# Scholarly Research Exchange

Volume 2008 • Article ID 818461 • doi:10.3814/2008/818461

# Research Article

# **Are Hofmeister Series Relevant to Modern Ion-Specific Effects Research?**

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Received 29 February 2008; Revised 17 April 2008; Accepted 5 May 2008

Ion-specific effects underlie a vast array of physicochemical and biological phenomena ranging from human physiology to biotechnology to ecology. These effects have traditionally been quantified by measuring the response of interest in a series of salt solutions at multiple concentrations; pH has consistently been shown to be of primary concern. However, salt-based approaches violate critical tenets of proper experimental design and introduce confounding errors that make it impossible to quantify ion-specific effects. For example, pH is a variable dependent on the type and concentration of ions in a solution, but is typically treated as an independent factor, thus confounding experiments designed to determine ion-specific effects. We examined the relevancy of ion-specific effects research in relation to these concepts and demonstrated how these ideas impact protein precipitation and enzyme activity. Based on these results, we present a conceptual and experimental framework of general applicability for proper quantification of ion-specific effects.

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Ion-specific effects underlie a vast range of physicochemical and biological phenomena [1] including, but not limited to, protein solubility/denaturation, enzyme activities, membrane transport, water activity coefficients, electrolyte activities, pH measurements, pH buffers, gel-coagel transitions, colloid stability, and biological growth. All modern investigations of ion-specific effects trace back to a series of studies by Hofmeister et al. published in the 1880s and the 1890s, who classified salts according to their ability to "saltin" or "salt-out" proteins from solution [2, 3]; a "Hofmeister series" is generally presented as a cat-/anion-specific ranking in relation to some measured response, for example,

$$SO_4^{2-} \approx HPO_4^{2-} > Cl^- > NO_3^- > ClO_3^-.$$
 (1)

Given the ubiquitous nature of these phenomena, Ninham [4] has stated that Hofmeister effects "are as important in the scheme of things as Mendel's work was to genetics."

No satisfactory explanations account for the physical basis underlying any Hofmeister series despite extensive experimentation over about the last 120 years. The classical framework to account for these forces derives from the theories of Born on the self-energy of ions; the DeBye-Huckel theory of correlations in electrolytes; the theory of the electrical double layer; the theory of Onsager for interfacial tensions, and the quantum mechanic; and Derjaguin, Verway, Landau, and Overbeek (DVLO) theory of colloidal particle interactions [4]. However, there is little agreement, except at very low salt concentrations, between what these theories predict and what is experimentally observed. Ninham and Boström [4, 5] assert that the failure of these theories is particularly disconcerting for the life sciences that rely upon an understanding of physical chemistry to explain ion-dependent phenomena.

The problems facing physical chemistry today are not limited to theories that cannot predict, but also include the experimental methods used to quantify these effects. These methods are deeply flawed, because they are based on several pervasive and fundamental misconceptions about the nature of ion-specific effects. In this paper, we discuss the serious and widespread problems with past and current ion-specific experimentation. Specifically, we argue that (1) the common understanding of pH-dependent effects and how these effects are elucidated is unsound, (2) the use of

salt-based solutions to intuit ion-specific effects is invalid, and (3) the standard "one-factor-at-a-time" experimental approach to an inherently multivariate question is improper. Here we present a conceptual and experimental framework for proper quantification of ion-specific effects that is of general applicability, and we discuss the relevancy of Hofmeister series research in relation to this frame of reference.

To explore pH (specifically, "relative proton activity") and the relevancy of this concept to ion-specific experimentation, we designed two experiments. The first measured the precipitation of two proteins, chicken ovalbumin and bovine serum albumin (BSA), in 1212 unique, 1 M solutions made of combinations of nine ions, which generated a wide range of pH values. The second measured bovine  $\beta$ glucuronidase (GUS) enzyme activity in 37 unique, 50 mM solutions of various combinations of three ions (Na<sup>+</sup>, OAc<sup>-</sup>, and PO<sub>4</sub><sup>3-</sup>). No additional ions were added—that is, pH was not "controlled" with buffers or "adjusted" with any ions. Because it is universally understood that protein precipitation and enzyme activity are directly dependent upon pH [6–9], then by definition these responses should be equivalent at any given pH value, regardless of the ions used to achieve that value. To wit, if these responses are truly pH-dependent, then a mixture of NO<sub>3</sub><sup>-</sup>, PO<sub>4</sub><sup>3-</sup>, and K<sup>+</sup> ions and a mixture of SO<sub>4</sub><sup>2-</sup>, Cl<sup>-</sup>, and Na<sup>+</sup> ions should produce the same result if these mixtures are molar equivalents at the same pH.

