionisation states, which do not necessarily track the bulk-phase water content on the same timescale as the water layer itself.

6.3 Distinguishing hysteresis from simple drift. It is important, for both mechanistic clarity and practical method development, to distinguish column hysteresis from the more commonly discussed problem of retention-time drift caused by insufficient equilibration. Drift is corrected simply by allowing more time (more column volumes) at a fixed composition; hysteresis, by contrast, implies that the pathway of approach -- not merely the dwell time at the final condition -- influences the outcome, and it can therefore persist even when generous, and apparently sufficient, equilibration periods are used, unless the column's approach history is also standardised. In gradient HILIC methods, this has practical implications: a repeatable, partial equilibrium state can often be established after purging with as little as five to twelve column volumes, but only if the direction and magnitude of the preceding gradient, and the equilibration time itself, are held rigorously constant from run to run.

6.4 Reported manifestations. Reports in the literature describe hysteresis-related effects including systematic differences in retention factor between the first and subsequent injections of a sequence, apparent "memory" of a column for the composition used in a previous day's analyses, and small but reproducible selectivity shifts that appear when a laboratory switches between isocratic and gradient modes on the same column without an intervening conditioning protocol. Notably, one investigation traced apparent retention-time irreproducibility in a HILIC system to the prolonged storage of an organic-rich mobile phase while connected to the instrument, rather than to the column packing itself, illustrating that hysteresis-like behaviour can also originate from slow compositional drift in reservoirs, tubing, and other wetted surfaces upstream of the column.

7. Comparative Perspective: HILIC versus Reversed-Phase Equilibration

It is instructive to place HILIC equilibration behaviour explicitly alongside that of RPLC, since much of the frustration reported by chromatographers transitioning between the two modes stems from an implicit expectation, formed through years of RPLC practice, that five column volumes or so of re-equilibration should always be adequate. In RPLC, retention arises predominantly from a comparatively fast, near-instantaneous partitioning of the solute into a covalently bonded, non-polar layer whose properties do not themselves depend on slow water uptake into a porous reservoir; consequently, re-equilibration after a gradient is typically limited mainly by convective flushing of the interstitial and extra-column volume, a process that is largely complete within five column volumes under most practical conditions.

HILIC, by contrast, layers a second, slower process on top of this convective step: the re-formation of the water-rich surface region itself, which is limited by diffusion into and out of the porous particle structure, as developed in Section 4. This is why HILIC re-equilibration is commonly reported as requiring two to four times as many column volumes as an equivalent RPLC method, and why some HILIC separations continue to display small but measurable retention drift even after apparently generous conditioning periods that would be considered excessive by RPLC standards. Recognising this difference explicitly, rather than assuming that RPLC-derived rules of thumb transfer directly, is one of the simplest and most effective ways to avoid early, avoidable frustration when developing a new HILIC method.

Table 2 summarises this comparison at a qualitative level, drawing together the points developed in Sections 2 through 6.

Table 2. Qualitative comparison of equilibration behaviour between RPLC and HILIC

Attribute	RPLC	HILIC
Dominant retention step	Fast, near-instantaneous hydrophobic partitioning	Slow water-layer partitioning plus adsorption/ion-exchange
Rate-limiting equilibration process	Convective flushing of extra-column and interstitial volume	Diffusion-limited water/buffer exchange within particle pores
Typical re-equilibration requirement	Approximately 5 column volumes	Approximately 8-20 column volumes (up to 50 CV at start-up)
Sensitivity to approach history (hysteresis)	Generally minimal	Can be significant unless approach pathway is standardised
Effect of temperature on equilibration	Modest	Substantial - higher temperature markedly accelerates diffusion

8. Factors Influencing Equilibration Time and Hysteresis

8.1 Stationary-phase chemistry. Bare silica and highly hydrophilic bonded phases generally sequester a thicker water layer and, correspondingly, tend to require longer equilibration than more moderately polar bonded phases such as amide or certain zwitterionic chemistries, which have been reported to offer improved reproducibility and comparatively faster equilibration alongside lower activity toward basic analytes [8]. Phases bearing residual ionisable groups (for example, unreacted silanols or bonded amino functionalities) add the buffer/ion re-equilibration step described in Section 4.3 on top of the underlying water-layer kinetics.

