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Application of the Osmotic Virial Equation in Cryobiology

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Abstract

The multisolute osmotic virial equation is the only multisolute thermodynamic solution theory that has been derived from first principles and can make predictions of multisolute solution behaviour in the absence of multisolute solution data. Other solution theories either (i) include simplifying assumptions that do not take into account the interactions between different types of solute molecules or (ii) require fitting to multisolute data to obtain empirical parameters. The osmotic virial coefficients, which are obtained from single-solute data, can be used to make predictions of multisolute solution osmolality. The osmotic virial coefficients for a range of solutes of interest in cryobiology are provided in this paper, for use with concentration units of both molality and mole fraction, along with an explanation of the background and theory necessary to implement the multisolute osmotic virial equation.

Keywords: osmolality, freezing point, phase diagram, multisolute solutions, solution theory, thermodynamics

Introduction

In many areas of biology, including cryobiology, the solution behaviour of both the extracellular and intracellular solutions plays an important role. The osmolality difference between the extra- and intra-cellular solutions drives the water flux across the cell membrane. Recently, a non-ideal replacement for the osmotic equilibrium equation was presented where it was demonstrated that the osmolality as a function of

concentration for the intracellular solution is needed in order to accurately determine the osmotically-inactive fraction of the cell volume [37].

In cryobiology, the freezing point depression of solutions is also important. The freezing point determines the temperature at which ice can first form in the extracellular solution, how much ice will form at equilibrium at a given temperature, and the amount of supercooling in the intracellular solution. For these reasons, cryobiologists are interested in predicting both the osmolality and the freezing point depressions of multisolute extra- and intra-cellular solutions.

There are many solutes of interest in cryobiology, from electrolytes to cryoprotective agents (CPAs) to macromolecules, and there are many combinations of these solutes. Since measuring the solution properties of all possible combinations is prohibitively time- and resource-consuming, much work has been done to predict the solution behaviour of these complicated multisolute solutions using a range of solution theories [9; 10; 20; 22; 23; 24; 31; 32; 33; 45]. The challenge is to develop a solution theory that is accurate for many solutions, preferably without the need to fit multisolute solution data. Solution theories have been developed for cryobiological solutions which are accurate for a certain subset of solutions, but these equations either require fitting of multisolute data [10; 23; 31; 32; 33; 45] or do not take into account all of the solute interactions [20; 22; 24]. Recently, a form of the multisolute osmotic virial equation was proposed in which the mixing rules are derived from thermodynamic first principles and require only single-solute information to predict multisolute solution behaviour [9]. The

word “virial” is derived from the Latin word for force or energy, “*vis*” [16]. The coefficients in the virial equation can be obtained from knowledge of the forces between molecules [36]. In the absence of knowledge of the intermolecular forces, the coefficients in the multisolute osmotic virial equation proposed by Elliott *et al*. [9] can be obtained by fitting single-solute data. The multisolute osmotic virial equation, in various forms, is a widely-used solution theory and has been shown to be accurate for a large range of solutes, including CPAs, small molecules, electrolytes, and macromolecules [8; 9; 11; 13; 17; 25; 29; 38; 44; 48; 49; 50]. The form proposed by Elliott *et al*. [9] was shown to accurately predict the osmolalities of solutions containing water plus (i) two small molecules, (ii) a protein and an ideal solute, and (iii) two proteins. It has also been expanded to solutions containing water with a CPA and an electrolyte and was shown to be accurate for these solutions [38; 44]. In the absence of multisolute solution data which can be fit to determine solution parameters, the multisolute osmotic virial equation proposed by Elliott *et al*. [9] should be used to predict solution behaviour. It is the only solution theory for which the mixing rules are derived from first principles and it has been shown to be accurate for a wide range of solutes and mixtures of solutes.

This paper provides a review of solution theories that have been developed for solutions of interest in cryobiology, focusing on application of the osmotic virial equation. Included in this paper is the necessary theory and background to understand the various solution theories in the literature and to apply the multisolute osmotic virial equation to multisolute solutions. Osmotic virial coefficients for a range of solutes are provided for use with concentrations in both molality and mole fraction, including updated

coefficients for dimethyl sulphoxide (Me_2SO_4), glycerol, bovine serum albumin (BSA), and ovalbumin (OVL). The purpose of this paper is to provide all the information and insight required for other investigators to use the form of the multisolute osmotic virial equation previously proposed [9] for a wide range of multisolute solutions.

Relationships between thermodynamic solution properties

Solution theories are written in terms of concentration, osmolality, osmotic coefficient, or water activity. These quantities are related and it is important to understand the relationship between them. Before reviewing the solution theories that have been developed for cryobiological solutions, the quantities of interest in cryobiology and the relationships between them will be outlined.