Contrary to the accepted paradigm, the results of our precipitation experiments indicated only a general and weak correlation between pH and ovalbumin/BSA precipitation (Figures 1(a) and 1(b)). No ovalbumin precipitated at pH > 5.4 and no BSA at pH > 2.6. At pH values below these thresholds, precipitations varied widely. For example, at pH 1  $(\pm 0.05)$ , precipitations for both proteins ranged from 0% to 100% depending on the specific mixture of ions in solution. Why do these results contradict a fundamental assumption of the purported effect of pH on protein precipitation? The answer is because this understanding is the result of a persistent conflation of the relationship between pH-specific and ion-specific effects [10]. Any given pH value can be achieved with multiple unique ion combinations, while any given combination of ions has only one pH value. Thus, pH is primarily a function of the type and concentration of the other ions in solution, that is, it is a *dependent variable*. This means that pH effects cannot be directly determined, are inherently correlative, and should be assumed to have no relevancy outside of the context of the particular ions used in any given experiment. Accordingly, the concept of an "optimal" pH is valid only in the context of the ions used in the experiment(s) that identified that particular "pH

Thus, from a strictly empirical viewpoint, pH is consistently misused. An experimenter who purports to "control" pH or to identify "pH-dependent" effects is actually using pH as an oversimplified proxy for independent ion effects. In contrast to protein precipitation, GUS activity in aqueous mixtures of Na<sup>+</sup>, OAc<sup>-</sup>, and PO<sub>4</sub><sup>3-</sup> at 50 mM *correlated* well with the known pH optimum of this enzyme at 4.4 (Figure 1(c)). However, because both pH and GUS activity

are inherently dependent on the ions used in this experiment, we cannot conclude that GUS will exhibit the same activity or pH optimum with a different set of ions.

Ion-specific effects can only be properly quantified when ions are treated as independent factors. *Interactions* between ions can only be quantified when multiple ions are treated as independent factors within a multivariate experimental design [11]. The nature of this experimental design space (Figure 2) is inherently geometric, is demarcated by the factor vectors, and can take two possible forms: (1) the primary dimensions are defined as single ion, concentration vectors, or (2) the primary dimensions are defined as mixtures (proportions) of ions across a single, total ion concentration dimension. Option 1 determines ion-specific effects strictly as a function of ion concentration and is independent of proportionality and total ion concentration (with the result that these two effects are potentially confounded). Option 2 provides separate estimates of ion proportionality and total ion concentration effects. The presence of significant ion proportionality and/or total ion concentration effects will preclude the use of option 1 for ion-specific effects experimentation. This begs the question as to which option represents the more correct approach.

To answer this, we quantified the ion proportionality and total ion concentration effects for ovalbumin precipitation and GUS enzyme activity. For ovalbumin precipitation, we designed four 2-ion experiments that included the saltspecific ion pairs: Na<sup>+</sup> and NO<sub>3</sub><sup>-</sup>; Na<sup>+</sup> and SO<sub>4</sub><sup>2-</sup>; NH<sub>4</sub><sup>+</sup> and  $\mathrm{NO_3}^-;$  and  $\mathrm{NH_4}^+$  and  $\mathrm{SO_4}^{2-}.$  Relative ion proportions were varied from 0 to 1 over a total ionic concentration that ranged from 1 M to 10 M. For GUS enzyme activity, we designed a 3-ion experiment that varied the proportions of Na<sup>+</sup>, OAc<sup>-</sup>, and PO<sub>4</sub><sup>3-</sup> from 0 to 1 with total ionic concentrations of 50 mM or 5000 mM. No buffers and/or "pH-adjustment" ions were used. Ovalbumin precipitation exhibited a complex, ion-specific response where total ion concentration was significant but considerably less important than ion proportionality (Figure 3). GUS enzyme activity was also complex and ion-specific, and exhibited a strong interaction between total ion concentration and ion proportionality. Maximum GUS enzyme activity was achieved in Na<sup>+</sup> and OAc<sup>-</sup> dominant mixtures at 50 mM total ion concentration, but at 5000 mM total ion concentration maximum activity was found in Na<sup>+</sup> and PO<sub>4</sub><sup>3-</sup> dominant mixtures (Figures 3(e) and 3(f)). GUS activity responded differently to the 3-ion mixture depending on the total ion concentrationthat is, proportionality effects interacted with concentration effects. Can this result be viewed and interpreted from the traditional salt-based perspective, that is, can we produce a Hofmeister-type ranking of anions? No, single ion main effects cannot be determined using only the design points found in salt-based experiments (Figure 3) and then explaining away "by assumption" any potential interaction effects with the common Na+ cation. Single ion effects can be determined from the linear component of a mixture analysis. However, the linear component is only a comparison of the effects at the vertices of the design space which, in the GUS experiment would be 50 mM Na<sup>+</sup>, OAc<sup>-</sup>, or PO<sub>4</sub><sup>3-</sup> (i.e., the base and the two acids) and is not amenable to a classical,

