3 Kosmotropic Chromatography of Proteins
Theory and Practice

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CONTENTS

3.1 Introduction 102
3.2 Kosmotropicity in Chromatography 103
3.3 Kosmotropic Salts in Hydrophobic Liquid Chromatography 103
3.4 Kosmotropic Salts and the Hofmeister Series in Three Chromatographic Regimes 106
3.4.1 Thermodynamic Aspects of the Hofmeister Series 108
3.4.2 Overview of Possible Mechanisms for the Hofmeister Series and Salting Out Effects 113
3.4.3 Theories Proposed to Explain the Hofmeister Series 114
3.4.3.1 Hydration Theories 114
3.4.3.2 Dipole Theories of Water 115
3.4.3.3 Electrostatic Theories 115
3.4.3.4 Internal-Pressure Theories 115
3.4.3.5 Theories Based on van der Waals Forces 116
3.4.3.6 Ion Pairing 116
3.4.4 A Quantum-Mechanical Approach to the Hofmeister Series 118
3.5 Chromatographic Implications of Kosmotropic Salts and the Hofmeister Series 119
3.6 Development of the Liquid Chromatographic Method for HIC Protein Separation 119
3.6.1 Optimization of the Separation in HIC 122
3.7 Conclusions 123
References 123

3.1 INTRODUCTION

During the late 1980s and early 1990s, there was a special interest in studying what was thought to be the ultimate effort to complete a theoretical and practical understanding of the chromatographic separation of proteins. As a result of these studies, fundamental achievements in the theory and practice of hydrophobic interaction, ion-pairing and reversed-phase chromatography were made. This work was conducted by various scientists such as Lloyd Snyder, Csaba Horváth, Georges Guiochon, Dan Martire, Fred Regnier and Barry Karger, among others. One of the most important subjects was an understanding of the practical consequences of the salting out effect.

Salting out is caused by different salts that decrease protein solubility in water. Salts that increase protein solubility are said to promote salting in. Salting out is a consequence of changes in the structure of proteins in an aqueous solution. It is useful in the chromatographic analysis of proteins using aqueous mobile phases and hydrophobic stationary phases [1].

Water has a unique, low entropy structure, governed by hydrogen bonding. It is known that ions are hydrated in aqueous solutions. Ion hydration causes changes in the structure of the aqueous environment. For example, the presence of ions is related to protein unfolding and protein separations through salting in and salting out processes. Hydrated ions have strong interactions with water molecules, and can change the structure of water. When hydrated ions increase the structuring of water, they promote protein salting out and are called "order makers" or "kosmotropes." Other ions decrease the structure of water and promote protein salting in. These are called "disorder makers" or "chaotropes." Both terms, "kosmotropes" and "chaotropes," originated from the Hofmeister series, which orders ions in terms of their ability to stabilize or destabilize proteins [2].

Kosmotropes are usually small ions with a high charge density. Chaotropes are large ions with a lower charge density. A general rule that helps to distinguish one type from the other is that kosmotropic ions show radii below 106 pm for monovalent cations, and below 178 pm for singly charged anions [2].

This review outlines the main theoretical aspects related to the effect of kosmotropic salts on chromatographic separations. It also describes the relation of the salting out effect to the Hofmeister series, and some practical high performance liquid chromatography (HPLC) applications. This review is intended not to be exhaustive but to provide a description of various chromatographic behaviors observed in the hydrophobic interaction chromatography (HIC) of proteins; and the most common analytical strategies for method development are discussed. Exhaustive reviews on specific subjects such as electrostatic interactions, Hofmeister effects on biological systems and the behavior of non-aqueous systems are available elsewhere [1, 2, 3]. Many publications on chromatography are related to kosmotropic effects that are applied mainly in the pharmaceutical industry [4], but they are omitted here with the explicit intention of addressing kosmotropicity as an important phenomenon in protein separations.

The objectives of this work are to review some theoretical models proposed to explain the kosmotropic effect, and to describe strategies for method development in order to improve kosmotropic chromatography. The amount of literature published on this subject is increasing. To date, most models proposed are far from being unifying rationales that explain the results and observations.

3.2 KOSMOTROPICITY IN CHROMATOGRAPHY

"Kosmotropicity" is a term to describe the effect of an aqueous solute capable of acting as an agent that increases the structure of water. The opposite effect is termed "chaotropicity." The most common kosmotropic agents are ammonium sulfate, potassium phosphate and sodium sulfate. The first attempts to explain kosmotropicity treated the electrostatic interactions between an ionic solute and its hydration sphere, mainly the anion. These interactions were supposed to favor an increase in water structure. More structured water consequently expelled a non-electrolytic analyte from the increasingly organized eluent, thereby decreasing its solubility [5]. However, recent evidence indicates that this explanation might be incorrect.

Kosmotropic chromatography, originally referred to as "salting out chromatography," is based on a decreased solubility of an analyte in the mobile phase caused by the presence of salts of specific types. The altered analyte solubility also causes an increased interaction with the stationary phase by a mechanism that is not fully understood, as is discussed in further sections. The salting out effect has hence been applied in order to modify the retention factor in separations of small and large molecules. Some separations use a single concentration of the salt, whereas others implement a salt gradient [5]. Its practical application resides in an empirical relation between the retention factor and the salt concentration. Salts commonly used as kosmotropic agents have been observed to follow a trend known as the Hofmeister series [6].

The Hofmeister series is related to kosmotropicity and chaotropicity. Štrop [6] and Loeser [7] observed that different salts showed the same tendency as that observed in the Hofmeister series in HIC and in reversed-phase liquid chromatography (RPLC), respectively. In general, chaotropic salts act by increasing the solubility of proteins (salting in), and kosmotropic salts act by decreasing it (salting out) [6, 7].

Several authors have tried to explain kosmotropicity in terms of electrostatic interactions [8, 9, 10], thermodynamic considerations [11], quantum-mechanical explanations [12, 13] and the simulation through molecular dynamics [14, 15, 16]. As evidence subsequently showed, structure-making in aqueous solutions by kosmotropes is not a consequence of the interactions with water molecules. Several results indicate that the main interaction occurs between a salt and an analyte, not because of a direct relationship with the hydration sphere of the salt. Anions have been observed to interact with proteins more strongly than cations because they are more polarizing [15]. This result opens a new way of describing the mechanism of interaction of kosmotropes with proteins.

3.3 KOSMOTROPIC SALTS IN HYDROPHOBIC LIQUID CHROMATOGRAPHY

This section addresses three types of chromatographic modes used in protein separations with kosmotropic additives: conventional reversed-phase liquid chromatography (RPLC), hydrophobic interaction chromatography (HIC) and ion-pairing chromatography (IPC). The use of kosmotropic salts is briefly presented in agreement with the nature of each technique.

A liquid-chromatographic system has a complication arising from a combination of several parameters. In RPLC these parameters include: the sample containing the analytes that must be separated, an aqueous solution to which non-ionic organic compounds (that can be polar or non-polar) might be added, the various salts that are used for the buffer eluent, the hydrophobic or hydrophilic nature of the adsorbate, and a variety of their concentrations. This complication has made it extremely difficult to propose a theoretical framework able to correlate all the experimental observations, and to make predictions. One such observation yet to be explained is the use of salts to modify the retention of the analyte upon a change in the analyte solubility.

The solubility of a non-polar analyte in an aqueous solution of an electrolyte might change from the salting effect. If solubility decreases with increasing concentration of the electrolyte in the mobile phase, salting out occurs. If solubility increases, then the non-polar analyte is said to be salted in [5, 17]. For the purpose of the definition, electrolytes and non-polar analytes are compounds that have large and small solubilities in water, respectively. The influence of an electrolyte in an aqueous solution of a non-polar analyte is expressed mathematically according to the Setschenow equation [5, 17]:

$$\log \frac{s_0}{s} = \log f_c = k * c_s \tag{3.1}$$

in which $\boldsymbol{s_0}$ and $\boldsymbol{s}$ are the solubilities of the non-polar analyte in water and the electrolyte in the solution, respectively; c_s is the concentration of the electrolyte in moles per liter; f_c is the activity coefficient of the non-polar analyte; and k is called the "salting parameter." A positive value of k indicates salting out whereas a negative value indicates salting in. Because $\boldsymbol{s_0}$ is constant in an aqueous solution, it follows from Equation (3.1) that the amount of electrolyte added is directly proportional to the amount of non-polar analyte precipitated and vice versa.

