Original Research Article

TITLE: Activity coefficient of solution components and salts as special osmolyte from Kirkwood-Buff theoretical perspective.

ABSTRACT

Background: There has been different interpretation of kosmotropes and chaotropes according to effect on aqueous solvent. The physicochemical characteristic of macromolecule is not always taken into account. Linking Hofmeister phenomenon with solution structure has not been a serious issue. Thus the objectives of this research are:1) To investigate issues concerning different ways of determining activity coefficient and activity of ionic osmolyte 2), to present a common theoretical basis for the interaction between reaction mixture components and Hofmeister phenomenon and 3) determine the preferential interaction parameters and the Kirkwood-Buff integrals.

Methods: A major theoretical research and partly experimental.

Results and Discussion: Some equations in literature gave different values of activity coefficient and activity of solution components. The preferential interaction by binding is positive with ethanol only and at its higher concentration in the presence of ideal solution of different concentration of calcium chloride. There was positive *m*-value with ethanol. It was negative *m*-value in the presence of preferentially binding species, calcium ion and ethanol as against the excluded chloride ion. There was negative and positive change of solvation preference and interaction parameter due respectively to ethanol only and a mixture of it and the salt.

Conclusion: Selected equations in literature may not give the same values of activity coefficient and activity of solution components. The presence of stabilising osmolyte, salt, and ethanol may not always yield positive *m*-values. The sign of the change of solvation preference with either binary or ternary mixture of osmolytes and, the cognate interaction parameter, may be a better indicator of the stability of a macromolecule. The kosmotropes and chaotropes may be cationic or anionic and their deficit or otherwise around the macromolecule and consequence, depend largely on net charge on the macromolecule at a given pH.

Keywords: *Porcine pancreatic alpha amylase; activity coefficient; preferential interaction parameter; change of solvation preference; m – value; ethanol; calcium chloride.*

1.0 INTRODUCTION

The term osmolytes have now become a general term used to specify any dissolved solute or cosolvent that can influence the stability and function of proteins and macromolecule in general. A well known mammalian xenobiotic osmolyte is ethanol whose effect on enzyme has been studied [1-2]. The interaction, binding mainly, and exclusion are of interest. There are two types of osmolytes which are mainly organic and inorganic in nature. There is also a current shift towards the study of inorganic cations and anions due to the known effects of the ions at low and high concentrations. The issue is the salting-in and salting-out effect of the salt at suitable concentration [3] which is usually high. These phenomena are encountered whenever separation or purification of macromolecules, proteins in particular, is of interest. However, the main concern in this research is the effect of the osmolyte at relatively low concentrations backed with theoretical background for interpretational purpose. Scholars have resulted to an age-long concept known as Hofmeister series [3]. Some scholars seem to question this approach, preferring what they consider as specific ion effect [4]. There is no as much interest in the fundamental theoretical background that can elucidate the effect of ethanol alone, and a mixture of it and calcium chloride. Since salt interact with macromolecule then the issue of relative deficit or enrichment around the macromolecule is where Kirkwood-Buff theory of solution structure becomes relevant. Interpretation based on Kirkwood-Buff theory and cognate interaction potentials have become imperative in this research. According to Harries and Rösgen (2008) [5], the so-called “structure making” (strongly hydrated ions or “kosmotropes”) are excluded from the surface of proteins at least at low concentration of the salt leading to stability or folding whereas the “structure breaking” (weakly hydrated ions or “chaotropes”) which preferentially bind to the protein should lead to dissolution of protein particularly at high salt concentration. This implies that chaotropes unlike kosmotropes may promote better interaction of the protein with the aqueous solvent otherwise the unfolded enzyme cannot perform its catalytic function. In this regard are “species to the left of Cl^- below, which are referred to as kosmotropes, while those to its right are called chaotropes as follows: $(CO_3^{2-}, SO_4^{2-}, S_2O_3^{2-}, H_2PO_4^-, F^-, Cl^-, Br^-, NO_3^-, I^-, ClO_4^-, SCN^-)$[3]. These terms originally referred to an ion’s ability to alter the hydrogen bonding network of water [3]. The kosmotropes, which were known as ‘water structure makers’, are strongly hydrated; they have stabilising and salting-out

effects on proteins and macromolecules" [5]. The implication is that the proteins or enzymes can be precipitated out of solution thereby losing catalytic function. This may not be impossible going by the claim in literature [6] that the less hydrated macromolecular species is the folded protein, for instance.

Calcium ion is a constituent of bone and teeth, and a cofactor of some protein such as pancreatic and salivary alpha amylase [7]. Apart from its known stabilising effect on alpha amylases [8], it also has the same effect on lipase BK-AB 18 [9], while its chloride counterpart activates alpha amylase [10]. Although interactions between different proteins may have been described in literature [11], interaction can also occur between the same macromolecule, between proteins and polymer substrate (*e.g.* polysaccharide), between polysaccharides leading to what have been referred to as solvation and self solvation as the case may be [12]. Interaction may be repulsive. The presence of osmolytes, salts as special inorganic osmolyte in this research, can alter the extent and strength of the different interaction but under the influence of pH status that determines charge distribution and net charge on a protein. Unlike organic osmolyte, salt presents two aspects, cation and ion, one of which is either preferentially excluded or bound while the other is affected differently as counterion. Thus this research is inextricably a major theoretical research and partly experimental. The objectives of this research are: 1) To present theoretical issues concerning different ways of determining activity coefficient and activity of ionic osmolyte 2), to present a common theoretical basis for the interaction between reaction mixture components and Hofmeister phenomenon and 3) determine partly by experiment the preferential interaction parameters, the corresponding KB integrals (KBIs), and relate same to the functional effectiveness of the enzyme.

2.0 Theory

2.1 Meaning of water activity

Water activity (a_w) is a very vital physical parameter that is useful for the interpretation of solution structure and cognate thermodynamic property in line with relevant theory. Cognate to water activity is also the activity coefficient not just for water alone but also for the solute. Activity and water content are not identical. The former describes the condition or relative availability of water for any number of actions and reactions in a material and may bear little or no relationship to the total amount of water present in a system. When water content and a_w are related, a useful construction, the sorption isotherm, is obtained which indicates the nature of the water binding that might be present

[13]. These immediate preceding statements are important because they show the importance of water in biochemical reaction catalysed by enzymes within and outside cellular environment.

2.2 The relevance of the Debye-Hückel inverse square length in the determination of activity coefficient

Although there are experimental methods for the measurement of activity coefficients, integrated volume method [14], measurement of electromotive force [15-16] *etc*, there are theoretical methods that are subject matter of this research. There may be methods for the determination of activity coefficient, but the method proposed by Lund [11] needs objective analysis. In Debye-Hückel (DH) equation of inverse square length (κ^2), given below, $e/\sqrt[2]{\varepsilon_0\varepsilon_r k_B T}$ at 37^oC, is $\cong 9.554\exp(-5)$. The ionic strength, I_m given as $\frac{\sum C_i Z_i^2}{2}$ where C_i and Z_i are the molal concentration and valence of the ion, is hardly $\geqq x.\exp(3)\,\mathrm{mol/kg}$ where x>1 but < 10. Therefore, κ may be « $31.6227766\times 9.554\exp(-5).\sqrt[2]{x}$. The inverse square length is given as

$$\kappa^2 = \frac{e^2}{\varepsilon_0\varepsilon_r k_B T}\sum C_i Z_i^2 \quad (1)$$

Where $\varepsilon_{0,}\varepsilon_r, k_B$, and T are the permittivity of free space, relative permittivity, Boltzmann constant and thermodynamic temperature respectively. Here it is not clear why ½ is omitted from Lund's presentation [11] unlike $I_e = \frac{1}{2}\sum C_i Z_i^2$ as observed in literature [17]. The equation for the determination of activity coefficient γ [11] is given as

$$k_B T \ln\gamma^{DH} = -\frac{Z^2 e^2 \kappa}{8\pi\varepsilon_0\varepsilon_r(1+\kappa d_{hc})} \quad (2)$$

Where γ^{DH} in Lund's notation [11] and κd_{hc}, are the Debye – Hückel activity coefficient and hard shell diameter of the ion respectively. The denominator, $(1+\kappa d_{hc})$ is for all practical purpose equal to one because κd_{hc} is < nanoscale magnitude. From the same equation $Z^2e^2/8\pi\varepsilon_0\varepsilon_r k_B T$ is ≈ 3.63 exp (-10) Z^2 (the unit is necessarily ignored). Hence the product of the latter and κ should be « 3.63 exp (-10). Consequently, $\gamma^{DH} \cong 1$ even if $\frac{1}{2}\sum C_i Z_i^2 \to \infty$. The implication is that wherever $\exp(\kappa r)$ appears, given any ambient condition and radii of chemical species, under mutual electrostatic perturbation for instance, the free energy may remain invariant regardless of the value of the ionic radius, r, in the general equation [11] such as

$$\frac{A(r)}{k_B T} = l_B Z_1 Z_2 \exp(-\kappa r)/r \quad (3)$$

Where, $A(r), l_B, Z_1$, and Z_2 are the free energy, Bjerrum length, valence of 1st ion and valence of 2nd ion respectively. This has to be the case if κr is $\equiv \sigma \exp(-b)$ where $\sigma > 1$ and b»1. Thus,

$$\exp(-\kappa r) = \left(\left(\frac{1}{e}\right)^{\sigma}\right)^{\exp(-b)} \quad (4)$$

Where, e is ≅ 2.718. The parameter, $\exp(\kappa r), \rightarrow 1$ as $b \rightarrow \infty$. The free energy of interaction otherwise referred to as potential energy of interaction, is outside the scope of this research but it cannot be ignored in the elucidation of the fundamental cause of preferential interaction.

