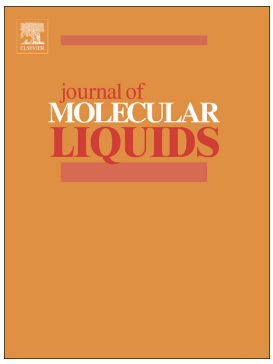

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The role of carboxyl groups upon the precipitation of albumin at low pH

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Abstract

Proteins play a major role in the organization of the living matter. Underlying this fact are their unique properties in aqueous solution, for which the available theories provide only a partial explanation. In order to throw some light into the behavior of proteins in aqueous solution, experiments on the precipitation of electropositive albumin by means of the sodium salts of sulfate, thiocyanate, nitrate, bromide and chloride upon varying the solution pH were undertaken. Results show that the protein undergoes a transition at approximately pH 3, characterized by a change on its precipitation profile. Its shape, of monotonic profile below pH 3, displays a non-monotonic behaviour with separated peaks at higher pH values, which increase in number and decrease in intensity with increasing pH. To elucidate these observations, additional precipitation experiments with sodium thiocyanate in the presence of a secondary salt were undertaken. It is shown that the secondary salt changes significantly the protein's precipitation profile, despite being present in very low concentration. Its influence on the precipitation depends in a great extent on the cation's valence, which induce effects proportional to x, x^2, and x^3 for monovalent, divalent, and trivalent cations, respectively. The observations reported can be explained under the assumption that i) the protein dissociates into distinct forms, as result of the dissociation of carboxyl groups, and ii) salt cations establish chemical equilibria with the negative charges of the dissociated carboxyl groups present in the emerging protein forms.

Introduction

Proteins have been recognized as a distinct class of molecules for at least two centuries[1]. They show diversity both in chemical structure and physical modification to an extent that lacks in any other class of substances[2]. Alone, they display the specific properties of the living matter, and are essential compounds for all known forms of life[2-4]. Underlying these attributes, are the numerous and unique protein properties in solution, for which a general theory affording a compelling clarification is still lacking.

Based on the assumption that the manifestations of proteins at neutral pH are the result of multiple events occurring simultaneously, and consequently difficult to unravel, we have begun a series of experiments on the salting out of bovine serum albumin (BSA) in which the pH was gradually changed. It has been known for a long time that the hydronium concentration considerably affects proteins properties in solution[4], and it was hoped in this way to elucidate this intriguing relationship[5]. Thus, in a previous work we have studied the precipitation of BSA by the action of inorganic salts at pH values between 2.4 and 3.1[5]. For these extremely acidic conditions the main conclusions were that i) The precipitation of albumin by the action of inorganic salts is intelligible under the assumption that the protein behaves like a common salt; ii) Anions are the main active agents in the precipitation of electropositive albumin; and iii) The effect of cations on the salting-out of BSA is only noticeable at pH values close to 3.0 and, remarkably, induce an effect which seems to result in the presence of distinct protein forms.

The previous observations and assumptions motivated the work here reported, which is based on the study of the precipitation of BSA at slightly higher pH values, and for which the dissociation of the carboxyl groups should be clearly noticeable. The obtained data are analyzed in light of the chemical theory, for which our previous results gave

strong support[5]. When justifiable, a comparison is given with the colloidal concepts, which, alongside the chemical theory, are the only ones of general character insofar advanced to explain the proteins behaviour in solution.

As a matter of fact, the protein behaviour in solution was an extremely debatable question in the late 19th and early 20th centuries[2, 4, 6-11]. Scientists discussed whether proteins were in true solution, in light of the chemical principles, or suspended particles, whose properties could be explained under the assumption of the colloidal ideas. According to some authors[4], the empirical colloidal concepts have prevailed due to the inability of the chemical school to reconcile their ideas with some experiments. In the present work, such an important question for our understanding of proteins will also be addressed.

Experimental

Materials:

Salts: The salts used were NaSCN (from Merck, > 98.5 %), $(NH_4)_2SO_4$ (from Merck, 99.5%), KCl (from Chem-lab, 99.5 %), NaCl (from Fisher, 99.5%), $CaCl_2$ (from Panreac, 95 %), $MgCl_2$ (from Sigma, 99%), and $InCl_3$ (from Sigma, 98%). Additional information can be found in the Supplementary Information.

BSA: The bovine serum albumin (Mw = 66.5 kDa) used was fatty acid free (< 0.02 %) from Fisher Scientific, lot 66-1384 and lot 66-1375, with purity > 98 %, ash content below 3 % (heavy metals < 10 ppm) and an isoelectric point (pI) of ~ 4.7. Additional information can be found in the Supplementary Information.

Others: Hydrochloric acid, 37 %, from Sigma-Aldrich, sodium hydroxide (from Panreac, 98 %), and potassium hydrogen phthalate (from Panreac, 99.8%). Additional information can be found in the Supplementary Information. The water used was ultra-

pure water, double distilled, passed by a reverse osmosis system and further treated with a Mili-Q plus 185 water purification apparatus.

Methods:

Precipitation experiments: Preliminary experiments were performed as detailed in the Supplementary Information. The precipitation data here reported, unless otherwise indicated, was obtained in the following manner: different solutions were prepared in 20 mL glass flasks, all containing the same quantity of protein acid, and, when adequate, of a secondary salt and increasing concentrations of the main precipitating agent. The final volume (3 or 5 mL) was completed by the addition of water. The required quantities of stock solutions of all components and water were dispensed with a Multipette Xstream pipette (Eppendorf, Hamburg, Germany). After the addition of all solutions, except for the protein stock solution, the mixture was homogenized. During the addition of the protein stock solution, the flask was gently mixed. After this procedure the flasks were maintained in an incubator (protected from light) at 25 ºC for 24 ± 2 h. Finally, the samples from each tube were transferred to quartz cuvettes, inserted in UV-1800 Shimadzu spectrophotometer with temperature control, let to stand for 30 seconds, and the absorbance at 350 nm was then read at least three times. Experiments performed at different days, with different stock solutions of all components, and also with different protein lots, allowed to estimate the error. The error was usually below 5 %. The major deviations were obtained at the very low concentrations of solution of Indium chloride, most likely due to the required sequential dilutions, but even in this case the error was below 9 %.

pH measurements: The pH control was performed as described in the Supplementary Information. The values of pH reported here for the precipitation curves are for those

solutions without salt, where reference was made to the titration curve (Figure S1 in the Supplementary Information). pH measurements were made at distinct salt concentrations in the presence of BSA at 10 $g.L^{-1}$. For all the figures presented the pH do not change considerably from the one reported. Their values in the absence or presence of salts amounts to the one presented ± 0.03 and ± 0.1, respectively.

Results and Discussion

In a previous work[5], dealing with the salting-out of the electropositive BSA at extreme acidic conditions, a suggestion has been made, according to which *bovine serum albumin dissociates into various forms as the result of the dissociation of its carboxyl groups*. In the following, experimental assays and evidences of this hypothesis are sought.

The transition at low pH

An attempt to verify whether the protein's precipitation profile varies considerably, or not, under the experimental conditions in which the dissociation of the carboxyl groups ought to become noticeable was carried out. The protein is composed of one hundred carboxyl groups whose pK_a values vary between 3.75 and 3.95[12]. Hence, we have undertaken experiments upon the precipitation of BSA by means of sodium salts of thiocyanate and sulfate, while varying the pH, whose results are shown in Figure 1.

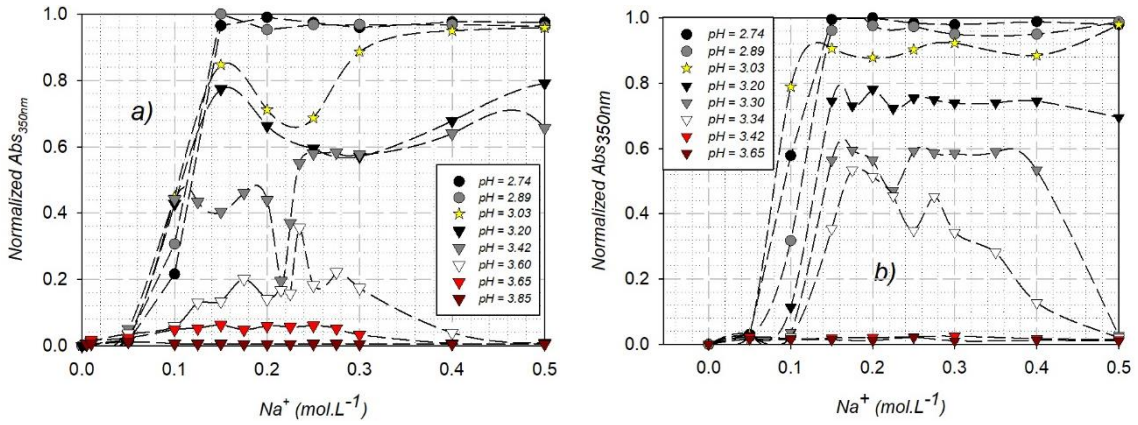

Figure 1. Precipitation of BSA (10 $g.L^{-1}$) by means of NaSCN (figure 1a - left) and Na_2SO_4 (Figure 1b - right) at different pH values, after incubation for 24 h at 25 ° C. Here, and in the remaining figures, the lines have no physical meaning and are only intended to assist in visualizing the data.