The effect predicted by the Setschenow equation is particularly evident when kosmotropic salts are used with the specific purpose of salting out proteins in HIC. This HPLC mode uses hydrophobicity as a property to purify and to separate macro biomolecules. It comprises chromatographic techniques that have in common the addition of salts to the mobile phase to help modulate or to modify hydrophobicity, and hence the retention of analytes on the hydrophobic sites of the stationary phase. The targets might be small molecules, non-ionic or ionic small molecules or biomolecules (proteins and DNA) that differ in their hydrophobicity. These techniques originated in the 1950s. The mechanism of the loss of solubility has since been related to the effects of ions, and was the first to be described as "salting out chromatography." Biomolecules are the target analytes to be salted out from the mobile phase in order to modulate their retention on the stationary phase as a function of salt concentration [18]. Figure 3.1 shows a classical separation of four biomolecules using HIC. This separation uses ammonium sulfate as a salting out additive and ammonium acetate as a buffer. The use of ammonium sulfate along with the acetate buffer can increase the retention factors, improving resolution and the time for the chromatographic test [18]. Figure 3.1 shows the separation of a mixture of biomolecules using 3 M ammonium sulfate [18].

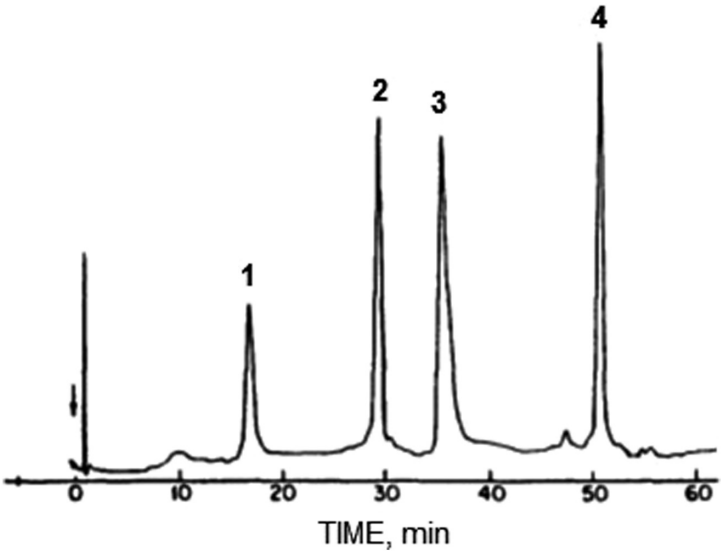

FIGURE 3.1 Hydrophobic interaction chromatogram of a protein mixture in linear gradient from 3 M ammonium sulfate + 0.5 M ammonium acetate, pH 6.0 to 0.5 M ammonium acetate, pH 6.0. Peak **1** corresponds to cytochrome-c, **2** ribunuclease, **3** lysozyme and **4** (α)-chymotrysinogen A. Reproduced from [18] with permission of the publisher.

Figure 3.1 is an example of the role that salting ions play in HIC separations. Among possible patterns, ions exhibit attractive forces with water molecules and with the organic modifiers added to the mobile phase. They also show adsorption on the stationary phase, but their effect is more strongly related to concentration. This effect arises from interactions of two types – non-specific [9, 19] and specific [4]. At small concentrations, non-specific interactions between the solute and the salting out ions are related to the charge of the ions. At any concentration, the ions produce double layer shielding [20, 21]. Other effects arise from electrostatic interactions between ions and solute molecules, or between the solute molecules and the stationary phases. These effects are independent of the type of salt, but dependent on concentration.

Specific effects of non-ionized species, such as hydrophobic interactions, appear at concentrations greater than 100 mM [21] and have been related to ions in the Hofmeister series through their increasing capacity to salt out proteins from aqueous solutions [15]. Other effects are the polarization induced by anions over adjacent water molecules, for instance, interference with the hydrophobic hydration of macromolecules and direct binding to the macromolecule. At concentrations below 100 mM in the presence of salts, hydrophobicity is of concern, because at that concentration IPC takes place.

IPC is a reversed-phase alternative that uses the addition of an ion-pairing reagent (IPR) to the mobile phase. The IPR contributes with counterions to form neutral ion pairs with the analyte ions. Ion pairs having a neutral electric charge are attracted to the stationary phase, retained and separated [22]. The formation of ion pairs was

proposed by Manning in 1969 as counterion interaction based on an infinitely long charged row. For polyelectrolyte solutions, ion pairs form at a limit of zero concentration [23]. Manning's conclusions support empirical observations in IPC about the retention behavior in a reversed-phase mode. In an IPC system at a given ionic strength, the addition of an IPR favors counterion interaction. As a consequence, the inverse of the product of the charges of the polyion and its counterion remain approximately constant. This causes deviations in the order of the salting out of various salts observed in RPLC and HIC, as confirmed by Florez and Kazakevich [24].

These authors studied the separation of ionic analytes using RPLC with kosmotropic and chaotropic additives. They observed that, under the conditions of their experiments, the salting out effect of a positively charged analyte had no relation to the effect of the additives (chaotropic or kosmotropic) on the separation of ionic analytes. This is the opposite behavior of that expected in protein separations with the same technique [24]. The relation of the salting effect to the Hofmeister series is discussed in the next section for RPLC, HIC and IPC. For the lattermost, recent evidence shows that salts fail to follow the Hofmeister trend in an IPC regime.

3.4 KOSMOTROPIC SALTS AND THE HOFMEISTER SERIES IN THREE CHROMATOGRAPHIC REGIMES

The effect of kosmotropic salts on the salting out of proteins from the mobile phase in RPLC follows the Hofmeister series. This behavior is more evident under HIC conditions and not evident in IPC. An important generalization about the behavior of kosmotropic salts is that large concentrations are necessary for their retention effects to be manifested in the stationary phase. These effects are generally in compliance with the salting out or salting in potential of ions. The trend was first described by Hofmeister in 1888 and the series contains both chaotropic and kosmotropic ions [5]. Here the concept of large concentrations of kosmotropic ions is negligible. It has been pointed out that salting out effects arising from Hofmeister behavior are observed in biological systems at concentrations of about 100 mM under physiological conditions. This opposes chromatographic systems using hydrophobic interaction, where concentrations can, however, be as great as 3 M and even 6 M [18].

A classification between chaotropic (anions) and kosmotropic (cations) ions arose initially according to an erroneous belief that anions have no hydration layer. Such an absence of a hydration layer allowed anions to affect the structure of the water more directly, for example by interrupting the network of hydrogen bonds. Anions could also cause protein unfolding and promote salting in effects. In contrast, cations that have a well-defined hydration layer would help reinforce the structure of the water and strengthen and stabilize the salting out effects of proteins and other macromolecules [15]. Although these effects occur commonly in multiple processes, the mechanisms that explain this behavior are poorly understood [1].

It is accepted that ions exert two opposing effects on proteins and charged macromolecules. One effect is the electrostatic interaction between the charges of proteins (or macromolecules) and the positive or negative ion. These interactions are non-specific in nature and favor a distribution of counterions around or near the surface of proteins (or macromolecules). The second effect is the solvation of ions located outside the

double electric layer, which is a specific interaction. This solvation favors ion hydration in the bulk solvent. Under these solvation conditions, movement of ions from the bulk solvent to the surface of the stationary phase is unfavorable. Even though this charge distribution decreases the electrostatic interaction of the protein with the stationary phase, retention can be modulated by controlling salt type and concentration [1, 25].