2.3 Other equations for the determination of water activity or the activity coefficient

Other mathematical models in the paper by Miyawaki *et al* [18], presented here primarily for the purpose of quick and immediate reference for feature research are Hildebrand and Scott's equation (a freezing point depression dependent approach) and equation according to Miyawaki *et al* [18] for the determination of water activity (a_W). These are respectively

$$\mathrm{In}a_w = \frac{-\Delta H_f(T_f - T)}{RT_fT} + \frac{\Delta C_f}{R}\left(\frac{(T_f - T)}{T} - \mathrm{In}\left(\frac{T_f}{T}\right)\right) \quad (5)$$

Where T, T_f, ΔH_f, and ΔC_f are the freezing point of solution, the freezing point of water, the latent heat of water, and the change of the specific heat of water, while R is the gas constant respectively.

$$a_W = (1 - \chi_S)\exp(\propto \chi_S^2 + \beta\chi_S^3) \quad (6a)$$

Where $\propto$, β, and χ_S are yet to be clearly defined parameters but, whose values are known for some compounds, and molar fraction of solute respectively. Equation (5) is dependent on predetermined experimental data, the freezing point of solution given known values of other parameters in literature. It seems it may be broadly applicable to any solution of whatever concentration, either infinitely dilute, dilute, concentrated or highly concentrated. However, Eq. (6a) is strictly for non-ideal solution [18] and may be applicable to both inorganic and organic aqueous solutions. If $\beta = 0$, the following may hold [18].

$$a_W = (1 - \chi_S)\exp(\propto \chi_S^2) \quad (6b)$$

The activity coefficients (γ_W) corresponding to Eq. (6a) and Eq. (6b) are given respectively by

$$\gamma_W = a_W/(1 - \chi_s) = \exp(\propto \chi_S^2 + \beta\chi_S^3) \quad (6c)$$

$$\gamma_W = \exp(\propto \chi_S^2) \quad (7)$$

But with ideal solution [18] as may be applicable to calcium chloride in this research, the equation may be

$$a_W = \chi_W = 1 - \chi_S \quad (8)$$

Another equation proposed by Troller [13] which seems not to indicate whether it is generalisable to both dilute and concentrated solution is given as

$$a_W = n_2/(n_1 + n_2) = \frac{p}{P_0} \quad (9)$$

Where n_1,n_2, P_0, and p are the number of moles of solute, solvent, partial pressure of pure water, and solution respectively. Equation (9) defines water activity in terms of solute concentration through its relation to Raoult's law [13]. There is nothing in literature to show that the equation is applicable to both dilute and concentrated solution.

2.4 Linking solute activity with solvent (water) activity.

In the paper by Timasheff [19] is the equation given as

$$\mathrm{In}a_1 = -C_3\phi_3/55.56 \quad (10a)$$

Where a_1, ϕ_3, and C_3 are the water activity, osmotic coefficient, and concentration of the solute respectively. The osmotic coefficient defined as the ratio between observed and theoretical osmotic pressure or the corresponding freezing point depressions [20], is therefore, given as

$$\phi_3 = -55.56\mathrm{In}a_1/C_3 \quad (10b)$$

Where it is immaterial whether or not the parameters, 55.56 and C_3 are either molal or molar concentration because they appear as ratio. As may be found in some standard text book [17],

$$\mathrm{In}\gamma_3 = (\phi_3 - 1) + (\phi_3 - 1)\int_0^{C_3} \frac{dC_3}{C_3} \quad (11a)$$

$$= (\phi_3 - 1)(1 + \mathrm{In}C_3) \quad (11b)$$

Recall that $a_3/C_3 = \gamma_3$ and substitute same and Eq. (10b) into Eq. (11b) to give

$$\mathrm{In}a_3 = \left(\frac{-55.56\mathrm{In}a_1}{C_3} - 1\right)(1 + \mathrm{In}C_3) + \mathrm{In}C_3 \quad (11c)$$

Where a_3, is the activity coefficient of the cosolute. Simplification and rearrangement gives

$$\mathrm{In}a_3 = \frac{-55.56\mathrm{In}a_1}{C_3}(1 + \mathrm{In}C_3) - 1 \quad (12a)$$

Rearrangement makes a_1 subject of the formula as follows.

$$a_1 = \exp - \left(\frac{(\mathrm{In}a_3+1)C_3}{55.56(\mathrm{In}C_3+1)}\right) \quad (12b)$$

2.5 Common ground for preferential interaction parameter and Hofmeister phenomenon

In the consideration of the link (or rather correlation) between solution structure defined according to KB theory and Hofmeister concept there is need to realise that interaction between

solute and macromolecule can either be repulsive (exclusion) or attractive (binding). This is contingent upon the physicochemical status of the macromolecule-electrostatic and hydrophobic characteristic occasioned by the type of amino acid residues both at the side chain and backbone. The potential energy and kinetic energy of interaction are applicable to stabilisation, destabilisation, salting-out, and salting-in process. The equations connected to this are to be considered elsewhere in the text. This constitutes the energetic aspect of the common ground for all forms of preferential interaction and Hofmeister phenomenon. Furthermore, Hofmeister phenomenon occurs at very high salt concentration for either salting-in or salting-out. The questions that are penitent are, is salting-in due to exclusion or binding; does salting-out occur due to exclusion or binding? While the experimental research does not cover salting-in or salting-out, there is a need to take the issue into cognisance as the effect of low concentration of calcium salt is investigated in this research. Incidentally there are conflicting views about what chaotropes and kosmotropes are.

According to Heitz et al [21] kosmotropes are small and highly charged ions which form stronger ion-water interactions than water-water hydrogen bonding interactions. This lowers the solution entropy. On the other hand chaotropes are large ions with a low charge density and weak hydration characteristics. For these ions there is a net increase in solution entropy because of weaker ion-water interactions [21]. According to Harries and Rösgen [5], the so-called “structure making” (strongly hydrated ions or “kosmotropes”) are excluded from the surface of proteins leading to aggregation and precipitation. But this should be at high salt concentration. This may not be the case at low salt concentration. The corollary is that the “structure breaking” (weakly hydrated ions or “chaotropes”) which preferentially bind to the protein should lead to dissolution of protein particularly at high salt concentration. The view of Chaplin (www1.lsbu.ac.uk) is that the terms 'kosmotrope' (order-maker) and 'chaotrope' (disorder-maker) originally denoted solutes that stabilized, or destabilized respectively, proteins and membranes; thus chaotropes unfold proteins, destabilize hydrophobic aggregates and increase the solubility of hydrophobes whereas kosmotropes stabilize proteins and hydrophobic aggregates in solution and reduce the solubility of hydrophobes.

In the light of the foregoing, there is a need to take appropriate position. Against the backdrop of Heitz *et al* position [21], there should be chaotropic cations, chaotropic anions, kosmotropic cations, and kosmotropic anions. All kosmotropes may be seen to possess higher charge density than the chaotropes. All multivalent cations and anions qualify as kosmotropes while all monovalent ions

qualify as chaotropes. Therefore, in terms of effect of ions on the aqueous solvent, in this research, calcium ion and chloride ion are respectively kosmotrope and chaotrope [21]. It seems the physicochemical state of the macromolecule (*e.g.* net charge, negative or positive) determines preferential interaction, either by binding or by exclusion of the two types of solute, the kosmotrope and chaotrope. For instance in an alkaline medium, a buffered solution, pH, 7.4, all acidic amino acid residues are ionised yielding carboxylic ions. Calcium ions should therefore, bind to such group, though it may be a kosmotrope. The chloride ion is rather excluded. The converse could have been the case in an acidic medium. At low salt concentration, the effect of ethanol may not be completely terminated as this research has shown. It is very likely that at higher concentration of the salt (but low concentration), total refolding may be achieved. If preferential exclusion is the only means of stabilising a protein, then only the chloride ion, the chaotrope, may account for the process. The order of effectiveness of activation found for some halide is $Cl^- > Br^- > I^- > F^-$ at a pH equal to 7. But at much higher concentration (not investigated in this research) there may be inhibition of biological function of the enzyme. For instance, at concentration higher than 0.005 mol/L calcium ion inhibited the function of human pancreatic alpha-amylase (alpha-1, 4-glucan 4-glucano-hydrolase, EC 3.2.1.1). This is where the effect of salting-out and salting-in becomes relevant. If salting-out is by exclusion, leaving higher water chemical potential around the protein, then there should be aqueous solvent concentration gradient; this may trigger diffusion of water towards the bulk, a translational gain in entropy [22] leaving the protein dryer as to promote aggregation or precipitation. If salting-in is by preferential binding, it is expected that the radial distribution function should be in favour of higher concentration of the ion around surface domain. Binding of cation on the surface of the protein and in particular movement of cations towards the protein may ultimately attract anions. If destabilisation or unfolding occurs, the unfolded state becomes more hydrated [12]. Coupled with aqueous solvent concentration gradient promoting diffusion of water from the bulk to the protein surface domain, there should be solubilisation or salting-in phenomenon. In this case there is translational entropy gain [22] of the aqueous solvent in opposite direction.

