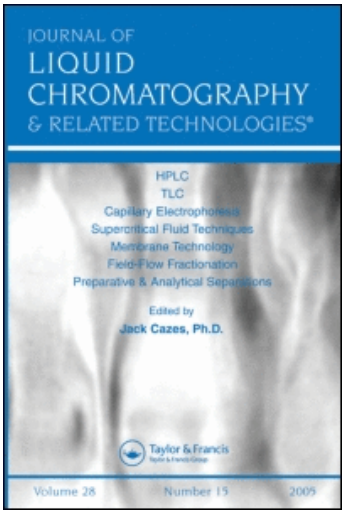

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Fundamentals of Reversed Phase Chromatography: Thermodynamic and Exothermodynamic Treatment

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Abstract: Reversed phase chromatography (RPC) is the most popular branch of HPLC for the analysis and purification of a wide variety of substances. Despite significant advances in both our knowledge and understanding of the fundamental principles governing the retention behavior in RPC, there is considerable debate in the literature regarding the mechanism of retention. This review addresses the theoretical foundation of the chromatographic technique, with an emphasis on thermodynamic and exothermodynamic treatment of retention equilibrium, as well as their implication on the mechanistic aspects of RPC retention. A unified and rigorous treatment based on the solvophobic theory is reviewed in terms of its ability to shed light on the physicochemical underpinnings of the retention in RPC, and to quantitatively predict the retention behavior of nonpolar compounds, acids and bases, and peptides and proteins. Also highlighted are areas of future challenges in the theory and practice of RPC, potentially leading to a better quantitative understanding and use of the popular technique.

Keywords: Solvophobic theory, Physicochemical parameters, Retention mechanism, Adsorption, Partitioning, Ion-pair, Chaotropicity, Hydrophobic effect, Enthalpy-entropy compensation, LFER

INTRODUCTION

High performance liquid chromatography (HPLC) is a high-resolution separation tool *par excellence* that has revolutionized myriad areas of science and

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technology.[1] It is undoubtedly the unsung champion of the modern biological sciences, enabling the intricacies of cellular biology to be at last discriminated in detail.[2] Without the recent advances in HPLC, modern biology, functional genomics, and proteomics would not exist. The incredible power of HPLC to discern the molecular diversity of biological phenomena is largely attributed to its ability to distinguish mass differences as little as 1 Da in a macromolecule, such as proteins when coupled with mass spectrometry,[3,4] to separate proteins that differ by only a single amino acid,[5] to separate conformational isomers of a long chain polypeptide,[6] and to resolve the different tertiary conformational structures of DNA fragments.[7] The exquisite sensitivity, the speed, and the impressive resolving power of modern HPLC find application in various fields, such as pharmaceutical, food and clinical analysis, pollution control, downstream processing, measurement of physicochemical properties of drugs as well as the separation of peptides, proteins, and nucleic acids.

At the heart of the HPLC revolution is the mode of separation that we are all familiar with—reversed phase chromatography (RPC). The seminal works of Horváth and co-workers[8–11] have contributed significantly to the theory and practice of RPC, resulting in its wide acceptance by the scientific community as a high resolution separation technique of choice. It is estimated that approximately 80% of HPLC analysis is conducted in this mode.[12] The success of this technique is attributed to the employment of microparticulate alkyl-silica monomeric bonded phases, such as octadecylated silica, which offers high separation efficiency combined with unparalleled convenience, versatility and reproducibility. The use of bonded hydrocarbonaceous stationary phases having a variety of functional groups and a wide choice of hydroorganic eluents to modulate retention offer a broad range of operating conditions to separate any kind of complex mixture containing small or large compounds of different polarity.[11]

Notwithstanding its wide popularity and remarkable success, RPC retention still remains one of the most challenging phenomena to model at the molecular level. Numerous studies have attempted to elucidate the precise origin of RPC retention. Many theories based on classical and statistical thermodynamics have been put forward to describe the fundamental principles governing RPC separation and to predict retention and selectivity of elutes with varying polarity. Despite the extensive theoretical treatment and widespread application of RPC in analytical and preparative HPLC, there is still considerable debate in the chromatographic community on several fundamental aspects of RPC retention. This can be attributed to the complexity of the retention process in general, arising from the interplay of myriad types of specific and nonspecific interactions between the eluite, the eluent and the stationary phase, and our lack of understanding of the stationary phase configuration. One of the long-standing issues is whether eluite retention in RPC is best described as an adsorption process or a partitioning process. Other areas of discord have focused on the key driving force in RPC

retention and the individual roles played by the mobile phase and the stationary phase in governing the retention behavior.

In this review, the current understanding of this major field of separation sciences is presented from both a thermodynamic and an exothermodynamic perspective, and some of the future challenges are discussed. An attempt is made to shed light on the controversy enshrouding RPC retention by discussing the merit of various points of contention and addressing discordant views arising from semantics or misinterpretation. The treatment based on Horváth's solvophobic theory, which is the most advanced of all theories proposed thus far for RPC retention, is reviewed in terms of its ability to quantitatively predict the retention behavior in RPC and to describe the mechanistic aspects of various interactions occurring in the retention process. Also described is its ability to link the thermodynamic equation with exothermodynamic relationships that are widely employed in RPC. Finally, the shortcomings of the solvophobic theory and future challenges in its development as a comprehensive theory to accurately describe all aspects of the complex nature of RPC retention are highlighted. The discussion is limited to RPC when operated under linear isocratic elution condition in analytical separations.

MOLECULAR INTERACTIONS IN RPC

Any theoretical treatment to describe the energetics of retention in RPC must consider all interactions, both specific and nonspecific, occurring in the process between the eluite of interest, the eluent, the surface as well as the grafted ligates of the stationary phase, as described in Table 1. These are weak in nature and do not involve covalent bonding. They include eluite–eluent interactions (e.g., ion-pairing of an ionizable eluite with a counter ion in the mobile phase), eluite–stationary phase interactions (e.g., interaction between an eluite molecule and residual silanols within the stationary phase or interaction between the eluite and the stationary phase ligate), and eluent–eluent interactions (e.g., hydrogen bonding between eluent molecules). Other competing interactions that may affect eluite retention in RPC, such as eluite–eluite interactions (e.g., interaction between various amino acid groups in a protein molecule or intermolecular interactions between charged proteins), eluent–stationary phase interactions (e.g., solvation of alkyl chains of the stationary phase), and ligate–ligate interactions (e.g., intermolecular interactions of alkyl chains in the stationary phase), must also be considered when appropriate. Among these, the least understood are eluite–eluite, eluent–stationary phase, and ligate–ligate interactions. Eluite–eluent interactions in RPC are believed to occur via both van der Waals and electrostatic interactions, while eluent–eluent interactions consist of hydrogen bonding and van der Waals interactions. Similarly,

Table 1. Typical molecular interactions observed in RPC employing monomeric alkyl-bonded stationary phases

Types of interactions	Nature of interactions	Examples
Eluite–eluite	van der Waals	Interaction between two eluite molecules due to London, Keesom or Debye forces
	Electrostatic	Intramolecular interaction between charged amino acids present in a protein molecule, or intermolecular interaction between two charged proteins
	Hydrogen bonding	Hydrogen bonding between acceptor and donor groups within a protein molecule
Eluite–eluent	van der Waals	Interaction between an eluite and an eluent molecule due to London, Keesom or Debye forces
	Electrostatic	Ion-pairing between the ionized eluite and the salt counter-ion in the mobile phase
Eluite–stationary phase	van der Waals	Interaction between an eluite and a ligate molecule due to London, Keesom or Debye forces
	Electrostatic	Interaction between a charge eluite and an ionizable group on the stationary phase surface
	Hydrogen bonding	Interaction between a basic eluite molecule and residual silanols
Eluent–eluent	van der Waals	Interaction between two eluent molecules due to London, Keesom or Debye forces leading to solvation of ligates
	Hydrogen bonding	Hydrogen bonding between eluent molecules such as water

(*continued*)

Table 1. Continued

Types of interactions	Nature of interactions	Examples
Eluent–stationary phase	van der Waals	Interaction between an eluent and a ligate molecule due to London, Keesom or Debye forces leading to solvation of ligates
	Electrostatic	Supression of residual silanols by charged ions in the mobile phase
Ligate–ligate	van der Waals	Intermolecular interaction between alkyl chains of the stationary phase due to London, Keesom or Debye forces

Eluite represents an analyte of interest in the chromatographic system, eluent represents the mobile phase employed and ligate denotes the grafted alkyl chain on the bonded RPC stationary phase.

eluite–stationary phase interactions can occur via one or more of the above mentioned interactions. In most instances of linear chromatography, it is often assumed that the eluite is present at an infinitely dilute state such that eluite–eluite interactions can be neglected. However, in some cases, such as in applications employing overloaded (nonlinear) chromatographic conditions, or with protein adsorption, this may not be true and, thus, result in deviations from expected behavior.

The importance of each of these interactions is obvious in the context of eluite retention. Figure 1 illustrates the range of action of van der Waals, electrostatic and hydrogen bond interactions. Their characteristics are briefly described below.

Hydrophobic Interactions

The term hydrophobic interactions has been extensively used in the scientific literature to denote the intermolecular forces resulting in the association of nonpolar molecules or the binding of hydrophobic moieties in aqueous solution, and suggests that solvent water plays a major role in these phenomena.[14] These are long-range Lifshitz-van der Waals interactions (London, Debye and Keesom), such as dipole-dipole and dipole-induced dipole interactions, that decay exponentially up to a distance of $\sim$1000 Å. Such interactions are believed to play an important role in the architecture

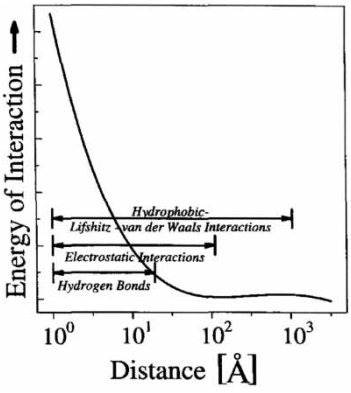

Figure 1. The dependence of the energy of interaction on the distance between the interacting species involved in RPC retention. When the distance is relatively large in atomic units, the primary interactions are based on long-range van der Waals interactions. As the approach distance decreases, the energy term exhibits a greater electrostatic component. On further closure of the approach distance, hydrogen bond effects occur. The short-range van der Waals attractions mediated by fluctuating electrical charges occur at closer association. At very small approach distances, interactions are dominated by strong repulsion and a larger increase in the energy. From Ref.[2].

and dynamics of various biological systems,[15] such as the maintaining of the three-dimensional stability of proteins in aqueous solutions[16–18] and site-specific protein-DNA complexes[19] and the self-association of phospholipids and other lipids to form biologically active membranes in living systems.[20,21] Another related term often used to describe interactions in biological systems is called the "hydrophobic effect", which is defined as an unfavorable interaction of nonpolar substances or moieties of the molecules with water, resulting in their low solubility.[20,21]

Calorimetry has been employed extensively to study the thermodynamic aspects of hydrophobic interactions. Simple model systems, such as the dissolution in water of nonpolar liquid,[16,22] gaseous[23,24] and solid[25,26] substances, have been employed to evaluate the thermodynamic parameters associated with hydrophobic interactions. Both calorimetric and solubility data obtained with these model systems have revealed a significant change in entropic and heat capacity terms at room temperature. Additionally, hydrophobic interactions have been shown to be driven by entropy change at low temperatures and enthalpy change at high temperatures when studied in a range of experimental temperature, resulting in a nonlinear (curved) van't Hoff or solubility plot. Such findings have provided the basis of a more detailed understanding of the influence of temperature on hydrophobic interactions and of the hydrophobic effect, at large.

Since RPC employs a strongly hydrophobic stationary phase surface for separation, it is widely believed that retention and selectivity in RPC is strongly governed by the magnitude of hydrophobic interactions between the nonpolar eluite molecules and the hydrophobic ligates at the stationary phase surface. However, it must be recognized that in RPC the eluent is rarely plain water but a hydroorganic mixture or a polar organic solvent. Therefore, the use of the term "solvophobic" interactions instead of hydrophobic interactions is more apt to describe the net interactions between the nonpolar eluites and the hydrophobic stationary phase proper.

Hydrogen Bond Interactions

Hydrogen bonds are of polar origin and are usually stronger than other noncovalent bonds. The silicious bonded stationary phase surface in RPC is predominantly hydrophobic in nature but may have residual silanols present on the surface that can participate in hydrogen bonding with the eluites. This type of interaction between the eluite and the residual silanols on the stationary phase surface is often referred to as silanophilic interaction. In polypeptides and proteins, both backbone amides and side-chain moieties bearing OH, NH_2, COOH, or SH groups can contribute as hydrogen bond donors or acceptors. These groups in proteins and polypeptides as well as acidic or basic eluites are capable of hydrogen bonding with themselves, with the surrounding solvent or with the stationary phase, and are of the type N–H . . . O, O–H . . . O, N–H . . . N, or O–H . . . N, etc. Although dissociation energies for these bonds are high, they do not appear to play a significant role in the retention process due to the very short distance of the order of 1.5–5 Å over which they act. Therefore, they are weaker and shorter range forces compared to charge-dipole interactions. Additionally, hydrogen bond effects involving eluite and stationary phase ligate can be attenuated by hydrogen bonding between hydrogen donor and hydrogen receptor moieties of the eluite and the ambient water molecules. Also, intramolecular hydrogen bonding in polypeptides and proteins is greatest among internalized amino acid residues, and becomes more limited as the accessibility of these residues to the surrounding water environment increases. The addition of organic solvents to water in RPC could, therefore, influence the hydrogen bonding ability of water, potentially resulting in retention and selectivity changes. As the hydrogen bond has many features in common with electrostatic or polarized dipole interactions, it follows that it strengthens at lower temperatures.

Electrostatic Interactions

Like hydrogen bond interactions, electrostatic or coulombic interactions are polar in nature and occur between charged or ionizable groups present in the eluite or on the surface of the stationary phase. Electrostatic interactions between oppositely charged species are effective over distances of the order of 10 to 100 Å. Unlike other types of interactions, coulombic forces can be attractive in case of oppositely charged groups at the surface, or repulsive in case of identically charged groups of interacting molecules. For simple charged molecules, the magnitude of electrostatic interaction is easy to calculate but this becomes significantly complicated for proteins and polypeptides that are amphoteric in nature. The presence of salts in the medium also directly influences the magnitude of electrostatic interactions between two species. It is well known that different salts can influence the ion-exchange chromatographic behavior of polypeptides or proteins due to the chaotropic or cosmotropic nature of the component cations and anions.[27–29]

Metal Ion Coordination Interactions

In some chromatographic systems, attraction between negatively charged eluites is also affected through cross-linking, i.e. chelate development, of multivalent cations. This interaction can be visualized in terms of the ability of a metal ion to accept a pair of electrons, and thus act as a Lewis acid, from an eluite, which is an electron donating base with an accessible lone pair of electron. Coordination interactions of metal ions with polypeptides and proteins are of this nature. Such interactions are typically not observed in RPC but are fairly common in immobilized metal affinity chromatography.

STATIONARY PHASE MODELS

RPC employs a wide variety of monomeric or polymeric hydrocarbonaceous stationary phases, which traditionally consist of a microparticulate silica support with covalently bound alkyl or aryl functions at the surface. Among the alkylated silica-based stationary phases, octadecyl (C_{18}), octyl (C_8), and butyl bonded (C_4) sorbents are the most widely used for the separation of compounds. While monomeric stationary phases offer the highest separation efficiency, polymeric stationary phases are more stable and resistant to hydrolytic degradation when in contact with aqueous mobile phases due to their cross-linked network. Since the silanol groups at the surface of the silica cannot be reacted completely, the unreacted silanols on the surface of monomeric stationary phases may give rise to heterogeneous surface, and as a result a mixed retention mechanism involving hydrophobic and silanophilic

interactions may be present.[30] To reduce silanophilic interactions, end-capping of the surface silanols is normally carried out.

Clearly, the bonded phase is a complicated, heterogeneous media whose chemical composition and configuration may vary with the mobile phase composition, the nature of the silica support, the bonding density, and the alkyl chain length. The structure and composition of bonded phases in the presence of different eluents has been the subject of intense study and discussion in the past.[11,31–37] At least six different models have been proposed to describe the stationary phase configuration in RPC and they have been discussed by Vailaya and Horváth.[38] Each of these models represents a different view of the retention mechanism in RPC. They invoke either the partition or the adsorption process. Partitioning entails creation of a solute-sized cavity in the organic phase, transfer of a solute molecule from the aqueous phase into the organic phase, and closing of the cavity left behind by the solute molecule in the aqueous phase. On the other hand, adsorption is a surface phenomenon in which the solute molecules migrate from the liquid phase to the solid–liquid interface and displace the physically adsorbed molecules of the solvent.

The first model called the bulk liquid hydrocarbon layer model was proposed by Lochmüller and Wilder[34] to describe the sorptive behavior of alkyl-silica bonded stationary phases in RPC. It assumes the bonded phase to consist of "liquid-droplet" like clusters offering a bulk liquid-like environment for the partitioning of small eluites. Thus at the molecular level, eluite retention in RPC involves the creation of eluite-sized cavity in the liquid-like stationary phase, the transfer of the eluite into the cavity, and the subsequent closing of eluite-sized cavity in the mobile phase.

Martire and Boehm[31] suggested that the alkyl-silica bonded phases should not be modeled as a bulk liquid but as a liquid-crystalline hydrocarbon layer. This model is a refinement of the bulk liquid hydrocarbon layer model as it includes the stationary phase organization in terms of the bonded chain length, the intrinsic chain stiffness and surface coverage, as well as the configuration of the chains in various mobile phases. The liquid-crystalline hydrocarbon layer is an anisotropic condensed phase with an order intermediate between that of a liquid phase and a crystalline phase. The model draws from related lattice statistics developed to treat other liquid-crystalline systems, fatty acid monolayers, and the amorphous region in diblock copolymers.[39–42] Eluite retention in RPC is believed to occur via the partition mechanism when the eluite fully penetrates the liquid-crystalline hydrocarbon layer.

Dill[32,43] proposed an amorphous-crystalline hydrocarbon model, also called the interphase model, to describe the retention behavior in RPC. He argued that the molecular organization of the bonded phase resembles neither the all-trans crystalline state of n-alkane chains nor the randomly structured liquid state, and not even a liquid-crystalline state of intermediate order.

Instead, the chromatographic surface in RPC may be likened to the interphase between lamellar crystalline and adjoining amorphous regions in a semi-crystalline polymer. The interphase is comprised of chains which have one end anchored at the interface so that they are characterized by a gradient of disorder that joins regions of high order and of liquid-like disorder. The proportion of *gauche* bonds varies along the length of a chain as it traverses the interphase layer. Such a resemblance for hydrocarbon chains in amphiphilic phases, such as lipid monolayers and bilayers as well as micelles and microemulsions, was first pointed out by deGennes.[44] According to the amorphous-crystalline hydrocarbon layer model, bonded chains of RPC stationary phases will have greater orientational order near their anchored ends than near their free ends. This variation of properties with distance from the interface contrasts with bulk liquid phases, whose properties by definition are invariant. Eluite retention in RPC is governed by the partition mechanism when the bound eluite is fully embedded within the amorphous-crystalline layer stationary phase.

In order to distinguish between the partition and the adsorption mechanism in RPC, Dill considered an adsorption model for the stationary phase, called the adsorptive hydrocarbon monolayer model, and contrasted it with the amorphous-crystalline hydrocarbon layer model.[32] The model was developed to describe adsorption in RPC when the density of bonded nonpolar functions is high enough for the chains to interact laterally among themselves, thus disallowing the penetration of eluite molecules into the hydrocarbon layer on the stationary phase support. In this case, the tips of the alkyl-silica bonded chains offer a hydrocarbonaceous surface for the adsorption of the eluites.

Yet another model for the stationary phase in RPC, called the isolated solvated hydrocarbon chains model, was proposed by Horváth and co-workers to describe retention in RPC.[8] It assumed that two distinctive stationary phase configurations are possible depending on the composition of the eluent. In organic-rich eluents, the bonded chains are extended and assume a fur-like configuration. Retention in RPC occurs by penetration of the eluite molecule within the interligate space followed by interaction with the lateral surface of the ligates, or association with the tips of the bonded chains, or both. In water rich mobile phases, the bonded chains are in close contact with each other and form a stack configuration. It is assumed that the ligate density is small enough so that the chains may be considered as isolated. Regardless of the stationary phase configuration and the orientation of eluite binding, RPC retention is driven by the magnitude of the contact area that is formed upon binding of the eluite molecule with the isolated solvated ligates of the stationary phase.

The last model of the stationary phase, which is referred to as the collapsed hydrocarbon chains model, was proposed by Kazakevich and co-workers recently.[45] Using low temperature nitrogen adsorption experiments

to measure excess adsorption isotherms, they argue that the effective molecular volume of bonded chains are similar to that of the corresponding liquid alkanes and that the bonded phase in RPC is mainly in a dense, liquid-like arrangement. They suggest a stationary phase model of RPC that is essentially collapsed, regardless of the chain length of the ligate or the concentration of the organic modifier in the mobile phase, with a layer of the organic phase adsorbed on top of the collapsed chains. According to this model, retention in RPC is envisioned to occur first by partitioning of the eluite from the mobile phase into the organic layer adsorbed on the collapsed stationary phase chains, followed by its adsorption on the surface of the collapsed chains.

OVERVIEW OF RPC THEORIES

Several theories have been proposed to describe the retention behavior of neutral and ionizable eluites in RPC. They can be broadly classified into two categories: classical and statistical thermodynamics. Both categories of theories have represented a clear view of the retention in RPC by accounting for most types of intermolecular interactions occurring during separation, however, neither has been able to accurately describe the retention behavior of all eluite under all possible conditions. This reflects the complexity of the RPC retention process and the lacuna in our understanding of the process at the molecular level. A brief review of the theoretical treatments put forward by various investigators is presented below.

Classical Thermodynamics

Horváth's Solvophobic Theory

It is long believed that hydrophobic interaction is the key driving force governing the retention in RPC. But most treatments of the hydrophobic effect based on a thermodynamic analysis of solute transfer between water and a nonpolar liquid or on the statistical thermodynamic analysis of aqueous solutions, while illuminating in many respects, were not readily applicable to explicate retention in RPC. Moreover, in RPC the eluent is rarely plain water but a hydroorganic mixture or a polar organic solvent. The solvophobic theory of Sinanoğlu[46] developed initially to describe the solvent effects on the energetics of various molecular associations in solution was adapted by Horváth et al.[8–10,47,48] to provide the first rigorous treatment of the retention energetics in RPC. The theory is based on classical thermodynamics and views the retention in RPC as association *in vacuo* of the eluite with the stationary phase and subsequent transfer of

participating species into the mobile phase. Since eluite retention in RPC is determined largely by the energy balance of eluite–stationary phase, eluent–eluent and eluent–eluite interactions, the solvophobic theory puts these interactions in a rigorous thermodynamic framework and allows one to calculate the retention energetics in RPC with nonpolar stationary phases from measurable properties of the eluite, the eluent, and the stationary phase. The theory is the most advanced of all theoretical treatments to describe the RPC retention process. It employs readily available physicochemical data to quantitatively predict the retention and selectivity of both neutral and ionizable eluites as a function of operating variables, such as temperature, eluent property and eluite molecular structure, on a given stationary phase. The theory has also been successfully adapted to many biological and chemical processes such as denaturation of DNA,[49,50] hydrophobic interaction chromatography,[51,52] behavior of proteins in aqueous salt solutions,[53,54] adsorption on activated carbon from dilute solution,[55,56] drug-biomolecule associations and denaturation of proteins,[57] octanol-water partitioning,[58] dissolution of nonpolar gases in water[52] as well as ion-pair solvent extraction.[59] In the following, both the framework of the solvophobic theory and its applicability to RPC retention will be reviewed in detail.

Jaroniec's Adsorption/Partition Theory

Jaroniec and Martire[60–62] proposed a theory for RPC retention that used a combination of adsorption and partition models. This approach, which viewed RPC as a process of formation of eluent-surface stationary phase via displacement mechanism and the subsequent distribution of the eluite between the mobile phase and the stationary phase via a partition mechanism, led to a general expression for the dependence of the retention factor upon the modifier content in the mobile phase, from which the limiting equations based on either the adsorption or the partition model may be deduced. These equations have however offered, at best, qualitative prediction of retention and selectivity in RPC.