During chromatography a protein can exist either in the mobile phase or be adsorbed in the stationary phase (folded in the mobile phase or unfolded in the stationary phase). Differences arise from the surface which the proteins are interacting with, and from the presence of salts that can alter the equilibrium in any direction (non-specific effects in the mobile phase and specific effects in the stationary phase). The way in which the salts displace the solubility equilibrium to favor protein salting out is not clear. The interaction involved in the solubility (and in the salting out) of proteins was studied by Kirkwood [1]. This author proposed that a salt ion has a repulsive interaction with a charge inside a low relative permittivity cavity in the protein. At low salt concentrations, solvation interactions dominate (known as Debye–Hückel interactions). The salts displace the equilibria toward the dissolution phase, causing salting in due to an attractive interaction with the charge inside the cavity. At large concentrations, the hydration effect of the ions drives the equilibria toward repulsive interaction, causing the protein to be displaced from water to the solid stationary phase. That is, decreasing the solubility and causing salting out. Kirkwood's findings were studied more rigorously in terms of the work required for the attractive and repulsive interactions [25]. The chemical potential of the protein depends on two kinds of work. One is the amount of work necessary to charge the salt ions around the protein's low dielectric cavity. The other is the work necessary to charge the protein in the presence of the electrically charged salt ions [1]. The latter is the salting in work. The former is the salting out work, also called the "Kirkwood term" [25]. The results allowed the relation of the Kirkwood term (W^{K}) in Equation (3.2) to the work of charging the ions around the cavity of small relative permittivity of the protein [25]. This term, plus the term corresponding to the Debye–Hückel interactions (ΔG_{DH}), yielded the following expression for the solubility of a protein [25]:

$$-k_B T \ln\left(\frac{S}{S_0}\right) = \Delta G_{DH} + W^k \tag{3.2}$$

In Equation (3.2) $\boldsymbol{k_B}$ is the Boltzmann constant, T is the temperature in K, S is the solubility of the protein in g/L in the presence of the salt, S_0 is the solubility of the protein in g/L in the absence of the salt, ΔG_{DH} is Gibbs energy of the interactions of Debye–Hückel type and W^k is the Kirkwood term. Equation (3.2) describes a diphasic behavior with increasing salt concentration. It shows that, initially, increasing the concentration of a salt, measured according to the ionic strength, I, increases the solubility of a protein (salting in) favored by the term ΔG_{DH}. Further increasing the salt concentration results in the solubility reaching a maximum and beginning to decrease, as the term W^k becomes dominant. Most salts at small concentration ($I < \sim 1$ M) have a salting in effect on proteins, consistent with the term ΔG_{DH}, and show a salting out behavior as concentration increases [1, 25]. An example of this behavior is shown in the next section in Figure 3.3.

However, Kirkwood's treatment does not explain observations about the behavior of electrolytes with themselves. In order to account for the behavior of electrolytes, electrostatic interactions must be considered: for instance, the formation of cation–anion pairs, and the interaction of the ion pair with the solvent. Although two 1:1 electrolytes are expected to behave similarly, it is known that the pH or electrolyte activity depends on the specific type of cation–anion pair and its interactions [26]. An example of such interactions are those of the cation–anion pair with solvent molecules and also with the adsorbent. Local interactions between an ion and a solute (or part of it) are also possible. Another possible interaction is with an interface, which means that an ion might be specifically adsorbed. All these possible interactions change the surface tension of the solvent. In consequence, surface tension changes with the surface concentration of the ion pairs. This has been confirmed by X-ray diffraction [26]. At the interface between the liquid and vapor of a saturated solution, anion and cation concentrations are different from those in the bulk solvent [27]. It was observed that there is an excess of anions with respect to cations. X-ray experiments with potassium halides showed that the ratio of iodide to potassium was greater than the ratio of bromide to potassium, revealing specific effects. This trend notably follows the observed effects of the Hofmeister series [27].

Electrostatic theories fail to explain several observations. For example, how the salting effects alter when the electrolyte is altered, or how a Born radius varies with temperature (a small Born radius is related to weak electrostatic interactions in bulk water) [26]. The paradigm used in electrostatic theories fails to take into account the existence of dipole–dipole interactions, or the short-range interactions of van der Waals forces. In order to take these interactions into account, the following approach has been proposed [26].

The solvent is considered to be a continuum that is characterized with a relative permittivity and lacks its own structure in the calculations. Short range forces in the solvent can be attractive or repulsive and can affect the solution. Calculations in a solution phase require a model of many bodies to describe all possible interactions. Among the most relevant interactions are those of Debye (induction), Keesom (orientation) and London (dispersion). Debye and Keesom interactions arise from interactions between permanent dipoles, ions and induced dipoles. London forces are quantum mechanical in nature, and relate to polarizability and ionization energy of the ions, reflecting their specific nature. In summary, the problem is manifold because of the presence of the structure of the solvent [26].

When the solvent is water, such effects follow the Hofmeister series [26]. Other kinds of kosmotropic salts that follow the Hofmeister series include ionic liquids [28–35]. In the following sections, we try to present several theories which attempt to explain mechanistically the interaction of ions in the Hofmeister series with different analytes. This will shed some light on the HIC of macromolecules.

3.4.1 Thermodynamic Aspects of the Hofmeister Series

Kosmotropic ions are supposed to induce local water structuring through hydration. This structuring depends on the size of the ions and on electrostatic and quantum-mechanical interactions. The short-range interactions involving the ionic

species depend on van der Waals forces between the hydrated ions and the surfaces (due to the superposition of the hydration layers). The long-range electrostatic interactions depend on the nature of the solvent and its interaction with the molecules that might be present, such as proteins, other biomolecules and impurities (dissolved gases) [26, 27].

The efficiency of the most common ions as promoters of salting out (kosmotropicity) follows these decreasing orders of the Hofmeister series [26, 27]:

For anions,

$$OH^- > SO_4^{-2}, CO_3^{-2} > ClO_4^- > BrO_3^- > Cl^- > H_3CCOO^- > IO_3^-, IO_4^- > Br^-, I^- > SCN^- > NO_3^-$$

For cations,

$$Na^+ > K^+ > Li^+ > Ba^2 > Rb^+ > Ca^2 > Ni^2 > Co^2 > Mg^2 > Fe^2 > Zn^2 >$$

$$Cs^+ > Mn^2 > Al^3 > Fe^3, Cr^3 > NH_4^+ > H^+$$

This order is not rigorous but is subject to variation due to the nature of the system and the type of effect studied. A pictorial view of the Hofmeister series for anions and cations, adapted from reference [36], appears in Figure 3.2.

Figure 3.2 shows the anions and cations in decreasing order of kosmotropic effect from top to bottom. The effect has been studied by several authors [17] and has special interest in the effect on protein stability under specific conditions [26]. As an example, Figure 3.3 shows the effect of salt concentration on thermal denaturation of poly(N-isopropylacrylamide), PNIPAM. The effect is shown on cooling PNIPAM in the presence of different salts [37].

The maximum chaotropic effect is shown by SCN^-, followed by I^-, Br^- and NO_3^-. The kosmotropic ions F^-, $H_2PO_4^-$, $S_2O_3^{2-}$, SO_4^{2-} and CO_3^{2-} show an approximately linear dependence of protein stability versus salt concentration. In the case of chaotropes, the stabilization of protein structure shows an increase in temperature at small salt concentrations. The open circles correspond to biphasic behavior points on a temperature gradient [37]. The trends observed are in agreement with Equation (3.2).

The terms "kosmotropes" and "chaotrope" conform to a supposition that chaotropic ions break the structure of water formed through hydrogen bonds. Chaotropes also destabilize the folded proteins promoting salting in. On the other hand, kosmotropic ions are strongly hydrated. These ions stabilize the folded proteins and macromolecules and produce salting out. This presumption is not substantiated, as experiments have shown no relation between ions and water structure whatsoever, as is discussed below.