Bringing this section to an end cannot be without earlier views such as the effect of surface tension increment of salts which promotes preferential interactions of the monovalent cations like sodium ions unlike divalent ions whose preferential interaction has no correlation with surface tension increment [23]**.** According to Arakawa & Timasheff [23], binding of divalent cations to the proteins

overcomes the salt exclusion due to the surface tension, leading to a decrease in the preferential hydration. It is not certain how this promotes salting-out (stability) or salting-in (instability). There is also the view that global changes in solvent structure enhancement or a breakdown of H-bond net work in water due to the presence of ions seems to be jettisoned in favour of the effects that the ions have on the local hydration of proteins. Whatever be the case, there should be attractive or repulsive interaction between the protein and the ions at given salt concentration as a basis for stabilisation at optimal concentration of salt being excluded and at a much higher salt concentration leading to salting-out by the same mechanism. But if destabilisation is the case, then the common basis is preferential binding with residual function at low salt concentration only. While total loss of function, may be due to salting-in, following exposure to very high salt concentration. Therefore, the connection or link between solution structure based on KB theory and Hofmeister concept is either electrostatic or hydrophobic or a combination of both that promote preferential interaction, which may be exclusion or binding.

2.6 Revisiting earlier theory

The main issue which stands in the previous paper is the fact that preferential interaction and the change in terms of binding or exclusion cannot be a measurable parameter and a slope (or a constant) at the same time [1]. Here there is need to reexamine the use of the equation in the paper by Shimizu [24]. The chemical potential in contention is as applicable to water. This according to Parsegian *et al*. [25] is given as $d\mu_w = -\nabla_w d\Pi$ where ∇_w is the molecular volume of water and $d\Pi$ is the incremental contribution to the osmotic pressure of the solution; however, Shimizu [24] and Timasheff [19] defined ∇_w as partial molar volume of species *i* and partial molar volume of water respectively.

Shimizu's position [24] implies that *i* can represent any chemical species, water, osmolyte (or cosolute), and protein in a ternary solution. This led to the incorrect sign of the calculated preferential interaction parameter, in terms of binding of ethanol to the protein. The conclusion that there was preferential exclusion need to be corrected even if there is support for it in literature which shows that the organic solvent, acetonitrile molecules, are preferentially excluded from the dried lysozyme, resulting in the preferential hydration [26]. This is more so, considering the fact that $\nabla_w d\Pi$ is a property of the aqueous solvent and the solution and it may not be equal to $d\mu_3$. Such does not exist in literature. A guiding principle is that water in any solution has activity < 0; its activity tends to 1

as $C_3 \rightarrow 0$, and its maximum value is 1. But the activity of the solute may be » 1 as $C_3 \rightarrow \infty$. However, there is no reason to give as to why ∇_w can be regarded as molar volume of water [25] and as partial molar volume considering the fact that the change in volume of a solution with every addition of a solute may be negative. On account of the preceding finding the equation in literature [1] is replaced with

$$\Delta\Gamma_{23} = \frac{\mathrm{In}K_{\mathrm{eq}(3)}}{\mathrm{In}a_3} \tag{13}$$

Where $K_{\mathrm{eq}(3)}$ and Γ_{23} are the equilibrium constant for whatever change and preferential interaction parameter for either binding or exclusion of the cosolute. Equation (13) can be used to calculate the values of the preferential interaction parameter of ethanol.

Also, arising from the different equations in literature [19] is the following derivable corollaries. Given that,

$$\Delta\Gamma_{21} = -\frac{RT}{\nabla_1}\frac{\mathrm{In}K_{\mathrm{eq}(1)}}{\Delta\Pi} = -\frac{m_1}{m_3}\Delta\Gamma_{23} \tag{14a}$$

Where Γ_{21}, m_1, m_3 and R are preferential interaction parameter for hydration, molal (or molar) concentration of water, cosolute, and gas constant respectively. The far-right end of Eq. (14a) is according to Timasheff [19]. It is on account of the suggestion that Γ_{21} and Γ_{23} are equivalents being linked in the equation $m_1\Gamma_{23} = -m_3\Gamma_{21}$. Such relation seems to arise from the perturbation of the chemical potential $\left(\partial\mu_3/\partial m_2\right)_{\mathrm{m_3}}$, which can be positive if the interaction between the cosolvent and the protein is unfavourable as applicable to stabilizers, or it can be negative if the interaction is favourable as applicable to destabilisers [19]. Thus the thermodynamic binding $(\partial m_3/\partial m_2)_{\mu_3} = \Gamma_{23}$, can be positive or negative; negative Γ_{23} means preferential exclusion of cosolvent leading to preferential hydration (positive Γ_{21}) as applicable to the effect of stabilisers [19]. On the other hand positive Γ_{23} which means preferential binding which leads to preferential dehydration or exclusion of water (negative Γ_{21}) is applicable to destabilisers. Since $\Gamma_{21} = \mathrm{In}K_{\mathrm{eq}}/\mathrm{In}a_1$, preferential hydration requires that $K_{\mathrm{eq}} < 1$ as long as a_1 is always < 1. Preferential exclusion of water requires that $K_{\mathrm{eq}} > 1$. This is similar to the analysis elsewhere [19]. The equilibrium for preferential hydration K_{eq}, is subsequently re-written as $K_{\mathrm{eq}(1)}$ in order to differentiate it from the equilibrium for preferential osmolation.

Nevertheless, it is necessary to redefine thermodynamic binding in terms of Kirkwood-Buff theory [27] about solution structure defined in terms of radial distribution functions $g_{2i}(r)$ between species 2 (biomolecule) and *i* (any chemical species referred to as cosolvent) in solution. The function, $g_{2i}(r)$ is a measure of the deviation from the random distribution of particles of type *i* from a central particle (the biomolecule), as a function of the distance (r) from the central particle, 2. The simplest interpretation is that when the ratio of the bulk concentration of *i* to its concentration around the surface domain of 2 is > 1, there is exclusion. On the other hand if the ratio is < 1, there is binding. In other words there may be no total absence of species, *i* around the protein surface domain.

Rearrangement of Eq. (14a) gives

$$\Delta\Gamma_{23} = \frac{RT}{\nabla_1}\frac{\ln K_{\mathrm{eq}(1)}}{\Delta\Pi}\frac{m_3}{m_1} = \frac{\ln K_{\mathrm{eq}(3)}}{\ln a_3} \tag{14b}$$

$$\ln a_3 = \frac{\nabla_1 \Delta\Pi m_1 \ln K_{\mathrm{eq}(3)}}{RT m_3 \ln K_{\mathrm{eq}(1)}} \tag{15a}$$

Equations (14b) and (15a) are premised on the fact that the same equilibrium constant may not be applicable to all solution components, the aqueous solvent (1), the macromolecule (2), and the cosolvent (3) when 2 is undergoing any change due to the presence of other solution components. This is to imply that equilibrium constant for preferential hydration and for preferential osmolation may be different. If the original equations are valid, it may be possible to calculate ∇_1 at different values of m_3 at a given temperature if $\Delta\Pi$ is known or theoretically determined using van't Hoff law if the concentration range is ideal. This is with reservation. Nonetheless, if the solution is ideal, then, $m_3 RT = \Delta\Pi$. Therefore, under ideal condition,

$$\ln a_3 = \nabla_1 m_1 \frac{\ln K_{\mathrm{eq}(3)}}{\ln K_{\mathrm{eq}(1)}} \tag{15b}$$

The implication of Eq. (15b) is that ∇_1 may be negative if a_3 is < 1 for an ideal case. But it is not certain experimental result may show similar sign, let alone the same magnitude. However, $\nabla_1 \Delta\Pi$ in Eq. (15a) can be replaced with $-RT\ln a_1$ such that

$$\ln a_3 = -\frac{\ln K_{\mathrm{eq}(3)}}{\ln K_{\mathrm{eq}(1)}}\frac{m_1}{m_3}\ln a_1 \tag{16a}$$

On the other hand, Eq. (15b) can be substituted into Eq. (16a) to give after rearrangement

$$\nabla_1 = -\frac{\ln a_1}{m_3} \tag{16b}$$

But the results from Eq. (16b) for a_1 may not be equal to the result from Eq. (12b). If so, the equivalence principle implied in the relation between Γ_{23} and Γ_{21} may not be compatible with Eq.