8.2 Particle and pore architecture. Larger pore diameters shorten the diffusional path required for water and buffer species to penetrate to the centre of the particle, and columns packed with such materials have been shown to reach full equilibration measurably faster than those with narrower pores [16]. Fully porous sub-2-micron and superficially porous ("core-shell") particle designs can likewise influence equilibration kinetics, generally by reducing the volume of stagnant intraparticle fluid that must be exchanged.

8.3 Temperature. Elevated column temperature increases the diffusion coefficients of water and buffer ions within the pore network and reduces mobile-phase viscosity, both of which accelerate the approach to equilibrium. McCalley's data indicate that full equilibration is reached more rapidly at higher operating temperatures across a range of HILIC stationary phases, making moderate temperature control (for example, thermostatting at 30-40 degrees Celsius) a practical, low-cost lever for reducing equilibration burden, provided the analytes and stationary phase are thermally stable under these conditions [16].

8.4 Buffer type and concentration. Because buffer ions must re-partition into the water-rich layer alongside water itself, buffer identity and concentration materially affect both the rate and the reproducibility of equilibration. Maintaining a constant, adequately concentrated buffer or additive (commonly cited targets are on the order of ten millimolar ammonium-based buffers, or approximately 0.2 percent volatile acid additive) throughout the gradient -- rather than allowing buffer concentration to vary as the organic/aqueous ratio changes -- has been repeatedly recommended as a means of shortening conditioning time and improving peak shape and retention stability, particularly for basic and acidic analytes.

8.5 Gradient slope, flow rate, and column volume accounting. Because HILIC equilibration times are inherently longer than those typical of RPLC, shallow gradient slopes reduce the frequency with which the column must re-equilibrate to widely differing compositions, while excessively steep gradients can outrun the column's capacity to track the notional mobile-phase composition with the true, near-surface composition. Equilibration is also more effectively described in column volumes than in absolute time, since it scales with the physical dimensions of the column and the volumetric flow rate; recommendations in the literature for adequate re-equilibration between injections typically range from roughly eight to twenty column volumes, with initial start-up equilibration sometimes recommended at fifty column volumes or more, depending on the stationary phase and the required level of retention-time precision.

8.6 Sample diluent and injection effects. Because HILIC retention depends on maintaining

a stable near-surface water content, sample diluents that are substantially more aqueous than the mobile phase can, especially at higher injection volumes, locally disturb the water-rich layer at the head of the column and produce peak-shape and retention artefacts that can be mistaken for equilibration failure. Matching the diluent as closely as practicable to the mobile phase, or minimising injection volume for weak (highly aqueous) diluents, is therefore a relevant, if secondary, contributor to overall method robustness.

9. Analytical and Experimental Evidence for the Bottleneck

A substantial and internally consistent body of experimental work now documents the equilibration bottleneck directly. Frontal-analysis and breakthrough-curve measurements have been used to quantify the water-rich layer's thickness and its dependence on mobile-phase composition, providing a direct physical measurement that parallels the indirect, retention-time-based evidence obtained from routine chromatographic runs [3,9]. Systematic column-volume studies, in which retention factor or retention time is monitored as a function of the number of column volumes passed after a compositional change, have shown that equilibration in gradient HILIC methods often proceeds in two phases: a comparatively fast, partial equilibration that is largely independent of stationary-phase chemistry and gradient slope, followed by a much slower approach to full equilibrium whose rate is strongly dependent on water-layer thickness, pore size, and temperature [16].

Precision studies built around these partial-equilibration protocols have reported retention-time relative standard deviations on the order of a few tenths of a percent when the equilibration period is kept strictly and reproducibly constant between runs, even when that period corresponds to only a modest number of column volumes. This finding is practically important because it demonstrates that full thermodynamic equilibration is not always a prerequisite for adequate chromatographic reproducibility, provided that the same, incompletely equilibrated state is reproducibly re-established prior to every injection.

Complementary evidence comes from spectroscopic and computational studies. Nuclear magnetic resonance measurements of water populations in silica- and zwitterionic-based stationary phases have distinguished mobile and immobilised water fractions whose relative proportions shift with mobile-phase composition on a timescale consistent with chromatographically observed equilibration behaviour, while molecular dynamics simulations independently predict the reduced mobility of interfacial water that underlies the diffusion-limited kinetics discussed in Section 4 [6,10]. The convergence of chromatographic, spectroscopic, and computational evidence lends considerable confidence to the water-layer-centred mechanistic account of the equilibration bottleneck that this review has adopted.