As has been shown, ions do not affect the properties of bulk water. Instead, their salting effect on the solubilization of proteins seems to be due to specific ion–protein interactions, even at small salt concentrations [38]. For example, the preferential accumulation of anions around a positively charged protein follows the order $SO_4^{2-} > SCN^- > I^- > Cl^-$. Except for SO_4^{2-}, the observed order is almost

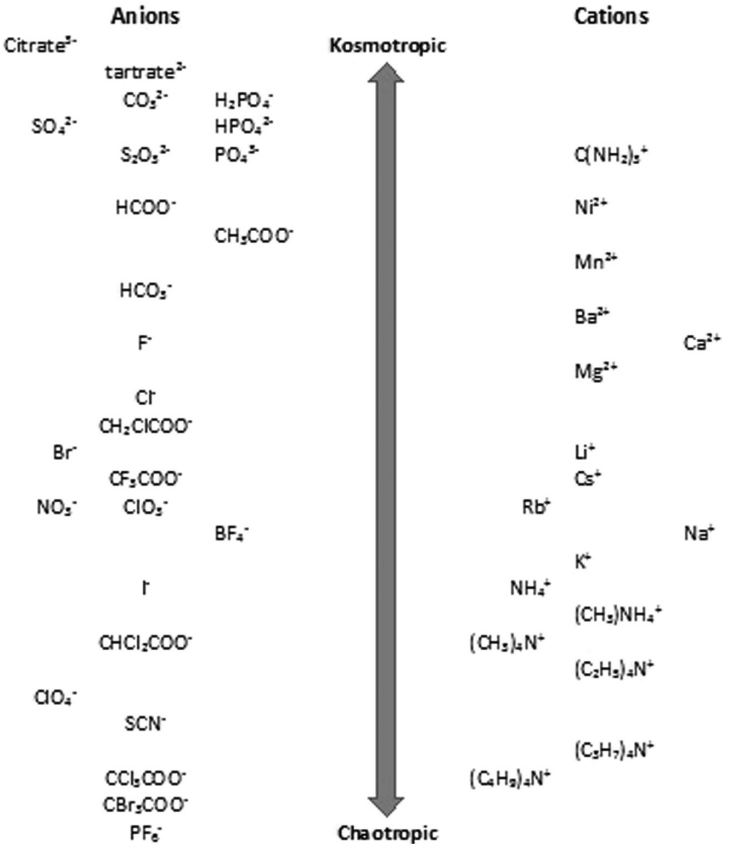

FIGURE 3.2 Hofmeister series for anions and cations in aqueous solution. Adapted from the scheme by Mazzini et al. [36].

the inverse of the Hofmeister series [38]. These selective effects are observed at concentrations of 50 mN and are manifested as dependent on the effective charge of the anion–protein interaction, but not on the cation–protein interaction [38]. This result demonstrates a progressively greater binding of the monovalent anion to the protein. The SO_4^{2-} ion, despite being strongly hydrated, interacts directly with the surface of a protein [38]. Polyvalent cations can bind strongly to acidic residues of the protein and reverse the net charge of the protein [39]. Figure 3.4 was adapted from reference [14]. It shows a possible mechanism according to which interactions of this kind arise between ions and a protein, with no direct interaction affecting the structure of water [14].

Figure 3.4 [14] indicates the possible ways in which cations and anions interact with water and protein dipoles. For example, kosmotropic anions polarize water molecules that are hydrogen-bonded to proteins on the positive end of the dipole. Zhang and Cremer obtained a similar result for the interaction of PNIPAM in Figure 3.3 in the presence of kosmotropic anions [15]. Anions polarize the

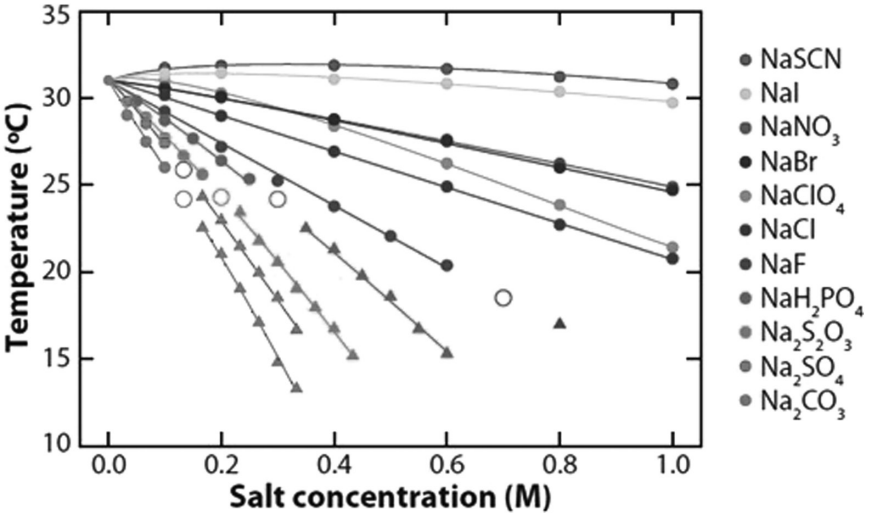

FIGURE 3.3 Effect of different sodium salts on the temperature of thermal denaturation on cooling of the macromolecule poly(N-isopropylacrylamide). At concentration 0.1 M, the specific effects of anions are manifested. For the chaotropic anions I^- and SCN^-, the characteristic stabilizing effect is manifested. The five kosmotropic ions show a linear dependence on the salt concentration. Taken from [37] with permission of the publisher.

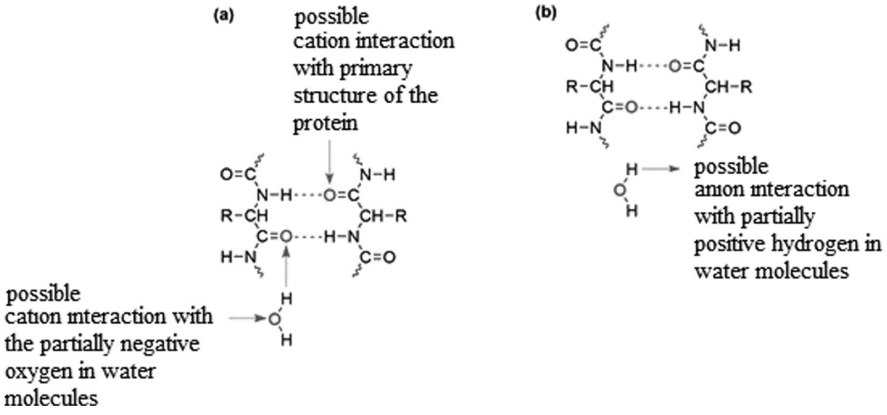

FIGURE 3.4 Possible mechanism of ion effects over the primary structure of a protein adapted from Xie and Gao [14]. (a) The cation can "solvate" carbonyl directly or indirectly by increasing the availability of water hydrogen, and (b) the anion competes with the carbonyl for hydration.

water molecules hydrogen-bonded to the amide moieties. Conversely, Xie and Gao included also the cation interaction with the oxygen atoms in water [14]. Experimental results support the possibility that ions do not affect the structural properties of the bulk water [14].

The time it takes for water dipoles to change their orientation in ionic solutions has been measured by spectroscopic techniques [3]. Measurements showed that,

outside the first solvation shells of ions, there is no influence of the ions on the rotational dynamics of water molecules. The correlation times for the water molecules in the first hydration layer of Cl^-, Br^-, I^- and ClO_4^- were smaller than for molecules in the bulk (in increasing order of ionic radii). These experiments clearly showed that these anions have no influence on the water dynamics in the bulk. Such a condition holds even at concentrations of both kosmotropic (SO_4^{2-}) and chaotropic (ClO_4^-) ions, up to 6 M. The conclusion was that ions do not cause a long-range effect of formation or destruction of water structure [3, 40].