(12b). This remains speculative for now. Besides, Eq. (16a) presents a contradiction because if a_3 should be directly proportional to m_3, then on the contrary increasing values of m_3 with decreasing values of a_1 may result in decreasing a_3. This is what it seems to be. However, in order to achieve total comprehension of Timasheff's equivalence principle, preferential interaction by osmolation is restated based on the rearrangement of Eq. (16a) as follows:

$$\frac{m_3}{m_1}\frac{\mathrm{In}K_{\mathrm{eq}(1)}}{\mathrm{In}a_1} = -\frac{\mathrm{In}K_{\mathrm{eq}(3)}}{\mathrm{In}a_3} = -\Delta\Gamma_{23} \tag{17a}$$

Taking 1st part of Eq. (17a) gives

$$\frac{\mathrm{In}K_{\mathrm{eq}(1)}m_3}{\mathrm{In}a_1 m_1} = -\frac{\mathrm{In}K_{\mathrm{eq}(3)}}{\mathrm{In}a_3} \tag{17b}$$

The position of negative sign is changed to give

$$-\frac{\mathrm{In}K_{\mathrm{eq}(1)}m_3}{\mathrm{In}a_1 m_1} = \frac{\mathrm{In}K_{\mathrm{eq}(3)}}{\mathrm{In}a_3} \tag{17c}$$

Negative $\mathrm{In}K_{\mathrm{eq}(3)}/\mathrm{In}a_3$ demands that, on the left hand side (LHS), $\mathrm{In}K_{\mathrm{eq}(1)}$< 1 and a_1<1; a_3>1 and $K_{\mathrm{eq}(3)} < 1$. Positive $\mathrm{In}K_{\mathrm{eq}(3)}/\mathrm{In}a_3$ demands that, on the LHS, $\mathrm{In}K_{\mathrm{eq}(1)} > 1$ and $a_1 < 1$; a_3>1 and $K_{\mathrm{eq}(3)}$ > 1 or $a_3 < 1$ and $K_{\mathrm{eq}(3)} < 1$. Meanwhile, suggestion has been made earlier in this research regarding the different equilibria, (de) hydration equilibrium and (de)osmolation equilibrium; taking the right hand side of E. (17c) as $\Delta\Gamma_{23}$,

$$K_{\mathrm{eq}(1)} = \exp\left(-\frac{\mathrm{In}a_1 m_1}{m_3}\Delta\Gamma_{23}\right) \tag{18}$$

Equation (17c) where $\mathrm{In}a_1$ is $\equiv -\nabla_1\Delta\Pi/RT$can be restated as

$$-\frac{RT\mathrm{In}K_{\mathrm{eq}(1)}m_3}{\nabla_1\Delta\Pi.m_1} = -\Delta\Gamma_{23} \tag{19}$$

But for an ideal solution of either osmolyte or salt solution, $\Delta\Pi = RTm_3$. Therefore, Eq. (19) can be rewritten as

$$-\frac{\mathrm{In}K_{\mathrm{eq}(1)}}{\nabla_1 m_1} = -\Delta\Gamma_{23} \tag{20}$$

Meanwhile the additives in this research are ethanol and calcium chloride. The pH determines the state of protonation or deprotonation. In this research the pH is 7.4 such that porcine pancreatic alpha amylase deprotonates because it has been shown to contain carboxylic amino acids [8]. Therefore, while ethanol, a polar cosolvent, can bind hydrophobically, as well as by polar-polar and polar-charge interaction, the cations and anions, the calcium ion and chloride ion respectively, may undergo, attractive and repulsive interaction with the holoenzyme. Then the question is, is the binding interaction of calcium ion destabilising while exclusion of the chloride is stabilising? The answer is

reserved for the result and discussion section. However, in terms of the interaction potential energy, there may be dipole-dipole interaction energy which may occur between polar groups of the protein and ethanol, ion-dipole interaction between mineral ion and the polar group of the protein given respectively as [11].

$$A(r)/k_{\mathrm{B}}T = -\left(l_{\mathrm{B}}Z_{\mathrm{A}}\mu_{\mathrm{B}}\right)^2/3\boldsymbol{R}^6 \quad (21)$$

Where l_{B}, $A(r)$, and Z_{A}, are the Bjerrum length, free energy (or effective potential) and valence of chemical species A (this implies that Z_{B} is the valence of chemical species B); μ_{B} and $\boldsymbol{R}$ are the magnetic moment for chemical species B and intermolecular distance;

$$A(r)/k_{\mathrm{B}}T = -\left(l_{\mathrm{B}}Z_{\mathrm{A}}\mu_{\mathrm{B}}\right)^2/6\boldsymbol{R}^4 \quad (22)$$

There is also the ion-ion interaction energy referred to as kinetic energy of interaction between carboxylate groups of the protein and the mineral ions given as

$$A(r)/k_{\mathrm{B}}T = l_{\mathrm{B}}Z_{\mathrm{A}}Z_{\mathrm{B}}/2\boldsymbol{R} \quad (23)$$

In the light of this research, there is need to revisit the KBI for solvation preference and solvation difference. The issue raised in previous publication [1] is that it is not certain if the change in solvation preference of proteins upon denaturation, $\Delta_{\mathrm{N}}^{\mathrm{D}}(G_{21} - G_{23})$ (taken as **A**) as function of [C_{os}] (or C_3) is similar to the solvation difference, $\Delta_{\mathrm{N}}^{\mathrm{D}}(G_{21}) - \Delta_{\mathrm{N}}^{\mathrm{D}}(G_{23})$ (taken as **B**). To the mathematicians, the commutative law may (but not with certainty) be applicable to the elucidation of the issue as follows: Given hypothetical case whereby the 1st $G_{21} = 6$, and the 2nd $G_{21} = 8$; the 1st $G_{23} = 2$, and the 2nd $G_{23} = 5$. Then **A i**s calculated as (8–5) – (6–2) = 3-4= –1; **B** is calculated as (8–6) –(5–2)=2-3= –1. It would appear therefore, that **A** and **B** are similar or equivalents. Besides it seems **A** can be interpreted as the change of the difference between KBI for hydration and KBI for osmolyte solvation (osmolation) while **B** is the difference between change of the KBI for hydration and change of the KBI for osmolation. This remains inconclusive. According to Rösgen *et. al.* [12], whether or not a cosolute is stabilising (with respect to either the native or denatured state) depends on the protein's preference to have positive correlations (preferential binding) either with water or with osmolyte. This preference determines the sign of the solvation expression, hydration or osmolation, $G_{21} - G_{23}$ or, equivalently, the preferential interaction parameter. The change in this preference is therefore, given as above. The parameter $G_{21} - G_{23}$ is also regarded as the difference between protein solvation by water and osmolyte and multiplication by [C_{os}] gives the preferential interaction parameter. Besides, **B** is said to determine whether the osmolyte is stabilising or destabilising [12]; this seems to point to the *m*-value

whose sign either positive or negative specifies respectively the effect of stabilising or destabilising osmolyte. Against this background, one can without definite motivation adopt one of the derived equations in literature [1].

$$\frac{m}{RT} = \frac{-\Delta_N^D \Gamma_{23}}{C_3 \exp\left(\ln C_3 - \frac{\mu_3 - \mu_3^0}{RT}\right)} \quad (23)$$

Where, $\Delta_N^D \Gamma_{23} = -C_3 \Delta_N^D (G_{21} - G_{23})$ and μ_3 and μ_3^0 are respectively, the chemical potential of the cosolute and the standard chemical potential. With the correct use of mathematical formalism, the *m*-values for ethanol and calcium salt can be determined and consequently $\Delta_N^D (G_{21} - G_{23})$ and $-\Delta_N^D \Gamma_{23}$ can also be determined.

The equivalent equation for $\Delta_N^D \Gamma_{21}$, can be derived based on Timasheff's [19] proposition as follows. In line with Timasheff's [19] notation

$$-\Delta_N^D \Gamma_{23} \frac{m_1}{m_3} = \Delta_N^D \Gamma_{21} \quad (24a)$$

Here, m_1, and m_3 are respectively concentrations of water and cosolute corresponding respectively to C_1 and C_3 in this research.

Rearrangement gives

$$\frac{-\Delta_N^D \Gamma_{23}}{m_3} = \frac{\Delta_N^D \Gamma_{21}}{m_1} \quad (24b)$$

Substituting the right hand side of Eq. (24b) into Eq. (23) gives

$$\frac{m}{RT} = \frac{\Delta_N^D \Gamma_{21}}{m_1 \exp\left(\ln C_3 - \frac{\mu_3 - \mu_3^0}{RT}\right)} \quad (25)$$

It is important to realise too, that

$$\frac{-\Delta_N^D \Gamma_{23}}{m_3} = \frac{\Delta_N^D \Gamma_{21}}{m_1} = \Delta_N^D (G_{21} - G_{23}) \quad (26)$$

3.0 MATERIALS AND METHODS

3.1 Materials:

The chemicals used were: Soluble potato starch from Sigma Chemicals Co, USA; ethanol, hydrochloric acid, and sodium chloride from BDH Chemical Ltd, Poole England; 3, 5-dinitrosalicyclic acid (DNA) from Lab Tech Chemicals India; Tris from Kiran Light Laboratories and BSA from Sigma USA; porcine pancreatic alpha amylase (PPA) (EC 3.2.1.1) from Sigma, Aldrich, US. All other chemicals were of analytical grade and solutions were made in distilled water.

3.2 Equipment:

pH meter (tester) from Hanna Instruments, Mauritius; electronic weighing machine from Wensar Weighing Scale Ltd, Chennai; Centrifuge, 300D model from China; 721/722 visible spectrophotometer from Spectrum Instruments Co Ltd, China.

3.3 Methods

The equilibrium constant (K_{eq}) for the process folded (F)→unfolded (U) is adapted from Pace equation [28] and modified Baskakov and Bolen equation [29] and are given as

$$K_{\mathrm{eq}} = \frac{U}{1-U} \tag{27}$$

Where U is given as

$$U = \frac{V_{\mathrm{N}} - V_{\mathrm{OBS}}}{V_{\mathrm{N}} - V_{\mathrm{D}}} \tag{28}$$

Where, $V_{\mathrm{N}}, V_{\mathrm{OBS}}$, and V_{D} are velocities of amylolysis by the native enzyme, the observed velocity for the treated enzyme, and the velocity of unfolded enzyme. However, V_{D} was obtained by extrapolation, the value of velocity of amylolysis as [Ethanol] →0. The activity coefficient is calculated using Eq. (6b) and Eq. (8) [18]. The activity is calculated using Eq. (12a) and equilibrium constant for the interaction of aqueous solvent is according Eq. (18).