In Figure 1, the quantity of salt is expressed in molarity of cation, for reasons discussed below. The anions, which are the main precipitating agents for electropositive proteins[5, 13], display consequently their precipitating power in distinct quantities in the results gathered in Figure 1. Even so, it can be seen the parallelism between the effects induced by both salts, which can be summarized in the following manner: below pH 3, the salt induces a monotonic precipitation profile, of sigmoidal shape, upon the precipitation of albumin. A striking change occurs at ca. pH 3, whereupon the precipitation profile exhibits a non-monotonic shape. Separated peaks are now distinguishable, which increase in number and decrease in intensity with growing pH. Below a certain hydronium concentration, the protein does not precipitate by the action of salt within the experimental conditions displayed, which is further confirmed by studies at higher pH values (data not shown).

In Table 1 the quantity of salt and hydronium required to bring about BSA precipitation are given in molar proportions *per* mole of protein. These values correspond to experimental data points taken from Figure 1a, but would qualitatively be similar if they were taken from Figure 1b. Also, the amount of precipitated protein is shown

comparatively, as the percentage of change in the Abs_{350nm}, either when the quantity of salt is doubled, while the hydronium concentration remains constant (top values in the corresponding cells), or when the hydronium concentration changes, while the quantity of salt remains constant (bottom values in the corresponding cells).

Table 1. Experimental conditions corresponding to data points in Figure 1a.

pH	$[H_3O^+]$ x 10^4 ($mol.L^{-1}$)	% of change in Abs_{350nm} (*)			molar ratio salt: H_3O^+: BSA		
		NaSCN 0.10 $mol.L^{-1}$	NaSCN 0.20 $mol.L^{-1}$	NaSCN 0.40 $mol.L^{-1}$	NaSCN 0.10 $mol.L^{-1}$	NaSCN 0.20 $mol.L^{-1}$	NaSCN 0.40 $mol.L^{-1}$
3.03	9.33	>100 [(a)] -4 [(b)]	56 -26 [(b)]	33 -3 [(b)]	665: 6.2: 1	1330: 6.2: 1	2660: 6.2: 1
3.42	3.80	>100 [(a)] -1.7	0 -37	43 -32	665: 2.5: 1	1330: 2.5: 1	2660: 2.5: 1
3.65	2.24	59 [(a)] - 85	15 -83	-67 -96	665: 1.5: 1	1330: 1.5: 1	2660: 1.5: 1

(*) – percentage of change in the Abs_{350nm} when the quantity of salt is doubled while the $[H_3O^+]$ remains constant (top values), and for the same quantity of salt when the hydronium concentration decreases (bottom values).

[(a)] - % change relatively to NaSCN 0.05 $mol.L^{-1}$; [(b)] - % change relatively to pH = 2.89 or $[H_3O^+] = 1.29 \times 10^{-3}$.

It can be seen from these numbers that copious amounts of salt are required to bring about a noticeable salting-out. It can also be seen from Table 1 the lack of proportionality between the amount of precipitated protein and the quantity of added salt: upon doubling the quantity of salt, stepwise from 0.05 $mol.L^{-1}$ to 0.4 $mol.L^{-1}$, while holding the pH at 3.03, the quantity of precipitated protein always increases, first considerably, then slightly, and then somewhat less; at pH 3.42, the quantity of the precipitated protein by means of the same quantities of added salt, first increases considerably, then remains constant, and after increases moderately; at pH 3.65 the quantity of the precipitated protein by the action of the same amounts of added salt, first increases moderately, then increases slightly, and finally decreases considerably. These behaviors suggest that the observed phenomena depends chiefly upon the pH, an

assumption which is further emphasized by the fact that a decrease in the quantity of hydronium brings about, as a general behaviour, a reduction in the amount of precipitated protein. This parallelism, however, is not strictly observed, and when it is, these quantities, *i.e.*, the hydronium and the precipitated protein, vary in distinct proportions. Taken together, these facts are intelligible under the assumption that the protein undergoes a change, in which the pH plays an important role.

These observations could be explained in the following manner: The electropositive protein is a macromolecular cation which can be present in multiple and distinct macromolecular cations. The dissociation into these forms is similar to those encountered in electrolyte chemistry, according to which the driving force for the speciation process is the hydronium concentration. Therefore, above a certain pH value, the undissociated protein dissociates into hydronium and macromolecular cations, which coexist in chemical equilibria between them. The pK_a for these various chemical processes varies within a very limited range of pH, corresponding to those expected for the dissociations of the carboxyl groups. The dissociations, likewise those encountered in salts, are stepwise processes, according to which the number of macromolecular cation species increase with increasing pH. Hence, their concentrations decrease for lower hydronium quantity, resulting in an increase upon the required quantity of salt to precipitate them. A threshold pH value is attained, above which the concentration of the various protein forms reaches a minimum, which are no longer observable by the precipitation conditions displayed.

The anion's series

The fact that BSA undergoes a transition within a low pH range, was perhaps firstly pointed out by Tanford[14]. The so-called "conformational change"[15] has been thereafter revealed by many other techniques, and for which, as we have seen above, precipitation

affords no exception. Neither would we expected otherwise. The protein is blind towards the technique, that is to say that it must respond in the same manner to varying experimental conditions. Therefore, the behaviour revealed by titration curves[12, 14], intrinsic viscosity[16], optical rotation[16], X-ray diffraction[17], circular dichroism[18], Raman spectra[19], amongst many others[15, 18, 20-24], finds correspondence with our observations, the elucidation of which shall by implication clarify the observations exposed by distinct techniques. Hence, the chemical interpretation of the transition demands further scrutiny.

From the previous considerations, we extended the studies to a wider number of salts, or rather, salt anions, which are the main precipitation agents for electropositive proteins[5, 13]. Figure 2 illustrates the precipitation experiments of albumin by means of sodium salts of chloride, bromide, nitrate, thiocyanate and sulfate at pH = 2.89 (Figure 2a) and pH = 3.42 (Figure 2b). Additional information can be found in the Supplementary Information (Figure S2).

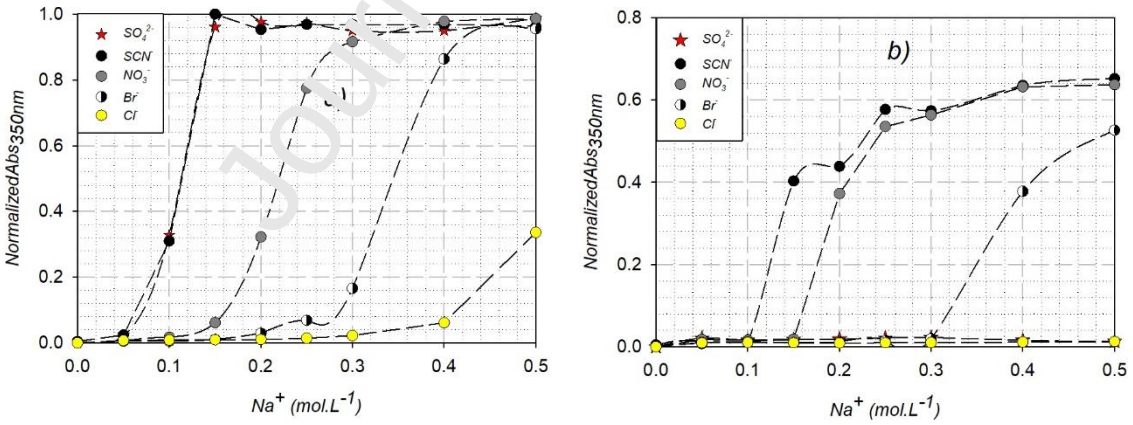

Figure 2. Precipitation of BSA (10 g.L^{-1}) by the action of Na_2SO_4, NaSCN, $NaNO_3$, NaBr and NaCl at different pH values (2a, pH = 2.89 and 2b, pH = 3.42), after incubation for 24 h at 25 ° C.

It can be seen from the results gathered in Figure 2, likewise from those in Figure S2 in the Supplementary Information that the amount of precipitated protein decreases with

increasing pH. The observed behaviour occurs with all precipitating agents, supporting the above conjecture, according to which the protein undergoes a change, which depends chiefly upon the hydronium concentration.

Some facts in Figure 2 are, however, apparently in conflict with the previous conjecture: the role of the sulfate anion, which seems to undergo an inversion of behaviour with pH, or the lack of observation of a transition by some salts, both of which suggest that the salts play a non-negligible role in the phenomena under consideration. In the following an attempt to clarify both observations is sought.

Ions, which are the active agents in the salting-out phenomena, display distinct capabilities in bringing a protein out of solution[5, 10, 13, 25-26]. The relative effectiveness of anions in precipitating an electropositive protein, which can be established in many distinct ways, was here appraised by the quantity of salted-out protein by means of the same amount of distinct anions, while holding constant the remaining variables upon which the precipitation depends on[5]. Therefore, upon the limits allowed by experiments, for the same pH, temperature, elapsed time before measurement, quantity and nature of counter-ions, and perhaps pressure, the higher the amount of precipitated protein the more effective the anion is in bringing the protein out of solution. Any attempt to establish the relative efficacy of anions in the salting-out phenomena, in which at least two of the foregoing parameters, including the quantity of salted-out protein[5], vary, may evidently lead to erroneous conclusions.