The thermodynamic aspects of the effects of ions on the structure of water were studied by Pielak et al. [41]. The bulk water was considered to be constituted of two species that exchange rapidly from one another – one less dense and more structured, the other denser and less structured. A *structure-making* solute, such as a kosmotropic salt, increases the fraction of the less dense species. This occurs at the expense of the denser species in the hydration of the solute. A *structure-breaking* solute has the opposite effect. Pielak et al. related this change in density to the change in partial molar thermal capacity with pressure at constant temperature. Table 3.1 shows the effect of different solutes on the structure of water, measured in calorimetric

TABLE 3.1
Solute Effects on Protein Stability and $(\partial C_p/\partial P)_T$ at 25°C

Compound	Effect on stability[a]	$(\partial C_p/\partial P)_T$ 10^{-6} at 25°C/J Pa^{-1} K^{-1}[b]
$(NH_4)_2SO_4$	+ +	4.58
NH_4Cl	+	3.67
guanidinium Cl	– –	2.62
guanidinium SCN	– – –	2.64
N-methylglycine (sarcosine)	+ +	1.96
urea	–	2.02
glucose	+	1.08
N-trimethylglycine (betaine)	+ +	0.966
trehalose	+	0.669
sucrose	+	0.710
glycerol	+,–	0.694
stachyose	+	0.537
melezitose	+	0.182
1,3-dimethylurea	–	–0.131
trimethylamine *N*-oxide dihydrate	+ + +	–0.859
1,3-diethylurea	–	–0.595
2-propanol	[c]	–0.268

[a] (+) indicates stabilizing, (–) indicates destabilizing. The number of symbols is related to the magnitude of the effect.

[b] Uncertainty is ± 0.02 10^{-6} J Pa^{-1} K^{-1}.

[c] Not applicable because 2-propanol is not commonly used to affect protein stability.

Adapted from J. D. Batchelor, A. Olteanu, A. Tripathy, G. J. Pielak, Impact of Protein Denaturants and Stabilizers on Water Structure, *J. Am. Chem. Soc.* 126(7) (2004) 1958–1961. doi:10.1021/ja039335h.

experiments. Values of $\left(\frac{\partial \bar{C}p}{\partial P}\right)_T > 0$ would imply that a solute breaks the structure of bulk water; the opposite sign means that structure is created [41].

In Table 3.1, $\bar{C}p$ is the partial molar thermal capacity at constant pressure. The sign of $\left(\frac{\partial \bar{C}p}{\partial P}\right)_T$ is related to the making of water structure by a solute [41]. When a solute creates water structure, $\left(\frac{\partial \bar{C}p}{\partial P}\right)_T < 0$, and when $\left(\frac{\partial \bar{C}p}{\partial P}\right)_T > 0$ the solute breaks water structure. The resulting sign of $\left(\frac{\partial \bar{C}p}{\partial P}\right)_T$ and its dependence on temperature should provide a direct test of the properties of the known effects of chaotropes and kosmotropes. The results obtained for the probe solutes show no obvious correlation between the stabilization effects and the sign of $\left(\frac{\partial \bar{C}p}{\partial P}\right)_T$. The conclusion is that the thermodynamic results do not prove any long-term effects of solutes on bulk water [3, 41].

In another set of experiments, Zhang and Cremer [15] followed the influence of Hofmeister anions on the phase transition of a surfactant monolayer. The anions' effects on the structure of the surfactant monolayer were followed by observing the adjacent water structure. The authors observed that the effect of the anions, from most to least ordered monolayer, agreed with the Hofmeister series as: $SO_4^{2-} > Cl^- > NO_3^- > Br^- > I^- > ClO_4^- > SCN^-$. Apparently, this observation is related to the ability of an individual anion to penetrate the polar region of the monolayer, causing hydrocarbon packing to disrupt [15]. These observations shed light on the possible mechanisms of protein folding in HIC. They might also explain the changes in retention observed in IPC due to ion pairing over the structure of bonded stationary phases.

These results (and the distinct effect of each salt) oppose the belief that the salting out effect arises from the effect of kosmotropic ions on the structure of water. These findings were corroborated by Leontidis et al. [42], who highlighted four key questions that still lack answers: 1) Is there a concentration threshold at which specific-ion effects to appear? 2) Are specific-ion effects really an interfacial phenomenon? 3) Are specific-ion effects based on local or collective interactions? 4) Does a unique ion parameter exist to correlate ion effects? [42] The next section outlines some possible mechanisms that propose different answers.

3.4.2 Overview of Possible Mechanisms for the Hofmeister Series and Salting Out Effects

Several models have been proposed to explain the Hofmeister effect. One model is known as *water-matching affinities* and was proposed by Collins [43]. It establishes that two oppositely charged ions with similar strengths in their interaction with water can form ion pairs, which dominate the ion-specific interactions. Collins showed that many properties of aqueous ionic solutions are a function of the charge density of the

ions [44–46]. An example of these is the strength of water–water interactions in bulk solution. Water–water interactions serve as a critical reference-energy level, and are comparable in strength with ion–water interactions [44].

Another property described by Collins is that chaotropes are monovalent ions of small charge density. This means that chaotropes are able to bind the immediately adjacent water molecules less strongly than water binds itself [43, 46]. Therefore, the polarizability of ions is considered to be important in specific-ion effects. For example, it is manifested through the ion dispersion forces, when the dispersion potential is treated at the same level as the electrostatic forces [36].

The Hofmeister effect has also been explained through the specific interactions between the ions and various surfaces, for instance, hydrophobic solids–water and air–water interfaces, in which the kosmotropes are repelled from the surfaces but the chaotropes are adsorbed thereon [3, 14, 16, 25, 41, 42, 47, 48]. The theoretical approaches that support these possible mechanisms are discussed next.

3.4.3 Theories Proposed to Explain the Hofmeister Series

This following section refers to treatment of the Hofmeister effect based on the approaches of Grover and Ryall [17] and of Lo Nostro and Ninham [26].

3.4.3.1 Hydration Theories

According to hydration theories [49], the salting out of a non-electrolyte by the presence of a salt is attributed to the preferential movement of water molecules to solvate the ions over non-electrolytes. In most cases, cations have a hydration layer greater than that of the anions. This suggests that salting out would be a consequence of the cations' solvation. In the same way, salting in would result from the anions' solvation. The net salting effect of a salt on the non-electrolyte solubility would be the result of these two opposing tendencies [49].

In electrolyte solutions, ion–dipole interactions are favored over dipole–dipole interactions. Because of this, the addition of a salt to the solution of a non-electrolyte causes ions and non-electrolytes to compete for water molecules. The water molecules of the hydration layers around the ions are immobilized. Therefore, the availability of water molecules to solubilize the non-electrolyte causes salting out [49]. This theory does not account for other effects, which are important when water serves as a solvent. Such effects include hydrophobicity, hydrophilicity and polarizability of the non-electrolyte, as well as the breakdown of the water structure.

There are two main drawbacks to the hydration theory. First, the theory implies that the predicted number of water molecules surrounding the ion (the hydration number) is a fixed number. This number is independent of the nature of the non-electrolyte that is being salted out, which contradicts experimental findings [50]. Second, these theories do not explain the dependence of the salting constant k from Equation (3.1) on the size of the non-electrolyte [51]. An additional observation is that hydration theories do not provide an explanation for salting in [27].

3.4.3.2 Dipole Theories of Water

The main idea of these theories is that the solubility of non-electrolytes depends on the variations in the specific effects between the salt ions and the water molecules. Non-electrolytes' solubility arises from the orientation of the dipoles of the water molecules around the ions. Ions of the same sign will orient water dipoles preferentially around polar non-electrolytes, causing salting in. Ions of the opposite sign will cause water dipoles to orient unfavorably, causing salting out [17, 52].