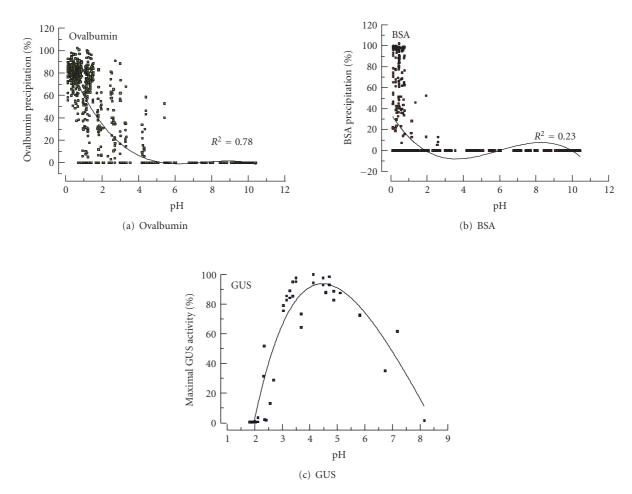


FIGURE 1: The percent precipitation of ovalbumin (a) and BSA (b) in 1,212 unique ion solutions at various pH values is represented by the green and red squares, respectively. The solid black lines are third-order polynomial fits of the data (R2 indicated on graphs). Proportions of NH<sub>4</sub><sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>, Li<sup>+</sup>, Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, PO<sub>4</sub><sup>3-</sup>, OAc<sup>-</sup>, and NO<sub>3</sub><sup>-</sup> were varied within an eight-dimensional mixture experimental design at a constant total ion concentration of 1 M. The weak correlation between pH and %-precipitation is clear. For ovalbumin, the precipitation at any given pH value less than ca. 5.5 (704 points) can vary greatly. At pH values greater than 5.5 (508 points) the solution dynamics are governed primarily by nonprecipitation inducing ions. For BSA, only ion blends with high proportions of one ion, NO<sub>3</sub><sup>-</sup>, resulted in precipitation. Note that these are strictly correlational analyses; pH is *not* an independent factor. (c) GUS activity, relative to maximum, was measured in 37 unique solutions with varying proportions of Na<sup>+</sup>, OAc<sup>-</sup>, and PO<sub>4</sub><sup>3-</sup>. Unlike the protein precipitation, there does appear to be a close correlation between pH and GUS enzyme activity in solutions of the three ions under study. However, the inherent dependency of pH precludes us from stating that GUS enzyme activity is "pH-dependent."

single-ion, Hofmeister ranking. The higher order nonlinear curvature observed in the 3-ion mixture for GUS activity is due to interactions, or more correctly blending effects, between the component ions. Thus, it is not possible to (1) translate the results of an ion-based, mixture-amount experiment to the simple linear ordering of a Hofmeister series, or (2) translate the results of a salt-based experiment into an ion-based, mixture-amount interpretation.

We concluded from these results that a properly designed experiment to determine ion-specific effects has two primary attributes: (1) all ions in solution are treated as independent factors/components,<sup>2</sup> and (2) ion proportional effects are independently estimated from total ion concentration effects. These two requirements are satisfied by the general mixture-amount approach [12, 13]. A mixture experiment varies proportions while holding amount (concentration)

constant; a *mixture-amount* experiment varies both proportions and total ion concentration independently (cf. Figures 2(c) and 2(d)). The recent development of a linear programming algorithm to calculate the salt/acid/base recipes required to achieve the specific ion solution formulations required by mixture-amount experimental designs [14]<sup>3</sup> facilitates the implementation of such experimentation.

The results reported here provide several clues to the underlying experimental problems with past studies to quantify ion-specific effects. One of the primary conceptual difficulties with all ion-specific research is the propensity to use *salts* to quantify the effects of *ions*. The words "salt" and "ion" are consistently conflated and are often used interchangeably: Hofmeister series are always ion-specific, but are derived from "salt solutions." This is not merely a semantic issue. A "salt solution" has a fixed ion