Under these circumstances, conclusions can be drawn about the effectiveness of anions with distinct valence, or between those of the same valence at distinct concentrations, which lacks correctness under those circumstances where the cation plays an active role upon the precipitation of proteins. It will be seen below that the cation has a central role upon the precipitation of electropositive albumin, starting at pH values whereby the

dissociation of the carboxyl groups ought to become noticeable. Consequently, the comparison between the efficacy of SO_4^{2-} in the salting-out phenomena and the monovalent anions, under the experimental conditions illustrated in Figure 2b may lead to wrong conclusions. Likewise at higher pH values, the comparison between anions of distinct valence, or between anions of the same valence at distinct concentrations, both of which often reported[25-27], will in all likelihood result in incorrect assumptions.

We have shown previously[5] that the sulfate anion displays a higher capacity than the above studied monovalent anions to bring the protein out of solution at extreme acidic conditions, whereby cations such as Na^+ play a negligible role upon the salting-out phenomena. This higher propensity of sulfate than the monovalent anions to precipitate proteins, is further supported by the results illustrated in Figure 2a, in which the effect of Na^+ is less pronounced, and under the same experimental conditions, including the amount of cation, half the quantity of SO_4^{2-} induces the same amount of precipitated protein as the strongest precipitating monovalent anion. We may then conclude from the preceding, that the sulfate is, amongst the anions studied, the strongest precipitating agent. The intriguing behaviour of the sulfate anion upon increasing pH, which is illustrated in Figure 2, must therefore be due to the role of cations, whose effects upon the salting-out of electropositive albumin increase with decreasing hydronium concentration. Following the same lines of thought, it can be seen from the results in Figure 2, as from those in Figure S2, that the order of efficacy for the monovalent anions in the salting-out of electropositive albumin can be unambiguously established. Therefore, anions have the property to precipitate albumin, whose efficacy in bringing it out of solution decreases in the following order: $SO_4^{2-} > SCN^- > NO_3^- > Br^- > Cl^-$.

The lack of observation of a change upon the precipitation behaviour by means of certain salts, is, according to the same lines of thought, due to the weak precipitating

power of certain anions, like bromide or chloride. It is, so to say, a limitation inherent to the technique. The conclusion is supported by earlier studies on *e.g.* intrinsic viscosity[16], or optical rotation[16], in which the transition experienced by the protein could be noticeable in the absence of salt.

Thus, the results illustrated in Figure 2 can be explained under the assumption that the protein undergoes a change, which depends chiefly upon the hydronium concentration. Anions, on the other hand, have the property to precipitate albumin, whose efficacy in bringing it out of solution is given by the above series.

Upon assumption of the colloidal ideas, the protein adapts itself differently in response to the presence of individual ions[28], the effects of which are believed to be ruled out by a universal mechanism hidden in the so-called Hofmeister series[25-27]. According to these ideas, ions can be organized in a definite order reflecting their efficacy on bringing about a protein out of solution. Anions are apparently more effective than cations[29], whose effects can be arranged in a decreasing order of efficacy as follows[30]: $SO_4^{2-} > Cl^- > Br^- > NO_3^- > SCN^-$. Either slight modifications[30], such as the switch between adjacent ions in the series, or considerable variations[30-31], such as a complete reversal of the same, have been reported. The results and interpretations here reported have shown otherwise.

According to our interpretation, albumin undergoes a change as the result of the dissociation of the protein, whereby distinct protein forms coexist in equilibria. Thus, the observed phenomena are due to chemical processes similar to those encountered in general chemistry. The driving force for the transition is mainly the hydronium concentration, while ions roles are intelligible under the assumption of classical physicochemical principles. Hence, the role of anions upon protein properties in general, and precipitation of electropositive proteins in particular, are ruled out by

electrostatic interactions, according to which the anion's valence, first, and the size of the same, thereafter, will determine anion's effectiveness in inducing precipitation. The effects are rationalized in the following way: The role of the valence is understandable in association with the Coulomb's law[32], according to which the magnitude of the electrostatic forces of attraction is directly proportional to the magnitudes of the charges of opposing character; the effect of the size is understood by the fact that the further is the electron away from the nucleus the more easily it combines with positive charges[4-5]. The anions size in aqueous solution, which should be a good proxy for the latter effect, has been obtained by Y. Marcus[33], who reported the following values: 18.1, 27.8, 29.0, and 46.6 $cm^3.mol^{-1}$ for Cl^-, Br^-, NO_3^- and SCN^- respectively, a trend which agrees with our observations and supports our arguments

The non-monotonic effect

After establishing that the transition is a phenomenon inherent to the protein and that depends chiefly on the pH, in agreement with earlier observations[12, 14-16], we looked in more detail into another phenomenon: The non-monotonic precipitation profile, bell-shaped, clearly discernible by the action of sodium thiocyanate at pH 3.65, illustrated in Figure 3. A similar sequence of events of salting-out followed by salting-in was reported more than 100 years ago[13], which we try to elucidate next.

In light of the concepts herein advocated, the electropositive protein dissociates into many distinct forms. Any of these carries globally a positive charge, differing amongst them in the value of its charge. In agreement with physicochemical principles, the assumption is made that the least positively charged, or otherwise, those containing more negative charges on their backbone, arising these from the dissociation of the carboxyl groups, are the least soluble ones, and consequently precipitated by smaller

amounts of salt. The assumption is also made that the precipitated protein forms are in equilibrium with the, so to say, macromolecular cations in solution, a premise supported by the reversibility of the precipitation often reported in the literature[13, 34-37]. Consequently, the observations in Figure 3, according to our interpretation, are the result of the precipitation of low-soluble protein forms which are in chemical equilibria with macromolecular cations in solution, some of which being abundant in ionized carboxyl groups.

The previous conjecture, if correct, can be experimentally tested by means of the following arguments: Throughout the reaction of the ionized carboxyl groups of the macromolecular cations in solution, by force of electrostatic interactions, the equilibrium can be perturbed towards a smaller amount of precipitated protein, with a consequent lowering of the precipitation profile. This effect, which depends on the charge, positive on this circumstance, of the added reagent body, will consequently be more effective if divalent than monovalent, and if trivalent than divalent.

The addition to the solution of a positively charged body carries inevitably with it the addition thereto of a negative one. According to the ideas here proposed, the negatively charged body will most likely react with the positive charges present in the backbone of the protein, with a consequent and parallel perturbation of the assumed chemical equilibria. A minimization of this effect is anticipated, if the main and the secondary negatively charged bodies are strong and weak interacting reagents, respectively.

As a result of the previous reasoning, we have undertaken experiments on the precipitation of BSA by sodium thiocyanate, under the same experimental conditions as in Figure 3, except that calcium or indium chlorides were also added, and whose results are illustrated in Figure 4.

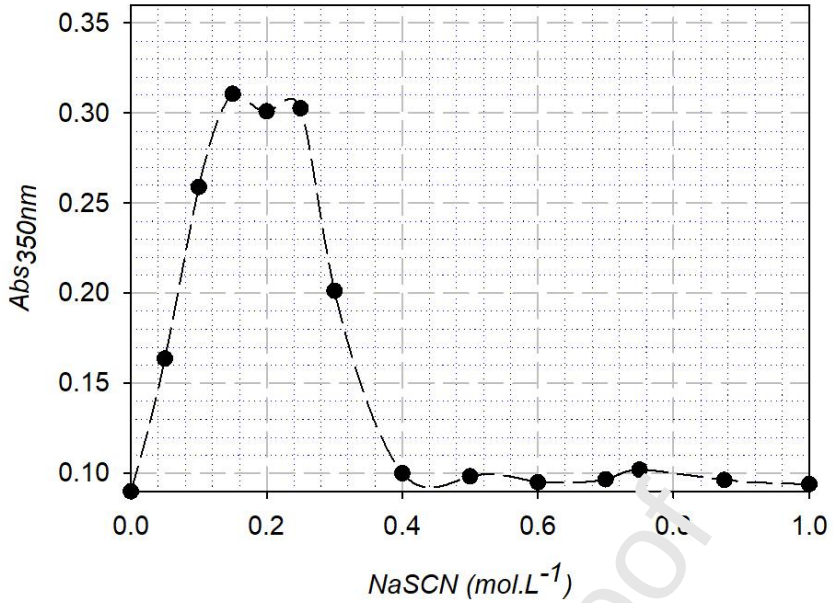

Figure 3. Precipitation of BSA (10 g.L-1) by means of NaSCN at pH 3.65, after incubation for 24 h at 25 °C.