Dipole theories of water give further insight than hydration theories. Dipole theories take into account two conditions: the polarizability of polar non-electrolytes, and the hydrophilic hydration near the ion. Both conditions have been proposed as a primary cause of salt effects. Although these explain the effects on polar solutes, they do not explain the variation in the effects on various non-polar solutes [17, 52].

3.4.3.3 Electrostatic Theories

Electrostatic theories are based on the influence of a solute on the relative permittivity of the solvent. The relative permittivity plays a fundamental role in the salting effects. Some studies describe how salting out occurs in a saturated solution of a non-electrolyte, if its relative permittivity is less than that of pure water. If the relative permittivity of the solution is higher, then the non-electrolyte is salted in [49]. This theory provides an explanation of the interactions and of the extent of salting out and sating in. For example, salting out by sodium chloride and potassium chloride have been described in terms of their salting parameter (k in Equation (3.1)). Theoretical values of k for both salts are in agreement with experimental values when the size and polarizability of the non-electrolyte solute are taken into account [50].

Electrostatic models are unable to explain some deviations from the Hofmeister series. For example, no explanation is provided for salting in caused by large ions, such as tetramethylammonium or naphthene sulfonate or salts of long-chain fatty acids [50]. This is to be expected since the theory considers only the attraction of water dipoles into the electrical field of the ions, and not the structural changes or the displacement of water molecules due to the presence of the ions [50]. A recent review stated that the current knowledge of electrostatic effects in complicated systems, such as protein clusters or protein aggregation, is deficient, and represents a challenge to theorists and experimentalists [1]. There is not a unique electrostatic model that can explain any experimental observation accurately.

3.4.3.4 Internal-Pressure Theories

These theories are based on the observation that the volume of a solution varies when a solute dissolves therein. These changes are related to the presence or absence of the dissolved salts. For example, the volume of water decreases upon dissolution of ethyl acetate. If salts are added, volume contractions are observed to increase in the same way as salting out occurs. The theory introduces the concept of an internal pressure, P_{int}, defined as $P_{\text{int}} = \left(\frac{\partial U}{\partial V}\right)_T = \left(\frac{T\alpha}{\kappa}\right) - P$, where T is the absolute temperature, $\alpha = \left(\frac{1}{V}\right)\left(\frac{\partial V}{\partial T}\right)_P$ is isobaric thermal expansion coefficient, $\kappa = -\left(\frac{1}{V}\right)\left(\partial V / \partial P\right)_T$ is the isothermal compressibility and P is the external

pressure. The internal pressure modifies the ion–solvent interactions and can produce a precipitation of a polar solute [26].

A subsequent theory [53] proposed a model that allows an explicit study of salt effects. According to this model, the neutral molecules of a solute merely occupy a volume in the bulk solution. Their presence exerts a pressure on the solvent molecules, which in turn modify the solvent–ion interaction. This change causes the precipitation of a solute. The degree of salting out or salting in of a non-polar solute is accordingly determined by the magnitude of the contraction or expansion of the solvent when ions are present [53]. As the compressibility of the electrolyte solution increases, salting out of the non-electrolyte also increases. The predicted and observed salt effects for non-electrolytes correlate well with the corresponding changes when salts dissolve in water [26, 50, 53]. But this theory is unable to account for the marked variation in the classification of the effects of similar electrolyte salting, and vice versa. According to this theory, the predicted order of salting out for several salts, such as sodium sulfate, sodium chloride, lithium chloride or ammonium nitrate, is almost the same for solutes as different as hydrogen, nitrous oxide and benzene [17, 50]. Practice demonstrates this to be untrue. The greatest deficiency of this theory is that, although it provides an effective explanation for non-polar electrolytes, it provides no explanation for the behavior of polar non-electrolytes [51].

3.4.3.5 Theories Based on van der Waals Forces

These theories are based on the van der Waals forces, which differ from the electrostatic forces mentioned above. Van der Waals forces might be attractive or dispersive [13]. Examples of van der Waals forces include Keesom forces due to the orientation of permanent dipoles. Another type are Debye forces, also known as induction forces because they arise from the interaction between a permanent and a temporary dipole. Apart from Keesom and Debye forces, there are London forces that are quantum-mechanical in nature. London forces consider the interactions between two instantaneous dipoles. The standard description of these interactions is based on perturbation theory for two bodies; however, it is not applicable in condensed media [13]. Although van der Waals forces mostly explain the salting in effect, these theories fail to explain salting out. For example, there is not yet an explanation for the low salting out caused by lithium and hydrogen ions [50].

3.4.3.6 Ion Pairing

Manning proposed that the attractions among opposite point charges, such as counterions, would be manifest to one another along a cylindrical geometry [23]. Counterions in such cylindrical geometry are referred to as “condensed.” Ion condensation is a mathematical artefact in Manning’s description. Such physical interaction between counterions happens in the local cylindrical geometry, while the relative permittivity of the bulk solvent remains constant [54]. Even though the model does not take into account the existence of repulsive interactions of short range, it has nevertheless proven to be useful because it defines a critical parameter ξ. This parameter corresponds to the minimum charge spacing necessary for counterion attraction, and is dependent on the relative permittivity of the solvent [54]. For water ξ=1, the relative permittivity is 78.5, and the distance between two opposite charges is 7.135 Å.

This distance is called the "Bjerrum length" [54, 55], which is the separation at which two elementary charges are attracted to one another by an electrostatic energy comparable in strength to the thermal energy scale [56].

According to this model, the presence of polyions in a solution contributes to deviations in the system from that of the bulk solvent, thus increasing the value of ξ. But the ions tend to condense so as to decrease the charge density in order to maintain ξ=1 [23, 54, 55]. In consequence, condensation also refers to a physical mechanism of attraction between ions that leads to aggregation. Manning demonstrated ion condensation by studying the variation of some colligative properties with increasing salt concentration. Condensation occurs between ions to maintain ξ=1 through the formation of ion pairs, as colligative experiments show [23].

Recent evidence in ion-pairing interactions confirm the Hofmeister behavior of added salts in RPLC and HIC [57]. However, in IPC there is no appreciable difference between kosmotropic or chaotropic agents. Cecchi emphasized that no existing theory (i.e. stoichiometric or electrostatic or thermodynamic) can explain all experimental observations. To encompass all possible phenomena, Cecchi proposed an extended thermodynamic approach, *extended* meaning that it considers all possible equilibria in the mobile and stationary phases, including electrostatic interactions (see Figure 3.5) [57].

Figure 3.5 has been adapted from reference [57]. It shows all the possible ion pair equilibria occurring in a chromatographic system. On the left side (indicated from *a* to *g)* are chemical equations representing each equilibrium. An ionic analyte

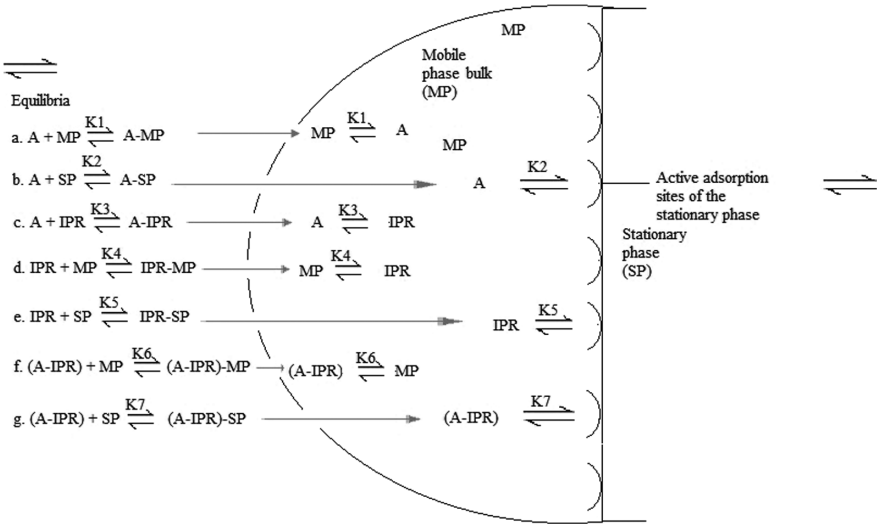

FIGURE 3.5 Equilibrium processes on the addition of an ion-pairing reagent (IPR). (Right) A diagrammatic representation of possible interactions of the analyte (A), the IPR and an analyte-IPR complex (A-IPR) in the mobile phase (MP) and the stationary phase (SP), with the corresponding equilibrium quotients. (Left) Chemical equations representing each equilibrium.