10. Mitigation Strategies

10.1 Extended and standardised pre-conditioning. The single most broadly applicable countermeasure is simply to allow, and rigorously standardise, an adequate conditioning period before analytical injections begin. Recommendations in the literature commonly specify an initial conditioning of approximately twenty to fifty column volumes when a column is first installed or after extended storage, with a smaller but still substantial re-equilibration of roughly eight to twenty column volumes between individual gradient injections. Because partial,

rather than full, equilibrium is often sufficient for acceptable precision, the critical requirement is not necessarily maximal equilibration time but exact reproducibility of whatever equilibration period is chosen.

10.2 Elevated-flow-rate conditioning. Because HILIC mobile phases are comparatively low in viscosity at high organic content, the conditioning step can often be performed at a flow rate substantially higher -- in some reports up to twice the analytical flow rate -- than that used during the separation itself, without exceeding system pressure limits. This approach delivers the required number of column volumes in less wall-clock time without altering the fundamental number of column volumes needed for equilibration, and it can meaningfully improve sample throughput in gradient HILIC workflows.

10.3 Continuous or closely spaced sample sequencing. Where feasible, running samples in a continuous, closely spaced sequence rather than with irregular gaps allows the column to settle into a stable dynamic equilibrium between injections, even if that dynamic state falls short of full thermodynamic equilibrium. Because this dynamic equilibrium is reproducible from injection to injection, acceptable precision can often be achieved with a shorter equilibration window than would be required to reach an absolute, history-independent baseline.

10.4 Temperature control. Operating at a moderately elevated, carefully thermostatted column temperature accelerates diffusion-limited equilibration steps and, by improving the reproducibility of the column's thermal state, also reduces one potential source of hysteresis-like behaviour. Temperature should nonetheless be validated for analyte and stationary-phase stability as part of method development, consistent with the robustness testing expected under ICH Q2(R1).

10.5 Buffer engineering. Maintaining a constant, adequately concentrated buffer or volatile additive across the entire gradient -- by adding it to both the aqueous and organic mobile-phase lines rather than to only one -- reduces the additional equilibration burden associated with buffer/ion re-partitioning and has been specifically recommended to avoid retention-time drift in gradient HILIC methods. Selecting a buffer with an ionic strength adequate for the analyte's charge state, while remaining compatible with mass-spectrometric detection where applicable, represents a practical balance that method developers must strike.

10.6 Stationary-phase selection. Where method flexibility permits, selecting a stationary phase engineered for a thinner or more kinetically labile water layer -- for example, certain amide-bonded or zwitterionic phases relative to bare silica -- can substantially reduce both the absolute equilibration time and the propensity for hysteresis, at the cost of some change in selectivity that must be re-validated. This trade-off between kinetic convenience and separation selectivity is a recurring theme in HILIC method development and underscores the importance of considering equilibration behaviour as a formal selection criterion alongside resolution and peak shape.

10.7 Method-level and instrumental compensation. Where physicochemical mitigation cannot fully eliminate residual drift, method-level compensation -- such as the use of internal standards with retention behaviour similar to the analyte, system-suitability criteria that explicitly monitor retention-time reproducibility across a sequence, and bracketing of sample runs with calibration or quality-control injections -- can absorb small, residual equilibration-related variability without compromising the validity of quantitative results. Modern chromatographic data

systems can also be configured to insert a fixed, non-negotiable equilibration hold after every gradient cycle, removing operator-dependent variability in equilibration time from the list of contributing factors.

10.8 Reversed-gradient and hybrid approaches. Recent exploratory work on reversed HILIC gradients, in which the organic content is increased rather than decreased over the course of the run, and on two-dimensional separations that combine HILIC with RPLC, illustrates that reformulating the gradient strategy itself -- rather than only adjusting equilibration time -- can sometimes sidestep the specific compositional transitions that are most prone to slow equilibration, while retaining useful selectivity for polar analytes.

11. Applications and Case Studies Illustrating the Bottleneck

11.1 Pharmaceutical impurity and residual solvent analysis. In regulated pharmaceutical testing, where HILIC is increasingly used for polar impurities, counter-ions, and certain residual-solvent or small polar excipient determinations, the equilibration bottleneck has direct implications for method validation. Because ICH Q2(R1) robustness testing requires that small, deliberate variations in operating parameters do not produce unacceptable changes in performance, a method whose retention time is sensitive to equilibration history is inherently more fragile under such testing than one that has been designed, from the outset, around a standardised and generously specified conditioning protocol.