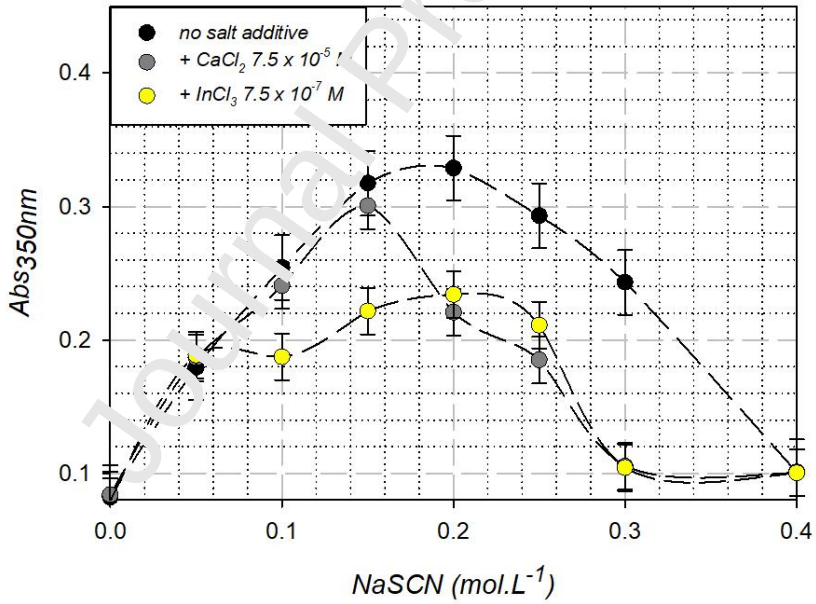

Figure 4. Precipitation of BSA (10 $g.L^{-1}$) by the action of NaSCN at pH 3.65, in the additional presence of $CaCl_2$ or $InCl_3$, after incubation for 24 h at 25 °C. The bars indicate the experimental uncertainty.

Here, it can be seen a slight but noticeable decrease in the precipitation profile in the presence of a secondary salt, as predicted. The observed effects appear to result chiefly

from the cation, according to the theory, although doubts may remain in this regard, and for which we hope to provide more explicit evidence latter on.

The facts illustrated in Figure 4 express particularities that need further scrutiny. Here, we restrict our analysis to the relative proportion of secondary salts required to induce similar effects, and to the distinct precipitation profiles induced by the secondary salts, a rationale for which, in light of the herein advocated concepts, are the object of our next considerations.

Hardy's valence rule

The precipitation of proteins by the action of ions, which we will assume in the following to be a chemical reaction, is a phenomenon of electrostatic nature[13, 38]. Therefore, the reaction between the negative charges of 1 mole of active macromolecular cation, *i.e.*, the positively charged protein in solution which contains dissociated carboxyl groups on their backbone, hereinafter referred to as MC, and n moles of divalent cations, below referred to as D^{2+}, can be formalized according to the equation:

$$MC + nD^{2+} \leftrightarrow MCD_n \qquad (1a)$$

and the associated rate equation, according to:

$$\nu_2 = k_D[MC][D^{2+}]^n \qquad (1b).$$

Here ν_2 is the rate of the reaction, and k_D the rate constant. Similar equations, but now for the reaction between 1 mole of MC and m moles of trivalent cations, which below are referred to as T^{3+}, holds:

$$MC + mT^{3+} \leftrightarrow MCT_m \qquad (2a)$$

$$\nu_3 = k_T[MC][T^{3+}]^m \qquad (2b),$$

whose symbols have similar meanings to the previous ones. In light of the foregoing, conclusions can be drawn about the proportion required by cations of distinct valences to react with MC, or, in other words, to induce similar effects in the precipitation profile. Two thirds of a trivalent cation are required for each divalent cation to induce a similar outcome, or according to the previous formalism, m = 2/3 n. Assuming that $\nu_2 = \nu_3$ and $k_D = k_T$, conjecture discussed later on, then for the same amount of active macromolecular cation, the relative proportion between divalent and trivalent cations to induce similar effects, will be given by the following relation:

$$[T^{3+}] = [D^{2+}]^{3/2} \qquad (3a),$$

or similarly,

$$[D^{2+}] = [T^{3+}]^{2/3} \qquad (3b),$$

Equation 3 can now be tested in predicting the experiments in Figure 4.

The effect induced by 7.5 x 10^{-5} mol.L^{-1} of a divalent cation on the protein precipitation profile, finds a theoretical correspondence in 6.5 x 10^{-7} mol.L^{-1} of trivalent cation, and 7.5 x 10^{-7} of the latter, in 8.3 x 10^{-5} of the former. The experimental values of 7.5 x 10^{-7}, and 7.5 x 10^{-5}, respectively, are as close to the theoretical ones as could be expected, as our next arguments try to support.

The slight deviations between the previous numbers, and between the precipitation profiles induced by cations of unlike valence can be attributed to at least two effects: i) to the anion of the secondary salt, which is assumed to play a non-negligible role on the phenomena upon consideration, and which is present at distinct concentrations; ii) and due to collision theory concepts[39], according to which the trivalent or divalent cation will react, respectively, with three or two carboxyl groups of the same, and not of distinct macromolecular cations. From such a consideration, which may have implications far beyond what is concerned here, conclusions can be gathered about the

previous assumptions, i.e., $\nu_2 = \nu_3$ and $k_D = k_T$, which certainly lacks precision. So, the similarity of the effects induced by means of the secondary salts, $CaCl_2$ and $InCl_3$, and illustrated in Figure 4, are as close by as allowed by the theory. Likewise, the accuracy in the prediction according to equation 3 are, in our view, a strong argument for the central role of the cation in the phenomenon under consideration.

Following identical reasoning, the relative proportion between monovalent (M^+) and divalent or trivalent cations to induce similar effects on the precipitation, is then given by the following relationship:

$$[M^+] = [D^{2+}]^{1/2} \qquad (4a),$$

or,

$$[M^+] = [T^{3+}]^{1/3} \qquad (4b),$$

respectively.

The experimental value obtained with potassium chloride (Figure S3 in the Supplementary Material) of 1.0 x 10^{-2} $mol.L^{-1}$, and following the same foregoing lines of thought, is as close by as could be expected to the theoretical one of 8.9 x 10^{-3}, which was obtained with equation 4, by arithmetic mean of the effects observed for Ca^{2+} and In^{3+}. The less pronounced salting-in effect induced by the potassium is perhaps due to the high quantity of the Cl^- anion in solution, which must play now a considerable role upon the phenomena.

From the preceding figures the following conclusions can be drawn: for pH values around 3.65, the albumin, which is brought out of solution by means of NaSCN, can be salted-in by addition thereto of a secondary salt. The observed influences of the secondary salt result chiefly from the cation, which depends to a great extent on its valence. The induced effects follow, within the experimental uncertainty, the proportion x; x^2; x^3 for monovalent, divalent, and trivalent cations, respectively.

The ratio $x; x^2; x^3$ is identical to Hardy's valence rule[38] established more than 100 years ago for the precipitation of proteins by means of inorganic salts. According to this rule, ions have the property of precipitating protein solutions, their precipitating capacities depending on the ion's valence according to that proportion[38]. The first theoretical explanation of Hardy's valence rule is due to Whetham[40] based on the colloidal theory, according to which the protein contains a double electrical layer, the existence of which leads to the stability of the system[10, 40]. The ions of opposite charge to the protein neutralize its charge thus increasing the energy of the protein's surface, with the consequent tendency of it to contract, to join with other identical ones to form large aggregates, which finally assume properties of matter in mass and, therefore, they are precipitated from the solution[10]. Whetham, by means of statistical arguments, succeed in demonstrating that the proportion $x; x^2; x^3$ could be rationalized in the light of the colloidal concepts[10, 40]. As we have seen previously, and which Robertson was perhaps the first one to call attention to[10], Hardy's valence rule can also be explained upon the assumption of the chemical theory.

The Chemical / Colloidal Debate

The observations summarized in the previous section seem, therefore, to belong to those circumstances which are intelligible upon assumption of the chemical or the colloidal theories. The facts, however, cover a limited range of experiments, the widening of which limits may clarify which interpretation holds for a wider range of observations. Hence, we have undertaken experiments on the precipitation of BSA under the same experimental conditions as before, except that the secondary salt was added at varying quantities. Figure 5 illustrate the results under the same experimental conditions as in Figure 3, with varying quantities of calcium chloride as the secondary salt.

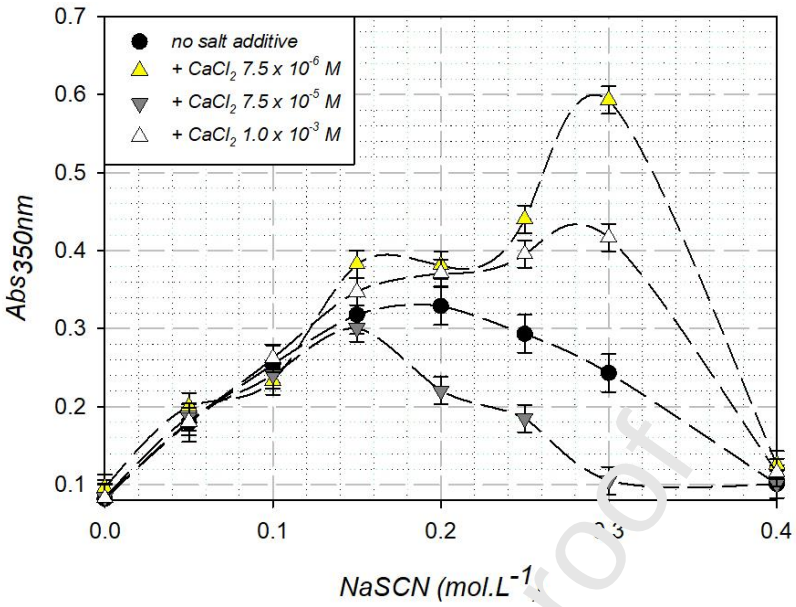

Figure 5. Precipitation of BSA (10 g.L-1) by NaSCN action at pH 3.65, in the additional presence of $CaCl_2$ at different concentrations, after incubation for 24 h at 25 °C. The bars are indicators of experimental uncertainty.