(A) can undergo ion pairing with an ion-pairing reagent (IPR) to form an ion pair moiety determined by the equilibrium constant K_3. Both A and the IPR can undergo, separately, a dynamic equilibrium with the mobile phase (MP). This equilibrium is determined by K_1, K_4 or K_6. Adsorption equilibria with active sites in the stationary phase (SP) are determined by K_2, K_5 or K_7.

Cecchi's model was successfully tested in retention modeling when IPR chaotropes were the chosen additives. Two main mechanistic differences between classic IPC and chaotropic IPC arise from Cecchi's treatment [57]: 1) the affinity of the chaotropic anion and its counterion for the stationary phase (adsorbophilicity) do not differ from one another; 2) the influence of classic IPRs on analyte retention is explained based on the lipophilic portion of the IPR. However, analyte retention with chaotropic salts is explained based on the electrostatic interaction between the chaotropic anion and its counterion [24, 57]. Cecchi's observations agree with Florez and Kazakevich [24], although no similar results are reported for kosmotropes.

According to Cecchi, no new theory has been developed to understand IPC fully, other than that extended thermodynamic approach. Efforts have been undertaken to revisit previous models to test potential new reagents such as chaotropic and kosmotropic salts [57].

3.4.4 A Quantum-Mechanical Approach to the Hofmeister Series

As was summarized in the preceding pages, the Hofmeister series has been known for over 130 years but has never been fully explained. The current explanations of the effects of kosmotropic and chaotropic salts rely on the structure of water and its capability of forming hydrogen bonds. The structure of water in solutions is supposed to be modified by electrostatic interactions with the solutes, including hydrogen bonding and van der Waals forces [12, 13]. Various authors agree that the Hofmeister effect might be explained based on a quantum-mechanical approach [12, 13, 58]. One possible explanation is based on the concept of a quantum vacuum [12].

The quantum vacuum is considered in a description of the Casimir effect. This effect arises from two repelling plates separated 10 pm from one another. The repulsive force drives the formation of a pressure as great as 1 bar, known as a quantum vacuum. Henry proposed that the salting effects and the changes in the structuring of water associated with the Hofmeister series can be explained based on interactions arising in a quantum vacuum [12].

An electrostatic interaction between a salt and a protein might exist because of the presence of opposite charges. From a quantum-mechanical point of view, when an electron of one species is attracted to the nucleus of another species it forms a virtual electron–positron pair. Interaction within this pair results in the disintegration of the approaching electron and the positron, releasing the electron of the pair and leaving a vacuum [12]. This vacuum is so energetic that a new electron fills it, resulting in a new interaction. This reasoning indicates that matter appears and disappears inside a vacuum with a period of attoseconds. Such a short time gives the idea of a continuous electrostatic exchange described as hydrogen bonding or van der Waals forces, which are the main driving forces for chaotropicity and kosmotropicity [12, 13, 58, 59].

3.5 CHROMATOGRAPHIC IMPLICATIONS OF KOSMOTROPIC SALTS AND THE HOFMEISTER SERIES

Apart from the variety of ways in which salts can interact with proteins mediating protein–protein interactions, they also exert an effect on their stability and solubility. These phenomena are due to an increase in the interfacial tension between the proteins and water [25], thus strengthening the hydrophobic interactions of a protein with the stationary phase [47]. These hydrophobic effects induced by the salts are the basis of HIC [48]. The addition of kosmotropic salts has a small effect of increasing resolution, while also improving peak shape and selectivity [5]. Therefore, in HIC the retention is modulated by varying the salt concentration in the mobile phase, in such a way that the protein retains its native structure to a larger extent. There is still no agreement between the theory and experiments of HIC that explains this due to the complexity of the chromatographic system [60].

There is a consensus that the chaotropic or kosmotropic ions from the Hofmeister series affects the solubility and stability of proteins in two possible ways. One, via a change in polarizabilty of the water-mediated ion–protein interaction. Another by direct binding of the ions to the protein [8]. Apart from the various ways in which salts can interact with proteins and mediate protein–protein interactions, salts exert an effect on the stability and solubility of proteins.

Cecchi showed that experimental results and molecular dynamic calculations suggest that chaotropic ions accumulate in two regions: at interface between the mobile phase and the stationary phase; and at the interface between the stationary phase and the protein surface [57, 60]. The surface of the stationary phase is different from the bulk of the mobile phase. These differences arise from the fact that the stationary phase is an inhomogeneous surface. In the stationary phase the electrolyte distribution can induce dipole moments which sum is not zero. Instead, the bulk of the mobile phase is symmetric with respect to electric vector intermolecular forces that sum zero. The experimental results showed that anions and cations partition differently between the surface of the stationary phase and bulk mobile phase. For instance, anions have been observed to accumulate at their interfaces. This tendency to accumulate at the interface follows the Hofmeister series [61]. Non-specific effects are observed for chaotropic salts at a small concentration and are related to chaotropic changes in the water structure and the salting in of solutes, because chaotropic ions are characterized by small electronic densities [57].

3.6 DEVELOPMENT OF THE LIQUID CHROMATOGRAPHIC METHOD FOR HIC PROTEIN SEPARATION

The development of HIC chromatographic methods for protein analyses has been studied by various authors [18, 62, 63, 64, 65]. The use of mobile phases containing kosmotropic salts, such as ammonium sulfate, has been suggested. Separations are performed with an inverse salt gradient, from high to low concentrations in ammonium sulfate. The use of the kosmotropic salt promotes hydrophobic interactions between the proteins and the stationary phases based on the effect predicted by the Hofmeister series [66].

The selection of the mobile phase conditions in HIC is an empirical process that can be simplified by applying RPLC gradient elution relationships. Karger et al. and others showed that retention and separation in the HIC of proteins can be explained by the linear solvent strength (LSS) gradient model for RPLC, shown in Equation (3.3) [67].

$$\log k^* = \log k_w - S\varnothing^* \tag{3.3}$$

In Equation (3.3) k^* is the retention factor in the gradient (the median value of k during gradient elution), $\varnothing^*$ is the median volume fraction of mobile phase B, k_w is the retention in pure water for a particular analyte and S is the negative slope $[-d(\log k)/d\varnothing]$ (the change of log$k$ with respect to %B). S is generally accepted to be approximately *0.25*(molecular weight)$^{1/5}$* for molecules with molecular weights over 400. The magnitude of S affects the retention factor k^*. Other values that affect k^* are the gradient steepness, the mobile phase flow rate, the column dead volume and the change in %B during the gradient ($\Delta\varnothing$) [67].