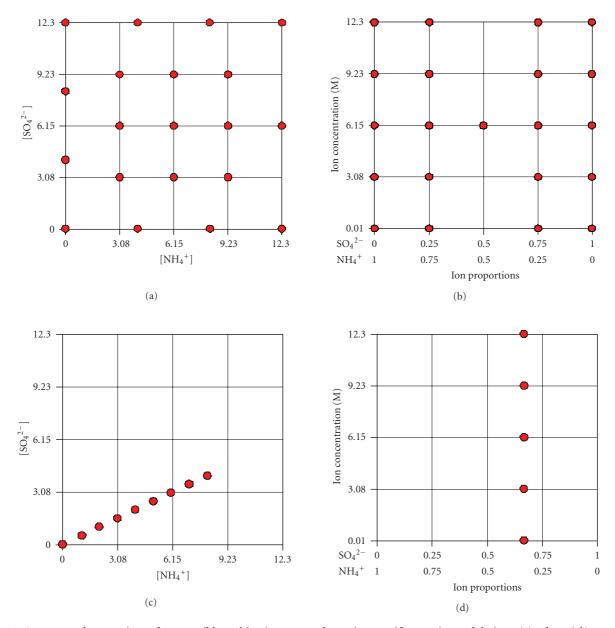


FIGURE 2: A conceptual comparison of two possible multivariate approaches to ion-specific experimental designs: (a) a factorial/regression-type design with factors defined as concentration ranges of individual ions, and (b) a mixture-amount approach wherein ions are treated as components of a proportional mixture that sums to unity across a total ion concentration range. Design points for (a) and (b) were selected using modified D-optimal criteria. For comparison, the standard approach, illustrated using  $(NH_4)_2SO_4$ , to ion-specific research utilizes salt-based solutions (c)-(d) with restricted ion ranges that sample the possible design space in an extremely poor manner. These salt-based designs are aliased for all factors/components; only the mean effect of the two ions can be quantified. Thus, the confounded designs represented by (c) and (d) are incapable of being used to quantify ion-specific effects and/or interactions between independent factors/components.

proportionality that is strictly a function of the parent salt.<sup>4</sup> From an ion-specific, experimental design perspective, this salt-dependent ion proportionality is decidedly arbitrary and is predicated upon the convenience of having a source of ions in an expedient, that is, crystalline form. Most importantly, experimental designs dependent upon salt-restricted ion proportionalities (Figures 2(c) and 2(d)) are not capable of differentiating between cation and anion effects. The "coion" introduced with a salt may exhibit significant interactive

effects and because the two ions are covariates added in a constant proportionality their effects are potentially confounded [11, 14]. We define "confounding" as two or more quantities varying together in a manner that makes it mathematically impossible to separately identify their unique effects. When "salts" are used to elucidate ion-specific effects, the component ions of the salt are always covaried. Thus, the main effect associated with the ion(s) of interest is possibly indistinguishable from, or confounded with, the effect(s) of