The independent variables were various concentrations of osmolyte, ethanol, a human xenobiotic cosolvent, thermodynamic temperature (310.15 K), and *p*H (7.4). The control reaction mixtures were without xenobiotic osmolyte-ethanol- and calcium chloride. Assay of alpha-amylase for the determination of the effect of ethanol and a mixture of it and the salt was according to Bernfeld (dinitrosalicylic acid) method [30]. A mixture of water and raw potato starch was the substrate. 0.01 g of PPA was dissolved in 20ml of distilled water to give 500 µg/mL while potato starch solution was prepared by mixing 1g in tris-HCl(aq) buffer (90 mL), 5 mL 6% (W/W) NaCl(aq) and 5 mL distilled water to give 1 g/100 mL. The enzyme, PPA (1 mL), was mixed with different concentration of aqueous solution of ethanol (0.5 mL) plus 0.5 mL of water and assayed for 5 minutes in a reaction mixture containing 1 mL of the substrate without any separate incubation of the enzyme in ethanol before assay. Then, without any separate incubation, assay was carried out for 5 minutes in a reaction mixture containing 0.5 mL ethanol, 0.5 mL calcium chloride, 1 mL substrate, and 1 ml enzyme giving in all cases, test and control, a total reaction mixture volume equal to 3 mL. Spectrophotometric readings were taken at 540 nm with extinction coefficient equal to 181.1 /M/cm.

3.4 Statistical analysis

The velocities of amylolysis were determined in triplicates. The mean values were used to determine the first-principle equilibrium constant (Eq. (27) and Eq. (28)). Microsoft Excel (2007) was used to plot the dependent variable versus independent variable.

4.0 RESULTS AND DISCUSSION

4.1 Preferential interaction of osmolyte with enzyme in a binary mixture of water and ethanol

The first additive investigated in the past [1, 31] is ethanol whose effect was investigated and analysed in terms of solution structure, the KBI, the preferential interaction parameter (Γ_{23}) and the *m*-values. The unfortunate mistake that did not affect the conclusion in the previous paper notwithstanding, there has been suggestion in the same published paper that, Γ_{23} or $\Delta\Gamma_{23}$, for the change, cannot be a measureable parameter and a constant quantity implied in the slope from linear regression ($\mathrm{In}K_{\mathrm{eq(1)}}$versus $\mathrm{In}a_{\mathrm{i}}$) under a given condition at the same time [1]. In the research, theoretical approach was used to calculate the partial molar volume of the cosolvent, ethanol. However, the method by Stothart [32] seems to overestimate the value of partial molar volume given as $\phi_3 M_3(\mathrm{or}\ \nabla_{\mathrm{i}})$, where ϕ_3 and M_3, are the partial specific volume and molar mass of cosolvent, ethanol, respectively. In this research $\Delta\Gamma_{23}$ is calculated using $(\mathrm{In}K_{\mathrm{eq(i)}}/\ \mathrm{In}a_{\mathrm{i}})$ instead of $(-RT\mathrm{In}K_{\mathrm{eq(i)}}/\ \mathrm{In}\nabla_{\mathrm{i}}\Delta\Pi)$ as in previous research [1, 31]. The result in this research (Table 1) shows that the preferential interaction of ethanol with the enzyme was positive as should be expected where $K_{\mathrm{eq(i)}} > 1$ and $a_{\mathrm{i}} > 1$, characteristics of the effect of ethanol. This is not withstanding the view that at low water content, the ethanol molecules are preferentially excluded from the enzyme surface that results in preferential hydration [2].

Table 1. Preferential interaction parameter of water and ethanol with the enzyme.

[Ethanol] (mol/L)	1.247	3.227733	5.27867
$\Delta\Gamma_{23}$	6.874	0.404	0.049
$\Delta\Gamma_{21}$	– 306.264	– 6.955	– 0.514

The parameters $\Delta\Gamma_{23}$ and $\Delta\Gamma_{21}$ are the preferential interaction parameters for osmolation and hydration respectively.

The positive value of $\Delta\Gamma_{23}$ means as expected, that ethanol interacted by binding to the protein; relative amount of ethanol on protein surface domain is > than in the bulk. This is the usual view of earlier investigators [19, 24]. There is a concomitant negative preferential hydration, dehydration or departure of water from the protein surface domain in line with result in literature [19]. What seems to be a paradox is that preferential solvation – the binding of ethanol – and expulsion of

438 water are decreasing in magnitude with increasing concentration of ethanol. Estimation of a_i seem to
439 confirm the equation by Miyawaki et al [18] as a valid means of estimating the activity coefficient of
440 non-ideal solution of a cosolvent such as ethanol whose concentration range adopted was > 1 mol/L.
441 To be more technical activity of ethanol instead of concentration may be more useful in elucidating the
442 observed paradox.

443 Although water is often regarded as a universal solvent but it is a commonplace observation
444 that water is not miscible with gasoline unlike ethanol. It should not be surprising that increasing a_3 of
445 ethanol may have enhanced the solubility of the bulky and characteristically hydrophobic water
446 insoluble potato starch whose hydrophilicity due to pockets of hydroxyl groups may not totally cancel
447 the effect of hydrophobes. Thus, while destabilising the protein, ethanol may have promoted the
448 partial solubilisation of the insoluble starch. As reported for chymotrypsin, at low water content, the
449 ethanol molecules may seem to have undergone partial preferential exclusion from the enzyme
450 surface giving rise to residual activity as previously reported for PPA [1]. It is therefore, imperative that
451 both substrate and the enzyme are considered in considering the effect of salt and osmolyte on any
452 reaction system.

453 **4.2 Preferential interaction of inorganic ion with enzyme in ternary mixture of water,**
454 **ethanol and calcium chloride.**

455 When the pH is > 7, protein containing acidic amino acid residues as side chain residues or
456 anywhere, may possess net negative charge due to deprotonation. This does not stop ethanol from
457 effecting a conformational change in the proteins' three dimensional structure, if not total unfolding.
458 Both calcium ion and ethanol may compete for available loci on the enzyme's surface domain. But the
459 chloride ion may be repelled for obvious reason. Therefore, for ethanol-calcium chloride system, there
460 is a tripartite preferential interaction regime comprising preferential solvation (or osmolation) by
461 binding relevant to both ethanol and calcium ion and exclusion by repulsion relevant to chloride ion.
462 Thus as Table 2a shows, there are different signs of preferential solvation or osmolation. The
463 positive $\Delta\Gamma_{23}$ at the lower concentration of ethanol and $CaCl_2$ may be as a result of the > effect of
464 preferential binding than exclusion of anion by repulsion unlike the situation at higher concentration of
465 the salt.

466 At higher concentration of ethanol, the $\Delta\Gamma_{23}$ values are positive even with increasing
467 concentration of the salt. This scenario seems to suggest that the exclusion of the chloride component

is unable to overcome the unfolding effect of ethanol and the effect due to binding of calcium ions. There is need to state that all animal-type alpha-amylases isolated so far display the unusual property to bind a chloride ion at a specific site that induces allosteric activation of the full amylolytic activity [10]. It has been shown that the chloride ion is responsible for the pKa shift of catalytic residues via interactions with active site carboxyl groups [10]. But it must be made clear that chloride cannot bind point with similar charge and where there is binding it must be at appropriate pH that can generate oppositely charge groups as may be found in basic amino acid residues as expected in this research.

Table 2a. Preferential interaction of inorganic ion with enzyme in the presence of ethanol and salt

[Ethanol]	$\Delta\Gamma_{23}$				
	$[CaCl_2(aq)]$/mmol/L				
(mol/L)	0.25	0.50	0.75	1.00	1.25
1.247	0.03449	– 6.63132 exp (– 4)	– 9.71983 exp(– 4)	– 0.02841	– 0.04352
3.227733	0.09859	0.08013	0.03724	0.03029	0.01533
5.27867	0.17815	0.14506	0.11952	0.08318	0.06243

The parameter, $\Delta\Gamma_{23}$, the preferential interaction parameters for osmolation.

However, in most protein stability studies, calcium ion is known to be a stabilizer. Studies have shown that some amylases have dependence on low concentration of calcium chloride while other amylases show dependence on higher concentrations [33]. AMY1 showed optimum activity at low calcium ion concentration, whereas AMY2 did so at relatively high calcium salt concentration. With soluble starch the calcium-dependent activities by the two enzymes were not significantly different [33]. It means that the remarkable calcium-dependent activity of AMYs may have resulted from the unique features of insoluble blue starch, one of the commercially modified starch materials [33]. Therefore, in this research the insoluble potato starch may have had effect on the amylolytic action of the enzyme in the presence of the salt. Besides, it is also known that addition of salts (NaCl(aq) and $CaCl_2$(aq)) has significant effect on structural stabilisation of *α*-amylase exposed to low pH [8]**.**

There is need however, to posit that preferential interaction by binding or exclusion may occur without the presence of formal charges, hence the action of osmolytes that may be polar but neutral can alter the structure of proteins either by binding or exclusion. In this research ethanol, a neutral molecule, binds to the enzyme which, as such, could not reach optimum catalytic action as previously reported [1]. Furthermore, a theoretical study has shown that in the imidazole unit of histidine the ring nitrogen has much higher metal ion (as well as proton) affinity as compared to the π-face. The interaction energies increase in the order of 1-M < 2-M < 3-M < 4-M < 5-M for all the metal ions

considered. Similarly, the complexation energies with the model systems decrease in the following order: $Mg^{2+} > Ca^{2+} > Li^{+} > Na^{+} > K^{+} \cong NH_4^{+} > NMe_4^{+}$. This suggests that nucleophiles otherwise called electron rich centres are subject to attack by cationic electrophiles such as calcium ions in this research even at neutral pH [34]. In addition to this is the report that Asn-100 is the most NH_2-terminal Ca^{2+}-binding residue of PPA in addition to Ca^{2+}-binding His-201 residue [35].