Ståhlberg's Theory

Reversed phase ion-pair chromatography is primarily used for the separation of mixtures of ionic and/or ionizable compounds, facilitated by the addition of amphiphilic ions to the mobile phase in order to enhance the retention of ionic sample components. Theoretical treatments of ion-pair RPC have involved the use of either stoichiometric models, such as Horváth's solvophobic theory, or non-stoichiometric models. The stoichiometric theories suggest that eluite ions and pairing ions form stoichiometric complexes either in the mobile

phase (ion-pair model) or at the stationary phase surface (dynamic ion-exchange model). The former model assumes the formation of an ion-pair in the polar eluent followed by the adsorption of this uncharged complex on the hydrophobic stationary phase. The latter model presumes that the amphiphilic ion-pair reagent molecule adsorbs together with their inorganic counter ions on the stationary phase and cause the column to behave as a dynamically generated ion exchanger. The retention of eluite ions is then assumed to be due to ion exchange with the inorganic counter ions. In a fundamental study on stoichiometric models, Knox and Hartwick[63] pointed out that formally both models lead to identical retention equations. Many variants and combinations of such stoichiometric models have been published. They have provided an easy-to-understand qualitative picture of solute retention for many chromatographers and promoted the practical use of ion-pair chromatography. However, in view of the presence of long-range electrostatic interactions, stoichiometric models of ion-pair chromatography may be inadequate because the equilibrium constant employed in these models vary on changing the electrostatic field. Therefore, a more comprehensive and accurate treatment based on the electrostatic model was proposed by Ståhlberg and co-workers[64,65] to describe the retention of ionizable eluites in RPC. In this model, the main factors that affect the retention of eluites are the surface charge density of the stationary phase and the ionic strength of the eluent. Nevertheless, stoichiometric models still remain very useful and instructive owing to its simplicity. They require no complex experiments or mathematic calculations, and allow an adequate description of the dependencies of retention on different characteristics of the eluent.

Statistical Thermodynamics

Several statistical thermodynamic investigations have been conducted to interpret the mechanism of eluite retention in RPC at the microscopic level. These theories typically evoke mean-field regular solution behavior which presumes random mixing between molecular components and limits the range of intermolecular interactions to nearest neighbor molecules and/or molecular segments. Generally, a lattice model has been utilized to estimate the configurational behavior of the mixture, taking into account differences in the molecular volume and geometry of the individual molecular components and differences between the mobile phase and the stationary phase. These theories are based on either Bragg-Williams (BW) random mixing approximation[66] or Bethe-Guggenheim (BG) quasi-chemical approximation,[67] which is a first correction to the BW approximation in lattice model based statistical thermodynamic theories of solution.

Regular Solution Theory

Regular solution theory[66,73] was adapted[68–72] to describe retention in RPC. In this theory, the partition coefficient is expressed in terms of Flory-Huggins interaction parameters for the eluite. Where enthalpic effects dominate, these parameters can be obtained from experimental data or from generalized thermodynamic functions expressed as Hildebrand's solubility parameter representing the square root of the cohesive energy density. They describe distribution processes in bulk liquids, where retention is assumed to depend on the free energy to create cavities for solvation of eluite molecules in the mobile and stationary phases. The theory describes the observed experimental trend, but is not suited for *a priori* predictions of retention versus eluent composition.

Martire's Theory

Based on Flory-Huggins' mean field lattice model for polymers in solution, Martire and Boehm[31] developed a molecular theory for RPC which takes into account the organization of the stationary phase chains. They considered the changes in the properties of the stationary phases under varying mobile phase conditions, and their influence on the retention behavior in RPC, by modeling the stationary phase as a liquid-crystalline hydrocarbon layer. Two regimes of the eluent composition were identified in which the stationary phase chains were believed to assume different geometric configurations. At high organic modifier concentration, the bonded chains are expected to be extended and oriented more or less normal to the surface, thus giving it a brush-like appearance and allowing complete penetration by the solvent and solute molecules. In contradistinction, the authors found that with water-rich mobile phases the stationary phase behaves as a quasi-liquid layer of recumbent alkyl chains that hinder solvent penetration but do not preclude solute penetration. They provided theoretical support for the latter geometric configuration under typical conditions in RPC with commonly used hydroorganic eluents and concluded that the retention process approaches that of classical liquid-liquid partitioning. By accounting for configurational entropy changes for completely flexible as well as rigid chains and upon equating the chemical potentials in the stationary and mobile phases, the equilibrium constant for distribution of the eluite between the two phases in RPC is derived in terms of binary interaction parameters of the solvents as well as the flexibility of eluites and bonded chains. Martire and Boehm[74] further refined their theory for the estimation of eluite retention in RPC by utilizing the BG quasi-chemical approximation, which is considered as a correction to the BW formalism. This was found to provide an improved microscopic description of the chromatographic retention. While the microscopic theories of RPC retention based on the application of mean-field BW and BG theories have been useful for interpreting and predicting experimental

observations and trends in the mobile phase composition dependence of eluite retention, they are too idealized to justify attempts to obtain quantitative agreement with experimental measurements of eluite retention.

Dill's Theory

Dill applied the mean field lattice theory to treat RPC both as a partition and an adsorption process.[32] He employed the lattice approach for solutes in amorphous-crystalline phases to treat partitioning in RPC by describing the grafted layer as small flexible chain molecules in different conformations, forming a layer of constant density. Unlike Martire and Boehm, his description of chain conformations is more detailed as he correctly precludes back folding of segments in a chain but neglects to consider the solvation of stationary phase ligates by mobile phase solvent. Dill treats the adsorption process in RPC separately from partitioning by assuming a planar interface surface between the stationary phase chains and the mobile phase, and envisioning the eluite molecules to interact at the interface with the tips of the chains, without intercalating between the chains. He employs the lattice monolayer approximation[75] for this treatment. Assuming a cubic lattice, the adsorption theory predicts that logarithmic retention factors in RPC depend linearly on the logarithmic equilibrium coefficients of the appropriate liquid-liquid partition system with a proportionality factor of $1/6$, since only one cubic face of the eluite surface is supposed to be in contact with the stationary phase. On the other hand, the lattice theory based on amorphous-crystalline hydrocarbon layer model predicts a similar linear dependence but with a proportionality factor of 1. Based on the comparison of the two treatments for partitioning and adsorption in RPC, Dill found that the retention in RPC was strongly correlated with liquid-liquid partitioning, thus concluding that partition is the primary mechanism of retention in RPC in a wide range of mobile phase conditions.[32] Dill's theory provides a sound interpretation of the stationary phase effects but only qualitatively explains the trends observed in RPC retention as a function of eluite, eluent, and stationary phase properties.

Klatte and Beck's[76–78] work on molecular dynamic simulations of RPC showed the existence of a number of specific interfacial effects that cannot be fully described by bulk partitioning models, such as Dill's lattice theory. Tijssen et al.[71] have also reviewed shortcomings of regular solution theories and other lattice models for the description of partitioning and adsorption in RPC.

Self Consistent Field Theory

Scheutjens and Fleer[79–81] combined DiMarzio-Rubin concept with Flory-Huggins mean field lattice theory for polymers in solution and extended it

to describe systems, such as adsorption of homopolymers on solid surfaces, that are inhomogeneous in one direction, for instance, perpendicular to a surface. This theory was further adapted to describe retention in RPC.[71,82] In this theory, all components including the grafted chains are allowed to adjust their local segment density to local conditions. This is in contrast to Dill's theory, where the segment density profile of the grafted chains is prefixed, and only the distribution of eluite in the grafted layer is found from statistical thermodynamics. An additional advantage over Dill's theory, where eluite and eluent are always monomeric, is that flexible oligomeric eluites and eluents are allowed, just as in Martire's theory. This allows one to study the retention of flexible chain molecules. In this theory, the effect of eluent quality, grafted chain length, and surface coverage on the segment density profile and shape effects besides residual adsorption effects are considered. The retention of monomeric and oligomeric eluites is studied as a function of eluent quality, grafted chain length, surface coverage, eluite chain length, and its composition and the retention factor is derived in these terms. The role of residual hydroxyls and the associated specific affinity for the solid surface and the case of mixed eluents is also treated in this theory. Although the theory accounts for specific interactions of the ligate chains and majority of the stationary phase effects, there are some experimental observations that cannot be explained on the basis of this theory, such as sharp breaks in the plots of logarithmic retention factors and the carbon number of the ligate chain. Furthermore, like all other statistical thermodynamic theories for RPC retention, it only offers a qualitative understanding of the retention process in RPC.

THERMODYNAMICS OF RPC RETENTION PROCESS

A comprehensive analysis of the retention in RPC entails the determination of the relative contributions of the enthalpy and the entropy to the free energy change of the eluites under consideration. In the following, a theoretical framework is presented first for the evaluation of thermodynamic parameters associated with the separation process in RPC from experimental data and the determination of retention factor for an ionizable eluite. Then, an expression for the dependence of the logarithmic retention factor on various chromatographic variables is derived within the hermeneutics of the solvophobic theory by taking into consideration all types of molecular interactions. The solvophobic theory is tested rigorously for its ability to predict retention and selectivity of neutral and ionizable eluites in RPC. Finally, the mechanism of RPC retention is discussed within the framework of the solvophobic theory.

Retention Equilibrium in RPC

The magnitude of eluite retention in linear elution chromatography is measured under isocratic conditions by the retention factor, k', that is evaluated directly from the chromatogram as[83]

$$k' = \frac{t_R - t_o}{t_o} \tag{1}$$

where t_R is the retention time of the eluite under consideration and t_o is the elution time for an "inert" tracer. The retention factor is related to the equilibrium constant, K, for the distribution of the eluite between the bulk mobile phase and the stationary phase as

$$k' = K\phi \tag{2}$$

where ϕ is the phase ratio of the column, i.e., the ratio of the volume of the stationary phase to that of the mobile phase. Since the chromatographic surface available for binding to eluites is believed to be heterogeneous, the measured retention factors represent average values. The Gibbs free energy change, ΔG^o, associated with eluite transfer from the mobile to the stationary phase is related to the corresponding equilibrium constant at temperature T as

$$\Delta G^o = -RT \ln K \tag{3}$$

where R is the universal gas constant. By combining Equations (2) and (3) with Gibbs-Helmholtz relation

$$\Delta G^o = \Delta H^o - T\Delta S^o \tag{4}$$

the dependence of logarithmic retention factor of the eluite on the temperature is obtained as

$$\ln k' = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R} + \ln \phi \tag{5}$$

where ΔH^o and ΔS^o are the standard enthalpy and entropy changes for the eluite transfer from the mobile to the stationary phase. When the chromatographic surface and the eluite and eluent solvent properties do not vary with the temperature, ΔH^o, ΔS^o and ϕ are also temperature invariant and the plots of $\ln k'$ versus $1/T$, the so-called van't Hoff plots, are linear. By fitting Equation (5) to the experimental van't Hoff plot, the enthalpy changes associated with the retention can be evaluated from the slopes and the entropy changes from the intercepts, provided the phase ratio of the column is known or can be estimated. On the other hand, when ΔH^o, ΔS^o and ϕ are temperature dependent, the retention data yield nonlinear van't Hoff plots. In this case the integrated form of Kirchoff's law can be employed to interpret the experimental data. Recognizing that changes in the properties of the eluites or the mobile phase proper

cause changes in the heat capacity, ΔC_p^o, of the process, which in turn results in variations in ΔH^o and ΔS^o, the following Equation can be derived for the dependence of the logarithmic retention factor on the temperature

$$\ln k' = \frac{\Delta C_p^o}{R}\left(\frac{T_H}{T} - \ln\frac{T_S}{T} - 1\right) + \ln\phi \tag{6a}$$

assuming ΔC_p^o is invariant with temperature. In this equation T_H and T_S are the temperatures at which ΔH^o and ΔS^o are zero, respectively. Equation 6a allows the evaluation of the three parameters, ΔC_p^o, T_H and T_S, from nonlinear van't Hoff plots by a least squares fitting procedure, provided the phase ratio of the column is known. Once these parameters are determined from experimental data, ΔH^o and ΔS^o can be evaluated by using the following equations:

$$\Delta S^o = \Delta C_p^o \ln\left(\frac{T}{T_S}\right) \tag{6b}$$

and

$$\Delta H^o = \Delta C_p^o (T - T_H) \tag{6c}$$

Alternatively, a quadratic form of equation can be employed to extract thermodynamic parameters from non-linear van't Hoff plots as

$$\ln k' = a + \frac{b}{T} + \frac{c}{T^2} + \ln\phi \tag{7a}$$

where the three parameters, *a, b* and *c*, can be evaluated by fitting Equation (7a) to the experimental data. The enthalpy, entropy and heat capacity changes can then be calculated with the parameters by using the following expressions:

$$\Delta H^o = -R\left(b + \frac{2c}{T}\right) \tag{7b}$$

$$\Delta S^o = R\left(a - \frac{c}{T^2}\right) \tag{7c}$$

and

$$\Delta C_p^o = \frac{2Rc}{T^2} \tag{7d}$$

Recent binding experiments of Sturtevant and co-workers[84,85] indicate that there are often significant discrepancies between van't Hoff enthalpies derived from temperature dependence of equilibrium constants and enthalpies determined directly from calorimetry. Precision calorimetric measurements with appropriate chromatographic sorbents will be required, therefore, to confirm the accuracy of thermodynamic quantities evaluated from van't Hoff plots. Recently, Huang et al.[86] have shown that adsorption energies obtained directly by

microcalorimetry measurements in hydrophobic interaction chromatography (HIC), a technique which shares many similarities with RPC, correlate well with the values calculated by the retention data in the literature via van't Hoff analysis.

The temperature dependence of retention factor as described in Equation (5) is valid for the retention of a neutral compound in RPC. A majority of eluites in RPC, however, are compounds that have ionizable functional groups, such as carboxylic acids, amino groups, or phenol groups. The retention of such compounds in RPC depends significantly on the degree of ionization of these compounds, and thus on the pH of the mobile phase. An early and very comprehensive study of the retention of ionizable compounds in RPC was published by Horváth et al.[9] They studied the effect of eluite ionization on the retention of weak acids, bases, and ampholytes and established equations that related the retention factor to the pH of the mobile phase. According to this treatment, for a simple monoprotic acid or base, the overall retention depends on three primary factors: the retention factor of the undissociated, i.e., neutral form, k_o, the retention factor of the ionized form, k_z, and the degree of dissociation of the acid or base, d as:

$$k' = \frac{k_o + k_z d}{1 + d} \tag{8a}$$

In the case of the monoprotic acid, k_o and k_z represent the retention factors of the undissociated acid and the conjugate base, respectively, and d is expressed as

$$d = \frac{K_a}{[H^+]} \tag{8b}$$

where K_a and $[H^+]$ are the acid dissociation constant and the concentration of the solvated proton in the eluent. In the case of the monoprotic base, k_o and k_z represent the retention factors of the neutral and fully ionized base, respectively, whereas d is expressed as

$$d = \frac{[H^+]}{K_b} \tag{8c}$$

where K_b and $[H^+]$ are the acid dissociation constant of the protonated base and the concentration of the hydrogen ion in the eluent. Thus, according to Equation (8a), the retention factor in RPC of a weakly acidic/basic eluite as a function of pH can be represented by a typical sigmoidal/antisigmoidal curve because the retention of the ionized species is much lower than that of the un-ionized species. The inflection point of the plot should agree with the acid-base pK_a value of the analyte.

Similarly, an expression for the retention factor of a diprotic acid can be derived as

$$k' = \frac{k_o + k_{z,1}d_1 + k_{z,2}d_1d_2}{1 + d_1 + d_1d_2} \tag{9}$$

where k_o and $k_{z,1}$ and $k_{z,2}$ are the retention factors of the undissociated, half dissociated, and fully dissociated diprotic acid and d_1 and d_2 are the corresponding degrees of dissociation in the mobile phase.

Likewise, the retention factor for zwitterionic eluites is given by

$$k' = \frac{k_o + k_{z,1}d_1 + k_{z,2}d_2}{1 + d_1 + d_2} \tag{10}$$

where k_o and $k_{z,1}$ and $k_{z,2}$ represent the retention factors of the zwitterionic, the anionic, and the cationic forms of the ampholyte and d_1 and d_2 are the corresponding degrees of dissociation.

Horváth et al.[9] were aware that the pH must be measured in the eluent used for separation. Since acid-base equilibria in the mixed solvents were more difficult to treat than in water, they limited their experiments to neat aqueous eluents.

Solvophobic Theory

When applied to RPC retention, the solvophobic theory adopts a thermodynamic cycle, as shown in Figure 2, and conveniently decomposes the RPC retention process involving an eluite A and a ligate L into two conceptual processes for the purpose of calculating the retention free energy. Thus, the standard free energy change associated with retention in RPC is expressed as

$$\Delta G^o = \Delta G^o_{solv} + \Delta G^o_{gas} \tag{11}$$

where ΔG^o_{solv} is the net standard free energy change due to solvation and ΔG^o_{gas} is the standard free energy change for the association of an eluite A

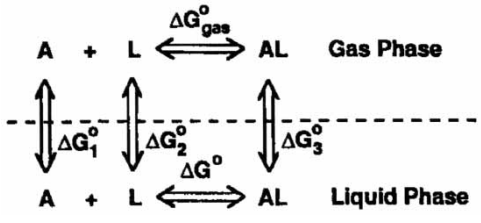

Figure 2. Thermodynamic cycle illustrating the hypothetical gas phase binding of an eluite A with the ligate L, and the solvation of the individual species in RPC. The net free energy of solvation, ΔG^o_{solv}, is given by $\Delta G^o_3 - \Delta G^o_1 - \Delta G^o_2$. Reprinted from Ref.[47].

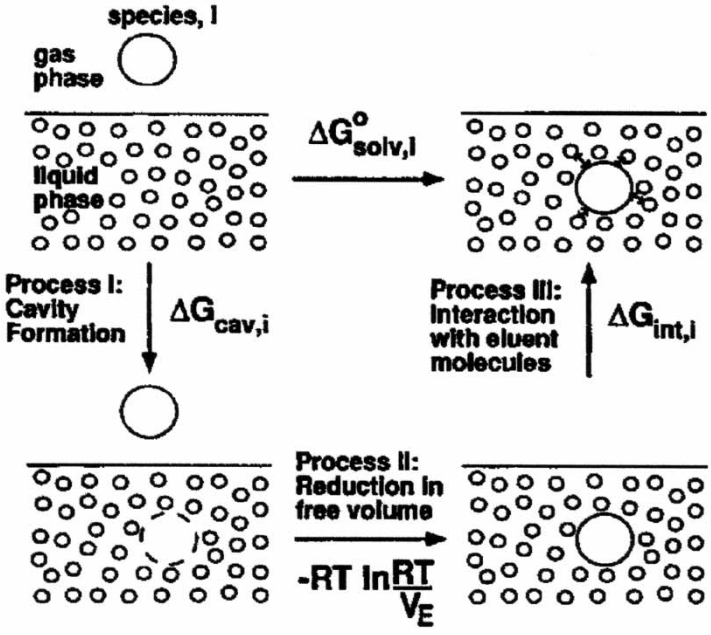

Figure 3. Schematic representation of cavity formation in the solvent, reduction in free volume and interaction of species, *i*, with neighboring solvent molecules in the process of solvation of the species. Reprinted from Ref.[47].

with the ligate L in the gas phase (vacuum). The former term indicates the mobile phase effect on the retention of an eluite A in RPC, whereas the latter term depends on the strength of eluite–ligate interactions and therefore represents the stationary phase effects.

The solvation process for each species consists of three steps as illustrated in Figure 3 – creation of a cavity in the eluent to accommodate the eluite molecule (eluent–eluent interactions), reduction in the free volume, and subsequent interaction of the eluite molecule placed in the cavity with the surrounding eluent molecules (eluent–eluite interactions). Its free energy can be expressed as[8,47]

$$\Delta G^{o}_{solv} = \Delta G_{cav} + \Delta G_{\text{int}} + \Delta\Delta G_{mix} + \Delta G_{red} - RT \ln \frac{RT}{PV} \qquad (12a)$$

where

$$\Delta G_{cav} = (\Delta G_{cav,AL} - \Delta G_{cav,A} - \Delta G_{cav,L}) \qquad (12b)$$

and

$$\Delta G_{\text{int}} = (\Delta G_{\text{int},AL} - \Delta G_{\text{int},A} - \Delta G_{\text{int},L}) \qquad (12c)$$

In Equations (12a), (12b) and (12c) the subscript AL refers to the eluite–ligate complex, $\Delta G_{cav,i}$ represents the free energy of cavity formation for species i, $\Delta G_{\text{int},i}$ is the free energy of species–eluent interactions, $\Delta\Delta G_{mix}$ is the net free energy of mixing of species and eluent molecules of different sizes, ΔG_{red} is the reduction in ΔG^{o}_{gas} due to the presence of the eluent, and the last term is the free volume change of the process.[87] In the last term, V is the molar volume of the eluent, and the pressure of the ideal gas is taken as 1 atm. It is assumed that the change in free volumes for the eluite–ligate complex and for the unbound ligate cancel each other.

The cavity formation term in Equation (12a) is the sum of hydrogen bonding and van der Waals interactions and is expressed as

$$\Delta G_{cav,i} = \Delta G_{vdw,ci} + \Delta G_{hb,ci} \tag{13}$$

where $\Delta G_{vdw,ci}$ is the free energy of van der Waals interactions and $\Delta G_{hb,ci}$ is the free energy of hydrogen bonding associated with the cavity formation of species i. Sinanoğlu has elegantly expressed the cavity term as a function of the molecular surface area of the eluite, A_A, and the surface tension of the eluent by

$$\Delta G_{cav,A} = \kappa^{g}_{E}\gamma_E A_A \tag{14}$$

where κ^{g}_{E} corrects the surface tension of the eluent, γ_E, to its micro-thermodynamic value by taking into consideration the curvature of the molecular cavity. This parameter depends on eluent properties as well as the ratio of the molecular radii of the eluite and the eluent. Similar expressions can be derived for the free energy of cavity formation of the ligate and the eluite–ligate complex. Combining all the expressions, the overall free energy change of cavity formation for RPC retention is given by

$$\Delta G_{cav} = \kappa^{g}_{E}\gamma_E \Delta A_C \tag{15a}$$

where

$$\Delta A_C = A_{AL} - A_A - A_L \tag{15b}$$

Thus, ΔA_C is the net surface area of the eluite and the ligate that is no longer accessible to the solvent upon association and is roughly twice the contact area upon binding. Since the retention process reflects the transfer of hydrocarbonaceous eluites from the eluent to the stationary phase, ΔA_C is negative and opposite in sign from previous treatment.[8] Equation (15a) is

much simpler than the earlier expression of the cavity term described in previous publications.[8–10,48]

$$\Delta G_{cav} = N\Delta A_C \gamma_E - 4.836N^{1/3}(\kappa_E^e - 1)V^{2/3}\gamma_E \qquad (15c)$$

The previously used parameter κ_E^e is replaced by κ_E^g, which has a precise relationship to κ_E^e.[87–89] The eluite–eluent interaction term in Equation 12a is the sum of van der Waals and electrostatic interactions and is expressed as

$$\Delta G_{\text{int},i} = \Delta G_{vdw,i} + \Delta G_{es,i} \qquad (16)$$

where $\Delta G_{vdw,i}$ is the free energy of van der Waals interactions and $\Delta G_{es,i}$ is the free energy of electrostatic interactions associated with the interaction of the eluent with species *i*. For the retention of hydrocarbonaceous and un-ionized eluites in RPC, the contribution of electrostatic interactions to the retention free energy is negligible compared to that of van der Waals interactions. However, in the case of ionizable eluites, such as monoprotic acids and bases, diprotic acids and zwitterionic solutes, the magnitude of electrostatic interactions largely depends on the charge distribution in the species. Therefore, this term appears to be highly significant and should be evaluated for the placement of both a permanent dipole and the corresponding ion into the cavity.

Sinanoğlu[46,90,91] used two different approaches to arrive at expressions for evaluating eluent–eluite interactions due to van der Waals forces. In the first approach, an effective Kihara potential was assumed for use in dilute solutions and $\Delta G_{vdw,A}$ was expressed in terms of ionization potentials, refractive indices, and other measurable properties of the solvent and the eluite. It was also assumed that the free energy changes for van der Waals interactions between the ligate and the eluent, $\Delta G_{vdw,AL}$, and between the eluite–ligate complex and eluent, $\Delta G_{vdw,L}$, cancel each other so that

$$\Delta G_{vdw} = -\Delta G_{vdw,A} = -0.60566 I_f D_A D_E (Q' + Q'') \qquad (17a)$$

where I_f is a function of ionization potentials for the eluent and the eluite, D_A and D_E are the Clausius-Mosotti function of refractive index for the eluite and the eluent, respectively, and Q' and Q'' are dimensionless functions that can be obtained by integrating the effective pair potential between the eluite and the eluent molecules over the total volume. The readers are referred to the article by Horváth and co-workers[8] for a detailed approach to measuring these parameters. Alternatively, $\Delta G_{vdw,A}$ can be expressed in terms of the surface area of the eluite as[46,90,91]

$$\Delta G_{vdw,A} = (\kappa_{AE}^g \gamma_{AE} - \kappa_A^g \gamma_A - \kappa_E^g \gamma_E) A_A \qquad (17b)$$

where γ_A is the surface tension of the eluite and γ_{AE} is the eluite–eluent interfacial tension; κ_A^g and κ_{AE}^g convert the respective surface or interfacial tension to the microthermodynamic value applicable to molecular

dimensions. κ^g_{AE} depends on the property of the eluent as well as the ratio of the eluite to the eluent molecular radii, while κ^g_A depends on the property of the eluite. For pure substances, κ^g_A and κ^g_{AE} can be readily evaluated from their vaporization[91] and liquid dissolution[52] data. Similar expressions can be written for the free energy of van der Waals interactions between ligate and eluent and between the eluite–ligate complex and the eluent. Combining all the expressions, the overall free energy change of van der Waals interactions in RPC is given by

$$\Delta G_{vdw} = (\kappa^g_{AE}\gamma_{AE} - \kappa^g_A\gamma_A - \kappa^g_E\gamma_E)\Delta A_C \tag{17c}$$

In deriving Equation (17c) it is assumed that[47]

$$\gamma_{AL} = \gamma_A = \gamma_L \tag{17d}$$

and

$$\gamma_{ALE} = \gamma_{AE} = \gamma_{LE} \tag{17e}$$

where γ_i and γ_{iE} represent the surface and species–eluent interfacial tensions, with i denoting the species A, L, or AL. Likewise, the corresponding κ^g values were assumed to be the same.