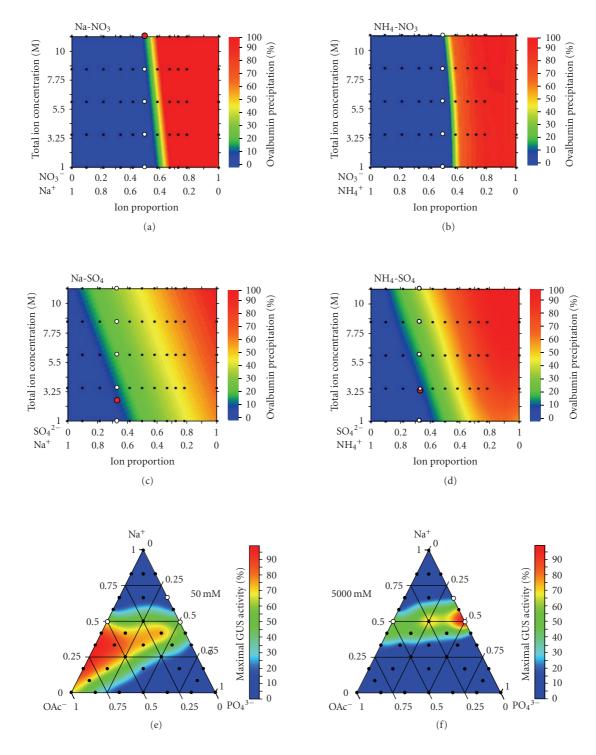


FIGURE 3: Contour plots of ovalbumin precipitation response for four cation-anion pairs, (a) Na-NO<sub>3</sub>, (b) NH<sub>4</sub>-NO<sub>3</sub>, (c) Na-SO<sub>4</sub>, and (d) NH<sub>4</sub>-SO<sub>4</sub>, from 1 to 10 M total ion concentrations. The salt-based experimental design points are indicated by the white dots; the ion-based experimental design points are indicated by both the black and white dots. Hofmeister's estimates of the minimum salt molarity necessary to induce egg white precipitation are indicated by the red dots. Note that Hofmeister was not able to induce egg white precipitation with solutions made with NH<sub>4</sub>NO<sub>3</sub> salts. It is clear that (1) total ion concentration plays a larger role in affecting ovalbumin precipitation for SO<sub>4</sub><sup>2-</sup> ion pairs than it does for either NO<sub>3</sub><sup>-</sup> ion pair, and (2) ion proportionality is highly important. Contour plots of bovine liver  $\beta$ -glucuronidase (GUS) enzyme activity across a three ion mixture experimental design space of Na<sup>+</sup>, OAc<sup>-</sup>, and PO<sub>4</sub><sup>3-</sup> at total ion concentrations of 50 (e) and 5000 (f) mM indicate that enzyme activity is ion-specific and exhibits a dynamic interaction with concentration. Synergistic blending between Na<sup>+</sup>-OAc<sup>-</sup> and Na<sup>+</sup>-PO<sub>4</sub><sup>3-</sup> was clearly observed with the strongest blending synergy (i.e., highest enzyme activity) occurring between Na<sup>+</sup> and OAc<sup>-</sup> at 50 mM and between Na<sup>+</sup> and PO<sub>4</sub><sup>3-</sup> at 5000 mM. Activity is reported in relative fluorescent units and is consistent between the two plots.

its coion. While it is true that the presence of a covariate does not a priori result in confounding, the two primary ways to avoid confounding are to eliminate the covariate or to show that the main effect and/or interactions of the covariate are constant and/or negligible between all factors that require independence. Otherwise, confounding must be assumed.<sup>5</sup> Thus, the only effect that can be quantified from a salt-based experiment is the combined effect of the two ions in solution at the proportionality dictated by the parent salt at multiple concentrations, or in other words, the mean effect of the salt's component ions at a fixed proportionality over a range of total ion concentrations.

To more fully explore ion proportionality effects on ovalbumin and BSA precipitation we examined nine ions, NH<sub>4</sub><sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>, Li<sup>+</sup>, Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, PO<sub>4</sub><sup>3-</sup>, OAc<sup>-</sup>, and NO<sub>3</sub><sup>-</sup>, within an 8-dimensional experimental design space with unconstrained ion proportionalities, that is, from 0 to 1, with the total ionic concentration held constant at 1 M. Interpreting effects in a mixture is very different from their interpretation in factorial/regression-type designs. Because total ion concentration is held constant in a mixture, ion-specific effects are actually "blending" vectors through a region of the mixture design space [15]. The relative proportion of a single ion varies along each vector; and the proportions of the remaining ions will sum to unity at a constant total ion concentration. The mixture responsetrace plots (blending vectors) clearly show the importance of proportionality in quantifying ion-specific effects (Figures 4(a) and 4(b)). Protein precipitation exhibits unambiguous ion-specific blending effects unique to each protein. For ovalbumin, Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, and NO<sub>3</sub><sup>-</sup> were the primary drivers of precipitation. Although mixtures and pure blends of NH<sub>4</sub><sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>, Li<sup>+</sup>, PO<sub>4</sub><sup>3-</sup>, and OAc<sup>-</sup> did not precipitate ovalbumin, their interactive/blending effects were ion-specific; NH<sub>4</sub><sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>, and Li<sup>+</sup> exhibited antagonistic blending effects (i.e., increasing proportions reduced precipitation) while PO<sub>4</sub><sup>3-</sup> and OAc<sup>-</sup> exhibited synergistic blending effects (i.e., at certain proportions they enhanced precipitation) when significant amounts of Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup> and/or NO<sub>3</sub><sup>-</sup> were also present. For BSA, NO<sub>3</sub> was the primary driver of precipitation with SO<sub>4</sub><sup>2-</sup> and Cl<sup>-</sup> having slight synergistic effects, although unable to precipitate BSA on their own. NH<sub>4</sub><sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>, Li<sup>+</sup>, PO<sub>4</sub><sup>3-</sup>, and OAc<sup>-</sup> all exhibited antagonistic blending effects. Because each ion mixture in the experimental design represents a geometric coordinate in the 8-dimensional space defined by the ion proportion vectors, the precipitation responses represent a sort of "precipitation map" through the experimental design volume (Figures 4(c) and 4(d)).