11.2 Metabolomics and biopharmaceutical characterisation. In untargeted metabolomics, where large batches of structurally diverse polar metabolites are analysed over long sequences, even small run-to-run retention-time drift arising from incomplete equilibration can compromise peak alignment and downstream statistical analysis. Consequently, metabolomics laboratories have been among the most active adopters of rigorous, fixed-volume conditioning protocols and of quality-control sample bracketing specifically to monitor for hysteresis-related drift across long analytical batches. Related considerations apply to the growing use of HILIC for intact glycan and glycoprotein characterisation, where reproducible retention is essential for consistent glycoform assignment.

11.3 Two-dimensional and orthogonal separations. In comprehensive two-dimensional liquid chromatography schemes that combine HILIC with RPLC, the first-dimension HILIC column is typically operated under conditions that must re-equilibrate very rapidly to keep pace with short second-dimension cycle times; equilibration behaviour is therefore not merely a matter of overall method robustness but a first-order constraint on which HILIC configurations are practically usable in such hyphenated workflows, favouring stationary phases and gradient designs that minimise the kinetic penalties discussed throughout this review.

12. Future Perspectives

Several directions appear likely to shape future work on the HILIC equilibration bottleneck. First, continued application of molecular simulation, ideally validated against increasingly sensitive spectroscopic probes of interfacial water dynamics, should refine the quantitative link between stationary-phase structural parameters (pore size, surface silanol density, bonded-phase chemistry) and the kinetic time constants that govern equilibration, moving the field from largely empirical column-volume recommendations toward more predictive, structure-based guidance. Second, there is scope for stationary-phase engineering explicitly

targeted at reducing water-layer thickness or increasing its exchange rate without sacrificing the selectivity that makes a given phase chemistry useful, potentially through more precisely controlled bonding density or pore-surface modification. Third, as two-dimensional and high-throughput HILIC applications continue to grow, method-level and software-level compensation strategies -- including automated, sequence-embedded conditioning protocols and real-time monitoring of system-suitability retention markers -- are likely to become standard features of commercial chromatography data systems rather than laboratory-specific workarounds. Finally, as regulatory expectations around method robustness and transferability continue to mature, explicit reporting of equilibration protocols (column volumes, flow rate, and temperature used for conditioning) as a required element of HILIC method documentation would likely reduce the incidence of transfer failures attributable to this well-characterised but frequently under-specified source of variability.

13. Statistical and System-Suitability Considerations

From a method-validation perspective, the equilibration bottleneck is best treated as a robustness variable in the same formal sense as flow-rate tolerance, column temperature tolerance, or mobile-phase pH tolerance under ICH Q2(R1). Rather than specifying only a nominal conditioning time or column-volume count, a well-designed HILIC method should explicitly state the acceptable range around that nominal value and should include, within its robustness study, a deliberate variation of equilibration time (or column volumes) to confirm that retention time, resolution, and peak shape remain within predefined acceptance criteria across that range.

System-suitability testing offers a complementary, ongoing safeguard. Because retention-time drift caused by incomplete or inconsistent equilibration is readily detected by monitoring the retention time of a well-retained system-suitability marker across a sequence, laboratories can build explicit control limits -- for example, a maximum permitted percentage relative standard deviation in retention time across bracketing standards -- directly into routine sequence acceptance criteria. Where such limits are exceeded, the appropriate corrective action is very often an extension or standardisation of the equilibration protocol rather than a change to the separation chemistry itself.

Statistically, because equilibration-related drift tends to manifest as a slow, monotonic trend across a sequence rather than as random scatter, ordinary replicate-based precision statistics (for example, simple relative standard deviation across all injections in a batch) can under-represent the practical risk if the trend is not detected early. Control-chart-style monitoring of retention time as a function of injection number, rather than a single aggregate statistic computed after the fact, is therefore a more sensitive means of detecting an emerging equilibration problem before it affects reportable results, and is increasingly recommended as good practice in HILIC-based regulated testing and in high-throughput metabolomics workflows alike.