The electrostatic term, $\Delta G_{es,i}$, in Equation (16) has different forms for small dipoles, for small eluites carrying an electronic charge, and for macromolecules such as proteins. Detailed expressions for the electrostatic term have been given in the literature for the different types of eluites and are reviewed below.

For a small dipole, the electrostatic free energy change is given by

$$\Delta G_{es,i} = -\frac{N}{4\pi\varepsilon_o}\left(\frac{\mu_i^2}{\nu_i}\right)\frac{D}{1-(\overline{\alpha}_i/\overline{r}_i^3)D} \tag{18}$$

where ε_o is the permittivity constant, μ_i, ν_i, $\overline{\alpha_i}$ and $\overline{r_i}$ are the dipole moment, molecular volume, average polarizability, and the average molecular radius of the species, i, respectively, and D is a function of the static dielectric constant of the eluent.[9] Combining the expressions for all species, the overall electrostatic free energy change for the association process involving a dipole is given by

$$\Delta G_{es} = \left(\frac{N}{4\pi\varepsilon_o}\right)\left(\frac{1}{2} - \frac{V_A}{V_{AL}}\right)\left(\frac{\mu_A^2}{\nu_A}\right)\left(\frac{D}{1-(\overline{\alpha}_A/\nu_A)D}\right) \tag{19}$$

where V_i is the molar volume of the species, i.

On the other hand, for small eluite molecules carrying an electronic charge, the electrostatic free energy change, $\Delta G^z_{es,i}$, at very low ionic

strength can be estimated form Debye-Hückel theory as

$$\Delta G^{z}_{es,i} = \frac{Z^2 e^2 N}{4\pi\varepsilon_0\varepsilon}\left(\frac{1}{b_i} - \frac{\eta}{1+\eta a_i}\right) \tag{20a}$$

where Ze is the electronic charge of the ion, ε is the dielectric constant, b_i is the ionic radius and a_i is the distance of closest approach. The Debye-Hückel screening parameter, η, is calculated as

$$\eta^2 = \frac{4\pi e^2 N I_p^2}{\varepsilon RT} \tag{20b}$$

where e is the elementary charge and I_p is the ionization potential of the medium. In Equations (20a) and (20b), the dielectric constant ε of the eluent is used for the ionized eluite. For the complex of the ionized eluite with the fixed ligate, however, the apparent dielectric constant of the stationary phase, ε^*, has to be used instead of ε. The overall electrostatic free energy change for an eluite carrying an electronic charge at low ionic strength in RPC retention can then be expressed as[9]

$$\Delta G^{z}_{es} = \frac{Z^2 e^2 N}{4\pi\varepsilon_o\varepsilon}\left[\frac{\varepsilon - \varepsilon^*(V_{AL}/V_L)^{1/3}}{\varepsilon^*(V_{AL}/V_L)^{1/3} b_A} - \left(\frac{\varepsilon-\varepsilon^*}{\varepsilon^*}\right)\left(\frac{\eta}{1+\eta a_A}\right)\right] \tag{21}$$

In deriving this expression, it is assumed that the stationary phase ligate has no charge and therefore, $\Delta G^{z}_{es,L} = 0$. Equation (21) is expected to be valid at salt concentrations up to 0.1 M, i.e. in the domain of Debye-Hückel theory. In chromatography, the ionic strength of the eluent is usually high so that the Debye-Hückel theory is not applicable. When the ionic strength is appreciable, the following equation[9]

$$\Delta G^{z}_{es} = \frac{Z^2 e^2 N}{4\pi\varepsilon_o\varepsilon}\left[\frac{\varepsilon - \varepsilon^*(V_{AL}/V_L)^{1/3}}{\varepsilon^*(V_{AL}/V_L)^{1/3}}\right] + \left(\frac{\varepsilon-\varepsilon^*}{\varepsilon^*}\right)(BI^{1/3} + CI)RT \tag{22}$$

should be used instead of Equation (21). Here I is the ionic strength, B is a charge dependent constant and C is a charge independent constant. Lietzke et al.[92] have tabulated the values of B and C for a variety of aqueous salt solutions.

An expression to describe the free energy of electrostatic interactions between a protein and a nonpolar ligate, ΔG^{p}_{es}, can be derived by combining the Debye-Hückel theory for the protein ion, which is applicable at low ionic strength, and Kirkwood's expression for the protein dipole, which is appropriate at high ionic strength, as[53]

$$\Delta G^{p}_{es} = \frac{Xm^{1/2}}{1+Ym^{1/2}} + Z\mu_p m - A \tag{23}$$

where m is the molality of the salt and μ_p is the dipole moment of the protein and the constants X, Y and Z are described in Ref.[53]. Both constants A and X are proportional to the net charge on the protein at low ionic strength and A is inversely proportional to the size of the macromolecule. When the protein has no net charge, i.e., at the isoelectric point, the first two terms on the right hand side of Equation (23) will vanish. Away from the isoelectric point, the relationship is nonlinear at low ionic strength, but with increasing ionic strength the second term approaches a constant value and Equation (23) becomes linear in salt concentration.

For ionized eluites, the free energy of gas phase adsorption will not be the same as that for a neutral eluite because the charged eluite may induce a dipole moment in the stationary phase. Thus the electrostatic effect on gas phase adsorption, $\Delta G^z_{gas,es}$, can be conveniently accounted for in the following expression for free energy of adsorption of an ionized eluite in the gas phase, ΔG^z_{gas}, as

$$\Delta G^z_{gas} = \Delta G^o_{gas} + \Delta G^z_{gas,es} \tag{24}$$

where ΔG^o_{gas} represents the free energy change for the adsorption of an unionized eluite in the gas phase.

The reduction term in Equation (12a) represents the reduction of gas phase van der Waals and electrostatic interactions in the presence of eluent molecules owing to three- and many-body interactions and is given by[91]

$$\Delta G_{red} = -I'_f M \frac{D_E}{1 + D_E} \Delta G^o_{gas} \tag{25a}$$

for an unionized eluite, where I'_f is a function of ionization potentials of the eluite, ligate, and the eluent, M is a dimensionless geometric function of the average radii of eluite and the distance between centers of eluite and ligate, relative to the ligate radius. These can be calculated from expressions given in Ref.[47]. For the ionized eluite this expression becomes[9]

$$\Delta G^z_{red} = -I'_f M \frac{D_E}{1 + D_E} \Delta G^0_{gas} + \frac{1 - \varepsilon}{\varepsilon} \Delta G^z_{gas,es} \tag{25b}$$

Thus, in the absence of silanophilic interactions, and assuming the free energy change of mixing, $\Delta\Delta G_{mix}$, to be negligible, one can derive expressions for the retention free energy change in RPC for the case of a neutral nonpolar eluite, a dipole, an ionized eluite, as well as a protein. These expressions in terms of the logarithmic retention factor can be written as follows:

In the case of the retention of a neutral nonpolar eluite in RPC, electrostatic interactions may be neglected to obtain the following expression for

the logarithmic retention factor

$$\ln k' = -\frac{N\Delta A_C \gamma_E}{RT} + \frac{4.836N^{1/3}(\kappa_E^e - 1)V^{2/3}\gamma_E}{RT} + \frac{0.60566 I_f D_A D_E (Q' + Q'')}{RT} - \frac{(1 - I_f' M D_E/1 + D_E)\Delta G_{gas}^o}{RT} + \ln \frac{RT}{PV} + \ln \phi \tag{26}$$

by combining Equations (2), (3), (11), (12a), (15c), (17a) and (25a).

The expression for the retention factor, k_o, of a dipole in RPC, takes the form

$$\ln k_o = -\frac{N\Delta A_C \gamma_E}{RT} + \frac{4.836N^{1/3}(\kappa_E^e - 1)V^{2/3}\gamma_E}{RT} + \frac{0.60566 I_f D_A D_E (Q' + Q'')}{RT} - \frac{(N/4\pi\varepsilon_o)(1/2 - V_A/V_{AL})(\mu_A^2/\nu_A)(D/1 - (\overline{\alpha}_A/\nu_A)D)}{RT} - \frac{(1 - I_f' M(D_E/1 + D_E))\Delta G_{gas}^o}{RT} + \ln \frac{RT}{PV} + \ln \phi \tag{27}$$

by adding to Equation (26) the electrostatic term expressed by Equation (19) for the dipole.

The retention factor of its ionized form, k_z, is given by combining Equations (2), (3), (11), (12a), (15c), (17a), (24) and (25b) with Equation (21) as

$$\ln k_z = -\frac{N\Delta A_C \gamma_E}{RT} + \frac{4.836N^{1/3}(\kappa_E^e - 1)V^{2/3}\gamma_E}{RT} + \frac{0.60566 I_f D_A D_E (Q' + Q'')}{RT} - \frac{(Z^2 e^2 N/4\pi\varepsilon_o\varepsilon)[(\varepsilon - \varepsilon^*(V_{AL}/V_L)^{1/3})/(\varepsilon^*(V_{AL}/V_L)^{1/3} b_A) - ((\varepsilon - \varepsilon^*)/\varepsilon^*)(\eta/1 + \eta a_A)]}{RT} - \frac{(1 - I_f' M(D_E/1 + D_E))\Delta G_{gas}^o}{RT} - \frac{\Delta G_{gas,ez}^z}{\varepsilon RT} + \ln \frac{RT}{PV} + \ln \phi \tag{28}$$

at low ionic strength, and by combining Equations (2), (3), (11), (12a), (15c), (17a), (24) and (25b) with Equation (22) as

$$\ln k_z = -\frac{N\Delta A_C \gamma_E}{RT} + \frac{4.836N^{1/3}(K_E^e)V^{2/3}\gamma_E}{RT} + \frac{0.60566 I_f D_A D_E (Q' + Q'')}{RT} - \frac{(Z^2 e^2 N/4\pi\varepsilon_0\varepsilon)[(\varepsilon - \varepsilon^*(V_{AL}/V_L)^{1/3})/\varepsilon^*(V_{AL}/V_L)^{1/3}]}{RT} - \left(\frac{\varepsilon - \varepsilon^*}{\varepsilon^*}\right)(BI^{1/3} + CI) - \frac{(1 - I_f' M(D_E/1 + D_E))\Delta G^0_{gas}}{RT} - \frac{\Delta G^z_{gas,ez}}{\varepsilon RT} + \ln\frac{RT}{PV} + \ln\phi \quad (29)$$

at high ionic strength.

Protein retention in RPC can be described by combining Equations (2), (3), (11), (12a), (15c), (17a), (24) and (25b) with Equation (23) as

$$\ln k' = -\frac{N\Delta A_C \gamma_E}{RT} + \frac{4.836N^{1/3}(\kappa_E^e - 1)V^{2/3}\gamma_E}{RT} + \frac{0.60566 I_f D_A D_E (Q' + Q'')}{RT} - \frac{(Xm^{1/2}/1 + Ym^{1/2}) + Z\mu_p m - A}{RT} - \frac{(1 - I_f' M D_E/1 + D_E)\Delta G^o_{gas}}{RT} - \frac{\Delta G^z_{gas,ez}}{\varepsilon RT} + \ln\frac{RT}{PV} + \ln\phi \quad (30)$$

It is noted that alternate expressions for the cavity term in Equation (15a) and the van der Waals interaction term in Equation (17a) may also be used to calculate the retention factor of eluites in RPC. In summary, Equations (26)–(30) describe the retention of nonpolar eluites, dipoles, ionized eluites as well as proteins as a function of eluent, eluite, and stationary phase properties. As can be seen from the above discussion, the solvophobic theory offers a powerful approach to the analysis of RPC retention with eluites of wide polarity in which electrostatic, hydrogen bonding and hydrophobic interactions are amalgamated.

Test of the Predictive Power of the Solvophobic Theory

In the following, several severe tests are presented for the solvophobic theory in analyzing and predicting the retention and selectivity of various eluites in RPC. As will be seen, the theory provides a quantitative understanding of the retention energetics in RPC. The predictive power of the solvophobic

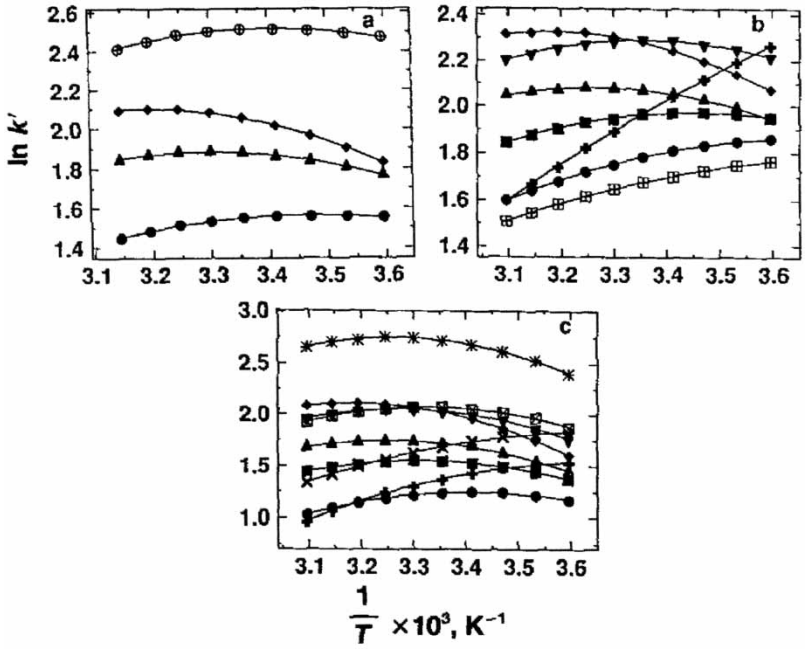

Figure 4. Graph illustrating curvilinear van't Hoff plots for the retention of dansyl amino acids in HIC. (a) Column: Spherogel (100 × 4.6 mm, 5 μm); mobile phase: 1.25 M ammonium sulfate in 0.05 M sodium phosphate, pH 7.0; flow rate: 1.5 mL/min; (b) Column: SynChropak propyl (100 × 4.6 mm, 6.5 μm); mobile phase: 0.7 M ammonium sulfate in 0.05 M sodium phosphate, pH 7.0; flow rate: 2.0 mL/min. (c) Column: TSK-GEL butyl-NPR (35 × 4.6 mm, 5 μm); mobile phase: 1.25 M ammonium sulfate in 0.05 M sodium phosphate, pH 7.0; flow rate: 1.0 mL/min; symbols represent dansyl derivatives of (+) glycine, (●) alanine, (■) α-amino n-butyric acid, (▼) norvaline, (▲) valine, (◆) leucine, (×) γ-aminobutyric acid, (⊕) phenylalanine, (⊞) proline, (✳) norleucine, (⊠) methionine; Solid lines represent the best fits of Equation (6a) to the data. Reprinted from Ref.[95].

theory can be attributed to its eclectic nature as well as its ability to describe RPC retention in terms of measurable physicochemical parameters.

Temperature Dependence

Our current understanding of the hydrophobic effect has been facilitated by observations made with the solubility of nonpolar substances in aqueous systems. Such systems have yielded nonlinear solubility curves indicating a large negative entropy change at room temperature and a large positive heat

capacity upon transferring a nonpolar solute into water. In contrast to electrostatic or hydrogen bond interactions, the magnitude of hydrophobic interactions are expected to first increase with increasing temperature driven mainly by the entropic effects, until it reaches a maximum arising from competing enthalpic effects, followed then by a decrease with increasing temperature dominated largely by enthalpic effects. Since the effect of temperature on the chromatographic retention factor is expressed by the van't Hoff equation, this concept would predict that the retention of an eluite in chromatographic systems where hydrophobic interactions are dominant should first increase with increasing temperature, reach a maximum, and then decrease with increasing temperature. Indeed, curvilinear van't Hoff plots shown in Figure 4 have been observed with the retention of dansyl amino acids in hydrophobic interaction chromatography (HIC), a technique similar to RPC but employing mildly hydrophobic stationary phases and aqueous salt solution to modulate retention.[93–95] The HIC retention process was found to be entropically driven at room temperature but enthalpically driven at higher temperatures. Furthermore, the magnitude of retention was determined to be linearly dependent on the nonpolar surface area of the dansyl amino acids, thus confirming the dominant role of hydrophobic interactions in governing the retention of eluites in HIC. Since hydrophobic interactions are believed to also dominate the retention behavior of nonpolar eluites in RPC, similar dependence of the retention factor on temperature would be expected.

On the contrary, van't Hoff analysis of small nonpolar eluites in RPC has yielded ambiguous results. The general observation is that retention decreases with increasing temperature in RPC when using a hydrocarbonaceous stationary phase, which manifests itself in the form of a linear van't Hoff plot.[96–98] Two features of RPC retention become apparent here when compared to HIC retention. First, RPC stationary phases employ alkyl chains (C_1 to C_{18}) grafted on silicious support containing residual silanols that may result in a mixed mode of interaction, whereas HIC employs butyl, octyl, or phenyl chains attached to a hydrophilic polymer backbone such as agarose or cross-linked dextran. Second, retention in RPC is usually modulated by hydro-organic mixtures, whereas aqueous solution with varying concentration of a salt is employed to drive HIC retention. Thus, the marked difference observed in the van't Hoff plots in RPC and HIC can be due to either the presence of an organic modifier in the mobile phase of RPC systems which tends to reduce the cohesive energy of water, or the counteracting effect of hydrogen bonding between the eluite and the residual silanols on the RPC stationary phase, or both. It would therefore be interesting to investigate model RPC systems where such effects can be minimized in order to better understand the role of hydrophobic interactions in RPC.

Unfortunately, very few studies have been conducted to explicate the effect of temperature on retention thermodynamics of small molecules using

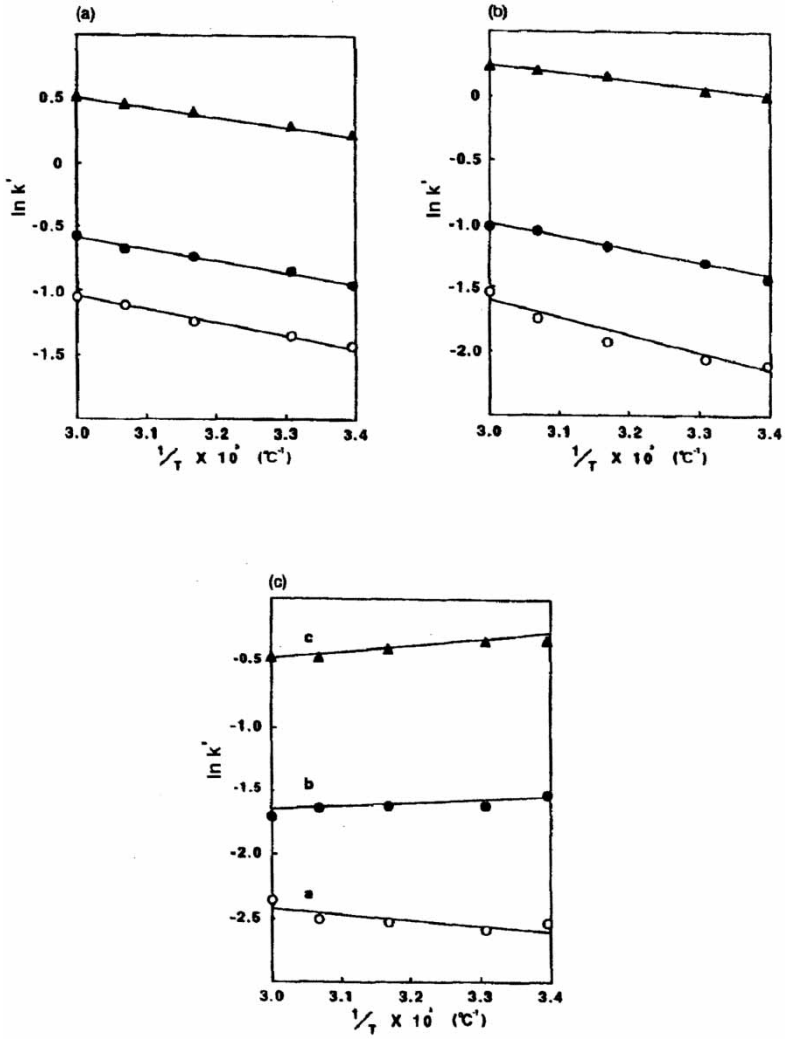

Figure 5. van't Hoff plots of (○) ethanol, (●) isopropanol and (▲) butanol on a porous copolymer stationary phase devoid of residual silanols using (a) water, (b) water: methanol (90:10, v/v) and (c) water:methanol (70 : 30, v/v) mobile phase systems. Reprinted from Ref.[99].

such model systems. If silanophilic interactions are the cause for the atypical van't Hoff behavior in RPC, then some insight may be gleaned from C_{18} derivatized polymer based columns such as PS-DVB since these lack free silanol groups. This purely hydrophobic stationary phase should reveal a curvilinear

van't Hoff plot or, at the very least, a positive temperature dependence of the retention factor. Such a behavior was in fact observed as shown in Figure 5 with the RPC retention of aliphatic alcohols in both purely aqueous mobile phase and 90/10 water/methanol system on a porous copolymer stationary phase devoid of residual silanols.[99]

Investigating the temperature dependence of the retention factor in water-rich mobile phases and the effect of organic modifier on van't Hoff curvature could also shed light on the role of hydrophobic interactions in RPC. But paucity of such data in water-rich mobile phases has generally precluded a comprehensive analysis of the van't Hoff behavior in RPC as a function of the organic modifier concentration. In one study, van't Hoff plot analysis was employed to investigate the retention of benzodiazepines in RPC.[100] It was found that the enthalpies of transfer were negative for all mobile phases examined, but that the entropy contribution to retention became more significant as eluent polarity decreased. Retention behavior with positive temperature dependence has also been revealed with a limited combination of solutes and eluents.[101] In another study, the van't Hoff plots for benzene on an octadecylsilyl silica gel column with a bonded ligand density lower than 2.84 $\mu mol/m^2$ were linear over a wide temperature range (268–353 K) but when the bonded ligand density was $\geq 3.06\,\mu mol/m^2$, nonlinear relationships were observed.[102,103] Recently, DeVido et al.[104] have also found that the retention of natural amino acids on low bonding density stationary phases using aqueous phosphate buffer is characterized by nonlinear (concave down) van't Hoff plots. These studies in RPC with water-rich mobile phase systems may allude to the hydrophobic effect as being the key driver in governing the retention of such eluites. However, the argument is not convincing enough owing to concerns raised by Coym and Chester[105] in a recent article. They indicate that in most investigations involving the thermodynamics of transfer of an eluite from the mobile phase to the stationary phase in RPC, phase ratio is nearly always assumed to be constant. When non-linear van't Hoff plots are observed, it is often attributed to the temperature dependence of enthalpy and entropy of transfer. However, when the possibility of a change in the phase ratio is considered, it is possible that non-linear van't Hoff behavior may or may not be due to changes in enthalpy or entropy. Coym and Chester presented mathematical evidence that phase ratio changes, if they occur, can cause deviation from linearity in a van't Hoff plot. Thus, temperature-dependent phase ratio changes, and not necessarily changes in the transfer enthalpy, may be responsible for the curved van't Hoff plots of the eluites observed in certain RPC systems.