From a strictly empirical point of view, we must now ask whether the hundreds of Hofmeister series generated since Hofmeister's seminal work in the late 1880's are valid.<sup>6</sup> First, let us examine this body of work in relation to the standard experimental approaches. Of primary concern is the issue of confounding. As already described, the vast majority of these studies have been performed with saltbased solutions at fixed ion proportionalities that exhibit potential confounding of ion main effects and interactions. In addition, buffer salts/ions are often introduced into

solution to control an inherently dependent variable (pH); these buffers are usually augmented with additional ions to adjust pH, which are generally ignored and also introduce further confounding. A subset of ions from these complex, multi-ion mixtures are then assigned to rankings of ionspecific main effects, that is, Hofmeister series. Regrettably, the experiments used to produce Hofmeister series never directly measure the main effects of any ions, but rather extrapolate these effects from responses to complex multiion mixtures. As described above, the critical assumption behind salt-based, ion-specific effects research is that the effects/interactions of all of the other ions in solution are constant and/or negligible. Because the results of the mixture experimentation revealed significant nonlinear blending effects for ovalbumin/BSA precipitation and GUS enzyme activity, we concluded that this assumption is not true. As we have no reason to believe these three phenomena to be unique, it is our contention that the standard, salt-based experimental designs used to quantify ion-specific effects are categorically invalid for this purpose. Logically, no true ionspecific effects can be, or have been, derived from these types of experiments. We must therefore conclude that the vast majority of salt-based experiments are invalid, placing a great deal of the extensive Hofmeister series and ion-specific effects literature in doubt.

The quantification of ion-specific effects is a recurring problem in many important disciplines. The ideal description of these effects should be complete, concise, unambiguous and amenable to statistical analysis. Implicit in this is the preservation of essential information from important characteristics of complex ion mixtures, including ion main effects, blending/interaction effects, and effects of total ion concentration. Regardless of the validity of the standard experimental approaches, we must ask whether the ranking of ions in a Hofmeister series is an inherently valuable exercise. These series are strictly focused on ordering the main effects of individual ions in relation to a given response. While it is certainly important to quantify the main effects of ions, it is equally important, if not more so, to quantify the interactions between ions. Certainly, the situations where true single ion effects are encountered, that is, solutions containing only one ion, are rare. The overwhelming majority of instances of ion-specific effect manifestations, for example, inside living cells, in industrial enzyme production, in carbon dioxide absorption by the world's oceans, and so forth, involve complex mixtures of ions with synergistic and antagonistic blending/interactions that are poorly described by the standard physicochemical theories and empirical methodologies. Two implicit problems with Hofmeister-type rankings are that (1) in and of themselves, they are poor approximations of ion-specific main effects and (2) they provide little to no insight into the complex and important milieu of ion mixtures. From a frame of reference predicated upon the fundamental tenets of the design of experiments [11], the reasoning behind the impetus to rank order single ion main effects is unclear. Is the  $SO_4^{2-}$  ion more effective at protein precipitation than the Cl<sup>-</sup> ion? It depends on the protein. It depends on protein concentration. It depends on the concentration