Finally, when transferring a validated HILIC method between laboratories, instruments, or column lots, it is advisable to treat the equilibration protocol as a transferable, explicitly documented parameter with the same status as the gradient program or detection wavelength, rather than as an implicit operational habit of the originating laboratory. Doing so reduces the likelihood that a technically valid method will appear to fail during transfer simply because the

receiving laboratory allowed a shorter, or differently timed, conditioning period than the one under which the original validation data were generated.

CONCLUSION

The equilibration bottleneck in HILIC is not an incidental inconvenience but a direct and predictable consequence of the mechanism by which HILIC achieves its useful selectivity: retention that depends on a water-rich, only slowly exchangeable surface layer will necessarily respond slowly to changes in mobile-phase composition. Understanding this bottleneck in kinetic terms -- as the composite outcome of water-layer adsorption/desorption, intraparticle diffusion, and buffer re-equilibration -- reframes what is often treated as an operational nuisance into a well-defined, physically grounded design constraint. Column hysteresis, in turn, reflects the same underlying slow kinetics expressed as a path-dependence rather than a simple time delay, and recognising this distinction is important both for diagnosing reproducibility problems correctly and for selecting the appropriate mitigation strategy. The practical countermeasures reviewed here -- standardised and adequately generous conditioning, elevated-flow re-equilibration, temperature control, buffer engineering, judicious stationary-phase selection, and method-level compensation -- are individually modest but collectively capable of rendering HILIC methods as robust and transferable as their reversed-phase counterparts, provided that equilibration behaviour is treated as an explicit and documented parameter of method development rather than an afterthought.

Ultimately, the maturation of HILIC from a specialist niche technique into a routinely used, regulator-facing analytical tool depends as much on disciplined operational practice around equilibration as it does on continued mechanistic and stationary-phase innovation. The evidence surveyed in this review indicates that the equilibration bottleneck, while real and mechanistically well-founded, is also thoroughly characterisable and, with appropriate protocol design, manageable in routine practice. Laboratories that adopt explicit, column-volume-based conditioning protocols, that validate equilibration time as a formal robustness parameter, and that monitor retention-time stability as an ongoing system-suitability criterion are well placed to obtain the full analytical benefits of HILIC selectivity without being unduly hindered by the kinetic limitations that have historically accompanied the technique.

Glossary of Key Terms

Term	Definition
Water-rich layer	A region of enriched, partially immobilised water associated with the polar stationary-phase surface in HILIC, believed to be the principal medium into which polar solutes partition.
Equilibration bottleneck	The characteristically slow re-establishment of a stable water-rich layer (and associated buffer distribution) after any change in HILIC mobile-phase composition.
Column hysteresis	Dependence of a column's retention behaviour on the direction and history of prior mobile-phase changes, rather than on the current composition alone.
Column volume (CV)	The internal volume of a chromatographic column, used as a normalised, flow- and dimension-independent unit for expressing conditioning and equilibration requirements.
Partial equilibrium	A reproducible, but not fully thermodynamically complete, steady state that a HILIC column can reach after a limited number of column volumes, sufficient for acceptable analytical precision if reproducibly applied.

Solvent-specific (ss) pH	A pH value measured directly in a predominantly organic mobile phase, which can differ materially from the aqueous (ww) pH of the buffer before mixing.
Van Deemter analogy	A conceptual borrowing from classical plate-height theory, used here to describe equilibration time as a sum of convective, diffusive, and interfacial-exchange contributions.

List of Abbreviations

Abbreviation	Definition
HILIC	Hydrophilic Interaction Liquid Chromatography
RPLC	Reversed-Phase Liquid Chromatography
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
Q2(R1)	ICH Guideline on Validation of Analytical Procedures
CV	Column Volume(s)
RSD	Relative Standard Deviation
MS	Mass Spectrometry
ESI	Electrospray Ionisation
NMR	Nuclear Magnetic Resonance
2D-LC	Two-Dimensional Liquid Chromatography
ss pH	Solvent-Specific pH (measured directly in the organic-rich mobile phase)
ww pH	Conventional Aqueous pH (measured in the aqueous buffer before mixing)

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Conflict of Interest

The author(s) declare no conflict of interest relevant to the content of this review.

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Data Availability

This is a review article; no new experimental or chromatographic data were generated. All information discussed is drawn from, and referenced to, the previously published literature cited herein.

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