The solvophobic theory, being general in terms of its applicability to hydoorganic mixtures when compared to the concept of the hydrophobic effect, provides an ideal framework to describe the retention of eluites in RPC as a function of temperature. According to the solvophobic

theory, Horváth et al.[8] expressed the standard enthalpy change for a dipole in RPC as

$$
\begin{aligned}
-\Delta H^o = & -\Delta H^o_{gas} + \Delta H_{vdw,A} \\
& + \left(\frac{N}{4\pi\varepsilon_o\varepsilon}\right)\left(\frac{1}{2} - \frac{V_A}{V_{AL}}\right)\left(\frac{D}{1-(\overline{\alpha}_A/\nu_A)D}\right)\left(1 - \frac{d\ln D}{d\ln T} + \upsilon_A T\right) \\
& - N\Delta A_C \gamma_E \left(1 - \frac{d\ln\gamma_E}{d\ln T} - \frac{2}{3}\upsilon_A T\right) - RT(1-\upsilon_E T) \\
& + 4.836 N^{1/3}(\kappa^e_E - 1)V^{2/3}\gamma_E\left(1 - \frac{d\ln\gamma_E}{d\ln T} - \frac{2}{3}\upsilon_A T - \frac{d\ln(\kappa^e_E - 1)}{d\ln T}\right)
\end{aligned}
\tag{31}
$$

by assuming the phase ratio to be temperature independent. In Equation (31), ε_i represents the liquid coefficient of thermal expansion for the species *i*. Equation (31) indicates that the enthalpy change in RPC retention is due to a balance of large enthalpy changes associated with the opposing forces of eluite–ligate, eluite–eluent, and eluent–eluent (cavity reduction) interactions. The explicit temperature dependence of enthalpy change in equation implies that van't Hoff plots for the retention factor would be curved. This curvature is predicted to be more pronounced in water-rich mobile phases and is expected to reduce in organic-rich mobile phases. Furthermore, the temperature dependence of electrostatic interactions represented by the third term in Equation (31) tends to counteract that of cavity reduction denoted by the fourth and the sixth terms in Equation (31). A closer examination of the temperature dependent terms of the equation shows, however, that in most cases the deviation from linearity is relatively small. The estimated variation of the temperature dependent terms for a typical eluite and water show that the expected change in the enthalpy over a temperature range of 50°C is less than 5%. Consequently, the enthalpy change in a temperature interval of chromatographic interest is probably small enough to yield linear van't Hoff plots in most cases. According to Equation (31) the enthalpy change could be negative if its value would be dominated by the second and/or fourth and/or sixth terms. The fourth term increases with the contact area, consequently, it could be larger when both the ligate and the eluite are large molecules. The magnitude of the sixth term is comparable to the other terms in Equation (31). If they are relatively small, however, the sixth term, because of its negative sign, could make enthalpy change negative. Depending on the magnitude of eluite–ligate, eluite–eluent, and eluent–eluent (cavity reduction) interactions, negative enthalpy change may be observed that is sufficiently large to overcome the corresponding entropy effects and dominate the retention energetics, thus explaining the unique van't Hoff behavior of RPC retention.

The effect of temperature on the retention of macromolecules, such as proteins and peptides, in RPC is more difficult to interpret owing to a complex mix of phenomena governing their retention energetics. As seen from Equation (30), both the hydrophobic and electrostatic interactions are significant and compete with each other as far as the temperature dependence of protein retention is concerned. Additionally, intramolecular interactions between amino acid groups in a protein molecule, as well as protein interactions in the presence of salts, may play a significant role in the retention process and must be accounted for in any interpretation of the van't Hoff plots. Indeed, conformational changes in proteins interacting with RPC surfaces have been well documented.[106-109] For instance, hen egg white lysozyme was shown by NMR and isotope-exchange techniques to unfold upon adsorption on the RPC surface.[110] Recently, Hearn and coworkers[111] have studied the retention of a pair of polypeptide isomers on an octylsilyl column in acetonitrile/water and methanol/water mobile phase systems and found that the van't Hoff plots are nonlinear, indicating a significant heat capacity effect. On the other hand, they observed nonlinear van't Hoff plots as shown in Figure 6 for the retention of N-acetyl-L-α-phenylalanine ethyl ester and several other polypeptides, such as bombesin,

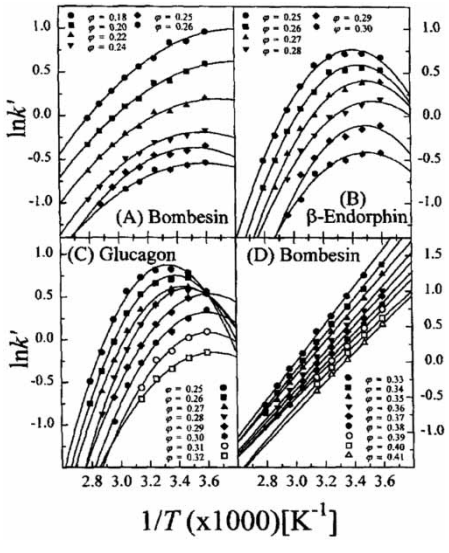

Figure 6. van't Hoff plots for (A) bombesin, (B) β-endorphin, (C) glucagon using acetonitrile-water mixtures containing 0.1% TFA, and for (D) bombesin using methanol-water mixtures containing 0.1% TFA, with a n-butyl RPC stationary phase. The plots show the experimental data and the lines of best fit according to Equation (7a). Reprinted from Ref.[112].

β-endorphin, and glucagons, with butylsilyl silica gel in acetonitrile/water mobile phase systems only.[112] It appears that the nature and composition of organic modifier in the mobile phase influences van't Hoff plot characteristics, even with relatively smaller molecules, such as N-acetyl-L-α-phenylalanine ethyl ester.

Effect of Molecular Size of the Eluite

Despite many simplifying assumptions, the equations derived from the solvophobic theory to describe RPC retention are quite involved, as can be seen from the expressions derived for a nonpolar eluite, a dipole, a charged eluite, and a protein in Equations (26)-(30). Nevertheless, under certain conditions when only one variable is changed some of the terms in these equations remain fairly constant so that further simplification is possible. To examine the dependence of the retention factor of a set of neutral and ionogenic eluites on their molecular structure when a fixed temperature, column, and eluent condition is used, we look at the molecular structure dependence of the individual terms in Equations (26) through (29). The magnitude of both eluite–eluent and eluite–ligate van der Waals interactions is expected to vary linearly with contact surface area at the same extent, so that changes in these two terms nearly cancel each other because of their opposite sign. Furthermore, electrostatic interactions between the eluent molecules and dipoles or ionized eluites is expected to be similar for closely related eluites and, therefore, remain constant as the contact surface area increases. Although the contact surface area between the eluite and the stationary phase is not known, as a first approximation it may be expected to increase proportionally with the increase in hydrocarbonaceous or nonpolar surface area, A_{np}, of the eluite as

$$\Delta A_C = \alpha A_{np} \tag{32}$$

Thus, Equations (27)–(30) may be simplified further as[8]

$$\ln k' = A' A_{np} + B' \tag{33}$$

where A' and B' are constants for closely related eluites at fixed eluent and stationary phase conditions. Thus, the solvophobic theory predicts a linear dependence of the logarithmic retention factor on the nonpolar surface area of the eluite for closely related eluites, according to Equation (33). Since A' is proportional to the surface tension of the eluent, plots of classes of eluites are expected to show identical slopes at fixed eluent condition. Furthermore, for a given set of closely related eluites, the slope A' is expected to be dependent on the eluent property. On the other hand, the intercept B' is expected to be different for different classes of eluites. Figure 7 illustrates the plots of logarithmic retention factor versus nonpolar surface area for the

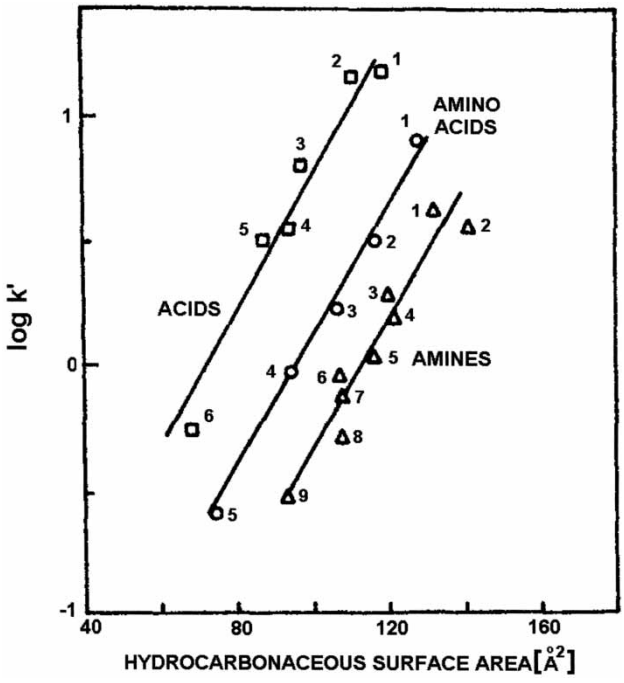

Figure 7. Graph illustrating the relationship between logarithmic retention factor and the hydrocarbonaceous surface area of different classes of eluites in RPC. Column: Partisil 1025 ODS; eluent: 1.0 M Na_2SO_4 in 0.1 M phosphate buffer, pH 2.05; flow rate: 1.0 mL/min; temperature: 25°C acids: (1) homovanillic acid, (2) phenylacetic acid, (3) 4-hydroxyphenylacetic acid, (4) mandelic acid, (5) 3,4-dihydroxyphenylacetic acid, (6) 3,4-dihydroxymandelic acid; Amino acids: (1) tryptophan, (2) phenylalanine acid, (3) tyrosine, (4) 3,4-dihydroxyphenylalanine, (5) 3,4-dihydroxyphenylserine; Amines: (1) phenylethylamine, (2) 3-O-methyldopamine, (3) phenylethanolamine, (4) tyramine, (5) normetanephrine, (6) dopamine, (7) norphenylephrine, (8) octopamine, (9) norepinephrine. Reprinted from Ref.[8].

retention of undissociated acids, zwitterionic amino acids, and protonated amines in RPC employing aqueous mobile phase and C_{18} stationary phase. As seen, the plots are linear with identical slopes but having different intercept values, thus confirming the prediction by the solvophobic theory. By fitting Equation (33) to the experimental RPC data, Horváth et al.[8] have evaluated the slopes of the linear plots and estimated the contact surface area to be approximately 35% of the hydrocarbonaceous surface area of the eluites in aqueous buffers. Belfort et al.[113] have also reported contact surface area values in the range of 20–30% of the total cavity surface area of an eluite for liquid phase systems consisting of activated charcoal and water. The different intercept values observed in Figure 7 have

been readily interpreted in terms of the relative magnitude of electrostatic interactions for these families of eluites.[8] For each family of eluites the magnitude of electrostatic interactions is the same, but it is different for different families of eluites. If the logarithmic retention factors of various eluites were to be corrected for electrostatic interactions and then plotted against the nonpolar surface area of the eluites, the solvophobic theory would predict all the eluites to fall on a single straight line. This is confirmed when retention factors of various classes of eluites are corrected for electrostatic interactions and plotted against the nonpolar surface area, as shown in Figure 8. The effect of eluent property on the slope of logarithmic retention factor versus nonpolar surface area plots is shown in Figure 9. When the eluent condition is varied, the surface tension would change so that the slopes of the linear plots for closely related eluites will no longer be identical according to the solvophobic theory. This is observed with experimental data plotted in Figure 9 for the retention of a set of alkylbenzenes in RPC when the organic modifier concentration is varied. Similar predictions could be made for the retention of peptides and proteins in RPC. Hearn et al.[112] have evaluated the free energy changes for the retention of polypeptides in RPC and found that they are linear functions of the hydrophobic surface area of the eluites, in accord with the solvophobic theory.

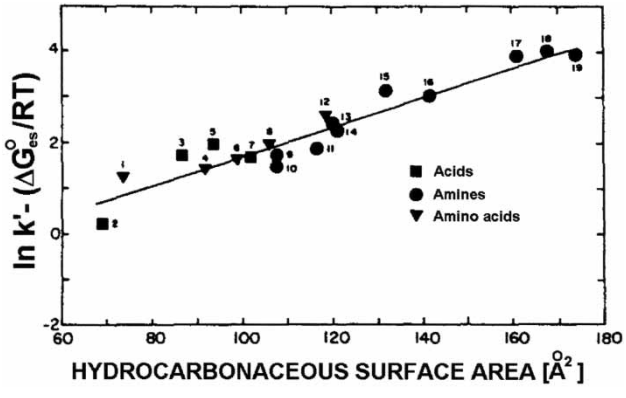

Figure 8. Graph illustrating the relationship between logarithmic retention factor corrected for electrostatic effects and the hydrocarbonaceous surface area of different classes of eluites in RPC. Column: Partisil 1025 ODS; eluent: 1.0 M Na_2SO_4 in 0.1 M phosphate buffer, pH 2.05; flow rate: 1.0 mL/min; temperature: 25°C; (1) anthranilic acid, (2) 3,4-dihydroxymandelic acid, (3) 3,4-dihydroxyphenylacetic acid, (4) 3,4-dihydroxyphenylalanine, (5) mandelic acid, (6) 4-aminophenylacetic acid, (7) vanillmandelic acid, (8) tyrosine, (9) norepinephrine, (10) octopamine, (11) normetanephrine, (12) phenylalanine, (13) phenylethanolamine, (14) tyramine, (15) phenylethylamine, (16) 3-O-methyldopamine, (17) paranephrine, (18) N-methylphenylethylamine, (19) ephedrine. Reprinted from Ref.[8].

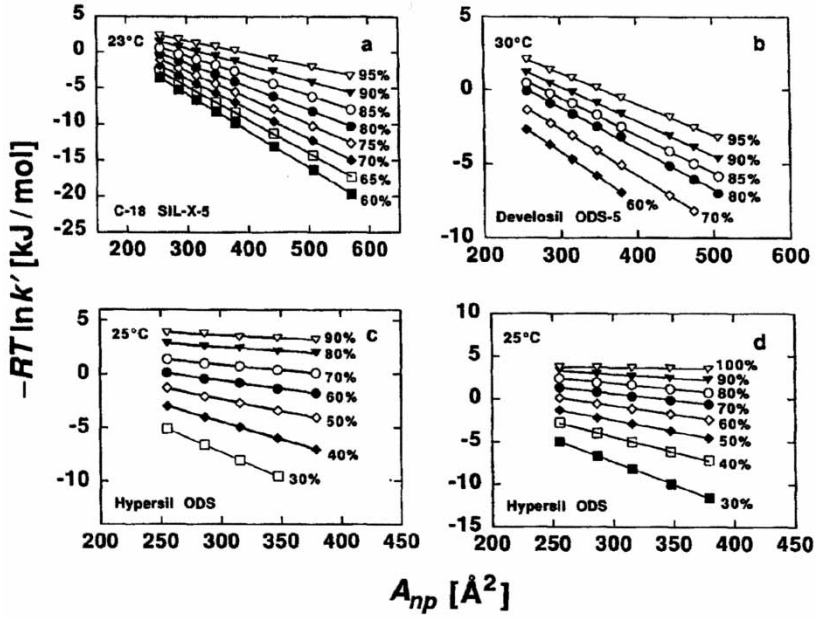

Figure 9. Effect of varying the concentration of (a) methanol, (b) acetonitrile, (c) tetrahydrofuran, and (d) 2-propanol in the hyrdoorganic mobile phase on the slopes of linear plots between logarithmic retention factor and nonpolar surface area of alkylbenzenes. From Ref.[47].

Equation (33) can manifest itself in other forms involving a different measure of the molecular structure. Linear relationships between logarithmic retention factor and carbon number of an eluite, or between logarithmic retention factor and the number of methylene units in an eluite, are well established for a set of closely related compounds and have been used often to describe hydrophobic interactions in RPC.

Prediction of Hydrophobic Selectivity

Hydrophobic selectivity, defined as the ability of the RPC retention process to selectively distinguish between nonpolar eluites, is measured in terms of an increment in free energy change due to an increment in the nonpolar surface area or the number of methylene units of an eluite. It is an important physicochemical parameter that characterizes processes driven by the hydrophobic effect. For instance, the hydrophobic selectivity was found to be the same for the HIC retention process and the dissolution of nonpolar gases in water, thus indicating mechanistic similarity of the two disparate processes.[52] It is therefore of interest to measure such parameters in RPC

in order to gain a better understanding of the role of hydrophobic interactions in the retention process. As discussed in the previous section, a linear relationship given by Equation (33) is observed between the logarithmic retention factor and nonpolar surface area for a set of closely related nonpolar eluites in RPC, when other chromatographic variables are kept constant. The hydrophobic selectivity is then given by A' and can be estimated from the slopes of the linear plots. In free energy terms, hydrophobic selectivity can be expressed as

$$\frac{\partial \Delta G^o}{\partial A_{np}} = a_g \tag{34}$$

According to the solvophobic theory, electrostatic interactions in Equations (26)–(29) may be considered negligible for closely related nonpolar eluites. Using alternate expressions for the cavity term [Equation (15a)] and the van der Waals interactions term [Equation (17a)] in Equations (26)–(29) and differentiating with respect to the nonpolar surface area, the following expression for hydrophobic selectivity in RPC can be derived as[47]

$$a_g = (\kappa_A^g \gamma_A - \kappa_{AE}^g \gamma_{AE}) + (1 - I_f' M \frac{D_E}{1 + D_E}) a_g^{gas} \tag{35}$$

where a_g^{gas} is the hydrophobic selectivity of the bonded stationary phase in gas chromatography. Here, the sign of the first term has been changed in light of the fact that ΔA_C is negative in sign according to Equation (15b) whereas A_{np} is positive in sign. It is further assumed that the κ^g values are invariant with nonpolar surface area. Equation (35) thus affords the estimation of hydrophobic selectivity in RPC as a function of eluent property, such as the surface tension. The first two terms can be determined from experimental data for the transfer of nonpolar compounds from the hydroorganic liquid phase, such as those used in RPC, to the gas phase, whereas the third and the fourth term can be measured from gas chromatographic experiments employing RPC stationary phases and nonpolar compounds.[47] Using such non-liquid chromatographic data, Vailaya and Horváth[47] estimated the hydrophobic selectivity in RPC employing C_{18} bonded phases for four commonly used hydroorganic mobile phase systems in the entire range of the organic modifier concentration. The a_g values estimated by the solvophobic theory were then compared to the a_g values obtained experimentally by analyzing a large body of RPC data from various laboratories. Figure 10 illustrates the comparison of theoretically and experimentally calculated hydrophobic selectivity values on C_{18} bonded phases for the four mobile phase systems. It is seen that the solvophobic theory predicts the hydrophobic selectivity in RPC reasonably well in the entire range of the organic modifier concentration for the four eluent systems.

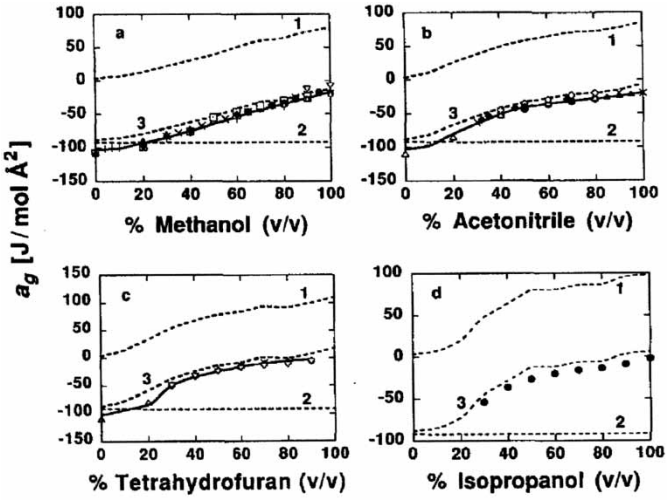

Figure 10. Test of the predictive power of the solvophobic theory for the estimation of hydrophobic selectivity in RPC employing C_{18} bonded phases and (a) methanol, (b) acetonitrile, (c) tetrahydrofuran, and (d) 2-propanol as the organic modifier. Plot 1 represents the first term while Plot 2 represents the second term in Equation (35). Plot 3 is the theoretically calculated value of hydrophobic selectivity using Equation (35). The symbols represent a large body of experimental RPC data obtained with nonpolar homologues in various laboratories. The solid curve represents the average arithmetic values of hydrophobic selectivity obtained with alkanes, alkanols, methyl alkanoates, alkyl chlorides, and alkyl benzenes on LiChrosorb RP18 and Hypersil ODS columns. Reprinted from Ref.[47].

Effect of Organic Modifier in the Eluent

When only the eluent composition changes, we can assume that the eluite and the ligate properties as well as eluite–ligate interactions are invariant so that Equation (27) for a neutral dipole can be simplified to[8]

$$\ln k' = A' + B'D + C'\gamma_E + D'(\kappa_E^e - 1)V^{2/3}\gamma_E + E' + \ln\frac{RT}{PV} \qquad (36a)$$

where

$$A' = \ln\phi - \frac{(1 - I_f' M(D_E/1 + D_E))\Delta G_{gas}^o}{RT} \qquad (36b)$$

$$B' = \frac{(N/4\pi\varepsilon_o)((1/2) - (V_A/V_{AL}))(\mu_A^2/\nu_A)(1/(1 - (\overline{\alpha}_A/\nu_A))}{RT} \qquad (36c)$$

with the approximation that $D = 1$

$$C' = -\frac{N\Delta A_C}{RT} \tag{36c}$$

$$D' = 4.836\frac{N^{1/3}}{RT} \tag{36d}$$

$$E' = \frac{0.60566 I_f D_A D_E (Q' + Q'')}{RT} \tag{36e}$$

When hydroorganic eluents are employed to modulate retention in RPC, all terms in Equation 36a are dependent on the eluent properties, with the exception of the first term. On the other hand, when salts are employed, only the surface tension may be assumed to change for the sake of simplicity. Figure 11 illustrates the variation of surface tension as a function of organic modifier concentration as well as salt concentration in aqueous solutions. Thus, the retention factor is expected to increase with salt concentration of the eluent and decrease with the water concentration when hydroorganic solvents are employed as eluents. The dependence of various terms in Equation (36a) on the organic modifier concentration in the hydroorganic eluent is also illustrated in Figure 12. Whereas both the surface tension and $\ln(RT/PV)$ decrease with increasing concentration of the organic modifier in the mobile phase, κ_E^e goes through a maximum and D is practically constant.

The effect of the organic modifier concentration in the eluent on the experimental retention factor of o-toluic acid in RPC employing octadecylsilyl

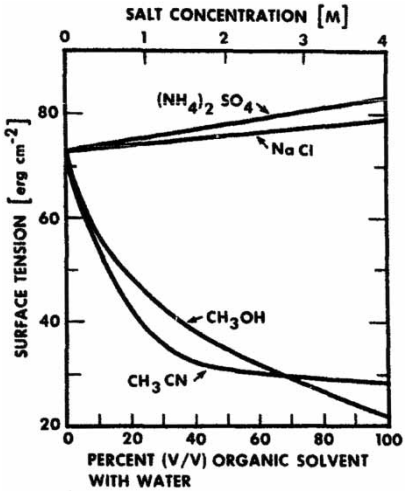

Figure 11. Dependence of surface tension on the composition of organic modifier and salt in aqueous solutions. Reprinted from Ref.[8].