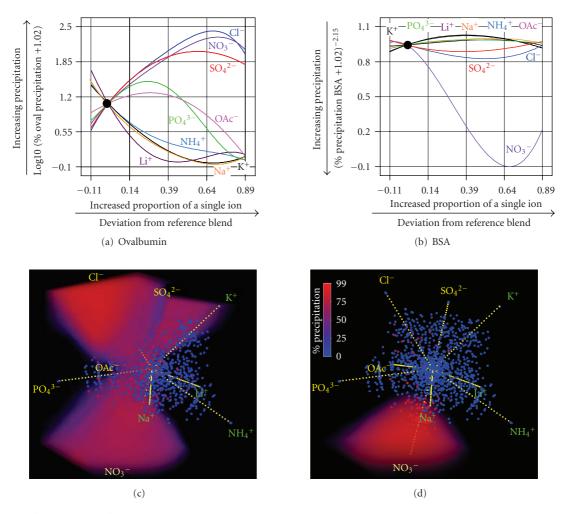


FIGURE 4: (a) An "effect plot" or "response-trace plot" of the 9-ion mixture experiment described in Figure 1 indicates that  $Cl^-$ ,  $NO_3^-$  and  $SO_4^{2-}$  are the primary drivers in ovalbumin precipitation at 1 M total ion concentration.  $PO_4^{3-}$  and  $OAc^-$  exhibit synergistic blending with  $Cl^-$ ,  $NO_3^-$ , and  $SO_4^{2-}$  but were not capable of precipitating ovalbumin on their own. All of the cations exhibit antagonistic blending with  $Cl^-$ ,  $NO_3^-$ , and  $SO_4^{2-}$ . (b) BSA precipitation was induced by  $NO_3^-$  only;  $Cl^-$  and  $SO_4^{2-}$  exhibit some synergistic blending, but were not capable of precipitating BSA at 1 M total ion concentration. None of the cations were responsible for BSA precipitation. Response-trace plots are used to simultaneously compare the effects of all the components in the mixture design space. The lines represent the effect of changing each mixture component along a line from a reference blend (i.e., all nine components at a proportion of 0.111; indicated by the black dot) to a single component's vertex while holding all other components in a constant ratio. As the amount of this component increases, the amounts of all the other components decrease, but their ratio to one another remains constant. When the eight-dimensional design volume is translated to Euclidean distance and collapsed to 3 dimensions for visualization, it immediately becomes apparent that the precipitation response for ovalbumin (c) is fundamentally different from BSA (d). The red-rendered regions indicate the response volume where each protein exhibits significant precipitation; the blue/red dots indicate the ion mixtures specified for the experimental design. The color scale is consistent between both (c) and (d) and applies to the volume renderings and the dots.

of the respective ions. It depends on the proportions and concentrations of the other cations and anions in solution. It depends on the dissolved gasses. It may or may not depend on the pH. It depends on temperature. These dependencies are conflated, confounded, lost or ignored in traditional Hofmeister series, but are fundamentally essential to realizing a deeper understanding of ion-specific effects.

While we have presented only two phenomena (protein precipitation and enzyme activity) directly affected by ions, the concerns raised in this paper are applicable to all ion-specific questions previously examined using salt-

based experimentation. Ninham and Boström [5] state "... physical chemistry and colloid and surface science today sit in splendid disjunction from modern cell and molecular biology" and express frustration regarding the "ancillary and irrelevant" role played by the physical sciences in the life sciences. Compared to strictly physical systems, biological systems include an additional layer of complexity; several of the inorganic ions have the dual role of satisfying mineral nutrition requirements and affecting cell, tissue, and organ physicochemical environments. Separating these two roles is a necessary requirement for understanding

ion-specific effects on responses such as growth, morphogenesis, osmoregulation, reproduction, and so forth, and cannot be accomplished using salt-based experimental approaches.

Although the failings of the present physicochemical theories of ion-specific effects have been, perhaps, correctly pointed out [3–5, 16], we submit that the empirically derived data traditionally used to test and/or parameterize these theories have certainly been inadequate for correctly describing ion-specific responses. It is true that a comprehensive physicochemical theory should predict the results of poorly designed experiments. It is also true that some conclusions drawn from salt-based experimentation could be similar to the conclusions drawn from correctly designed, ion-specific experiments. However, it is our contention that ion-specific effects can only be measured within the conceptual framework of correctly designed experiments; "valid" conclusions of ion-specific effects cannot be logically derived from the Hofmeister series generated from salt-based experiments. We submit that the results presented here are, to the best of our knowledge, the first report of unconfounded ion-specific effects. The onus is upon researchers of ion-specific effects to adopt the experimental concepts described here or to justify alternative approaches within the context of the constraints inherent to these types of experiments. By sampling ionspecific design space for proportional and concentration effects, the resulting empirical models should enhance the development and testing of mechanistic physicochemical theories. A deeper understanding of ion specific effects across disciplines, ranging from agriculture to human medicine to ecology will result.

#### **Notes**

- (1) The root cause of this experimental conundrum lies in the propensity to approach inherently multifactor problems from a *one-factor-at-a-time* (OFAT) perspective. This OFAT approach is generally the default experimental design choice and is based on the presupposition that we are aware of which factors will have the greatest effect. However, this approach is only valid if we are sure that a single factor will exert its effects independently of all other pertinent factors or that its effects are related to variations in these other factors in a very simple manner, that is, there are insignificant interactions (cf. [11]).
- (2) We use the term "independent factors" in this paper for conceptual clarity. However, factor independence only applies when ions are treated in a factorial or regression type design (i.e., experiments that systematically vary only ion concentrations). When proportionality is incorporated via a mixture design the factors are not truly independent since changing the concentration of one ion requires adjusting the other ions to maintain proportionality, that is, the proportions of the mixture components must sum to unity. Thus, mixture factors are referred to as "components" in recognition that they are not independent in the same manner as a traditional "factor."
- (3) A freeware program "ARS-Media" that can be used to calculate salt/acid/base recipes that satisfy ion concentration requirements has been developed by the authors

and is available on the internet at http://www.ars.usda.gov/services/software/download.htm?softwareid=148.