Freezing point depression and osmolality

Since osmolality and freezing point play such a large role in cryobiology, solution theories have been developed to predict both of these quantities for multisolute extra- and intra-cellular solutions. Osmolality and freezing point are related to each other, so once one is known, the other can be determined.

From the Gibbs-Duhem equation [36], the relationship between freezing point depression, ΔT_{FP}, of an aqueous solution and osmolality, π, can be obtained [9; 43] (see Appendix A).

$$\Delta T_{FP} = T_{FP}{}^{o} - T_{FP} = \left[W_1 / \left(\overline{s_1^{0^L}} - \overline{s_1^{0^S}} \right) \right] R T_{FP} \pi \qquad (1)$$

where $T_{FP}{}^{o}$ is the freezing point of the pure solvent (water), T_{FP} is the freezing point of the solution, W_1 is the molecular weight of water (kg/mole), $\overline{s_1^{0^L}}$ is the entropy per mole of pure liquid water (J/moleK), $\overline{s_1^{0^S}}$ is the entropy per mole of pure water in the solid phase (J/moleK), and R (J/moleK) is the universal gas constant. In the derivation of equation (1), the molar entropies of water, $\overline{s_1^{0^L}}$ and $\overline{s_1^{0^S}}$, are assumed to be constant. The values for the constants in equation (1) can be found in Table 1. Equation (1) can be rearranged to yield osmolality as a function of the freezing point depression:

$$\pi = \frac{T_{FP}{}^{o} - T_{FP}}{\left[W_1 / \left(\overline{s_1^{0^L}} - \overline{s_1^{0^S}}\right)\right] R T_{FP}} \qquad (2)$$

Winzor also published the nonlinear conversion between osmolality and freezing point depression (equation 2) [43], but in his paper the density of water is missing in the conversion between osmotic pressure and osmolality.

Equation (1) can also be used to convert osmolality to freezing point depression. Since the freezing point of the solution (T_{FP}) appears on both sides of equation (1), equation (1) is usefully rearranged so that T_{FP} appears only on the left hand side of the equation.

$$T_{FP}{}^{o} - T_{FP} = \frac{\left[W_1 / \left(\overline{s_1^{0^L}} - \overline{s_1^{0^S}}\right)\right] R T_{FP}^{0} \pi}{1 + \left[W_1 / \left(\overline{s_1^{0^L}} - \overline{s_1^{0^S}}\right)\right] R \pi} . \qquad (3)$$

By neglecting the last term in the denominator, the conversion between freezing point depression and osmolality, equation (3), is linearized yielding the widely used equation [1; 2; 18; 43]:

$$T_{FP}{}^{o} - T_{FP} \approx 1.86\pi$$

or

$$\pi \approx \frac{T_{FP}{}^{o} - T_{FP}}{1.86} \qquad (4)$$

Figure 1 shows that this simplification introduces over 7% error when the freezing point depression is 20 ^{o}C and over 18% error when the freezing point depression is 50 ^{o}C.

Osmotic coefficient and osmolality

The osmotic coefficient, Φ, is often used to express the osmolality of a solution. For a single-solute solution, the osmotic coefficient is defined as:

$$\Phi = \frac{\pi}{m} \qquad (5)$$

For a multisolute solution, the osmotic coefficient is defined as the osmolality divided by the total solute molality.

$$\Phi = \frac{\pi}{\sum_i m_i} \qquad (6)$$

Water activity and osmolality

In addition to the relationships between freezing point, osmolality, and osmotic coefficient, the relationship between water activity and osmolality is often needed. Many solution theories provide predictions of the solution behaviour in water activity, which is then converted to osmolality or freezing point.

Water activity, a_1, is defined through its relationship to chemical potential, μ_1 [36]:

$$\mu_1 - \mu_1^o = RT \ln a_1 \qquad (7)$$

where μ_1 is the chemical potential of water (J/mole), R is the universal gas constant (J/moleK), and T is temperature (K). The subscript *1* refers to the solvent (water) and the superscript *o* refers to the standard state.

Using the approach of Landau and Lifshitz [21]†, osmolality, π (osmoles/kg water), is defined by the following relationship [9]:

$$\mu_1 - \mu_1^o = - RTW_1\pi \qquad (8)$$

where W_1 is the molecular weight of water (kg/mole).