From the results illustrated in Figure 5 the following is seen: for pH values around 3.65, the precipitation profile of BSA by means of sodium thiocyanate can be changed by the addition thereto of calcium chloride. Sequential addition of the secondary salt can first bring about a further salting-out of the protein, followed by salting-in by further addition of salt. The protein can be salted-out again by further addition of salt. The quantity of secondary salt to induce such effects is substantially inferior to the amount of sodium thiocyanate, varying between two to five orders of magnitudes below.

The previous studies were extended to a larger number of salts, whose results are illustrated in Figure 6.

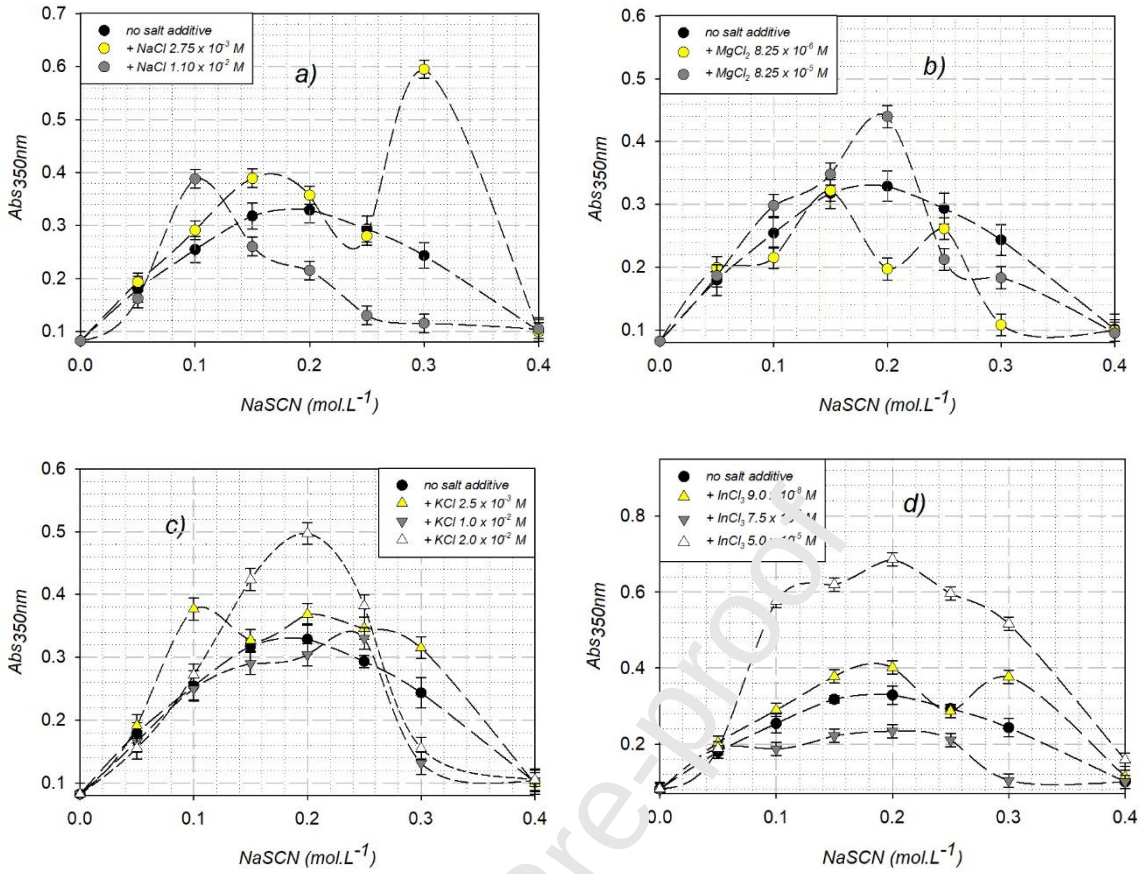

Figure 6. Precipitation of BSA (10 g.L-1) by NaSCN action at pH 3.65, in the additional presence of a secondary salt at different concentrations as indicated, after incubation for 24 h at 25 °C. The bars are indicators of experimental uncertainty.

It is seen from Figure 6 that the precipitation profile of albumin, which can be brought out of solution by relatively small quantities of NaSCN at pH 3.65, changes considerably by the addition thereto of a secondary salt. The effects induced by the secondary salt seem difficult to predict with multiple possible outcomes: The quantity of precipitated protein by means of NaSCN 0.1 mol.L^{-1} increases about 50% by the addition of NaCl in a quantity almost one order of magnitude below. If the quantity of NaSCN is triplicated, while holding the same quantity of NaCl, the amount of precipitated protein now decreases about 50%. If, on the other hand, we keep the same quantity of NaSCN and decrease the amount of NaCl four times, the amount of precipitated albumin increases considerably; The quantity of precipitated protein by

means of NaSCN 0.2 mol.L^{-1} increases about one third by the addition of $MgCl_2$ in a quantity which is more than three orders of magnitude below. If the quantity of $MgCl_2$ is decreased 10 times, while holding the same quantity of NaSCN, the amount of precipitated protein decreases more than one third. If now the quantity of sodium thiocyanate is increased 25%, while holding the same quantities of $MgCl_2$, the quantity of precipitated protein remains the same in the presence of the lower quantity of secondary salt, while decreases slightly for higher quantity of $MgCl_2$. By a further increase in NaSCN in the same amount, the quantity of precipitated protein, in the presence of the same quantities of $MgCl_2$ as previously, now always decreases, but more pronouncedly in the presence of lower quantities of secondary salt.

It is also seen from Figures 6c and 6d that sequential addition of the secondary salt can first induce an increase in the quantity of precipitated protein by means of NaSCN, followed by salting-in by further addition of salt, which, that by still further addition of salt, the protein can be again brought out of solution. The effects are more noticeable for salts composed of cations of higher valence (Figures 5 and 6d), for reasons previously considered, and the induced effects follow, within the experimental uncertainty, the proportion x; x^2; x^3 for monovalent, divalent, and trivalent cations, respectively.

According to the colloidal concepts, the effect of the secondary salt upon the protein precipitation can be either additive or competitive[41-42]. In the former circumstance, one might expect a continuous increase in the precipitation profile upon sequential addition of a secondary salt. From a competitive viewpoint, one might expect upon progressive addition of a secondary salt, firstly a decrease in the precipitation profile down to a minimum, followed by a continuous increase upon further addition of salt. The observations here reported show otherwise.

The experimental facts can be explained in the following manner: The electropositive albumin is a macromolecular cation, which is dissociable into multiple and distinct macromolecular cations that coexist in solution in chemical equilibria. The dissociations into these forms are stepwise processes that take place within a very limited range of pH values. The driving force for the dissociation is the hydronium concentration, likewise in similar processes encountered in general chemistry. Hence, above a certain pH value the carboxyl groups begin to dissociate, carrying with it the dissociation of the protein into macromolecular cations and hydronium.

The multiple emergent protein forms, which now contain negative charges on their backbone, are globally positively charged. Therefore, the relative proportions in which they co-exist in solution can be changed by means of both positively and negatively charged bodies, according to chemical equilibria concepts. In agreement with the same principles, the macromolecular cations can be brought out of the solution by means of inorganic salts. Hence, the precipitation of albumin can be, under certain circumstances, the result of the salting-out of manifold and distinct protein forms.

From the preceding considerations, the precipitation profile of the distinct protein forms can be perturbed by means of a cation, as long as the effect of the anion is minimized, in a manner which is at the moment impossible to predict, with multiple possible outcomes. The effects induced by distinct cations can, however, be anticipated, and follow the proportion x; x^2; x^3 for monovalent, divalent, and trivalent cations, respectively, and in all likelihood x^n, for cations with an n-fold valence. The accuracy on the prediction is limited not only by the eventual non-negligible role of the added anion, but also by probabilistic arguments[39], according to which an n-valence cation reacts with n-sites of the same, and not of distinct macromolecular ions; The formed

bodies have consequently distinct structure and solubility, depending upon the valence of the reagent cation.

Final remarks

In the late 19th and early 20th centuries[2, 4, 6-11], there were two different prevailing opinions regarding the behaviour of proteins in solution: the chemical theory, according to which proteins are in true solution whose properties can be explained upon assumption of classical physicochemical principles, such as electrostatic interactions between charged bodies, chemical equilibria concepts, and so forth; and the colloidal theory according to which proteins are suspended particles in solution whose properties are explained upon assumption of the surface phenomena concepts.