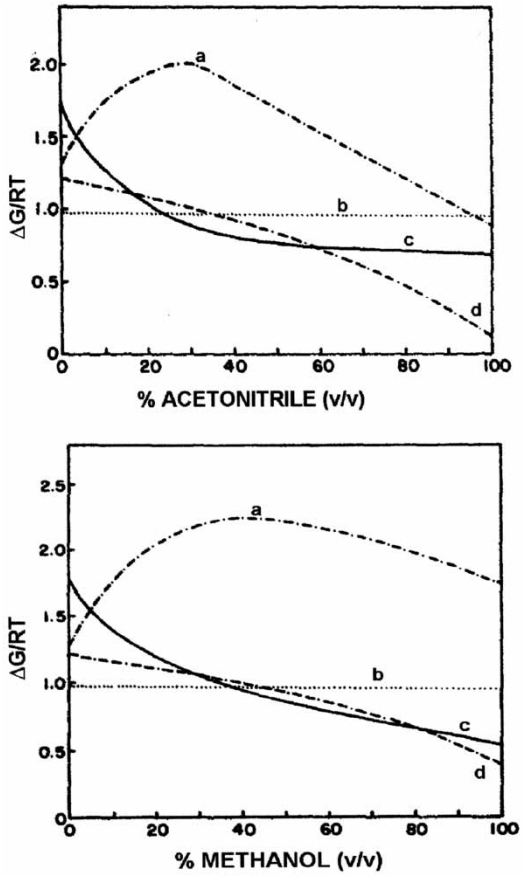

Figure 12. Graph illustrating the dependence of a) κ_E^e, b) D, c) γ_E/RT, and d) $\ln RT/PV$ on the composition of organic modifier in a hydroorganic mixture. Reprinted from Ref.[8].

stationary phase is shown in Figure 13 for acetonitrile/water and methanol/water mobile phase systems. Also illustrated by the solid curve is the solvent effect as predicted by the solvophobic theory by adding up the individual terms in Equation (36a). It is noted that in the absence of experimental ΔG^o_{gas} data, A' was first calculated by fitting Equation (36a) to the experimental RPC data obtained at a particular organic modifier concentration and this value was then used in subsequent calculations to estimate the solvent effect in the entire range of the mobile phase. As seen in Figure 13, the theoretically derived values are indeed in good agreement with experimental observation in the entire range of the organic modifier concentration for both the mobile phase systems. Miyabe and Takeuchi[114–116] have recently tried to interpret differences

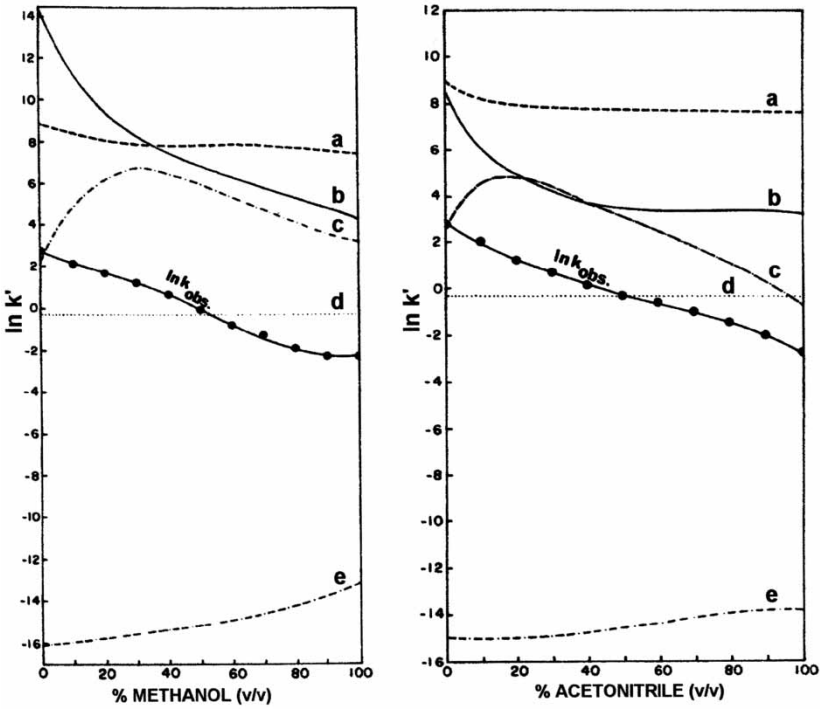

Figure 13. Retention factor of o-toluic acid as a function of the organic modifier concentration in RPC employing octadecylated silica column at 25°C. The solid line represents the logarithmic retention factor calculated using Equation (36a) whereas the symbol represents experimental data. Other curves represent the individual terms of Equation (36a) as a) ln (RT/PV), b) $C'\gamma_E$, c) $D'(\kappa_E^e - 1)V^{2/3}\ \gamma_E$, d) $B'D$ and e) E'. Reprinted from Ref.[8].

in the retention behavior of organic compounds in these two widely employed mobile phase systems on the basis of the solvophobic theory. They compared the adsorption characteristics of benzene, toluene, ethyl benzene, and naphthalene on an octadecylsilyl silica gel column and found that the absolute values of the adsorption equilibrium constant are smaller in acetonitrile/water systems than in methanol/water systems. By invoking the concept of contact surface area from the solvophobic theory, the investigators plotted values of the adsorption equilibrium constant against the corresponding hydrocarbonaceous surface area of the eluites at organic modifier concentrations varying from 20–100%. The ratio of ΔA_C to A_{np} was estimated from the slope of the linear plots for both mobile phase systems. Figure 14 illustrates the dependence of the ratio obtained with both methanol/water and acetonitrile/water systems on the concentration of the organic modifier. As seen, the ratio depends on chromatographic conditions employed, such as the type and concentration of the organic

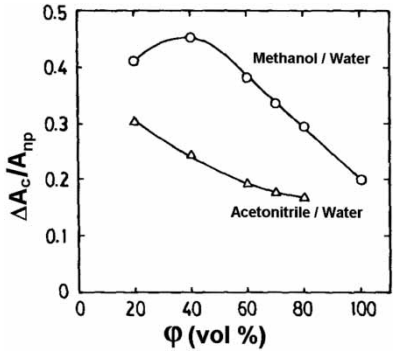

Figure 14. Dependence of the ratio of twice the contact surface area to nonpolar surface area of the eluite on the organic modifier concentration in the mobile phase. Reprinted from Ref.[115].

modifier in the mobile phase. The values of the ratio in acetonitrile/water systems are smaller than the corresponding values in methanol/water systems. Based on these results, they conclude that the magnitude of the interaction between C_{18} ligates and eluite molecules is weaker in acetonitrile/water system than in methanol/water system.

Similar behavior is expected for the dependence of retention factor of an ionized eluite on the organic modifier concentration. Increasing the concentration of the organic modifier in the mobile phase at constant ion-pairing salt concentration leads to decreasing eluite retention since the organic solvent lowers either the adsorption of the ion-pair to the stationary phase or the amount of dynamically adsorbed surfactants to the surface of the stationary phase.

Effect of Salt and pH

The effect of salt on the retention behavior of eluites in RPC depends on the type of eluites employed. For un-ionized eluites, the electrostatic term is practically independent of the salt concentration. When only the surface tension changes with the salt concentration, we can assume that the eluite and the ligate properties as well as eluite–ligate interactions are invariant so that Equation (27) for a neutral dipole can be simplified to[8]

$$\ln k_o = A' + B'\gamma_E \tag{37}$$

where A' is the sum of all terms which do not contain the surface tension and B' is associated with the cavity term. Equation (37) predicts a linear dependence of logarithmic retention factor on the surface tension of the eluent for a given eluite provided κ_E^e and the mole volume of the eluent remain constant. In view of the data in Figure 11, the surface tension of inorganic salt solutions in water

may be expressed to a good approximation as a linear function of the salt concentration and given by

$$\gamma_E = \gamma_o + \tau m \tag{38a}$$

or

$$\gamma_E = \gamma_o + \sigma I \tag{38b}$$

where γ_o is the surface tension of pure water, m is the molal salt concentration, I is the ionic strength, and σ and τ are the coefficients which depend on the nature of the salt. It is noted that the molal salt concentration and the ionic strength can be used interchangeably. Combining Equations (37) and (38b), the logarithmic retention factor for a neutral eluite is given by

$$\ln k_o = \ln k^o + B'' I \tag{39a}$$

where $\ln k^o$ is the logarithmic retention factor of the eluite at zero ionic strength and

$$B'' = \left(-\frac{N\Delta A_C}{RT} + \frac{4.836 N^{1/3}(\kappa_E^e - 1)V^{2/3}}{RT} \right)\sigma \tag{39b}$$

Equation (39a) predicts that the retention factor of a neutral eluite should linearly increase with the salt concentration. This is in agreement with general experience. As shown in Figure 15, the retention of aromatic acids and bases in RPC employing octadecylsilyl bonded phase and aqueous phosphate buffer showed linear dependencies on the salt concentration in the eluent. For ionized eluites, however, electrostatic interactions play a significant role in governing their retention behavior in RPC. Thus, Equation (29) for retention factor at relatively high salt concentrations can be further simplified and expressed in terms of the ionic strength as[9]

$$\ln k_z = \ln k^o + B'(BI^{1/3} + CI) + B'' I \tag{40}$$

where $\ln k^o$ is the logarithmic retention factor of the eluite at zero ionic strength, B' is a constant for a given eluite, salt, and column and B'' is given by Equation (39b). In deriving this expression it is assumed that the salt affects only the surface tension of the eluent according to Equation (38b). Furthermore, the electrostatic effect on gas phase adsorption as well as the associated reduction term was neglected based on the assumption that there are no charged groups present on the hydrocarbonaceous stationary phase surface. Figure 16 illustrates the difference in the behavior of neutral and ionized eluites as a function of the ionic strength of the eluent. For neutral eluites, the dependence is fairly linear as predicted by Equation (39a). On the other hand, retention of ionized eluites first decreases and then increases linearly with increasing ionic strength. The solid lines in Figure 16 were calculated by employing

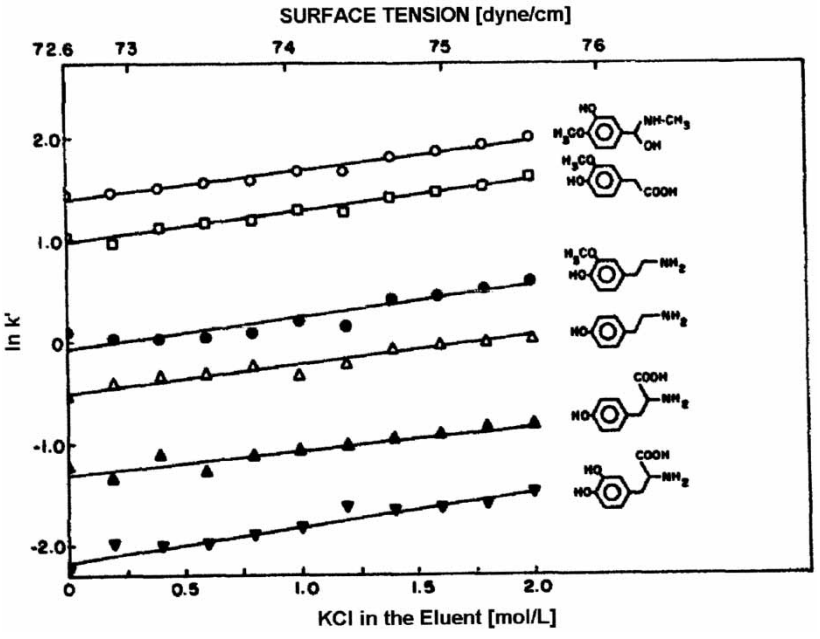

Figure 15. Effect of salt concentration on the retention factor in RPC. Column: Partisil 1025 ODS; Flow Rate: 1 mL/min; Temperature: 25°C. Reprinted from Ref.[8].

Equations (39a) and (40) of the solvophobic theory. As seen, the fit is fairly good between the theoretical and experimental values. Thus, in general, increasing the concentration of the ion-pairing agent in the mobile phase leads to an increase in eluite retention. Deviations from this behavior may be expected in the presence of silanophilic interactions.

The effect of salt on protein retention in RPC can be derived from Equation (30). Assuming again that electrostatic interactions between the protein molecules and the stationary phase surface are negligible, the retention factor of a protein can be expressed in terms of salt molality (or ionic strength) in the entire range of the salt concentration as

$$\ln k' = \ln k^o - \frac{[(Xm^{1/2}/(1 + Ym^{1/2})) + Z\mu_p m]}{RT} + B'm \tag{41a}$$

where

$$B'' = \left(-\frac{N\Delta A_C}{RT} + \frac{4.836N^{1/3}(\kappa_E^e - 1)V^{2/3}}{RT} \right)\tau \tag{41b}$$

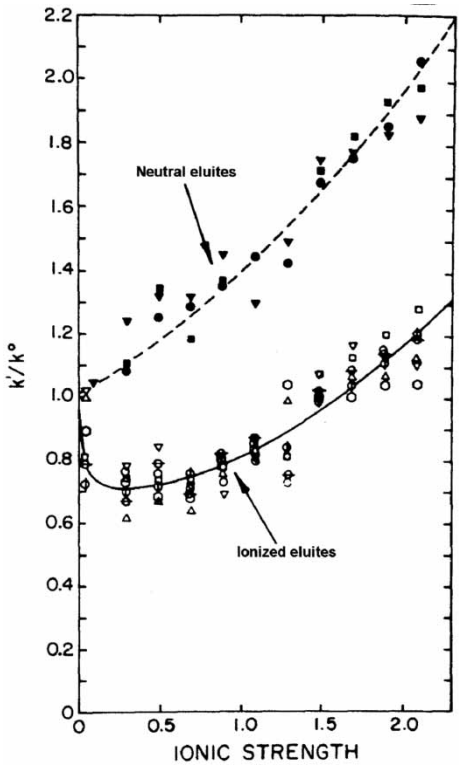

Figure 16. Dependence of normalized retention factor on the ionic strength of the eluent for a variety of un-ionized and ionized eluites. k^o represents the retention factor at zero ionic strength. The dashed line represents the fit of Equation (39a) while the solid line represents the fit of Equation 40 to the experimental data. Neutral eluites: 3,4-dihydroxyphenylacetic acid, homovanillic acid, vanillmandelic acid; Ionized eluites: normetanephrine, metanephrine, 3-o-methyldopamine, dopamine, adrenaline, tyramine, paranephrine. Reprinted from Ref.[9].

According to Equation (41a), the retention of a protein in RPC is determined by two antagonistic effects of the salts on electrostatic and hydrophobic interactions. At low salt concentration, the retention factor is expected to decrease with increasing salt molality because the binding of the eluite is reduced by electrostatic effects of the salt. Above a certain salt concentration, the salt dependence of logarithmic retention factor will be dominated by the hydrophobic interaction term [the last term in Equation (41b)], which is a linear function of the salt concentration. Such behavior has been observed for the retention of peptides and proteins on mixed mode support media, where both ion-exchange and hydrophobic interactions play a significant role in eluite binding.[117–121]

The effect of pH on the retention factor of ionogenic eluites, such as monoprotic acids and bases, in aqueous solutions is expressed by Equations (8a)–(8c). These Equations predict a sigmoidal curve for pH dependence. Figure 17 shows the plot of retention factor versus pH for experimental data measured with a number of monoprotic acids in RPC employing octadecylsilyl bonded phase and salts in phosphate buffers. In all cases, the curve is sigmoidal, in agreement with the prediction of Equations (8a) and (8b). By fitting Equation (8a) to the experimental data using least squares analysis, pK_a values of the eluites can be determined from the inflection points of the curves. These values have been found to be in good agreement with the pK_a values of the eluites in the corresponding mobile phase system determined titrimetrically,[9] further corroborating the theoretical treatment. Thus, Equation (8a) is accurate for aqueous solutions in the absence of additional ionic or hydrogen bonding between the eluite and the stationary phase.

In the presence of an organic modifier in the mobile phase, the influence of the solvent composition on individual retention factors of the neutral and ionized forms of the eluite as well as on the degree of dissociation of the eluite, *d*, must be considered. Van de Venne et al.[122] extended the work of Horváth and Melander to hydroorganic mobile phases. They demonstrated that the retention of carboxylic acids was directly related to the pH of

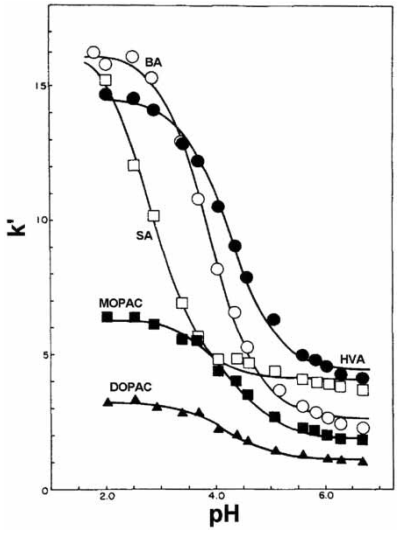

Figure 17. Retention of monoprotic eluites as a function of eluent pH in RPC. Column: Partisil 1025 ODS; Mobile phase: 1.0 M Na_2SO_4 in 0.05 M phosphate buffer; Temperature: 25°C; BA: benzoic acid; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; MOPAC: parahydroxyphenylacetic acid; SA: salicylic acid. The solid lines were obtained by fitting Equation (8a) to the experimental data. Reprinted from Ref.[9].

methanol/water mixtures when used as mobile phases, with pH measured in these mobile phases, by means of the pK_a of the acid in the same methanol/water mobile phases. They recommended the measurement of pH in the mobile phase after calibration with standard buffer solutions of the same solvent composition as the mobile phase. Schoenmakers and co-workers[123–125] discussed different approaches to pH measurement but recommended measuring before mixing the buffer with organic solvent. However, it should be recognized that in this approach the pK_a values obtained from the inflection point in the plot of logarithmic retention factor and pH do not have a physical significance and differ from the expected thermodynamic pK_a values of the eluite. Kele and Guiochon[126] and Neue et al.[127] found that amines in 65% methanol mobile phases buffered with phosphate at pH 7 measured before the addition of methanol were not as protonated as expected from its aqueous pK_a. This was attributed to the increase of the pH of the phosphate buffer and the decrease of the pK_a of the amine caused by the addition of the methanol, which essentially suppressed the ionization of the eluite by preferentially solvating it. Sýkora et al.[128] observed apparent shifts of the retention versus pH plots towards pH values more acidic than the true pK_a value of neutral bases. These shifts are a combination of the two individual shifts caused by the change in the dissociation of the buffer, which produces a mobile phase pH change, and by the change in the pK_a of the basic eluite caused by the addition of the organic modifier.[129–132]

Several investigators have observed deviations from the theory for weakly amine containing basic eluites in RPC employing octadecylated silica gel column with acetonitrile/water system and have attributed this to additional interactions of the eluite with strongly acidic residual silanols on the chromatographic surface.[128,133–135] The ionization of these silanols depends on the pH of the mobile phase. Negatively charged silanols should increase the retention of positively charged eluites and decrease the retention of negatively charged eluites. Neue and co-workers[136] have recently derived a general expression for the retention factor of ionizable eluites in RPC by extending Horváth's formalism to take into account the influence of the organic modifier and the ionization of surface silanols and successfully applied it to describe the retention of several ionizable eluites.

Kazakevich and co-workers[137] focused on the effect of the nature and concentration of different types of acid counter ions on the retention of primary and secondary nitrogen containing compounds in RPC. They observed that trifluoroacetic acid and perchloric acid behaved differently from phosphoric acid at low pH as gleaned from Figure 18. With the use of trifluoroacetic acid and perchloric acid the retention of basic eluites increased with decrease in pH whereas with phosphoric acid a plateau was observed, suggesting an influence of the type and concentration of the ion-pairing agent on the retention behavior. They described their findings in terms of chaotropicity,[138,139] a measure of a counter ion's ability to change

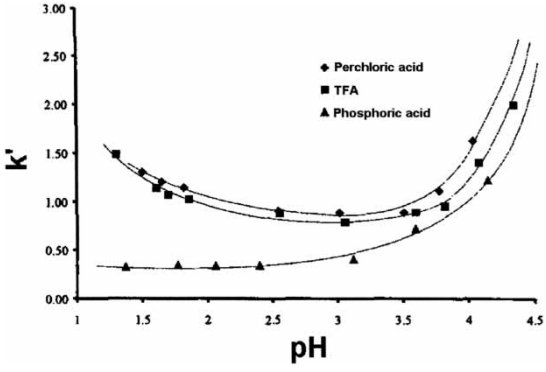

Figure 18. Retention of 4-ethylpyridine as a function of pH using three different acidic ion-pairing agents. Column: 150 × 4.6 mm Zorbax XDB-C_{18}; Mobile phase: 10:90 acetonitrile:disodium hydrogenphosphate (10 mM) buffer adjusted with acidic ion-pairing agent; Flow rate: 1.0 mL/min; Temperature: 25°C; UV detection: 254 nm. Reprinted from Ref.[137].

the structure of water surrounding the eluite in the direction of greater disorder. This causes disruption of the solvation layer of an ionizable eluite via preferential interactions and leads to greater retention of the eluite. The perchlorate, trifluoroacetate and dihydrogen phosphate counter ions of the acids fall into this class. The chaotropic effect is dependent not only on the pH of the solution but also on the concentration of the free counter ion. Thus, it can be observed in experiments conducted with varying concentrations of the chaotropic counter ions at constant pH. This concept was applied to describe the effect of counter-ion type and concentration on the retention of β-blockers in RPC.[140,141]

Protein separation in RPC is most commonly achieved at low pH in the presence of small amounts of trifluoroacetic acid or phosphoric acid in the eluent. Under these conditions, the surface silanols are not ionized and the protein is thought to form an ion-pair with the acid, thus becoming almost a neutral eluite. In the case of very hydrophobic proteins such as membrane proteins and glycoproteins, formic acid at high concentration has been found suitable for such applications. The addition of chaotropic salts to the eluent can also result in increased peak sharpness and weaker retention. For instance, it has been demonstrated that the addition of hydrophilic anionic ion-pair agents, such as perchlorate ion, to low-pH mobile phases can decrease the hydrophilicity of cationic side chains on proteins.[142] This can lead to an enhancement in resolution as well as a change in selectivity. They have found that a phosphoric acid/perchlorate/acetonitrile mobile phase exhibited differences in selectivity from the aqueous trifluoroacetic

acid/acetonitrile mobile phases traditionally used for protein and peptide separations.

Prediction of Retention Factor

The most exacting test of the solvophobic theory can be executed by examining its ability to accurately estimate the magnitude of the retention factor of an eluite in RPC. In the absence of adsorption data with RPC stationary phases in the gas phase, which is needed to estimate the free energy change of eluite–ligate van der Waals interactions, ΔG^o_{gas}, in Equation (12a), the original treatment of the solvophobic theory focused mainly on the solvent effects on eluite retention in RPC. Gas chromatographic data with RPC stationary phases have now become available,[143] thus affording a stringent test of the solvophobic theory by allowing the estimation of retention factors in RPC using readily available physicochemical parameters of eluite, eluent and the stationary phase from sources other than RPC. Miyabe and Suzuki[48] measured the equilibrium constants for the adsorption of several organic compounds on C_{18} bonded phase in RPC employing methanol/water mobile phase systems as well as in gas chromatography. Using Equation (11) they calculated the free energy change associated with the solvent effects, ΔG^o_{solv}, from experimental RPC (ΔG^o) and gas chromatographic data (ΔG^o_{gas}), and compared it with ΔG^o_{solv} values estimated by the solvophobic theory using Equation (12a) and readily available physicochemical parameters. Based on the results listed in Table 2 they concluded that the difference in the adsorption characteristics of gaseous and liquid phase adsorption systems employing octadecylated silica columns could be quantitatively interpreted on the basis of the solvophobic theory as a phenomenon attributable to solvent effects on liquid-phase adsorption. The correlation between experimentally observed and theoretically calculated ΔG^o_{solv} values was reasonable for small eluites like toluene, pentane and cyclohexane, but poor for eluites with long alkyl chains, such as n-hexane, n-heptane and n-octane. They attributed this discrepancy to calculation errors in the estimation of parameters of the solvophobic theory, namely, the ratio of ΔA_C to A_{np}, molecular size and κ^e_E. It is noted, however, that the authors considered the free energy of reduction, ΔG_{red}, to be negligible in their calculation of ΔG^o_{solv} according to Equation (12a). But this term could be as much as 25% of ΔG^o_{gas} in methanol/water mobile phase systems owing to the influence of three- and many-body interactions that considerably reduce the gas phase van der Waals interactions in the presence of eluent molecules.[47,89] By accounting for this term, better correlation is obtained between observed and calculated ΔG^o_{solv} values. The correlation for compounds with longer alkyl chains is still poor. This is because a shape of an eluite and a cavity in which the eluite would be placed was assumed to be a sphere because of

Table 2. Comparison of ΔG^o_{solv} values observed experimentally by subtracting the retention free energy change of nonpolar compounds on C_{18} bonded phase in gas chromatography (ΔG^o_{gas}) from the corresponding retention free energy change in RPC (ΔG^o), and calculated theoretically by applying Equation (12a) of the solvophobic theory. The individual free energy terms for eluite–eluent electrostatic interactions, volume reduction, eluent–eluent van der Waals and hydrogen bond interactions (cavity term) and eluite–eluent van der Waals interactions are also listed. The free energy of mixing in Equation (12a) has been neglected in the calculation. All free energy terms are in kJ/mole units. Data taken from Ref.[48]

				Prediction by the solvophobic theory					
								Calculated ΔG^o_{solv}	
Compound	ΔG^o	ΔG^o_{gas}	Observed ΔG^o_{solv}	ΔG_{es}	$-\ln(RT/PV)$	ΔG_{cav}	ΔG_{vdw}	Miyabe[a]	Vailaya[b]
Benzene	−1.8	−15.1	13.3	0.00	−16.5	−16.4	45.0	12.1	8.4
Toluene	−3.2	−18.4	15.2	0.02	−16.5	−18.8	52.8	17.5	12.9
Ethylbenzene	−4.3	−20.6	16.3	0.02	−16.5	−21.0	60.5	23.0	17.9
p-Xylene	−4.6	−21.0	16.4	0.02	−16.5	−21.0	64.0	26.5	21.3
n-Pentane	−5.8	−11.6	5.7	0.00	−16.5	−20.2	43.8	7.0	4.1
n-Hexane	−7.2	−14.3	7.1	0.00	−16.5	−22.2	54.5	15.6	12.0
n-Heptane	−8.5	−17.2	8.7	0.00	−16.5	−24.4	65.6	24.7	20.4
n-Octane	−9.9	−20.2	10.4	0.00	−16.5	−26.4	77.4	34.5	29.5
Cyclohexane	−6.1	−14.7	8.6	0.01	−16.5	−18.9	45.1	9.7	6.0
Chlorobenzene	−3.0	−19.2	16.2	0.38	−16.5	−18.4	55.3	20.7	15.9

[a]Neglecting the free energy of reduction term in Equation (12a).
[b]Assuming $\Delta G_{red} = 0.25\,\Delta G^o_{gas}$.

convenience of calculations. The shape and curvature of an eluite and a cavity surface are, however, irregular depending on properties of the eluite. Thus, calculation errors resulting from the assumption in shape of the eluite and a cavity increased with an increase in alkyl chain length in an eluite. If the parameters of the solvophobic theory can be estimated by taking into account a real shape of an eluite and a cavity, better correlation may be obtained between the calculated and observed ΔG^{o}_{solv} values for compounds with longer alkyl chains.