In HIC, the retention k depends on the concentration of kosmotropic ammonium sulfate. This dependence is shown in Equation (3.4) [18, 67].

$$\log k = \log k_0 + A_{\mathrm{HIC}} C_{\mathrm{AS}} \tag{3.4}$$

In Equation (3.4) C_{AS} is the concentration of ammonium sulfate, k_0 is the value of k when $C_{AS}=0$ and A_{HIC} is the slope d(log$k$)/d(C_{AS}). As C_{AS} increases, k also increases. C_{AS} must decrease during the gradient; otherwise proteins will remain retained in the column. That is the reason why inverse gradient elution is preferred. Equation (3.4) can be transformed into Equation (3.5) based on ammonium sulfate in order to be analogous to the LSS gradient model as:

$$\log k = \log k_{2.5} - S_{\mathrm{HIC}} \varnothing_{\mathrm{HIC}} \tag{3.5}$$

$k_{2.5}$ is the value of k for 2.5 M ammonium sulfate, $\varnothing_{\mathrm{HIC}}$ is defined as $[-(C_{AS}-2.5)/2.5]$ and S_{HIC} is $-2.5A_{HIC}$. In a linear inverse gradient from 2.5 M to 0 M ammonium sulfate, $\varnothing_{\mathrm{HIC}}$ varies from 0 to 1. For example, if C_{AS} gradient values are 2.5 M, 1.25 M and 0.0 M, $\varnothing_{\mathrm{HIC}}$ is equal to 0.00, 0.50 and 1.00, respectively. Other kosmotropic salts, such as ammonium acetate and sodium formate, give similar values of A_{HIC} and S_{HIC} at higher concentrations, but are not frequently used [67].

Equation (3.5) for HIC is equivalent to Equation (3.3) for a RPLC gradient, because they follow similar qualitative and quantitative relationships in relation with k. For this reason, RPLC gradient elution rules can be applied directly to HIC method development for protein analysis. For instance, for the analysis of proteins in the range of molecular weight $10^4 \leq M \leq 10^5$, S_{HIC} values range from 4 to 9. In contrast, S values in RPLC for the same analytes are between 25 and 80. These S_{HIC} values are almost an order of magnitude smaller than S values for RPLC. This means that, based on the LSS gradient model, steeper gradients can be used in HIC with shorter gradient times compared to RPLC for the same analytes [67].

Recent interest in the HIC analysis of proteins has sought to provide generic guidelines for method development. This includes the use of other kosmotropic salts besides

ammonium sulfate, as well as determining the proper salt concentration, salt gradient and gradient steepness. For instance, Fekete and coworkers studied salt type and concentration to optimize HIC mobile phase for the analysis of monoclonal antibodies [64, 68].

These authors tested the influence of 2 M ammonium sulfate on the selectivity for antibody separation, and used it for comparison with other salts such as sodium chloride, ammonium acetate and ammonium formate. What the authors found is that similar selectivities can be achieved with either salt by adjusting their kosmotropic strength [68]. This is done by determining the kosmotropic salt concentrations at which the selectivity is similar. A summary of the chromatographic results for sodium chloride compared to 2 M ammonium sulfate is shown in Table 3.2.

From Table 3.2 it can be seen that to obtain the same selectivity as 2 M ammonium sulfate it is necessary to have 5 M sodium chloride in the mobile phase. At the same time, selectivity can be manipulated by changing the concentration of Cl^-. Similar selectivities to that observed with 2 M ammonium sulfate can be obtained with sodium formate and sodium acetate in the mobile phase in concentrations between 5.0 and 5.5 M. These results are in line with the Hofmeister series [64].

From a method development point of view, salts should be interchangeable if their positions in the Hofmeister series are close to each other. At the same time their salting out strength should be adjusted for their concentration. The adjustment could be determined using Equation (3.4) by determining A_{HIC} from a plot of log$k$ vs %B. Similarly S_{HIC} can be determined from Equation (3.5) by plotting log$k$ vs ϕ. However, the effect of salt type and concentration cannot be determined in advance, and should be determined experimentally [64, 68].

Another alternative for HIC method development has been described by Tyteca et al. The authors separated cytochrome c, ribonuclease A and lysozyme using an ammonium sulfate gradient from 1.5 M to 0.5 M. Their results show that these gradients do not behave linearly with respect to the LSS gradient model proposed by Snyder due to a mixed mode of interaction. At higher salt concentration at the beginning of the gradient, the linearity improves according to the theory. The best results were observed at a concentration of 1.8 M at the beginning of the gradient [65].

TABLE 3.2
Observed Selectivity for Different Concentrations of Sodium Chloride as an Alternative Kosmotropic Salt Compared to 2 M Ammonium Sulfate

Salt	Concentration/(M)	Selectivity factor between two antibodies
NH_4SO_4	2	1.1
NaCl	5	1.1
NaCl	4	1.6
NaCl	3	2.8

Adapted from M. Rodriguez-Aller, D. Guillarme, A. Beck, S. Fekete, Practical Method Development for the Separation of Monoclonal Antibodies and Antibody-Drug-Conjugate Species in Hydrophobic Interaction Chromatography, Part 1: Optimization of the Mobile Phase, *J. Pharm. Biomed. Anal.* 118 (2016) 393–403. doi:10.1016/j.jpba.2015.11.011.

Protein retention in HIC not only depends on the kosmotropic salt concentration, selection of salt type, concentration and gradient program, but also on the stationary phase to be used. Hydrophobic stationary phases, as well as salt concentration, can induce conformational changes of proteins as well as aggregation [62, 63]. Stationary phases in HIC can be either amphipilic [62] or hydrophilic, with hydrophobic ligands linked by spacer arms. HIC stationary phases differ in chemical nature, in surface concentration of the ligand and in the chemistry and particle size of the matrix. A comprehensive description of the main stationary phases used in HIC can be found in [64]. The stationary phase's hydrophobicity can change with the ionic strength of the mobile phase, affecting retention due to conformational changes of proteins [62] as well as aggregation [63].

3.6.1 Optimization of the Separation in HIC

The LSS model is a function of experimental conditions that affect protein separation. These conditions, such as salt gradient, flow rate and column dimensions, have a predictable effect on the separation (at constant temperature and mobile phase pH). The effect of one or more of these conditions can be predicted by the LSS model in order to optimize a method for protein separation. This can be done by computer simulation, performing two gradient runs with different slopes, thus predicting the separation as a function of gradient conditions. For computer simulation, retention times and peak widths are entered into software. The software uses the specified conditions to determine k_w and S for each protein to be separated. Once k_w and S for each component are determined, the software displays its predicted chromatograms, tables or resolution plots. Such results can be obtained using software such as DryLab, ChromSword, ChromSmart, ACD/LC Simulator, Osiris or Preopt-W [67].

For instance, reference [69] describes the use of DryLab 2010 in order to optimize salt gradient steepness in the HIC analysis of antibody-drug conjugates. The computer-assisted optimization led to a better selectivity with a multilinear salt gradient, steeper at the beginning and flatter at the end. Another work is described in reference [70]. The initial calibration runs were performed for the separation of monoclonal antibodies using two isocratic conditions and extrapolating to a proper gradient combined with a suitable stationary phase. A resolution map was constructed indicating the proper chromatographic conditions [70].

Computer optimization also offers the advantage of performing an automatic search of the best combination of initial and final %B in the gradient, and of the time gradient when other conditions, such as temperature, are changed. Reports based on computer simulation include HIC gradients which do not exceed 10–30 min and temperatures of 20°C and 40°C [68]. The experimental retention time, peak widths and peak tailing were imported into DryLab 2010. The software then converts the retention times into retention factors. The final results were a significant decrease in the time of the analysis [64, 68].

Snyder shows that DryLab is also able to perform the selection of stationary phases along with flow rate and pressure. Using the conditions described above allows the practicing analytical chemist to develop a chromatographic method in a very short time [67]. Other discussions on protein separations are available in references [71–73].

3.7 CONCLUSIONS

Kosmotropicity is an important phenomenon in protein separations yet to be elucidated. The main conclusions from this review are as follows:

1. There is no complete theoretical framework that considers all variables involved in the salting effects due to kosmotropicity or chaotropicity.
2. The kosmotropicity and chaotropicity of salts have wide applicability for the optimization of protein separations and of small molecules.
3. The absence of a theoretical framework is no limitation for the development and optimization of new analytical methods, especially in the pharmaceutical industry.

What began as an empirical area of research to improve HPLC in the late 1980s is still an unfinished compendium of theoretical explanations that has found successful continuous applications, especially in protein and biomolecules separations.

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