During those years in which the colloidal ideas were developed[43], some of the most outstanding results in the realm of the natural sciences have been achieved[44-45]. Perhaps encouraged by van't Hoff great accomplishments, which proved that thermodynamic laws are not only valid for gases, but also for dilute solutions, or by the outstanding achievements by Einstein[45], which was able to show that the molecular-kinetic theory of heat developed in the domain of gas theory, could as well be extended to liquid phases, scientists adopted the newly born empirical formulas of adsorption[43], originally developed for gases[43, 46], to explain the behaviour of proteins in aqueous solution. More based on the inability of the chemical school to reconcile their ideas with some experimental facts[4], than on any convincing experimental evidence or mathematical proof, the empirical colloidal concepts have grown into a widely accepted and apparently established discipline to explain protein-related phenomena[4], an idea which prevails nowadays.

Any general theory, so to speak, to be considered as such, should not hold within a limited range of observations, but instead throughout the whole range of experiments. In

this regard, the so-famous precipitation experiments of Hofmeister[25-26] have contributed decisively for the acceptance of the colloidal theory to explain the protein behaviour in solution[4], or rather, to reject the chemical theory, for the simple fact that the ion series observed by Hofmeister[25-26] and similar ones[2, 9, 13] were impossible to bring together with the stoichiometric properties of the ions.

No one can explain adequately the Hofmeister series. Any, so to say, Hofmeister series, find exceptions[30] raising some doubts about their existence, an idea which has been suggested a long time ago[47]. In the present work, which was partially based on these controversial opinions, a careful study upon the precipitation of albumin was undertaken. Of utmost relevance was the conclusion that Hofmeister as well as others after him, ignored some of the parameters upon which the precipitation depends on[5], which led, in all likelihood, to wrong assumptions. Of course, the protein is blind towards the technique, or in other words, it must respond in the same manner to varying experimental conditions, and the same wrong assumptions are reached by means of distinct techniques if such experimental parameters are ignored, like the elapsed time before measurement.

The precipitation studies were here extended to other protein-related phenomena, which have also contributed to the acceptance of the colloidal ideas. Some important observations/conclusions have been reached, which can be resumed in the following manner: (i) the effects of anions upon the precipitation of albumin can be understood in light of the chemical theory; (ii) The Hardy valence rule[38] holds both for the ion that has charge opposite to the protein surface, as is widely recognized[48], and for the ion that has the same sign of the protein surface, a fact we have never seen reported previously; (iii) Both salting-in and salting-out effects by means of the secondary salt were observed, raising in this way questions about the stabilizing/destabilizing power of the electrolytes

upon the protein surface[40, 48]; (iv) It was unambiguously shown that Hardy's valence rule[38] can be explained upon the assumption of the chemical theory; (v) An unprecedented rationale for bell-shaped precipitation curves, or the so-often called non-monotonic effect, in light of the chemical concepts was presented, which was supported by experimental facts; (vi) The transition al low pH[14] was explained under assumption of an new protein dissociation concept, according to which the biomolecule dissociates into manifold compounds in a "salt-like" manner. Many protein phenomena observed by others, such as the increasing amount of evidence which suggests that proteins exist in solution as dynamic ensembles of interconverting structures[49-50], can be explained upon assumption of such concept.

Conclusions

In view of the foregoing, we can conclude that the colloidal theory does not accurately explain the protein manifestations in solution here reported, and the chemical theory is, at the moment, the only one that explain the observed facts.

All the observations presented in this work are strong arguments in support of the suggested concept according to which *bovine serum albumin dissociates into various forms as the result of the dissociation of its carboxyl groups*. According to these ideas, the protein properties in aqueous solution can thus be understood under two general assumptions:

(i) Chemical entities differing slightly in structure or charge have drastically distinct biological activity, physical and chemical properties, and

(ii) Le Chatelier's principle, according to which chemical equilibrium is a dynamic state, whose position can shift towards different directions in response to perturbations to changing the conditions.

Since proteins are all formed by the same building blocks, the described ideas are expectedly of general validity for different proteins. It is hoped that the observations reported, and interpretations proposed foster additional studies in this field under a new light, which can have an everlasting impact on protein research, in particular, and upon biosciences, in general.

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Author statement

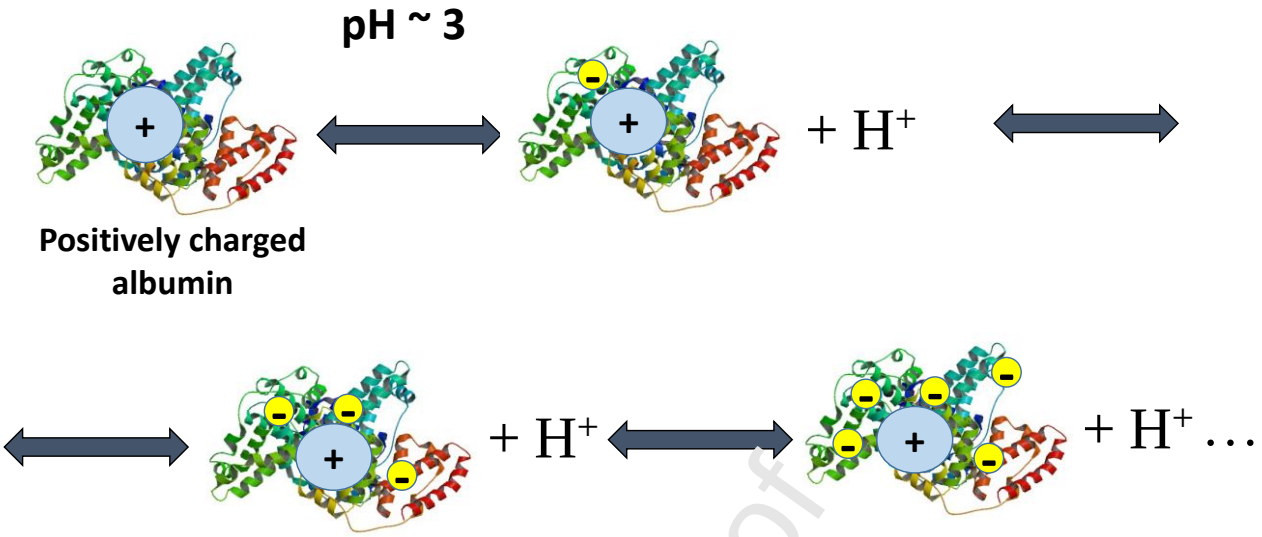
Pedro P. Madeira: Conceptualization, Methodology, Validation, Investigation, Writing - Original Draft

Mara G. Freire: Resources, Writing - Review & Editing, Funding acquisition

João A. P. Coutinho: Resources, Writing - Review & Editing, Funding acquisition

Declaration of competing interest

There are no conflicts of interest to declare.



Highlights

- Salting-out of BSA reveals that the protein undergoes a transition at pH 3
- The protein dissociates into distinct forms
- Salt cations establish chemical equilibria with the protein
- The data supports the protein's chemical behaviour in solution.

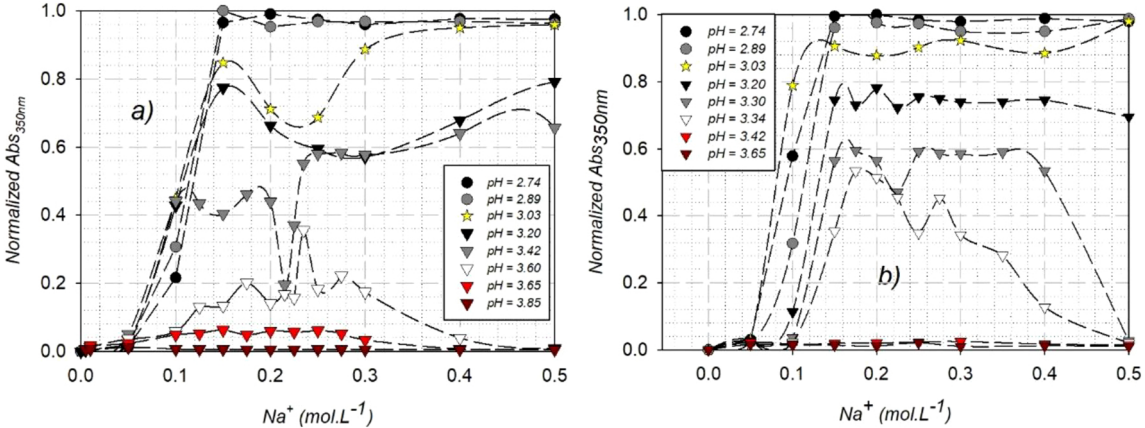
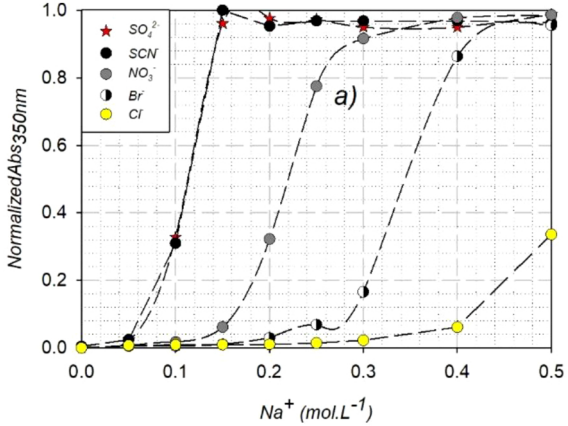

a)
b)
Normalized Abs350nm
Na+ (mol.L-1)
pH = 2.74
pH = 2.89
pH = 3.03
pH = 3.20
pH = 3.30
pH = 3.34
pH = 3.42
pH = 3.60
pH = 3.65
pH = 3.85