Effect of Stationary Phase

A number of experimental observations have been made with respect to the effect of the stationary phase properties on the retention of eluites in RPC. Some of these observations are conflicting while others cannot be readily explained. As a result, chromatographers have struggled to describe these effects on the basis of a theory. The mean field lattice theory based on statistical thermodynamics has had some success in predicting the trends of some of the observed effects, although only qualitatively. The solvophobic theory, on the other hand, has predominantly been used to describe solvent effects in RPC so that the effect of stationary phase has not received much attention in the past.

There is growing experimental evidence that the retention of eluites in RPC is affected by changing the bonding density, alkyl chain length as well as the carbon content of the bonded stationary phase. Linear relationships between logarithmic retention factors and the chain length of the ligate have been obtained with many homologous series by some investigators,[34,144–147] whereas others have demonstrated nonlinearity of the plots for several homologous series when eluites having a wide range of alkyl chain lengths were included.[148–150] These contradictory results have led to two schools of thought. The first believes that only a part of the long alkyl chains of the bonded stationary phase contributes to the retention in RPC, whereas the second proposes that the entire length of alkyl chains contributes to hydrophobic interactions between eluites and ligates.

Miyabe et al.[151–153] have tried to explain the stationary phase effects on the basis of the solvophobic theory by conducting studies with a series of compounds on a silica gel bonded to alkyl ligates of various lengths, carbon content and density. Using C_1, C_4, C_8 and C_{18} bonded phases to investigate the effect of chain length and four C_{18} bonded phases with different bonding densities to investigate the effect of bonding density, they found that the absolute value of the equilibrium constant for RPC retention increased with increasing chain length of the alkyl ligate, carbon content and the density of the chains as shown in Figures 19a–c. However, a critical level of the carbon content, depending on the size of the eluite

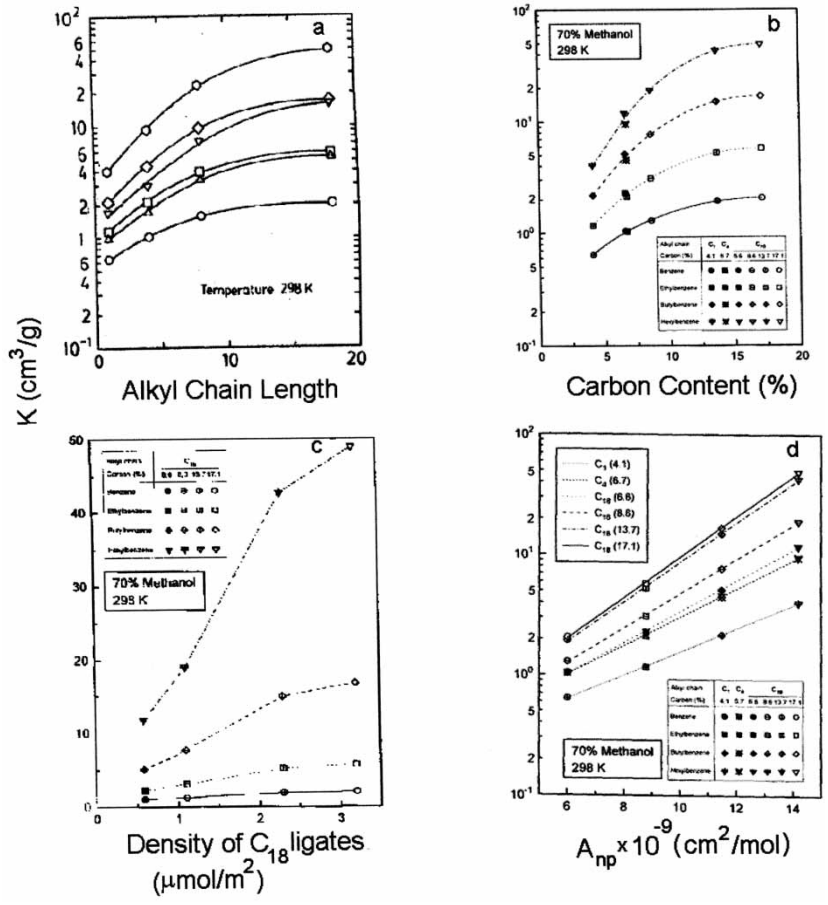

Figure 19. Correlation between RPC equilibrium constant and the a) alkyl chain length of the bonded stationary phase (Ref.[150]), b) carbon content of the bonded stationary phase (Ref.[115]), c) ligate density of C_{18} bonded stationary phase (Ref.[115]) and d) hydrocarbonaceous surface area of the eluite (Ref.[115]).

molecule and the alkyl ligate, was observed above which the slope of the curves gradually decreased and retention no longer changed with increasing alkyl ligate density. Similar behavior was also observed for bonded phases with varying ligate chain length and the retention factor became constant above the critical chain length slightly greater than C_8. On the other hand, the correlation of retention factor with the density of C_{18} ligates was almost linear, although retention on the bonded phase with the highest ligate density was lower than the value predicted by extrapolation of the linear plot for the other three phases. This seems to suggest that small eluites may

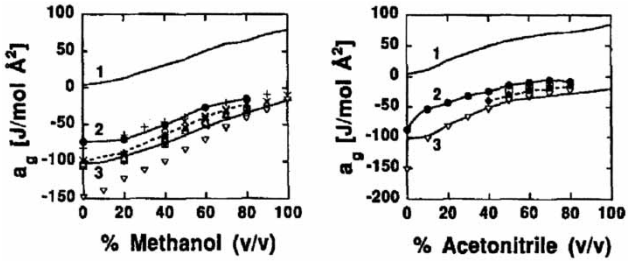

Figure 20. Effect of different stationary phases on hydrophobic selectivity of nonpolar eluites in RPC using methanol/water and acetonitrile/water mobile phase systems. For methanol/water system: Symbols represent experimental RPC data for (□) C_{22}, (◆) C_{14}, (△) C_{10}, (×) C_8, (▲) C_6, (+) C_4, (●) C_1 bonded phases, and (▽) poly(styrenedivinylbenzene) stationary phase; Plot 1 represents the sum of 1st, 2nd and 4th terms in Equation (35); Plots 2 and 3 show the respective variation of hydrophobic selectivity with methanol concentration for the retention on C_1 and C_{18} bonded phases, while the dashed line represents that for the retention on C_6 bonded phase; For acetonitrile/water system: Symbols represent experimental RPC data for (◆) C_8, (●) C_4, (□) phenyl bonded phases, and (▽) poly(styrenedivinylbenzene) stationary phase; Plot 1 represents the sum of 1st, 2nd and 4th terms in Equation (35); Plots 2 and 3 show the respective variation of hydrophobic selectivity with acetonitrile concentration for the retention on C_4 and C_{18} bonded phases, while the dashed line represents that for the retention on C_8 bonded phase; Reprinted from Ref.[47].

interact with only one alkyl ligate chain even when the density of the ligates is high and that all C_{18} ligates do not necessarily contribute to the retention of the eluites at ligate densities higher than $\sim 2.3\ \mu mol/m^2$. This is consistent with the view that the eluite molecules penetrate into the layer of alkyl chains. Based on these results the authors concluded that only part of the long alkyl ligates may contribute to the retention behavior in RPC.

These findings suggest that the contact surface area upon binding may depend on the stationary phase properties. Figure 19d illustrates the linear plots between equilibrium constant for RPC retention and hydrocarbonaceous surface area of a set of alkyl benzenes on various bonded phases. The slope of the straight lines is slightly different for the six bonded phases, confirming the hypothesis that eluite retention in RPC depends somewhat on the nature of the modified chromatographic surface, the alkyl chain length, and the C_{18} ligate density. Miyabe and Guiochon[153] estimated the ratio of ΔA_C to A_{np} from the slopes of linear plots in Figure 19d. As expected, the ratio was slightly different for different stationary phases -0.2 for C_1, 0.25 for C_4, 0.30 for C_8 and 0.35 for C_{18}. This variation may arise from the difference in the manner of steric interactions between the eluite molecules and the alkyl ligates. The eluite molecules may be able to penetrate into a layer of long alkyl ligands, whereas only planar interactions would take place on the surface of C_1 silica gel.[148,154] Based on these studies it appears likely that

no more than about 10–18% of the hydrophobic surface area of the eluite molecules can interact with the long alkyl chains of the bonded stationary phase because of steric hindrance when the density of the bonded ligates is high.

So how significant is the stationary phase effect on eluite retention in RPC in comparison to that of changing the organic modifier concentration in the mobile phase? In order to answer this question, Vailaya and Horváth[47] analyzed a large body of experimental RPC data obtained with bonded stationary phases of varying ligate chain length, such as C_1, C_4, C_6, C_8, C_{10}, C_{14}, and C_{22}, as well as other stationary phases, such as poly(styrenedivinylbenzene) and phenyl bonded phases. The change in hydrophobic selectivity as a function of stationary phase property was then compared to the change in the same due to variation in the composition of the organic modifier in the mobile phase. Figure 20 illustrates this comparison for acetonitrile/water and methanol/water mobile phase systems in the entire range of the organic modifier concentration. It is seen that RPC data obtained with various alkyl bonded phases exhibit similar dependence of hydrophobic selectivity on the organic modifier concentration. The curve, however, shifts upward with decreasing length of the alkyl ligate. In the case of methanol/water mobile phase system, the relative shifts from plot 3 representing data on C_{18} bonded phase is about 25 and 8 J/molÅ^2 for C_1 and C_6 bonded phases, respectively, whereas the shift is quite insignificant for C_8, C_{10}, C_{18} and C_{22} bonded phases. In contradistinction, hydrophobic selectivity values associated with the changes in organic modifier composition in the mobile phase vary from -103 J/molÅ^2 in neat water to -17 J/molÅ^2 in neat methanol, a three-fold change compared to the stationary phase effect. Similar results are obtained with acetonitrile/water mobile phase system. Thus, the hydrophobic selectivity is more fundamentally affected by changes in the mobile phase than by changes in the chain length of the bonded stationary phase. This unequivocally confirms the dominating role of mobile phase in governing the selectivity of nonpolar eluites in RPC.

Correlation Between Octanol/Water Partitioning and RPC Retention

According to the solvophobic theory, the free energy change for the partitioning of a solute between octanol and water phases can be expressed in terms of interfacial surface tension and molecular surface area, A_A, as[38]

$$\Delta G^o_{ow} = (\kappa^g_{AO}\gamma_{AO} - \kappa^g_{AW}\gamma_{AW})A_A + RT \ln \frac{V_W}{V_O} \tag{42}$$

assuming the electrostatic interactions in the two phases to be negligible. In Equation (42), γ_{AO} and γ_{AW} represent solute–octanol and solute–water

interfacial tensions and V_O and V_W are molar volumes of octanol and water, respectively. The κ^g's convert the respective interfacial tension to the microthermodynamic value applicable to molecular dimensions. For hydrocarbonaceous solutes, $A_A = A_{np}$. For most hydrophobic compounds, A_A can be considered to be proportional to A_{np}. Thus, Equation (42) predicts a linear relationship between free energy change associated with octanol/water partitioning and the nonpolar surface area of the solute for a set of hydrophobic compounds, with the slope of the linear plot determined by interfacial tensions. It is seen that Equation (42) bears a striking semblance to Equation (26) so that an expression can be derived that relates the free energy change of RPC retention to that of octanol/water partitioning. This relationship is expected to be linear owing to the linear dependence of both the free energy changes on the nonpolar surface area of the hydrophobic compounds. This is indeed observed when experimental RPC retention data are plotted against octanol water partition coefficients for a set of hydrophobic solutes.

MECHANISM OF RPC RETENTION

Investigating the mechanistic aspects of RPC retention is important from the view point of gaining a better understanding of the fundamental principles underlying the retention process. This in turn facilitates the development of novel stationary phases and eluent systems that offer unique selectivities, thus leading to an advancement of the separation technique. The adsorption/partitioning controversy as well as the mechanistic interpretation of the solvophobic theory is reviewed in the following to shed light on the mechanism of RPC retention.

Adsorption or Partition?

In practice, partition and adsorption processes are quite different in a physical sense. Adsorption of a substance from a solution takes place at the solid–liquid interface whereas partition involves its transfer from the bulk solution into another immiscible solvent. This distinction between the two has warranted an inquiry into the nature of retention in RPC within such a classification. Therefore, the question whether retention in RPC is governed by adsorption or partitioning has attracted considerable attention in the past and continues to be an area of active research. Notwithstanding numerous articles on this topic in recent times, progress has been painfully slow. The fact that no conclusion has been drawn yet regarding the mechanism of RPC retention based on this distinction is reflective of the complexity of the retention process and our limited knowledge about the configurational state of the grafted chains on

the siliceous supports commonly used in RPC. The chromatographic surface is different from that of an adsorbent, such as activated charcoal, and its surface density is much less than that of a bulk hydrocarbon liquid. It can be highly solvated by components of the mobile phase and have residual silanols, making it act neither as a pure adsorbent nor as a pure liquid organic phase. Thus, it appears that the mechanism of RPC retention cannot be ascribed to either of the processes alone in their ideal form. Furthermore, it is important to recognize that the retention of an eluite in RPC is due to the statistical average of various plausible binding orientations of the eluite with the stationary phase ligates as it traverses through the column under high pressure, and that the measured retention factors represent average values of the corresponding free energy of binding. It is therefore not surprising that most of the studies investigating the adsorption or partitioning mechanism in RPC have concluded that the retention mechanism is most likely a combination of the two.[38] Perhaps, the uniqueness of RPC retention calls for its mechanism to be interpreted not in terms of adsorption or partitioning, but in terms of the underlying molecular interactions. A brief review of the adsorption/partition controversy in RPC is presented below.

The RPC retention process has predominantly been modeled by liquid/liquid partition systems because there is considerable experimental evidence that RPC retention shares some significant similarities with partitioning. Lochmüller and Wilder[34] speculated the formation of liquid-like clumps of dodecyl and longer chain moieties on the siliceous surface of bonded phases which the eluites penetrated and suggested that this was the reason why the retention of simple aromatics followed a partitioning behavior. Similarly, Tan and Carr[155] employed hexadecane/mobile phase liquid/liquid systems to model the retention in RPC. They measured the standard free energy of transfer for a methylene group from the mobile phase to liquid hexadecane and the corresponding standard free energy of transfer from the mobile phase to the RPC stationary phase and concluded that the two were similar in magnitude at most operating conditions. The excellent correlation between octanol/water partitioning and RPC retention for a large number of eluites and the subsequent use of RPC retention for the measurement of hydrophobicities of novel compounds as an alternative to the traditional and time-consuming shake-flask method also corroborated the similarity of the two disparate processes and led to the belief that the retention in RPC is governed by the partition mechanism.

But some significant differences between liquid/liquid partition and RPC retention processes have also been recognized and this has led to the development of liquid-crystalline and amorphous crystalline hydrocarbon partition views of the stationary phase for modeling the retention in RPC. While there is a good correlation between RPC retention and octanol/water partitioning, chromatographic and spectroscopic studies[156–160] suggest that the chemical properties of RPC bonded phases differ significantly from those of

bulk alkane phases equilibrated with common mobile phase mixtures. There is growing evidence that mobile phase molecules are intercalated among the bonded chains and that these chains are configurationally strained due to the bonding density and the chain length. In addition, the residual silanol groups on the silica support are believed to play a significant role in solvent enrichment and retention of basic compounds.[156,157] This may suggest that polar solutes are able to specifically interact with such components in the stationary phase that impart a polar nature to it, either via hydrogen bonding with residual silanols on the chromatographic surface or via van der Waals with adsorbed mobile phase molecules. When neither of these are present, such as in the case of a C_{18} derivatized PS-DVB column, retention data has been shown to correlate well with alkane-water partition coefficients but not with octanol-water partition coefficients.[161] Although partition models may provide a satisfactory representation of the experimental RPC data obtained on C_{18} column with benzyl alcohol, 2-phenylethanol and 2-methyl benzyl alcohol at low concentration, they fail to represent the curvature of the equilibrium data at higher concentrations of the eluites or describe the temperature dependence exhibited by the experimental data.[162]

Other investigators have explained the observed deviation of RPC retention data from those of alkane/mobile phase liquid/liquid systems in terms of the participation of an additional mechanism, based on adsorption, in the retention of the eluite of interest.[33,34] These are predominantly hypothetical conjectures without any conclusive experimental evidence. For instance, by comparing the partition coefficients and selectivities at 25°C for methyl-substituted benzene solutes in alkane/methanol:water system to RPC retention factors obtained on various bonded phase at 25°C using methanol:water mobile phase, Lochmüller and Wilder[34] concluded that RPC stationary phases with dodecyl and longer bonded chains exhibited partition behavior owing to the similarity of the energetics of the two systems, whereas bonded phases with chain length less than 12 carbon atoms, which showed weaker retention, exhibited an adsorption mechanism or some blend of adsorption and partition behavior. On the other hand, Carr et al.[33,155,163] used an analytical test based on Dill's lattice theory to distinguish between partition and adsorption mechanisms in RPC. Assuming a cubic lattice model, they proposed that the change in free energy of partitioning is six times the change in free energy of adsorption and introduced the parameter F that measures the extent of deviation of RPC retention data from partition data as

$$F = \frac{a_g^P}{a_g} \tag{43}$$

where a_g is the RPC retention free energy change per unit surface area or methylene group and a_g^P is the normalized free energy change associated

with the corresponding partitioning process. RPC retention is then governed by the partition mechanism if $F = 1$ and the adsorption mechanism if $F = 6$. Based on this test, Carr and co-workers[33] concluded that the retention of small nonpolar eluites, such as alkylbenzenes, in RPC employing organic modifiers in the range from 0–70% is governed by the partition mechanism because of its similarity with the energetics of the transfer of alkylbenzenes from hydroorganic liquid phase to bulk hexadecane. At higher organic modifier concentrations, retention behavior in RPC deviated from partition like behavior and this was attributed to either a shift to adsorption mechanism, or to the sorption of organic modifier by the stationary phase or simply to experimental artifacts.

The inadequacy of Carr's analytical test based on Equation (43) to distinguish between adsorption and partition mechanism in RPC becomes apparent upon closer examination of the parameter F. Since it is a ratio of two quantities, when the denominator, i.e., a_g approaches zero, F becomes statistically meaningless. In such cases, F may not be a good measure of the deviation of retention in RPC from partition. Indeed, most experimental RPC data show that at very high organic modifier concentrations a_g values approach zero. This introduces considerable amount of uncertainty in the evaluation of F thus casting serious doubt on the results of studies that employ F analysis to conclude that the retention in RPC employing organic-rich eluents is driven by the adsorption mechanism. A more pressing concern is the validity of the underlying assumption for the F analysis—Is the adsorption process really six times less favorable energetically than the partition process? A comparison of thermodynamic data obtained with partition and adsorption processes indicates that the free energy changes per unit nonpolar surface are essentially the same for the two processes, in conflict with the predictions of the lattice theory.[38] This questions the validity of using F analysis to distinguish between adsorption and partition mechanism in RPC.

Vailaya and Horváth[38] recently compared the energetics of nonpolar compounds in oil/water partitioning, adsorption on activated charcoal from dilute aqueous solution and RPC retention within the hermeneutics of the solvophobic theory and found that a clear distinction between adsorption and partition mechanism in RPC may not be apparent from thermodynamic analysis. They established that the physicochemical principles underlying such apparently disparate processes are fundamentally identical and that the quite similar selectivities exhibited by such processes for nonpolar substances can be attributed to the comparable magnitude of van der Waals forces governing solvent-solvent, solute-solvent and solute-stationary phase interactions in the case of nonpolar compounds in aqueous systems. However, energetic differences between partition, adsorption and RPC retention processes are expected in cases when complex compounds are employed that may be involved in electrostatic and/or hydrogen bonding interactions between the

eluite and the stationary phase, in addition to other interactions that are similar for all three processes. For instance, the selectivities toward polar substances are expected to differ due to the considerable energetic differences between the partitioning of the polar moieties in organic phases, i.e., octanol and hexadecane, and between their interactions with bonded stationary phases in RPC and with activated charcoal in adsorption. This finding is in conflict with the prediction of the lattice theory and exposes its limitation in providing a consistent framework for the treatment of both partition and adsorption processes. It further raises the question whether this approach is suitable to distinguish between partition and adsorption mechanisms in RPC.

While a detailed analysis of the similarities and differences between RPC retention and the corresponding liquid/liquid partition system may shed some light on the mechanistic aspects of RPC retention, it fails to adequately explicate the physicochemical principles governing the retention process at the molecular level. Any mechanistic interpretation of the RPC retention process must go beyond the macroscopic view and delve deeper to explicate the retention behavior at the microscopic, i.e., molecular, level. This is afforded by the application of the solvophobic theory to describe the retention in RPC.

Mechanistic Interpretation by the Solvophobic Theory

The solvophobic theory offers both a macroscopic and a microscopic view of the retention process. At the macroscopic level, the RPC retention process is viewed as the association of an eluite with the ligate in the gas phase followed by the solvation of all related species. This breakdown of the retention process into sub-processes is thermodynamically consistent and offers an approach to predict retention in RPC. Being eclectic in nature, the framework of the solvophobic theory draws upon numerous well-established theories for the treatment of individual sub-processes and specific interactions therein. This formalism captures the microscopic details of the various molecular interactions involved in the retention process, thus offering valuable insight into the mechanistic aspects of RPC retention at the molecular level. Rather than addressing the mechanism of retention in RPC in terms of adsorption or partition, a macroscopic concept originating from Dill's lattice theory, the solvophobic theory focuses instead on capturing the influence of the magnitude of various molecular interactions on the retention behavior of different types of eluites. This is evident form the approach employed by the solvophobic theory to treat hydrophobic interactions that are believed to dominate the retention behavior of most eluites in RPC. At the macroscopic level, the magnitude of hydrophobic interactions is assessed from the change in surface tension of the eluent that is required to eluite the compound retained on a nonpolar surface. At the microscopic level,

the surface area dependence of the free energy of transfer is employed to describe the magnitude of hydrophobic interactions. The duality of these macroscopic and microscopic phenomena thus provides a rigorous basis to elaborate the hydrophobic processes involved in the molecular interactions of nonpolar eluites with nonpolar surfaces.

In summary, the elegance of the solvophobic theory is demonstrated by the fact that it not only presents a macroscopic view of the RPC retention process in terms of conceptual sub-processes for the purpose of calculating free energy change of retention, but also offers a quantitative understanding of the fundamental physicochemical principles underpinning RPC retention at the microscopic level by accurately expressing various molecular interactions in terms of easily measurable eluite, eluent and stationary phase properties.

Criticisms of the Solvophobic Theory

When the solvophobic theory was first adapted in 1976 to describe retention in RPC, column technology was still evolving and a chromatographer's tool chest consisted of only a few bonded stationary phases with low carbon loading. The effects of stationary phase properties on eluite retention were not well understood then. Chromatographic separations were typically controlled by varying the amount of organic modifier or salt in the mobile phase. The broad range of operating conditions offered by the different types of mobile phase systems was found to be adequate for achieving the desired separation of any mixture of compounds on just one column in a majority of cases. It was clear that the solvent effects played a significant role in the retention behavior of a wide variety of compounds. Recognizing this remarkable feature of RPC, Horváth and co-workers adapted Sinanoğlu's solvophobic theory, developed essentially for solvent systems, to describe the solvent effects in RPC. The theory confirmed the dominant role of the solvent in RPC retention by accurately predicting the effects of salt molality, organic modifier concentration and eluite molecular structure on the retention behavior of various eluites in RPC using a fixed stationary phase. With rapid advancement in column technology, more types of stationary phases became available. These newly introduced columns offered unique selectivities, which could not be explained readily. Sometimes, a reversal of the order of elution was observed and, at other times, better resolution between two eluites could be achieved by merely switching from one type of bonded stationary phase to another. These observations seemed to suggest that the stationary phase also played a significant role in governing the retention behavior of eluites in RPC. Since the original treatment of the solvophobic theory did not explicitly focus on the effect of the stationary phase property on RPC retention, it has led some chromatographers to believe that the

solvophobic theory is either inadequate[71,164] because it does not adequately take into account the stationary phase effects in RPC or inaccurate[165] because it treats the stationary phase as a passive entity, i.e., playing a relatively minor role. In their view the solvophobic theory considers the mobile phase mainly responsible for driving eluites towards the stationary phase, and no account is taken of possible eluite–stationary phase interactions. Another criticism of the solvophobic theory is that it treats separation in RPC as an adsorption, rather than partition, process when experimental data overwhelmingly show similarities between RPC retention and partitioning systems. This has led to the belief that the solvophobic theory underestimates the effects of cavity formation in the stationary phase.