4.3 Preferential interaction of water with enzyme in a ternary mixture of water, ethanol and calcium chloride.

Solvation (osmolation), either preferential binding or preferential exclusion are the two thermodynamic events which occurs whenever a solution of a macromolecule is introduced into a single solution of an osmolyte. They may also be referred to as preferential hydration change and preferential osmolation change; these changes are very likely if a second osmolyte is introduced into the solution containing the first osmolyte. As Table 2b shows, there was preferential dehydration of the enzyme at the lowest concentration of the salt and ethanol. This is to imply that the thermodynamic preferential exclusion process that leads to preferential hydration could not compensate for the preferential dehydration resulting from the binding of other solution components. But with increasing concentration of the salt, there was generally increasing preferential hydration. At higher concentration of ethanol (Table 2b), there is increasing magnitude of dehydration of the protein and a diminishing magnitude of the same parameter with increasing $[CaCl_2(aq)]$. This is a manifestation of the effect of the limited effect of the salt in opposing the effect of ethanol. This is similar to the report that trimethylamine-*N*-oxide (TMAO) opposed the effect of urea on lactate dehydrogenase [36].

Table 2b. Preferential interaction of water with enzyme in the presence of ethanol and salt

[Ethanol] (mol/L)	$\Delta\Gamma_{21}$ $[CaCl_2(aq)]$/mmol/L				
	0.25	0.50	0.75	1.00	1.25
1.247	–7665.785	73.691	72.004	1578.394	1934.577
3.227733	– 21909.530	– 8904.046	– 2758.517	–1682.689	– 681.338
5.27867	– 39591.389	– 16118.845	– 8853.671	– 4621.536	– 2774.886

The parameter, $\Delta\Gamma_{21}$, is the preferential interaction parameter for hydration. The values of $\Delta\Gamma_{21}$ can be determined by two ways either via $\mathrm{In}K_{\mathrm{eq}(1)}/\mathrm{In}a_1$ or – 55.56 $\Delta\Gamma_{23}/[CaCl_2]$.

There is need however, to state that water of protein hydration is different from protein preferential hydration because the former is the mass of water that, at any instant, travels nonrandomly in the same direction as the protein in a transport process [19] while the latter can be

smaller than, equal to or greater than the former. Preferential hydration may be a function of osmolyte/cosolute concentration [19]. Besides, alcohols lower the dielectric constant of the solution. As the dielectric constant decreases, the solution becomes a poorer solvent for the protein. Consequently, there is a relatively favorable protein-protein interaction that may lead to precipitation [37]. This may reduce velocity of the amylolysis as reported in previous research [1]. By the same mechanism, organic solvents like ethanol, a fluidiser, in this research decrease the strength of hydrophobic interactions, within the three dimensional (3-D) structure, leading to decreased protein stability.

Furthermore, the mechanism of salt induced refolding can be explained on the basis of neutralisation of protonated side chains in an acidic medium[8]; intuitively one can posit that in an alkaline medium, deprotonation yielding anionic groups in side chains can also be neutralised by the cations from the inorganic salt as in this research.

4.4 Number of water molecules and ions surrounding protein

Here, as in earlier publication [1], Shurr *et al* [38] definition of $N_{2\mathrm{i}}$ as either $N_{2\mathrm{i}}$ or N_{23} which respectively denotes the total number of water and osmolyte molecules in a domain of sufficient size surrounding a single isolated macromolecule and the parameter $\Gamma_{2\mathrm{i}}$ which is either Γ_{21} or Γ_{23} represents the excess water or osmolyte in the vicinity of the macromolecule is adopted. From the plot of $\Delta\Gamma_{23}$ versus [$CaCl_{2(aq)}$], the slope seems to imply that there is increasing deficit in the number of water molecules surrounding a single isolated protein with increasing concentration of ethanol (Table 3). This is expectedly applicable to the KBI for hydration. The values from intercept (FI) seem to imply that there was increasing interaction of ethanol with protein by binding with increasing concentration of ethanol.

Table 3. Number of water molecules and ions surrounding protein influenced by the presence of ethanol in the reaction mixture and corresponding Kirkwood Buff integrals.

[Ethanol] (mol/L)	From $\Delta\Gamma_{23}$ versus [$CaCl_{2(aq)}$]			From $\Delta\Gamma_{21}$ versus 1/[$CaCl_{2(aq)}$]	
	$\Delta N_{21}(\mathrm{FS})$	$\Delta G_{21}(\mathrm{FS})$	$\Delta N_{23}(\mathrm{FI})$	$\Delta N_{23}(\mathrm{FS})$	$\Delta N_{21}(\mathrm{FI})$
1.247	− 4055.88 $r^2 = 0.941$	− 73 $r^2 = 0.941$	0.047 $r^2 = 0.941$	− 0.0534 $r^2 = 0.958$	4616 $r^2 = 0.958$
3.227733	− 4778.16 $r^2 = 0.937$	− 86 $r^2 = 0.937$	0.117 $r^2 = 0.937$	− 0.1222 $r^2 = 0.994$	5211 $r^2 = 0.994$
5.27867	− 6500.52 $r^2 = 0.994$	−117 $r^2 = 0.994$	0.205 $r^2 = 0.994$	− 0.20788 $r^2 = 0.999$	6711 $r^2 = 0.999$

From the plot of $\Delta\Gamma_{23}$ versus [Ethanol], the slope gives $\Delta N_{21} = -93.507$ and $\Delta G_{21} = -1.544$; from the intercept as [Ethanol] $\rightarrow$ 0, $\Delta N_{23} = 7.916$; from the plot of $\Delta\Gamma_{21}$ versus 1/[Ethanol], $\Delta N_{23} = -9.552$ and $\Delta N_{21} = 125.6$. The parameters, $\Delta\Gamma_{21}$, $\Delta\Gamma_{23}$, and ΔG_{21} are preferential interaction parameters for

hydration, osmolation and KBI for hydration. FS and FI designate values from slope and intercept respectively.

The increasing negative values of ΔN_{23} from the plot of $\Delta\Gamma_{21}$versus 1/[$CaCl_{2(aq)}$] seem to suggest that there was exclusion; only one of the three species, chloride ions, calcium ions and ethanol, can be excluded given the ambient pH condition. The chemical species is chloride ions. This is mainly the implication of the first principle whereby whenever there is exclusion there may be hydration [19] otherwise the results (Table 3) remains the outcome of mathematical abstraction because the slope from the plot of $\Delta\Gamma_{23}$ versus [$CaCl_{2(aq)}$] gives values of $\Delta\Gamma_{21}$ nearly similar to those from the plot of $\Delta\Gamma_{21}$versus 1/[$CaCl_{2(aq)}$] but of opposite sign. Meanwhile Rösgen *et al*. [12] claimed that three concentration regimes, extremely low salt concentration, low-to-intermediate salt concentration, and high salt concentration exert different effects on KBI: The effects are respectively high affinity specific binding and long-range Debye-Hückel electrostatic effects, indirect electrostatic effects and solvation effects. At low-to-intermediate salt concentration there may be departure from ideality leading to screening of the net charge of protein polyatomic surface as well as long range electrostatic effects. As the charges on the protein are increasingly screened with increasing ionic strength of the salt, the chemical potential of the protein is reduced because of increasing binding of the ions rather than exclusion. At higher salt concentration electrostriction and solvation effects (hydration) dominate [12].

On the basis of the preceding analysis and discussion, one can deduce that dehydration at high concentration of ethanol in this research and very high concentration of salt at a given pH leads to a tendency to protein association and ultimately precipitation. This is where electrostriction phenomenon becomes very relevant. It is the pull of the dipolar water molecules into the field, the field created by electrostatic field generated by the protein atom partial charges leading to a thermodynamic equilibrium between a water shell in the field and the rest of water outside the field [39]. The water molecules are confined to smaller surface area and depth leading to density > bulk density [39]. The biologically useful implication is that the electrostricted water molecules are more stable than the bulk water easily vulnerable to the thermal perturbation of solution. This is to say that the electrostricted water can easily form a more stable hydrogen bond with incoming bulk water, the water of preferential hydration for instance. This enhances the chemical potential of the enzyme or protein in general. The presence of ethanol partially altered the water hydration status leading to residual amylolytic activity as previously reported [1]. At this point it is clear that protein water of

584 hydration is mainly populated by electrostricted water. A decrease in the density of the water of
585 hydration leads to total or partial loss of biological function of the enzyme due to decrease in the
586 chemical potential of the protein as to be less available for function. Salts containing cations with a
587 high surface charge density and/or anions with a low surface charge density tend to destabilize
588 proteins in solution [40]. This, once again, represents another view regarding kosmotropes and
589 chaotropes. But this depends on the prevailing pH that determines the net charge of the protein.
590 Thus the strength of interaction is to a large extent regulated by electrostatic interactions, governed by
591 key parameters such as pH and salt concentration [41]. Thus salting-in and salting-out potential of
592 any inorganic salt, the cations and anions components in particular, depend on the pH of the medium.
593 Also, electrostatics appears to be a common background for the application of Kirkwood Buff theory
594 and Hofmeister series for the elucidation of effect of both organic and inorganic solute on protein
595 solution behaviour, increase/decrease in its chemical potential, aggregation/precipitation, and
596 dissolution/salting-in. Calcium ions possess high charge density characteristic of group II elements. It
597 is more hydrated than the chloride component. At pH > 7, PPA may possess net negative charge
598 such that the cations could not have been excluded from the protein surface if it is regarded as a
599 kosmotrope in line with the definition of Rösgen et al, [12]. As stated elsewhere in the text, the
600 chloride ion should rather be excluded leading to hydration. The presence of ethanol opposes the
601 effect of the chloride ions.