- (4) While it is true that the dissociation of salts varies widely, and that complex mixtures of ions will create complex associations, or "species," of ions in solution, for the sake of argument we will consider all salts as dissociating into their parent ions in solution. Speciation and ion dissociation levels are a function of several properties such as the proportion and concentration of all ions in solution, temperature and partial pressure of dissolved gases. Thus, as with pH speciation is a dependent variable.
- (5) This confounding can be somewhat mitigated if the covariate ion is added at small concentrations, for example,  $\mu$ M amounts, to waters with a large concentration, for example, hundreds of mM or greater, of that ion, such as Na<sup>+</sup> or Cl<sup>-</sup> in seawater. However, this case is still technically confounded and these types of experiments still operate under the *assumption* of negligible covariate effects.
- (6) It should be noted that Hofmeister never actually reported a "Hofmeister series" of ions in the traditional sense (e.g.,  $SO_4^{2-} > Cl^- > NO_3^-$ ), although he did introduce the concept of ranking the *salts* based on common cat/anions. Hofmeister categorically stated that "... the protein precipitating effect of the salts depends both on the acid and the base of the salt," acknowledging that there is an inherent interaction of the ion pair contributed by the salt. It appears that Lilli [17] was the first to publish what became the standard Hofmeister series type of ranking. Hofmeister's results were subsequently extrapolated to produce the ion-specific series, divorced from salt-specific effects, which were named in his honor but were not supported by his experimental designs.

### **Acknowledgments**

The authors would like to thank B. Ninham, J. Cornell, B. Schecher, R. DeLoach, A. Parsegian, W. Smith, L. Kochian, W. Kunz, J Watterson, T. Beck, K. Larsson, D. Chapman, J. Wee, S. Lapointe, B. Kirkpatrick, G. Kirkpatrick, K. Bowman, J. Leblond, R. Shatters, J. Mozuruk, F. Niedz, and P. D'Aiuto for helpful comments on this manuscript. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. Both authors contributed equally to this work.

#### References

- [1] K. D. Collins and M. W. Washabaugh, "The Hofmeister effect and the behaviour of water at interfaces," *Quarterly Reviews of Biophysics*, vol. 18, no. 4, pp. 323–422, 1985.
- [2] F. Hofmeister, "Zur Lehre von der Wirkung der Salze," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 24, no. 4-5, pp. 247–260, 1888.
- [3] W. Kunz, J. Henle, and B. W. Ninham, "Zur Lehre von der Wirkung der Salze' (about the science of the effect of salts): Franz Hofmeister's historical papers," *Current Opinion in Colloid & Interface Science*, vol. 9, no. 1-2, pp. 19–37, 2004.

- [4] B. W. Ninham, "The present state of molecular forces," Progress in Colloid and Polymer Science, vol. 133, pp. 65–73, 2006.
- [5] B. W. Ninham and M. Boström, "Building bridges between the physical and biological sciences," *Cellular and Molecular Biology*, vol. 51, no. 8, pp. 803–813, 2005.
- [6] R. K. Scopes, Protein Purification: Principles and Practice, Springer, New York, NY, USA, 3rd edition, 1994.
- [7] B. Honig and A. Nicholls, "Classical electrostatics in biology and chemistry," *Science*, vol. 268, no. 5214, pp. 1144–1149, 1995
- [8] I. M. Rosenberg, Protein Analysis and Purification, Birkhäuser, Boston, Mass, USA, 2nd edition, 2005.
- [9] J. Loeb, "The proteins and colloid chemistry," *Science*, vol. 52, no. 1350, pp. 449–456, 1920.
- [10] P. A. Stewart, "Independent and dependent variables of acidbase control," *Respiration Physiology*, vol. 33, no. 1, pp. 9–26, 1978
- [11] R. A. Fisher, *The Design of Experiments*, chapter 6, Oliver & Boyd, Edinburgh, UK, 1935.
- [12] J. A. Cornell, Experiments with Mixtures: Designs, Models, and the Analysis of Mixture Data, John Wiley & Sons, New York, NY, USA, 3rd edition, 2002.
- [13] W. F. Smith, Experimental Design for Formulation, ASA-SIAM Series on Statistics and Applied Probability, SIAM, Alexandria, Va, USA, 2005.
- [14] R. P. Niedz and T. J. Evens, "A solution to the problem of ion confounding in experimental biology," *Nature Methods*, vol. 3, no. 6, p. 417, 2006.
- [15] D. R. Cox, "A note on polynomial response functions for mixtures," *Biometrika*, vol. 58, no. 1, pp. 155–159, 1971.
- [16] B. W. Ninham, "Physical chemistry: the loss of certainty," Progress in Colloid and Polymer Science, vol. 120, pp. 1–12, 2002.
- [17] R. S. Lillie, "The influence of electrolytes and of certain other conditions on the osmotic pressure of colloidal solutions," *American Journal of Physiology*, vol. 20, no. 1, pp. 127–169, 1907.