The most prolific opponent of the solvophobic theory is Carr,[165,166] who started out as an advocate of the theory but switched camps in 1990. In a series of papers[33,155,163,165,167] entitled "The revisionist look at solvophobic driving forces in RPC" and spanning fifteen years, Carr and his co-workers have relentlessly criticized the solvophobic theory and claimed that it is inaccurate. Their assumption of the solvophobic theory is that it predicts a) the net free energy change due to solvent effects to be much greater than the net free energy change due to stationary phase effects and b) the RPC retention free energy change to be driven mainly by entropic effects. They attack this view of the solvophobic theory by analyzing the energetics of RPC retention in terms of mobile and stationary phase effects as well as by evaluating the enthalpic and entropic contributions to the retention free energy change and observing contradictory results. Unfortunately, their criticism of the solvophobic theory is based on a fallacious argument. First, they create a straw man by misinterpreting and misrepresenting the concepts of the solvophobic theory. Then they attack the "distorted" view and claim that the solvophobic theory is essentially incorrect for RPC retention. What they have failed to recognize is that their conclusions about the energetics of the RPC retention process which seem to contradict their "distorted" view of the solvophobic theory are, in fact, consistent with the "actual" view of the solvophobic theory. The ensuing discussion should hopefully dispel some of the misinterpretations of the solvophobic theory and clarify the concepts of this powerful theoretical treatment.

Ranatunga and Carr[165] estimated the contribution of the mobile phase and the stationary phase to the retention energetics by using a thermodynamic cycle that views the RPC retention process as a sum of two sub-processes—eluite transfer from the mobile phase to the gas phase followed by eluite transfer from the gas phase to the stationary phase. These investigators are oblivious of the fact that their proposed thermodynamic cycle for dissecting the RPC retention process is quite similar to that used by the solvophobic theory. However, unlike the latter, Carr's proposed cycle is thermodynamically inconsistent since it assumes the octadecylsilyl bonded stationary phase in RPC to be like a bulk hexadecane liquid phase, already acknowledged

to be a poor model for bonded phases. By employing experimental data for the transfer of eluites from the hydroorganic mobile phase to the gas phase as well as from the gas phase to bulk hexadecane phase, Ranatunga and Carr found that most of the free energy of retention in RPC arises from the net attractive processes in the stationary phase, not from net repulsive processes in the mobile phase, and that the retention process is enthalpically driven, in contradistinction to their distorted view of the solvophobic theory. This led to their dismissal of the solvophobic theory as being essentially incorrect for the description of RPC retention.

The employment of bulk liquid hexadecane model to describe the effects of bonded hydrocarbonaceous stationary phases may offer some insight into the energetics of RPC retention, owing to the similarity between RPC and partitioning systems. Using this approach, Carr concluded that the net free energy change associated with the stationary phase effect is much greater than that associated with the mobile phase effect. This finding is essentially correct but is not new as it has been known to the scientific community for decades.[12] Nor is Carr's finding in conflict with the prediction of the solvophobic theory as per his claim. In fact, the solvophobic theory unequivocally predicts that the net free energy change associated with the stationary phase effect is much greater than that associated with the mobile phase effect in RPC. Clearly, there is no argument that eluite interactions with the hydrocarbonaceous bonded phases under conditions of gas chromatography are quite strong. This was precisely the reason why the use of long chain alkyl functions covalently bound to silica via siloxane bridging groups was first explored in gas chromatography[168] before such stationary phases became popular in liquid chromatography. Even Horváth and Melander[12] recognized this phenomenon when adapting the solvophobic treatment to describe retention in RPC. To explain their point they construed a "Gedanken" experiment using the same octadecyl-silica column first in gas chromatography, then in RPC with water/acetonitrile mixtures as eluent. The expected results of such an experiment for the retention factors of a single eluite, such as toluene, at room temperature in GC and RPC, both employing a C_{18} stationary phase, are depicted in Figure 21a. It is seen that the retention factor in gas chromatography would be higher than 1500, implying that such results in practice would have to be obtained from high temperature data extrapolated by a van't Hoff plot. Such a large number for the retention factor indicates a very strong van der Waals interaction between the eluite and the bonded stationary phase ligate in gas chromatography. On the other hand, the retention factor in RPC depends on the eluent composition and, as shown by the solid line in Figure 21a, it changes from about 100 to about 0.1, i.e., it decreases by almost three orders of magnitude when plain water is replaced by plain acetonitrile as the eluent. The actual retention in RPC can be considered as a result of the eluite–ligate van der Waals interaction in gas chromatography and the energetically

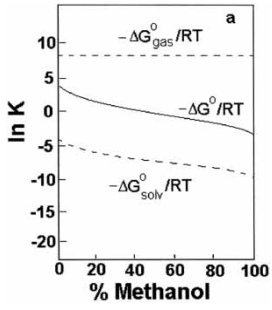

Figure 21a. Estimated equilibrium constant at room temperature for the retention of toluene on octadecylated silica gel column in gas chromatography with helium as carrier gas and in RPC with acetonitrile/water mixtures as the mobile phase. Also shown is the net effect of the mobile phase, $-\Delta G^{o}_{solv}/RT$, which essentially reduces the magnitude of eluite–stationary phase van der Waals interactions in the gas phase.

reverse effect of the eluent, shown by dashed lines in Figure 21a. According to this illustration, the eluent apparently reduces the strength of the direct interaction between the eluite and the stationary phase ligate. This finding of the Gedanken experiment was later confirmed by Miyabe and Suzuki[48,143] who investigated the effect of solvent on adsorption characteristics in liquid-phase chromatography by comparing experimental adsorption data for several organics in both gas- and liquid-phase systems using octadecylsilyl silica gel columns. Their results in free energy terms are summarized in Table 2. As seen, the free energy change is much more favorable for eluite retention on octadecylated silica gel in the gas phase than in RPC employing methanol/water mobile phase system. For instance, the ratio of adsorption equilibrium constant of ethyl benzene in RPC to that in the corresponding gaseous system was less than 1/700. What then justifies the statement based on the solvophobic theory that retention in RPC is driven by solvent effects, especially the favorable reduction of the cavity size in the eluent upon eluite binding to the stationary phase, like in other manifestations of the hydrophobic effect?

The answer to this question is obtained when the retention process in RPC is viewed microscopically in terms of the individual terms representing various molecular interactions according to Equation (12a) of the solvophobic theory, rather than when it is viewed macroscopically, as Carr did, in terms of just the stationary phase and mobile phase effects. In Figure 21a, the dashed line representing retention in gas chromatography is simply a measure of the strength of eluite–ligate van der Waals interactions in the gas phase. However, the dashed curve representing the net mobile phase effects in RPC is actually the sum of four major effects due to solvation: 1) reduction in cavity surface area exposed to the mobile phase upon binding, i.e.,

eluent–eluent interactions associated with van der Waals and hydrogen bonding, 2) eluite–eluent interactions due to van der Waals and electrostatic, 3) reduction in free volume, i.e., the entropy change between gaseous and liquid states, and 4) reduction of eluite–ligate van der Waals interactions in the gas phase due to the presence of eluent molecules. Thus, by comparing the net stationary phase effect to the net mobile phase effect, one is actually comparing the magnitude of eluite–ligate van der Waals interactions to the magnitude of the sum of interactions in the eluent comprising free volume reduction, eluite–eluent interactions and eluent–eluent interactions and reduction in eluite–ligate interactions. This comparison is unequal and leads to ambiguity. Using this comparison, Carr arrived at the erroneous conclusion that the stationary phase effect dominates the retention in RPC. The correct approach to determining the key driving force in RPC retention, however, is to compare the magnitude and sign of the free energy change associated with each of the molecular interactions with one another. Figure 21b shows the individual terms representing the solvent effect in RPC retention, according to Equation (12a) of the solvophobic theory. The ordinate of the axis measures in $\ln K$ units the various solvent related free energy terms for the retention of an eluite, such as toluene, on an octadecylsilyl column with a water/methanol eluent system. The free energy change for eluite–eluent van der Waals interactions is expected to be positive (so that the corresponding $\ln K$ term would be negative) since such interactions have an

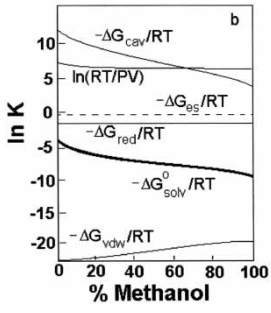

Figure 21b. Graph illustrating the individual contributions of various terms in Equation (12a) (in dimensionless free energy unit) to the net solvent effects, ΔG^{o}_{solv}, in RPC as a function of the methanol concentration in the hydroorganic eluent for the retention of toluene on an octadecylated silica column at room temperature. ΔG_{vdw} = free energy change of eluite–eluent van der Waals interactions, ΔG_{es} = free energy change of eluite–eluent electrostatic interactions, ΔG_{cav} = free energy change associated with cavity reduction in the eluent (eluent–eluent van der Waals and hydrogen bond interactions), ln (RT/PV) = reduction in free volume and ΔG_{red} = free energy change associated with reduction of eluite–ligate van der Waals interactions in the gas phase.

unfavorable effect toward eluite retention in RPC. Similarly, the decrease in the cavity size upon binding of the eluite to the stationary phase will have a negative free energy change associated with it, and thus a positive $\ln K$ value, as it favors the binding process. On the other hand, the magnitude of eluite–eluent electrostatic interactions is expected to be negligible for toluene. The greatest single term in Figure 21b is clearly the magnitude of eluite–eluent van der Waals interactions. It is in fact much larger in magnitude than eluite–ligate van der Waals interactions shown by the solid line for retention in gas chromatography in Figure 21a, and has an opposite sign. If the solvent effect was solely due to the sum of eluite–eluent van der Waals interactions and the free volume term and that the cavity effects were negligible, then the strength of eluite–ligate van der Waals interactions would be so greatly reduced by the solvent effect that there would be no retention in RPC, i.e., the eluite would prefer to stay in the eluent due to the overwhelmingly favorable eluite–eluent van der Waals interactions. However, it is clear from Figure 21b, that the magnitude and change in the cavity term dominates the solvent effect and makes the overall retention process favorable. The data in Table 2 for toluene and other organic eluites confirm this finding. As seen, the eluite–eluent van der Waals term is by far the greatest in magnitude and is opposite in sign to eluite–ligate van der Waals, cavity reduction and volume reduction terms. It is important to recognize that the strength of eluite interactions with the stationary phase in polar solvents is largely influenced by the balance of opposing forces arising from various molecular interactions as shown in Figure 22. The net free energy change associated with eluite–ligate interactions in the gas phase, the reduction in the size of cavities, and the reduction in free volumes is counter balanced by the free energy change arising from the van der Waals and electrostatic interactions between the eluite and the eluent. Consequently, the difference between often large opposing energies will yield the free energy change for RPC retention.

In his criticism of the solvophobic theory, Carr also assumed that the solvophobic model predicts the RPC retention behavior to be entropically driven. This is again a misrepresentation of the solvophobic theory. As discussed earlier in the section on temperature dependence, the solvophobic theory predicts that the enthalpy change is negative and its variation in the temperature range of chromatographic interest is small so that linear van't Hoff plots with negative temperature dependence are observed in most cases. The investigation of the effect of temperature on RPC retention and the individual roles of entropy and enthalpy changes upon eluite binding as a function of the organic modifier concentration is complicated, however, because of paucity of data and lack of model systems. Dissecting retention free energy change into its enthalpic and entropic components may provide valuable insights into the energetics of RPC retention at the macroscopic level, but assessing the individual enthalpic and entropic

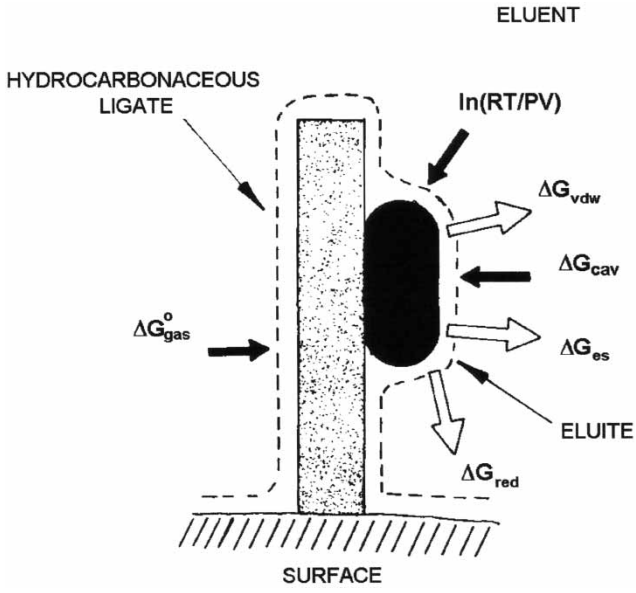

Figure 22. Schematic illustration of the association between an eluite and a ligate of the hydrocarbonaceous bonded stationary phase in RPC. The arrows represent the forces acting on the two interacting species—solid arrows indicating the forces that favor the binding of the two species and open arrows symbolizing the forces that counteract this phenomenon. ΔG^0_{gas} = free energy change associated with eluite–ligate van der Waals interactions in the gas phase, ΔG_{vdw} = free energy change of eluite–eluent van der Waals interactions, ΔG_{es} = free energy change of eluite–eluent electrostatic interactions, ΔG_{cav} = free energy change associated with cavity reduction in the eluent (eluent–eluent van der Waals and hydrogen bond interactions), $\ln(RT/PV)$ = reduction in free volume and ΔG_{red} = free energy change associated with reduction of eluite–ligate van der Waals interactions in the gas phase. The magnitude of the interactions between the eluite and the ligate, which ultimately determines eluite retention in RPC, is given by the difference between the two opposing effects, i.e. by the balance of all forces acting upon the two species.

contributions of the various molecular interactions involved will undoubtedly offer a better understanding of the overall retention process.

In summary, the macroscopic view of the solvophobic theory predicts that the net free energy change associated with the stationary phase effect is much greater than that associated with the mobile phase effect. But by further dissecting the mobile phase effect at the molecular level, the solvophobic theory clearly identifies the dominant role played by the favorable energetics of cavity reduction in overcoming the unfavorable energetics of eluite–eluent van der Waals interactions. Thus, the solvophobic theory offers a valuable insight on the magnitude and role of various molecular interactions in RPC,

based on which one may indeed conclude that the energetics of RPC retention is largely determined by the favorable gain in energy associated with the decrease in cavity size upon binding of the eluite to the stationary phase. This favorable effect is especially pronounced in processes employing pure water, owing to the extraordinarily high surface tension of water, thus justifying the distinguishing name of the hydrophobic effect. It is, therefore, not surprising that even today, despite the availability of more than 400 commercial brands of C_{18} bonded phases and many more stationary phases containing other functional groups,[153] C_{18} bonded phases continue to enjoy wide popularity among chromatographers for most of the separation problems. Since most C_{18} bonded phases behave in a very similar fashion with minor differences, any one of the many commercially available stationary phases can be employed to solve 90% of the difficult separations encountered in the laboratories. Of course, the choice of the bonded phase depends on a chromatographer's preference based on years of past experience.

EXOTHERMODYNAMIC RELATIONSHIPS

Exothermodynamic relationships are empirical correlations of thermodynamic parameters that typically fall outside the formal structure of thermodynamics. They are employed to explicate the role of molecular structural parameters in chemical equilibria and rate processes and thus to shed light on the underlying physicochemical phenomena. They have been used extensively in RPC to describe various linear relationships observed between the logarithmic retention factor and the operating variables. Recently, the architecture and commonality of many of these exothermodynamic relationships were delineated and organized in a systematic fashion, thus providing a unified framework for the interpretation and analysis of a large body of retention data.[169] The interrelationship of some of the important exothermodynamic relationships as well as the theoretical interpretation of the necessary conditions for their existence is briefly described below.

Linear Free Energy Relationships

A.J.P. Martin[170] introduced the concept of linear free energy relationships in liquid chromatography by expressing the additivity of the free energy increments of structural elements of the eluite molecules. This explained for the first time the remarkable power of chromatography in separating closely related biopolymers, such as proteins and peptides that differ only in a single amino acid. It was indeed very important to recognize that the difference in the free energy change for two eluites that differ in a structural element is proportional to the corresponding free energy change for that

structural element but not the rest of the molecule. This is mathematically expressed as

$$\Delta G^o = \sum_{1}^{z} \Delta G_j^o \tag{44}$$

where ΔG_j^o is the free energy change associated with each structural element j of an eluite containing z such elements.

Free Energy and Molecular Structure

For an eluite that has a reoccurring structural element, Equation (44) can be simplified to

$$\Delta G^o = a_g X + b_g \tag{45}$$

where X is the reoccurring molecular property, such as the number of methylene units or carbon number in a molecule, N, or the nonpolar surface area of the molecule, A_{np}, and a_g and b_g are group molecular parameters. When X is taken as the nonpolar surface area of the molecule then a_g represents the free energy change per unit nonpolar surface area, i.e., the hydrophobic selectivity, and b_g represents the free energy change contribution by polar groups. Combining Equations (2), (3) and (45), the logarithmic retention factor in RPC can be given by

$$\ln k' = aX + b \tag{46}$$

where a and b are proportional to a_g and b_g. A great deal of experimental data have corroborated the applicability of Equation (46) with N or A_{np} as the molecular property in the RPC of homologues and hydrocarbonaceous eluites.[8,47,149,171–174]

Quantitative structure-activity relationships (QSARs) have also been employed to gain insight into the retention mechanism. In general, QSAR methods involve the use of multivariate statistical methods to build linear models relating retention to a property such as chemical structure. Breneman and Rhem[175] recently used solute descriptors obtained from transferable atom equivalent-derived surface property indices to predict retention factors for a set of high-energy materials.

Linear Solvation Energy Relationships

Linear solvation energy relationships (LSERs) were originally developed to rationalize and deconvolute the chemical factors that contribute to the mechanism of various chemical systems.[176,177] This approach has been extensively employed to investigate the retention mechanism in RPC[178–181] and to predict the retention behavior in RPC using training sets of experimental

data.[182–184] The LSER approach considers the free energy of retention to be the sum of weighted solute descriptors, with the weighting factors representing the differences of the mobile and stationary phase contributions. Mechanistic information can then be derived from the magnitude of the obtained weighting factors. Based on this statistical approach, the free energy for phase transfer can be linearly correlated with various fundamental molecular solute descriptor properties. When applied to RPC, the general Equation for $\log k'$ can be written as[183]

$$\log k' = \log k'_o + M(\psi_s - \psi_m)V_2 + S(\pi^*_s - \pi^*_m)\pi^*_2 + A(\beta_s - \beta_m)\alpha_2 + B(\alpha_s - \alpha_m)\beta_2 \quad (47)$$

where the subscripts s and m denote bulk stationary and mobile phase properties, respectively; the subscript 2 denotes a solute property such as cavity or molecular volume V_2, dipolarity/polarizability ($\pi_2{}^*$), hydrogen bond acidity (α_2) or hydrogen bond basicity (β_2). The coefficients M, S, A, B and $\log k'_o$ are fitting parameters independent of the solute and the nature of the chromatographic phases. When applied to a fixed pair of mobile and stationary phases, Equation (46) reduces to

$$\log k' = \log k'_o + mV_2 + s\pi^*_2 + a\alpha_2 + b\beta_2 \quad (48)$$

where m, s, a and b are fitting coefficients characteristic of the pair of chromatographic phases. The coefficients are evaluated by simultaneous multifactor least square regression of experimental data. Many approaches have been put forward to measure, calculate or estimate the solute parameters. Most often McGowan's method is used to calculate molecular volume and gas chromatographic measurements are employed to obtain the rest of the parameters.[185,186] When properly employed, such approaches might be useful in predicting retention factors of eluites and revealing certain mechanistic aspects of RPC retention. Figure 23 illustrates the applicability of the LSER in predicting retention data in RPC for a set of eluites. In terms of mechanistic studies the results have generally identified both the cavity term and the hydrogen bond acceptor basicity as major factors in governing retention behavior in RPC, while solute dipolarity/polarizability and hydrogen bond donor acidity have been found to play a minor role.[182,183] The usefulness of LFERs is however limited by the quality of the data employed for regression analysis. This approach is also subject to spurious statistical artifacts that may result in misinterpretation.

Linear $\ln k' - \ln k'$ Relationships

A linear exothermodynamic relationship for the retention of a set of eluites on two stationary phases has been established for comparing the energetics of eluite retention in RPC employing different columns.[187] If the energetics

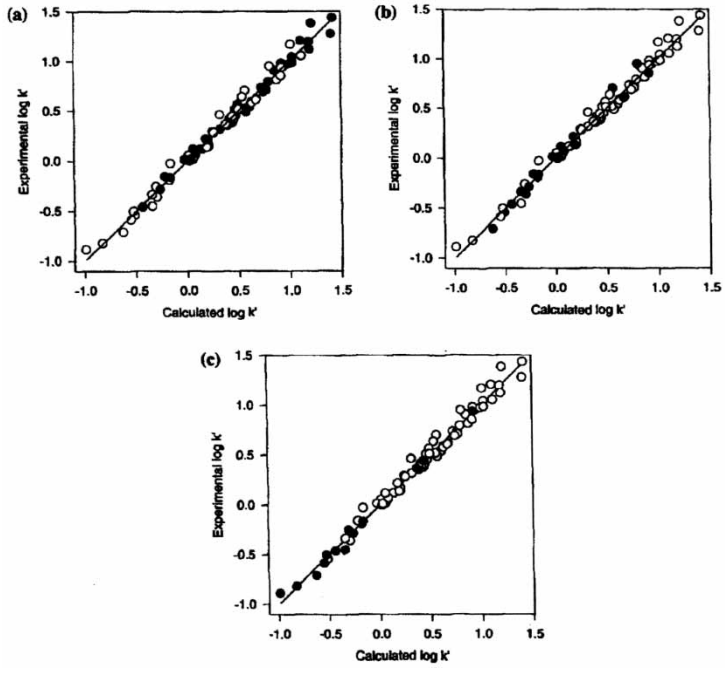

Figure 23. Correlation between experimental and LSER calculated logarithmic retention factors in RPC employing Zorbax Rx-C_{18} stationary phase. The following training data sets were employed: a) aromatic (●) and aliphatic (○) eluites, b) hydrogen bond donors (●) and non-hydrogen bond donors (○) and c) strong hydrogen bond acceptors (●) and the remainders (○).

of eluite retention on the two columns are similar then plots of $\ln k'$ obtained with a set of eluites on one column against that obtained with the same set of eluites on another column are linear at a fixed temperature and eluent condition. Such $\ln\ k' - \ln\ k'$ plots serve as useful diagnostic for the mechanism of complex chromatographic process over a broad range of conditions. Vailaya and Horváth[169] established the criterion for the observation of linear $\ln k' - \ln k'$ plots to be the existence of a linear relationship between the free energy change and a molecular structure property for a set of closely related eluites. Thus, the linear exothermodynamic relationship between logarithmic retention factors obtained on two columns at fixed temperature and eluent conditions can be readily derived by combining the expression in Equation (45) for each column with Equations (2) and (3) and writing in terms of group molecular parameters as

$$\ln k_1' = \frac{a_g^1}{a_g^2} \ln\ k_2' + \ln\ \phi_1 - \frac{a_g^1}{a_g^2} \ln\ \phi_2 + \left(\frac{1}{RT}\right)\left(\frac{a_g^1 b_g^2}{a_g^2} - b_g^1\right) \qquad (49)$$

where 1 and 2 denote the two columns. A similar expression can be derived for the linear relationship between logarithmic retention factors obtained with a set of eluites on a column employing two different eluent conditions. Horváth and co-workers[187] demonstrated that $\ln k' - \ln k'$ plots were linear with a slope of unity for various sets of homologues on most pairs of alkyl bonded phases with medium or long chain ligates, whereas the slope of the linear plots was not equal to one when stationary phases with short alkyl chains or adamantly ligates were involved. Furthermore, they found that retention data on column pairs obtained with eluites of wide ranging polarity in eluents rich in organic modifier did not exhibited linear $\ln k' - \ln k'$ plots. These findings were explained by Vailaya and Horváth[169] on the basis of Equation (49.) At a fixed eluent condition the hydrophobic selectivity for RPC retention on various alkyl bonded phases with ligate chain length greater than C_8 is almost identical, resulting in linear $\ln k' - \ln k'$ plots with a slope of unity. On the other hand, hydrophobic selectivity values of poly(styrene-divinylbenzene) stationary phases and alkyl bonded phases with ligate chain length less than C_8 are very different from those for alkyl bonded phases with ligate chain length greater than C_8. Thus, at a fixed eluent condition linear $\ln k' - \ln k'$ plots with a slope different from unity is expected when the column pair under investigation involves an alkyl bonded phase with ligate chain length less than C_8 and an alkyl bonded phase with ligate chain length greater than C_8 or an alkyl bonded phase with ligate chain length greater than C_8 and a poly(styrene-divinylbenzene) stationary phase. When eluites of wide ranging polarity are analyzed in RPC, nonlinear $\ln k' - \ln k'$ plots are expected due to the interplay of silanophilic and hydrophobic interactions. In such cases, Equation (45), and hence Equation (49), may not be valid anymore.