602 **4.5 The *m*-values arising from cosolutes' and aqueous solvent's interactions**

603 Based on the method applied in the determination of the equilibrium constant (K_{eq}) for
604 unfolding, it was observed that its reciprocal values were decreasing with increasing concentration of
605 ethanol, due perhaps to the fact that the residual velocities of amylolysis (the range [1, 31] is shown
606 below Table 4) was also increasing with the increasing concentration of ethanol. The native velocity of
607 amylolysis was 97.70 U/mL (1U = micromoles maltose released/mL enzyme in the reaction mixture/ 5
608 min.). But the fact that velocities were less than normal implies that the enzyme was partially
609 destabilised by ethanol. Going by the definition of *m*-value, the capacity of a soluble solute to unfold
610 or refold, there seem to be a paradox considering the fact that, those positive *m*-values (Table 4)
611 suggest that ethanol assumed the status of a protecting cosolute contrary to its known effect.
612 Therefore, there may be alternative explanation which rests squarely on the effect of ethanol on the
613 insoluble potato starch. Ethanol seemed to have increased the solubility of the insoluble starch. The

negative free energy seems to suggest that unfolding is rather very feasible as [C_{os}] →0. Resistance to unfolding or folding entails preferential hydration if there is a protecting osmolyte. As stated earlier increasing concentration of ethanol enhanced the solubility of starch, a sugar, which though a substrate, belong to a chemical species that can be described as osmolyte; sugars generally are protecting osmolyte in nature. This may account for the positive *m*-values. The larger value of negative free energy due to interaction with water alone seems to indicate there is a greater tendency for unfolding.

Table 4. The *m*-values arising from cosolutes' and aqueous solvent's interactions with the enzyme, in a reaction mixture, containing ethanol.

Interaction with ethanol	$m-$value (JL/mol^2)	$\Delta G_{C_3\rightarrow 0}$ (J/mol)	Interaction with water	$m-$value (JL/mol^2)	$\Delta G_{C_3\rightarrow 0}$ (J/mol)
	1077.888 $r^2 = 0.928$	– 5598.315 $r^2 = 0.928$		4479.167 $r^2 = 0.782$	– 21088.446 $r^2 = 0.782$

Here, the Table of values is as a result of plotting ln (1/$K_{eq(i)}$) versus [C_{os}] where $K_{eq(i)}$ and C_{os} are the equilibrium constant for any process in the presence of any osmolyte, i and the concentration of any osmolyte respectively. The lower case alphabet, i, in parenthesis, as subscript, can be water (1) or ethanol (3) in this case. This effectively corrects previous error [1] arising from the mistake in plotting ln ($K_{eq(i)}$) versus [C_{os}] [1]. The parameter, $\Delta G_{C_3\rightarrow 0}$ is the KBI for hydration as [C_{os}] →0. Here, the subscript, 'os' denotes osmolyte such as ethanol in this research. The residual activity range is 36.18-57.62 corresponding to ethanol concentration range equal to ~1.25-5.28 mol/L [1].

Like the report for PPA, previous research with another enzyme, alpha chymotrypsin, has shown that chymotrypsin shows significant residual activity in the water-poor ethanol [33]. The difference lies in the different substrates for the enzymes. At low water content, the ethanol molecules are preferentially excluded from the enzyme surface [33], a paradox considering the known effects of ethanol but seem to agree with the positive *m*-value in this research. Positive *m*-value implies that the cosolute is a stabiliser. If ab initio, $K_{eq(i)} < 1$, the measured binding stoichiometry of the ligand (or the calculated preferential binding parameter as adopted in this research) must be negative – preferential exclusion [19]. The contrary is the case with ethanol as cosolvent alone which gave values of $K_{eq(i)} > 1$. The fact that the $K_{eq(i)}$ values due to the presence of ethanol, is decreasing with increasing [Ethanol] though yielded positive $\Delta\Gamma_{23}$ (Table 1), nevertheless gave positive *m*-value as against negative *m*-value because ln(1/$K_{eq(i)}$) versus [Ethanol] expectedly showed positive correlation with coefficient of determination ~ 0.92.

4.6 The *m*-values arising from calcium chloride and aqueous solvent's interactions with the enzyme

Further consideration for the determination of *m*-value due to combined effect of ethanol and calcium chloride, demands that one takes into cognisance of the fact that the magnitude is purely concentration range dependent; it could be large or small. This is clearly illustrated before now in Table 4 in which the concentration regime of ethanol is > 1 mol/L unlike here in Table 5 in which the concentration of calcium chloride is of the millimolar scale. With a mixture of ethanol and calcium chloride, and increasing concentration of the latter and values of $K_{eq(i)}$ a plot of $\ln(1/K_{eq(i)})$ versus [$CaCl_2$(aq)] should naturally give a negative slope-a negative *m*-value. The negative sign of *m*-value means that there may have been preferential binding [12]. This cannot be doubted because both ethanol and calcium ion can bind at the prevailing favourable pH. The deduction one can make, however, is that binding of mineral cation does not always lead to destabilisation, but on the contrary stabilisation is the case as exemplified with calcium salt in this research where it is unmistakingly shown with appropriate use of equations for the determination of parameters. The positive values of the free energies as $CaCl_2$(aq)→0 means that refolding may be less feasible without the salt in the presence of ethanol.

Table 5. The *m*-values arising from cosolutes' and aqueous solvent's interactions with the enzyme, in a reaction mixture, containing calcium chloride and ethanol.

[Ethanol]/mol/L	Interaction with $CaCl_2$(aq)		Interaction with water	
	$m-$value (JL/mol^2)	$\Delta G_{C_3\rightarrow 0}$ (J/mol)	$m-$value (JL/mol^2)	$\Delta G_{C_3\rightarrow 0}$ (J/mol)
1.247	~ – 1.408exp (+6) $r^2 = 0.931$	907.695 $r^2 = 0.931$	~ 1.882 exp (5) $r^2 = 0.941$	~ – 121.198 $r^2 = 0.941$
3.227733	~ – 1.915exp (+6) $r^2 = 0.928$	2493.584 $r^2 = 0.928$	~ 2.22 exp (5) $r^2 = 0.937$	~ – 301.706 $r^2 = 0.937$
5.27867	~ – 2.738exp (+6) $r^2 = 0.982$	4368.284 $r^2 = 0.982$	~ 3.017 exp (5) $r^2 = 0.994$	~ – 528.629 $r^2 = 0.994$

The equation from the plot of intercept (obtained from the plot of $\ln\frac{1}{K_{eq(3=salt)}}$ versus [Salt], and where [Salt]→0) versus [Ethanol] gives $m-\text{value} = 858.700\text{JL/mol}^2$ and $\Delta G_{C_3\rightarrow 0} = -201.137\text{J/mol}$; the results from the plot of intercept (obtained from the plot of $\ln\frac{1}{K_{eq(1)}}$ versus [Salt] and where [Salt]→0) versus [Ethanol] are $m-\text{value} = -100.569\text{JL/mol}^2$ and $\Delta G_{C_3\rightarrow 0} = 10.315\text{J/mol}$.

The preferential interaction of water with the enzyme presents different scenario. The values of $K_{eq(i)}$ showed increasing trend (data not shown directly) with increasing [$CaCl_2$(aq)]. Consequently, a plot of $\ln(1/K_{eq(i)})$ versus [$CaCl_2$(aq)] gives positive slope-the positive *m*-value. This, according to Rösgen et al [12], implies preferential exclusion. But what is excluded? What seems to be preferentially excluded is the chloride ion because the net charge of PPA under alkaline medium is negative. Realising that both folded and unfolded protein are hydrated though unequally, more with

672 unfolded than with the folded [12], the negative free energies as [$CaCl_2$(aq)] →0 (that is unfolding is
673 more feasible as [$CaCl_2$(aq)] →0), indicates that the greater tendency to unfolding promoted greater
674 hydration. There was neither total unfolding nor total refolding.

675 The plot of intercept/*RT* (obtained from the plot of $\ln\frac{1}{K_{eq(3=salt)}}$ versus [Salt], and where
676 [Salt]→0) versus [Ethanol] gives a negative free energy-the intercept- and positive *m*-value as shown
677 below Table 5. This implies that there was stabilising effect of the cosolvent contrary to known effect
678 of ethanol while the negative free energy aspect means that unfolding seems more feasible in the
679 absence of ethanol. From the plot of intercept/*RT* (obtained from the plot of $\ln\frac{1}{K_{eq(1)}}$ versus [Salt])
680 versus [Ethanol], the positive free energy shown below Table 5, seem to suggest that unfolding due to
681 water alone as [Ethanol] →0 is thermodynamically not feasible, though there is a view that water is
682 not the only factor that induces unfolding [42]. This against the backdrop of the view that water, on
683 purely thermodynamic grounds, but for reason that is not very clear, is unlikely to be the denaturing
684 agent in aqueous solutions of denaturant. As usual, the corresponding negative *m*-value points to the
685 fact that there was a destabilising effect of the cosolute.