Figure 1


a)
SO_4^{2-}
SCN^-
NO_3^-
Br^-
Cl^-
$NormalizedAbs_{350nm}$
$Na^+ (mol.L^{-1})$
0.0
0.2
0.4
0.6
0.8
1.0
0.1
0.3
0.5

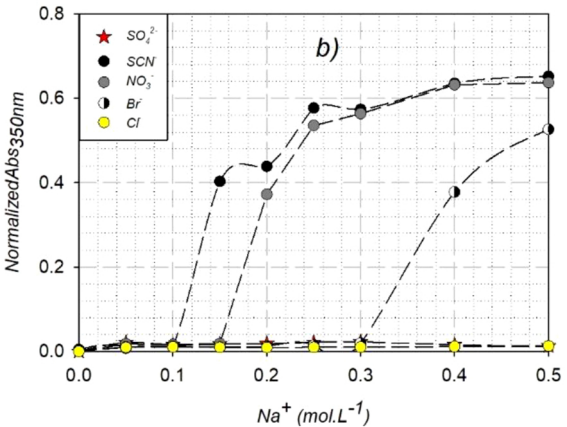

b)
SO_4^{2-}
SCN^-
NO_3^-
Br^-
Cl^-
$NormalizedAbs_{350nm}$
$Na^+ (mol.L^{-1})$
0.0
0.2
0.4
0.6
0.8
0.1
0.3
0.5

Figure 2

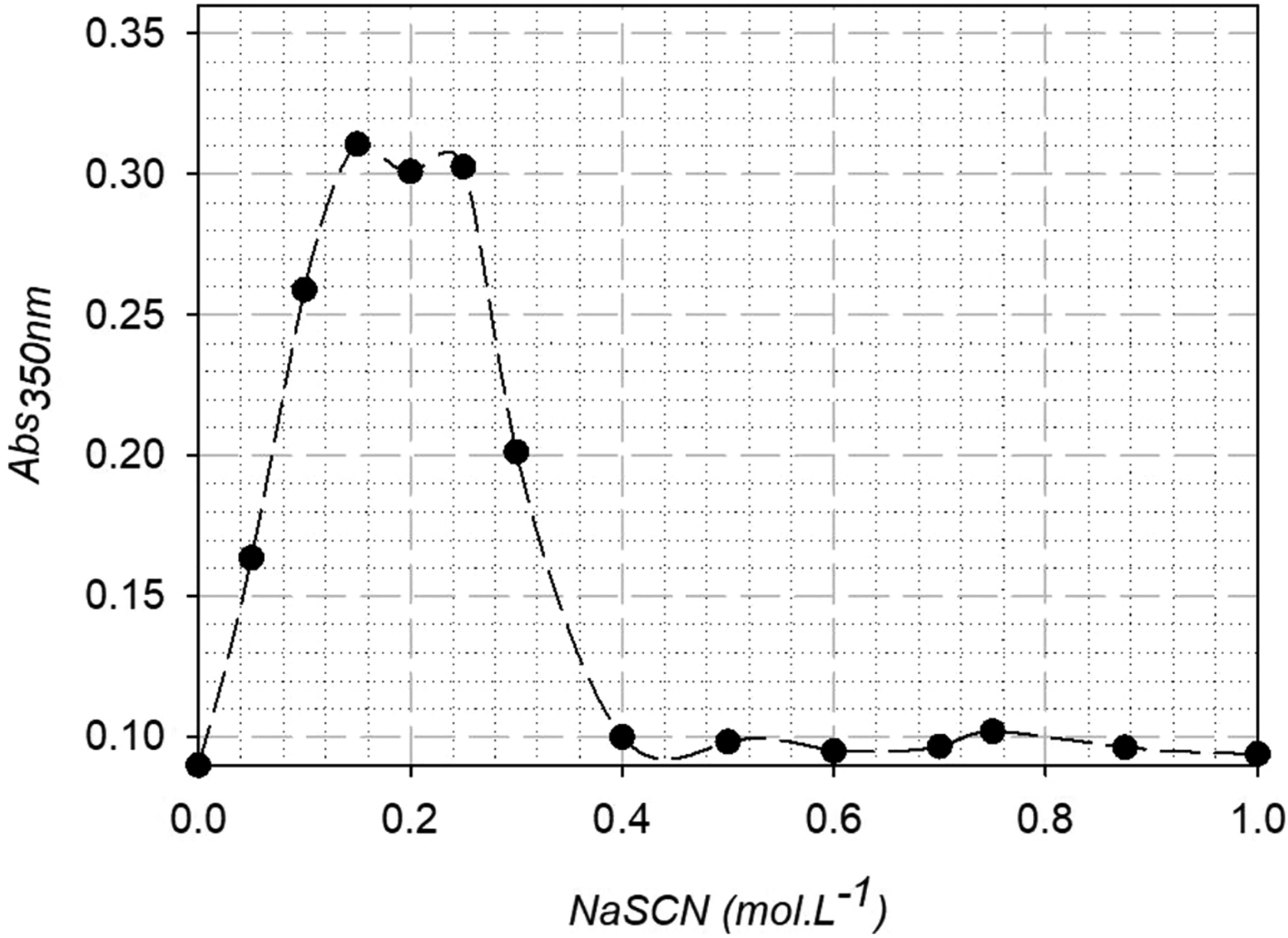


Figure 3

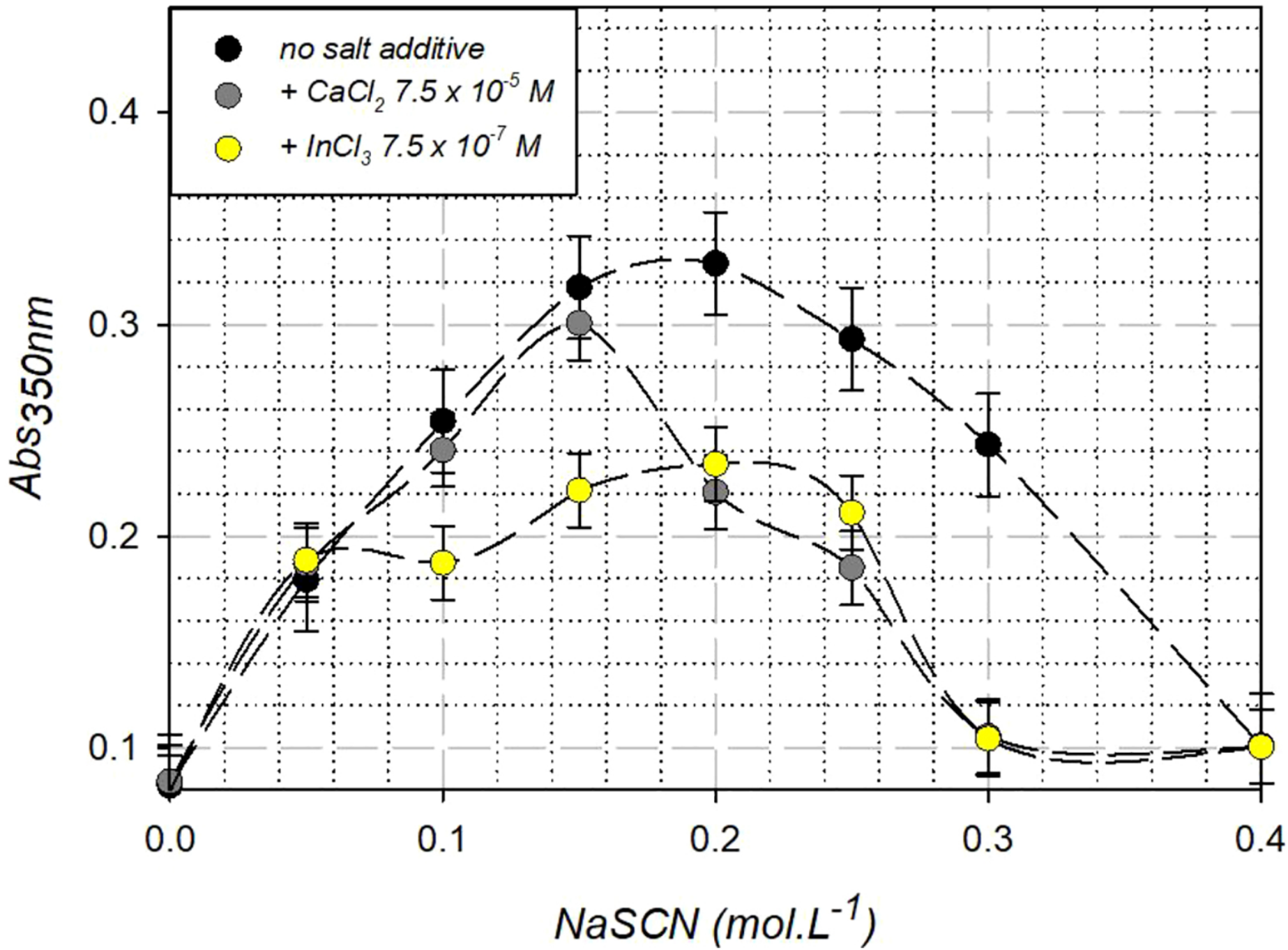

no salt additive
+ CaCl2 7.5 x 10-5 M
+ InCl3 7.5 x 10-7 M
Abs350nm
0.1
0.2
0.3
0.4
0.0
0.1
0.2
0.3
0.4
NaSCN (mol.L-1)