Collander Equation

RPC is widely used in place of octanol/water partitioning to measure the hydrophobicities of pharmaceuticals. A linear relationship between logarithmic retention factor in liquid chromatography and the corresponding liquid–liquid partition coefficient was first proposed by Collander.[188] Numerous studies have since confirmed this linear relationship between RPC and octanol/water partitioning for a given set of solutes. Again, this can also be readily explained by evoking the linear dependence of free energy associated with RPC retention and octanol-water partitioning on a molecular property of hydrophobic compounds, such as the nonpolar surface area.[169] Thus, the relationship can be expressed in terms of group molecular parameters as

$$\ln k' = \frac{a_g}{a_g^{ow}} \ln K_{ow} + \ln \phi + \left(\frac{1}{RT}\right)\left(\frac{a_g b_g^{ow}}{a_g^{ow}} - b_g\right) \qquad (50)$$

where the superscript *ow* represents octanol/water partitioning, a_g and a_g^{ow} are the hydrophobic selectivities in RPC and octanol/water partitioning systems, and b_g and b_g^{ow} are constants. The validity of Equation has been tested as shown in Figure 24 by the good agreement between the slope values from $\ln k' - \ln K_{ow}$ plots of experimental data derived from a wide variety of sources and the a_g/a_g^{ow} values calculated from hydrophobic selectivities in RPC and octanol/water partitioning systems, respectively.

Relationships Between Thermodynamic Quantities and Other Chromatographic Variables

Most exothermodynamic relationships in RPC are manifestation of a linear variation of the retention free energy with a molecular property of a set of eluites and its combination with Gibbs-Helmholtz relationship between retention free energy change and temperature. RPC retention process involves other variables, such as organic modifier, φ, and stationary phase ligate chain length, N_l. As a first approximation, linear free energy relationships may be assumed with respect to each of these chromatographic variables in a manner similar to Equations (44) as

$$\Delta G^o = a_g^{\varphi}\varphi + b_g^{\varphi} \tag{51}$$

$$\Delta G^o = a_g^{N_l} N_l + b_g^{N_l} \tag{52}$$

Thus, the logarithmic retention factor of an eluite in RPC would vary linearly with the organic modifier composition in the mobile phase or the alkyl chain length of the stationary phase ligate if Equations (51) and (52) are valid.

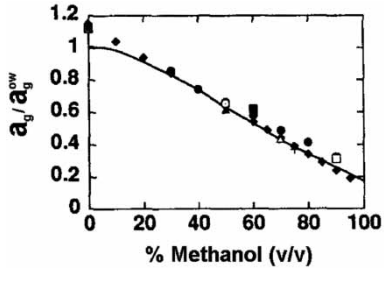

Figure 24. Test of the validity of linear relationship between RPC retention and octanol/water partitioning according to Equation (45). Solid line represents the ratio of hydrophobic selectivity values of RPC retention and octanol/water partitioning determined individually from experimental data. Symbols represent the slope values of linear $\ln k' - \ln K_{ow}$ plots of data from various sources. Reprinted from Ref.[38].

Although experimental RPC data obtained in a wide range of organic modifier concentrations yield nonlinear $\ln k' - \varphi$ plots, it has been well established that Equation (51) accurately describes the retention behavior in RPC over a limited range of organic modifier concentration.[70,189,190] Similarly, experimental retention data obtained with nonpolar eluites on various bonded phases employing methanol/water mobile phase systems appear to support Equation (52) in a small range of ligate chain length, although at higher ligate chain length the retention factor levels off.[145,146,191]

Linear Entropy and Enthalpy Relationships

Martin's additivity relationship for free energy can be extended to other thermodynamic quantities associated with RPC retention, such as enthalpy and entropy as

$$\Delta H^o = \sum_1^z \Delta H_j^o \tag{53a}$$

$$\Delta S^o = \sum_1^z \Delta S_j^o \tag{53b}$$

and in cases where heat capacity change is significant as

$$\Delta C_p^o = \sum_1^z \Delta C_{p,j}^o \tag{53c}$$

Such additivity relationships have been employed to relate thermodynamic quantities for processes involving hydrophobic interactions to molecular structure.[192,193] In a fashion similar to Equation (45), enthalpy, entropy and heat capacity changes associated with a set of closely related eluites can be expressed in terms of a reoccurring molecular property as

$$\Delta H^o = a_h X + b_h \tag{54a}$$

$$\Delta S^o = a_s X + b_s \tag{54b}$$

$$\Delta C_p^o = a_c X + b_c \tag{54c}$$

where a_h, a_s and a_c are enthalpy, entropy and heat capacity changes per reoccurring structural unit, while b_h, b_s and b_c represent corresponding thermodynamic quantities for the nonreoccuring structural elements. Equations (54a) and (54b) have abundant experimental support with RPC retention data. For instance, plots of retention enthalpy and entropy changes against the number of methylene groups or the carbon number of homologous eluites are linear.[147,194] Equation (54c), however, may not hold for eluite retention in RPC employing water-lean eluents since the heat capacity change in such systems is negligible. On the other hand, RPC retention data with proteins and

peptides have confirmed the validity of Equations (54a), (54b) and (54c) when eluite hydrophobic surface area is taken as the molecular property. For instance, Hearn et al.[112] have evaluated thermodynamic parameters, such as free energy, enthalpy, entropy and heat capacity changes, for the retention of polypeptides in RPC and found that they are linear functions of the hydrophobic surface area of the eluites.

Enthalpy-Entropy Compensation

Enthalpy-entropy compensation is another exothermodynamic relationship that manifests itself as a linear dependence of the enthalpy change on the corresponding entropy change, upon changing an experimental variable of the process under investigation.[195,196] It is expressed as

$$\Delta H^o = T_C^X \Delta S^o + \Delta G_{T_C^X} \tag{55}$$

where T_C^X is the compensation temperature, $\Delta G_{T_C^X}$ is the free energy change at the compensation temperature and the superscript X denotes the experimental parameter chosen to obtain a set of enthalpy and entropy pairs form van't Hoff plots, such as a molecular property of the eluite, organic modifier or salt concentration in the eluent, or ligate chain length of the bonded stationary phase.[169] It has been extensively used as a diagnostic tool for the mechanistic identity of various processes. Chemical reactions and equilibrium processes having similar compensation temperatures are considered fundamentally related and are called isokinetic and isoequilibrium processes, respectively.[195] Since enthalpy and entropy values are typically evaluated from van't Hoff plots, they are subject to errors of determination, sometimes leading to spurious artifacts. It has been shown that when plots of ΔG^o against ΔH^o are employed, statistical compensation is minimized.[197,198]

Enthalpy-entropy compensation has been applied for the mechanistic study of RPC data obtained with various hydrocarbonaceous bonded stationary phases under a wide range of operating conditions as far as the eluites and the composition of the eluent is concerned.[187,199–201] The results of these studies indicate virtually indistinguishable compensation temperatures, thus suggesting that the intrinsic mechanism of interaction of small eluites with bonded stationary phases is the same. On the contrary, compensation temperatures in chromatographic systems employing polar stationary phases and nonpolar eluites are significantly lower, indicating a different mechanism. Enthalpy-entropy compensation effects have also been observed in the interaction of polypeptides with hydrophobic ligates of the RPC stationary phase.[112]

Vailaya and Horváth[169] have established the physicochemical basis of enthalpy-entropy compensation when closely related eluites are examined by evoking linear relationships in Equations 54a and Equations 54b-c using

A_{np} for X. In such cases, the parameters of Equation (55) become

$$T_C^{A_{np}} = \frac{a_h^{A_{np}}}{a_s^{A_{np}}} \tag{56a}$$

and

$$\Delta G_{T_C^{A_{np}}} = b_h^{A_{np}} - T_G^{A_{np}} b_s^{A_{np}} \tag{56b}$$

Equation (56a) provides a molecular interpretation of the compensation temperature and establishes a criterion for the existence of enthalpy-entropy compensation. Miyabe and Guiochon[202,203] have recently confirmed the validity of Equation (56a) by analyzing RPC data obtained in THF/water mobile phase system with alkyl benzenes. It is interesting to note that the compensation temperature manifests as the temperature at which van't Hoff plots of eluites that vary in a reoccurring structural unit intersect at a common point. The criterion for its occurrence is the simultaneous existence of a linear dependence of the logarithmic retention factor on both the reciprocal temperature and the molecular structure of the eluite.

Miyabe and Guiochon[203] have extended the concept further to establish a link between linear $\ln k' - \ln k'$ relationships for any two chromatographic systems and enthalpy-entropy compensation as

$$\ln K_2 = \left[\frac{a_s^2 T_1 (T_C^2 - T_2)}{a_s^1 T_2 (T_C^1 - T_1)}\right] \ln K_1 + \frac{1}{RT_2}\left[\left(\frac{a_s^2 T_1 (T_C^2 - T_2)}{a_s^1 T_2 (T_C^1 - T_1)}\right) \times \frac{T_2}{T_1}(b_h^1 - T_1 b_s^1) - (b_h^2 - T_2 b_s^2)\right] \tag{57}$$

where the superscripts and subscripts 1 and 2 denote two different conditions employed in the investigation. Here K_1 and K_2 are the retention equilibrium constants of two chromatographic systems at temperatures T_1 and T_2. Thus, Equation (57) explains the correlation of the retention behaviors in two chromatographic systems with the slope expressed as a function of compensation temperatures, experimental temperatures and group molecular properties. The validity of Equation (57) has been confirmed using RPC retention data in two chromatographic systems that differed 1) only in temperature, 2) only in the sets of eluites employed, 3) only in the mobile phase employed and 4) randomly in any of the above mentioned variables.[203]

Generalized Compensation Model

As mentioned earlier, enthalpy-entropy compensation is a manifestation of the simultaneous existence of linear $\ln k' - (1/T)$ and $\ln k' - X$ relationships, where X is a molecular property such as the eluite carbon number, N, or the

nonpolar surface area A_{np}. On a theoretical basis, Vailaya and Horváth[169] demonstrated that when two linear free energy relationships co-exist, they result in the observation of a common intersection point in each of the plots of the free energy change against one of the variables. For instance, in the case of the chromatographic variable pair $[1/T, N]$, when linear relationships exist between ln k' and $1/T$ as well as between ln k' and N, then extrapolated van't Hoff plots for all the eluites under investigation intersect at one temperature marked by the compensation temperature T_C^N. Similarly, ln $k' - N$ plots at various temperatures using the same data set have a common intersection point given by the carbon number of the eluite, N_C^T. Such common intersection points in linear free energy plots can therefore be considered as characteristics of compensation. By drawing analogies and extending the concept of enthalpy-entropy compensation to other chromatographic variables, Vailaya and Horváth[169] developed a generalized compensation model for RPC retention as illustrated in Figure 25. Combinations of any two variables from a set of four—$1/T$, N, φ and N_l—yield 12 cases of compensation. Indeed, analysis of a large body of experimental RPC data obtained with two chromatographic variables has confirmed the existence of common intersections points in linear free energy plots. As seen in Figures 26 and 27, generalized compensation effects are observed in at least eight cases when methanol/water mixtures and four cases when acetonitrile/water mixtures were employed as mobile phase systems.

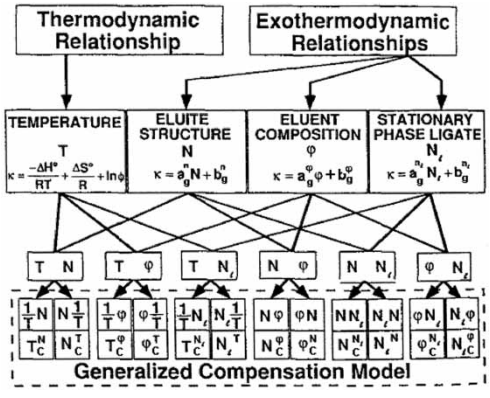

Figure 25. Schematic illustrating the generalized compensation model in RPC. Compensation effects are obtained by combining any two of the four linear free energy relationships for the temperature, eluite structure, eluent composition, and stationary phase property. These combinations lead to twelve compensation parameters, at which the linear free energy plots intersect at a common point. Enthalpy-entropy compensation is a special case of generalized compensation when the van't Hoff relationship is combined with any other LFER. Reprinted from Ref.[169].

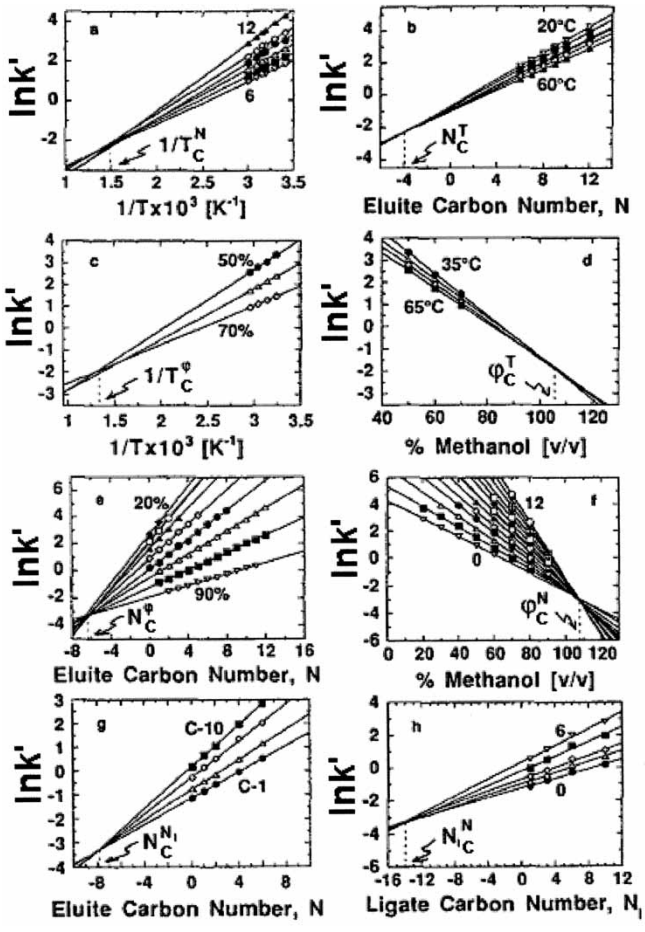

Figure 26. Illustration of common intersection points in eight free energy plots in RPC employing methanol/water mixtures. a) van't Hoff plots with eluite carbon number, N, as the parameter, b) $\ln k' - N$ free energy plots with temperature, T, as the parameter, c) van't Hoff plots with methanol concentration, φ, as the parameter, d) $\ln k' - \varphi$ free energy plots with the temperature, T, as the parameter, e) $\ln k' - N$ free energy plots with methanol concentration, φ, as the parameter, f) $\ln k' - \varphi$ free energy plots with eluite carbon number, N, as the parameter, g) $\ln k' - N$ free energy plots with ligate carbon number, N_l, as the parameter and h) $\ln k' - N_l$ free energy plots with eluite carbon number, N, as the parameter. Reprinted from Ref.[169].

LINK BETWEEN CLASSICAL THERMODYNAMICS AND EXOTHERMODYNAMIC RELATIONSHIPS

In the past, the use of exothermodynamic relationships in RPC has predominantly focused on the interpretation of thermodynamic parameters in order to gain

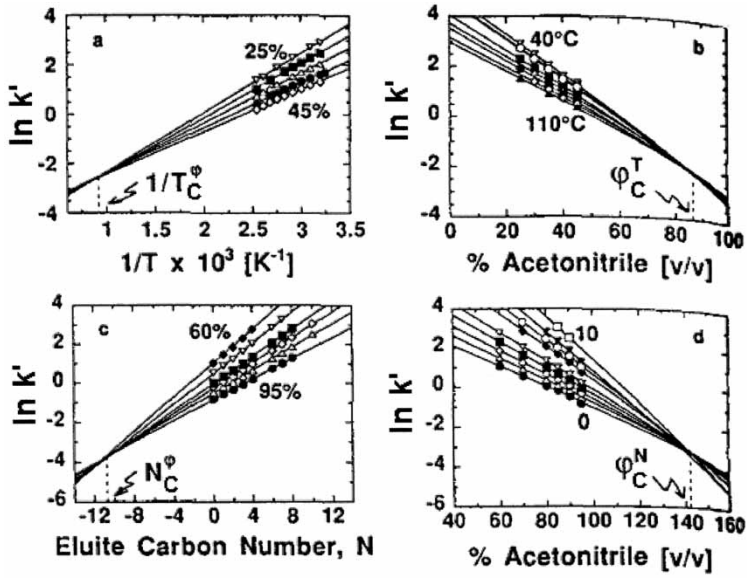

Figure 27. Illustration of common intersection points in four free energy plots in RPC employing acetonitrile/water mixtures. a) van't Hoff plots with acetonitrile concentration, φ, as the parameter, b) $\ln k' - \varphi$ free energy plots with the temperature, T, as the parameter, c) $\ln k' - N$ free energy plots with acetonitrile concentration, φ, as the parameter and d) $\ln k' - \varphi$ free energy plots with eluite carbon number, N, as the parameter. Reprinted from Ref.[169].

mechanistic insights into the retention process. Yet very few studies have actually investigated the criteria for their existence or provided a quantitative interpretation of the correlation between various exothermodynamic relationships. It is clear that such empirical relationships have often been treated as separate entities and accepted at face value without attempting to understand the physicochemical basis of their origin. Nor have they been linked to relationships based on classical thermodynamics. This review presents for the first time the interconnectedness of various exothermodynamic relationships observed in RPC. It is revealed that all exothermodynamic relationships are simply different manifestations of Martin's Equation for free energy additivity and therefore are linked to each other. The use of group molecular parameters such as hydrophobic selectivity, which is unique for systems driven by hydrophobic interactions, establishes the fundamental basis for the existence of such exothermodynamic relationships based on the molecular property. Most importantly, the solvophobic theory unequivocally establishes a link between classical thermodynamics and exothermodynamic relationships in RPC. Using a thermodynamically consistent cycle to break down the RPC retention process into sub-processes, the theory couches the retention free

energy change in terms of chromatographic variables, such as organic modifier or salt concentration in the eluent, stationary phase property and the molecular property of the eluite, and establishes the physicochemical underpinning of the RPC retention process within the realm of classical thermodynamics. Thus, the solvophobic theory expresses the logarithmic retention factor in RPC as a function of the contact surface area of the eluite, the salt or organic modifier concentration in the eluent, the stationary phase property and other physicochemical parameters that can be obtained from the literature. As shown earlier, the expression can be further simplified to yield practically linear relationships between logarithmic retention factor and various chromatographic variables under certain operating conditions in RPC of model compounds. This establishes the physicochemical basis for the occurrence of various linear free energy relationships shown in Figure 26 and justifies their use in the organization and interpretation of retention data in RPC.

FUTURE CHALLENGES

Although the solvophobic theory offers a sound physicochemical interpretation of the RPC retention process in most cases, it is unable to capture the complexity of the chromatographic process in its entirety. In order to develop a more comprehensive theory of RPC retention, certain modifications will have to be made in the solvophobic theory that will result in better quantification of the stationary phase effects and protein retention. It is also recognized that the predictive power of the solvophobic theory can be futher improved by reducing the errors associated with the estimation of the parameters, such as the ratio of ΔA_C to A_{np}, molecular size and κ_E^e.

Quantifying Stationary Phase Effects

The solvophobic theory accounts for the stationary phase effects in RPC via the ΔG_{gas}^o term in Equations 26–30. However, it is assumed that the configurations of the stationary phase ligate chains in the gas phase and the liquid phase are the same. Thus, no effect on the configurational state of the ligates due to the possibility of selective adsorption of the organic modifier onto the stationary phase is taken into consideration, *i.e.*, eluent–stationary phase interactions have been neglected. Furthermore, the solvophobic theory recognizes the lack of knowledge of the molecular geometry of the binding process in RPC and assumes that there are a number of different ways for the eluite to bind to the stationary phase ligate. From a thermodynamic point of view, the retention free energy change is minimized when the contact surface area between the eluite and the ligate is maximum and this determines the orientation of binding. For instance, in polar eluents, the

polar groups of the eluite are expected to be preferentially oriented away from the hydrocarbonaceous ligate and toward the eluent. This energetically favored orientation of binding could significantly affect the selectivity of closely related eluites. For instance, enhanced shape selectivity offered by some stationary phases has been known to resolve certain compounds, such as geometric isomers and optically active compounds, which do not differ significantly in polarity.[204] Although absolute retention is influenced by mobile phase composition, shape selectivity is relatively insensitive to changes in this parameter. The solvophobic theory is unable to account for differences in retention that result for isomers or for shape selectivity differences observed among various C_{18} columns. Thus, a great deal of investigation is required to gain insight into the actual molecular orientation of binding. The knowledge gained can be used to better estimate the contact surface area parameter in the solvophobic theory, resulting in improved correlation between experimentally observed and theoretically calculated retention factors. As Miyabe and Guiochon[153] have suggested recently, the ratio of ΔA_C to A_{np} is slightly different for bonded stationary phases having varying lengths of the ligate chain, possibly indicating the difference in the manner of steric interactions between the eluite molecules and the alkyl ligates.

Unfortunately, progress in this direction is greatly hampered by our scant knowledge of the topography of the stationary phase surface and the arrangements of hydrocarbonaceous ligate chains. Over the last twenty years, much has been written on the conformational order of bonded alkyl chains as a function of solvent parameters, stationary phase grafting procedure, surface coverage and temperature.[205–217]. Most of the studies have used spectroscopic methods to study the role of eluent–stationary phase interactions in solute retention in RPC. It has long been speculated that hydrophobic alkyl chains are fully extended in the organic rich mobile phase but collapse or lie down onto each other and onto the silica surface in water rich mobile phases. In many cases, nonpolar solvents have been reported to disorder the alkyl chains of the stationary phase, as a result of deep intercalation or partitioning of the solvent into the stationary phase layer.[206,213,218] In contrast, polar solvents have been reported to either have no effect on the stationary phase order[219,220] or increase alkyl component order relative to water.[221–223] In water, some researchers have described the stationary phase as being collapsed in which the alkyl chains act to exclude water from the interior of the phase.[224–226] Recent investigation using Raman spectroscopy have, however, shown no evidence that the stationary phase ligates were collapsed or extended as a function of the mobile phase composition even when a pure aqueous mobile phase was used.[227] In stark contradistinction, some others have concluded that all alkyl bonded phases exist in a collapsed state in all mobile phase conditions, based on low temperature nitrogen adsorption measurements and excess adsorption isotherm

studies.[45,228] Evidence also exists for a phase transition of the bonded phase chains at a temperature similar to the metling temperature of neat octadecane. These conclusions are based on differential scanning calorimetry experiments[229] and on breaks or non-linearities in a van't Hoff plot.[230,231] However, spectroscopic studies seem to indicate that a gradual change in the stationary phase conformation, rather than an abrupt transition, actually occurs.[232] Thus, there does not appear to be a consensus, and the mechanistic interpretation of the stationary phase configuration is still only speculative.

Predicting Retention of Biologicals

A full understanding of the chromatographic binding of proteins requires a detailed knowledge of the chemical and physical nature of both the mobile phase and the stationary phase as well as information about the types of interactions involved between the proteins, the eluent and the ligate. While little is known about the three-dimensional molecular structure of proteins and polypeptides at the chromatographic surface, experimental data with proteins suggest that they interact with the chromatographic surface in an orientation-specific manner[233–235] It is generally believed that proteins unfold upon binding under the harsh conditions in RPC employing organic-rich eluents. Furthermore, salts are often employed to modulate retention of proteins in RPC. It is known that certain salts, such as $MgCl_2$ and $CaCl_2$, preferentially interact with the proteins, thus resulting in a retention behavior different than expected. Thus, retention of proteins and peptides in RPC is strongly influenced by a combination of complex variables – steric hindrance, electrostatic and hydrophobic interactions, and conformational

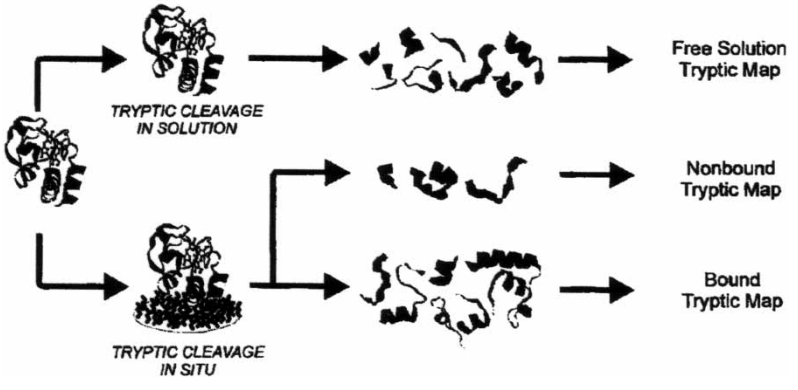

Figure 28. Schematic representation of the in situ proteolytic digestion procedure for proteins adsorbed to hydrocarbonaceous bonded stationary phases in RPC.

changes. Owing to this complexity, the ability of the solvophobic theory to adequately describe and predict the molecular details of peptide and protein interactions in RPC is limited from a quantitative view point. Modeling of the interactions of peptides with RPC stationary phases using molecular dynamics could potentially shed some light on the role of hydrophobic interactions in these processes.[236] Other elegant procedures, such as the procedure developed by Hearn et al. (see Figure 28) to identify chromatographic binding domains of proteins in RPC via proteolytic cleavage[237] or the use of probe molecules in carefully designed chromatographic experiments,[238] may also reveal valuable information regarding the three-dimensional structures of proteins, their orientation as well as the extent of unfolding upon binding.

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