686 **4.7 Change of solvation preference and change of preferential interaction parameter with**
687 **ethanol as the only cosolvent.**

688 The concern of scientist is to establish the direction of change either unfolding or rigidification
689 (refolding). Against what is expected of a stabilising osmolyte, it seems ethanol had greater
690 preferential binding ($\Delta_N^D\Gamma_{23}$) to the native state than the unfolded ensuring the partial unfolding of the
691 native state (Table 6). If the native state had greater number of cosolvent bound to it, then it has
692 greater number of excluded or displaced solvent, water, if consideration is given to the general
693 principle of Timasheff [19]. But it is known too that the unfolded is more hydrated than the folded
694 protein [12]. This may account for decreasing loss of water of preferential hydration [Table 6]. The
695 change in solvation preference, $\Delta_N^D(G_{21}-G_{23})$ of proteins upon denaturation is cognately linked
696 to $\Delta_N^D\Gamma_{23}$. Therefore, the parameters exhibit the same trend.

697 **Table 6. Change of solvation preference and change of preferential interaction parameter in**
698 **terms of *m*-values with ethanol as cosolvent.**

[Ethanol] mol/L	$\Delta_N^D\Gamma_{23}$	$\Delta_N^D\Gamma_{21}$	$\Delta_N^D(G_{21}-G_{23})$
1.247	**–** 0.501	–22.321	–0.402
3.228	**–**1.140	–19.628	–0.353
5.279	**–**1.620	–17.049	–0.307

The parameter $\Delta_N^D\Gamma_{23}$ is the change of preferential osmolation; $\Delta_N^D\Gamma_{21}$ is the change of preferential hydration; $\Delta_N^D(G_{21}-G_{23})$ is the change of solvation preference. Values were approximations to three decimal places.

4.8 Change of solvation preference and change of preferential interaction parameter with a mixture of ethanol and aqueous solution of calcium chloride.

According to Asciutto *et al* [42] and Rösgen *et al* [12] it is the competition between protein hydration and ion solvation that determines whether a salt stabilizes or destabilizes the peptide. The sign observed in Table 7 seem to support the proposition that the stabilising tendency of a cosolute (with respect to either the native or denatured state) depends on the protein's preference to have positive correlation either with water or cosolute; this preference determines the sign of the solvation expression $G_{21}-G_{23}$. However, the latter does not represent the change $\Delta_N^D(G_{21}-G_{23})$. The important issue is that calcium salt assumed a protecting role because all the parameters shown in Table 7 possess positive values. In the presence of protecting osmolytes, however, the protein changes its solvation preferences several fold as the osmolyte concentration is increased [12]. Unlike this suggestion elsewhere [12], the increasing value of $\Delta_N^D(G_{21}-G_{23})$ indicates that the protein transition becomes more sensitive to the presence of increasing concentration of the salt. Where there is protective outcome of a cosolute there may be preferential hydration. The presence of the salt enhanced the function of the enzyme but the concentration of the salt was not sufficient to enable total reversal of the effect of ethanol.

Table 7. Change of solvation preference and change of preferential interaction parameter in terms of *m*-values with a mixture of ethanol and aqueous solution of calcium chloride.

$[CaCl_2(aq)]$ (mmol/L)	[Ethanol] mol/L								
	1.247			3.228			5.279		
	$\Delta_N^D\Gamma_{23}$	$\Delta_N^D\Gamma_{21}$	$\Delta_N^D(G_{21}-G_{23})$	$\Delta_N^D\Gamma_{23}$	$\Delta_N^D\Gamma_{21}$	$\Delta_N^D(G_{21}-G_{23})$	$\Delta_N^D\Gamma_{23}$	$\Delta_N^D\Gamma_{21}$	$\Delta_N^D(G_{21}-G_{23})$
0.25	0.146	3.235 exp (+4)	582.227	0.198	4.400 exp (+4)	791.878	0.283	6.291 exp (+4)	1132.199
0.50	0.299	3.318 exp (+4)	597.200	0.406	4.514 exp (+4)	812.425	0.581	6.455 exp (+4)	1161.576
0.75	0.447	3.310 exp (+4)	595.724	0.604	4.471 exp (+4)	804.796	0.869	6.436 exp (+4)	1158.462
1.00	0.683	3.795 exp (+4)	683.057	0.929	5.162 exp (+4)	929.016	1.328	7.380 exp (+4)	1328.300
1.25	0.949	4.217 exp (+4)	758.947	1.290	5.735 exp (+4)	1032.233	1.845	8.200 exp (+4)	1475.850

The parameter $\Delta_N^D\Gamma_{23}$ is the change of preferential osmolation; $\Delta_N^D\Gamma_{21}$ is the change of preferential hydration; $\Delta_N^D(G_{21}-G_{23})$ is the change of solvation preference. Values were approximations to three decimal places.

4.9 Validation of derived equations for the determination thermodynamic activity

This research seems to have provided immediate opportunity to validate Eq. (12a) or Eq. (12b) because as the values in Table 8 show, there is no large difference between values obtained from calculations using different equations, Eq. (8) and Eq. (12b). It need to be stated that while Eq. (8) is intended strictly for ideal solution, Eq. (12b) may be a general one applicable to both ideal and nonideal solutions. Calculation may take some time, but the use of equations as in this research may be useful for the assessment of equipments used to determine water activity in food and drug preparations. According to Miyawaki *et al* [18], water activity is reflective of the macroscopic state of water in food and affects various rate processes such as browning, oxidation, and degradation of nutrients, enzyme reaction, and especially the growth rate of microorganisms. Therefore, the concept of water activity is very important in relation to food preservation [18]. As expressed in this research, the pH of any preparation, food, drug, *etc* must be taken into account because the ionisation state or what Miyawaki *et al* [18] called molecular specificity of the solute materials, in addition to polar groups can influence the hydration of the mixture components and ultimately water activity. Salt as a preservative, a special osmolyte, and being neutral is added to food material or solution where it alters water activity just as in this research where calcium salt had effect on the enzyme's amylolytic activity through its preferential interaction and effect on water activity.

Theoretical determination of activity coefficient by different methods may not give the same results. As shown in Table 8, the values of activity coefficients obtained using Debye-Hückel-Davis [43] and Lund's methods [11] are not the same. Since an activity coefficient is an important factor in the determination of the effect of solution structure on the function of enzymes as well as its purification it is important its value does not differ widely from experimentally measured values. There is a report which indicates that Debye-Hückel-Davis result [43] is very similar to experimentally measured values [16].

Table 8. Thermodynamic activities and activity coefficients from two different methods

Equations	[$CaCl_2$(aq)] (mmol/L)				
	0.25	0.50	0.75	1.00	1.25
	a_1				
Eq. (8)	0.9999955	0.999991	~0.999987	~0.999982	~0.999978
Eq. (12b)	0.99999546	~0.9999909	~0.9999863	0.9999816	0.99997695
Methods	γ				
DH-Davis	~0.937801	~0.91408497	0.896637	0.88241099	0.87023061
Lund	0.99999737	~0.99999629	0.99999545	~0.99999475	~0.99999413

The parameter, a_1 is the activity of water in salt solution and γ is the activity coefficient. DH-Davis stands for Debye-Hückel-Davis method [43].

Before, informed conclusion on the outcome of this research, results and discussion, there is need for a concise summary as follows. Some theoretical methods in literature were analysed and found to give different results for activity coefficient and activity. An equation linking the activity of water to the activity of solute was derived; the equation gave results that are very similar to results from conventional methods for ideal solution (but may not be limited to ideal solution). With ethanol, the preferential interaction parameter (Γ_{23}) was expectedly positive with corresponding negative preferential hydration, $-\Gamma_{21}$. Calcium salt, at higher concentration, showed sign of exclusion at a lower concentration of ethanol unlike at higher concentration. This led to negative preferential hydration. There were a negative number of water molecules signifying a deficit of water molecules around the protein surface domain. The *m*-value with ethanol alone was unexpectedly positive which may be as a result of increasing solubility of raw starch with increasing concentration of ethanol; unfolding propensity (negative $\Delta G_{C_3 \rightarrow 0}$) seems paradoxically feasible as [Ethanol] $\rightarrow$0. With the presence of a mixture of ethanol and calcium salt, the *m*-values were negative in sign as to imply that there was destabilisation of the enzyme; positive values of $\Delta G_{C_3 \rightarrow 0}$ indicates that unfolding is not feasible when [$CaCl_2$(aq)] $\rightarrow$0 but feasible in the presence of water and calcium chloride only. This is another paradox given known effect of calcium ion even if a holoenzyme was assayed. Indeed results from intercepts may represent a departure from practical or experimental reality in all its ramification, including the ambient condition. The negative change of solvation preference and the corresponding change of interaction implied that there was partial destabilisation of the enzyme in the presence of ethanol only giving rise to residual amylolysis. With aqueous mixture of ethanol and calcium chloride, there was positive change of solvation preference as was the case with interaction parameter. This was a sign of partial stabilisation which sustained residual amylolysis.

5. CONCLUSION

Selected equations in literature may not give the same values of activity coefficient and activity of solution components. The presence of stabilising osmolyte, salt and ethanol may not always yield positive *m*-values. The sign of change of solvation preference with either binary or ternary mixture of osmolytes, and the cognate interaction parameter may be a better indicator of the stability of a macromolecule. The kosmotropes and chaotropes may be cationic or anionic and their deficit or otherwise around the macromolecule and consequence, depend largely on net charge on the macromolecule at a given pH.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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