Figure 4

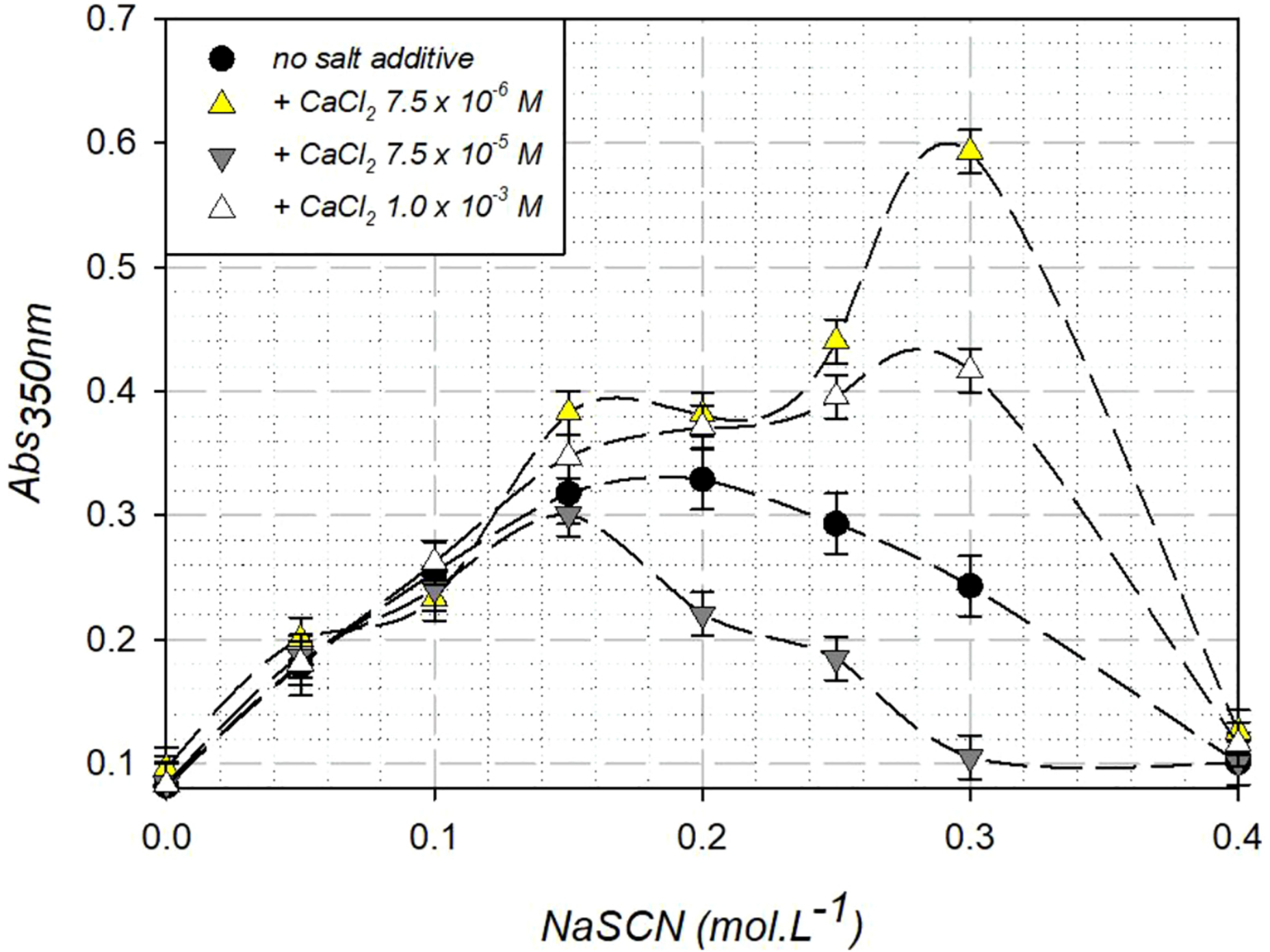
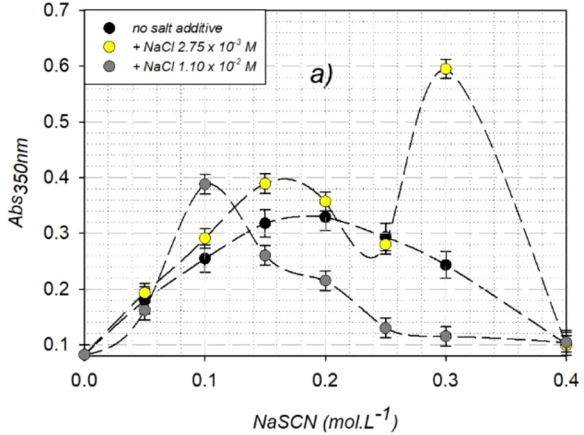
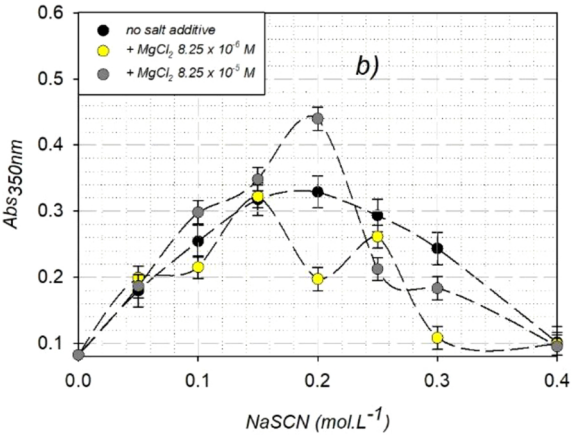
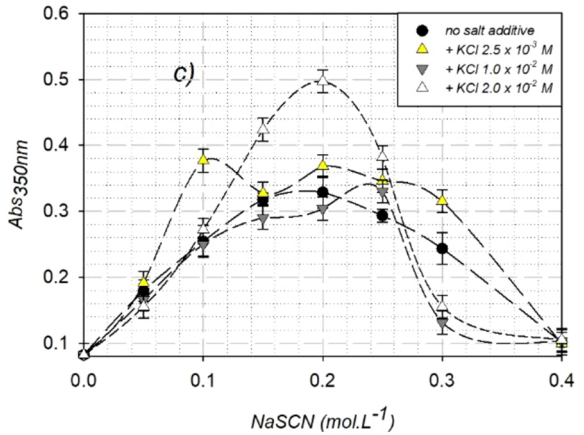

no salt additive
+ CaCl2 7.5 x 10-6 M
+ CaCl2 7.5 x 10-5 M
+ CaCl2 1.0 x 10-3 M
Abs350nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.1
0.2
0.3
0.4
NaSCN (mol.L-1)

Figure 5


no salt additive
+ NaCl 2.75 x 10-3 M
+ NaCl 1.10 x 10-2 M
a)
Abs350nm
NaSCN (mol.L-1)


no salt additive
+ MgCl2 8.25 x 10-6 M
+ MgCl2 8.25 x 10-5 M
b)
Abs350nm
NaSCN (mol.L-1)


no salt additive
+ KCl 2.5 x 10-3 M
+ KCl 1.0 x 10-2 M
+ KCl 2.0 x 10-2 M
c)
Abs350nm
NaSCN (mol.L-1)

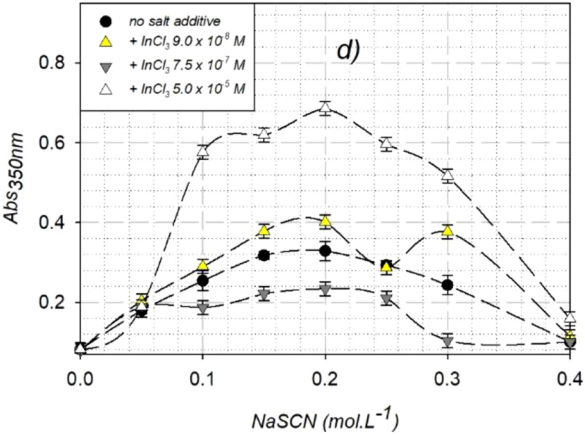

no salt additive
+ InCl3 9.0 x 10-8 M
+ InCl3 7.5 x 10-7 M
+ InCl3 5.0 x 10-5 M
d)
Abs350nm
NaSCN (mol.L-1)

Figure 6