Comparing equations (7) and (8) gives the following relationship between water activity and osmolality:

$$\pi = - \frac{\ln a_1}{W_1} \qquad (9)$$

The relationship between osmotic pressure, Π, and osmolality is:

† While many solution theories are written from an *a priori* assumption of dependence on mole fraction, Landau and Lifshitz [21] had a different *a priori* assumption involving dependence on molality.

$$\Pi = RT\rho_1\pi \qquad (10)$$

where ρ_1 is the density of water (kg/m^3). Thus the relationship between osmotic pressure and water activity is:

$$\Pi = -RT\frac{\ln a_1}{\upsilon_1} \qquad (11)$$

where υ_1 is the molar volume of water (m^3/mole).

In order to determine the osmolality, osmotic coefficient, freezing point depression, or activity of all the solutions of interest in cryobiology, either measurements or predictions from an accurate solution theory are needed. Measuring all possible multisolute solutions is prohibitively time- and resource-consuming, so much work has focused on developing predictive multisolute solution theories for cryobiological solutions [8; 9; 11; 13; 17; 25; 29; 38; 44; 48; 49; 50]. An overview of some of the most commonly utilized approaches is presented below.

Multisolute solution theories used in cryobiology prior to the introduction of the multisolute osmotic virial equation

Types of solution theories

Within cryobiology, there are several different types of solution theories. The first is ideal, dilute solution theory in which interactions between solute molecules are not taken into account. This approach is often valid at very low solute concentrations, when the solute molecules are not interacting with each other. Additionally, some molecules (such as methanol) can be approximated as ideal solutes over a larger concentration

range, up to almost 20 molal (i.e. interactions between methanol molecules do not contribute significantly to the solution behaviour). However, the ideal, dilute approach does not work well for the majority of solutes past very low concentrations, including most CPAs, electrolytes, alcohols, and macromolecules.

In order to account for the non-ideal behaviour of solutions, other solution theories have been developed. These include the empirical fitting equations and solution theories developed from thermodynamic principles. The empirical solution theories require parameters that are obtained by fitting multisolute solution data in order to predict multisolute data. The fitting parameters capture the non-ideal behaviour of the solutes which arise from the interactions between the solute molecules. The parameters are unique for each particular solution and must be obtained from multisolute data for each new combination of solutes. These solution theories provide accurate results for the specific solutions for which the fitting parameters can be obtained; however, they can only be used to make predictions of solution behaviour for which multisolute solution data are available. Examples of this type of solution theory are the equations developed by Pegg [31; 32; 33], Woods *et al.* [44; 45], and Fahy [10].

Multisolute solution theories that have been developed from thermodynamic principles and applied to cryobiological solutions include the van Laar equations and the multisolute osmotic virial equation. The van Laar equations have been applied to predict the behaviour of red blood cell cytoplasm [22; 23; 24]. The van Laar equations use the van der Waal’s mixing rules, which are not accurate for many liquids, including solutions

containing macromolecules or electrolytes [36]. The van der Waal's mixing rules can be removed from the van Laar equations, but this requires the use of empirical constants, which restricts the usage of the van Laar equations to solutions for which multisolute solution data are available. Conversely, the multisolute osmotic virial equation is also developed from thermodynamic principles [9] and can be used to accurately predict the solution behaviour for a broad range of multisolute solutions using only singe solute data.

The following section outlines some of the solution theories that have been applied in cryobiology to capture the non-ideality of the multisolute solutions, including the assumptions used in the equations and the limitations to each approach.

Empirical solution theories

Pegg [31; 32; 33] fit equations to data for melting point as a function of concentration for specific ternary and quaternary solutions in order to obtain empirical parameters for specific combinations of solutes. The empirical parameters are typically functions of the mass ratio of the first solute to the second solute, (i.e. the R-value). Fitted equations for mixtures of Me_2SO_4 + NaCl + water, glycerol + NaCl + water, and PG + glycerol + NaCl + water [31; 32; 33] were generated. The equations are in terms of total solute mass fraction $\left(\sum_i X_i\right)$ and are of the general form:

$$T_m = a\left(\sum_i X_i\right) + b\left(\sum_i X_i\right)^2 + \ldots \qquad (12)$$

where T_m is the melting point of the solution (^{o}C), a and b are fitting parameters which are typically functions of the R-value, $\sum_i X_i$ is the total solute mass fraction (g/100g of solution), where *i* refers to each solute, and X_i is the mass fraction of solute *i* (g/100g of solution). The polynomial expansion in total solute mass fraction is truncated after sufficient parameters are included to describe the multisolute melting point data. The non-ideal solution behaviour is captured by the fitting parameters which account for the interactions between all of the solute molecules.

Woods *et al*. also used this approach to develop equations to predict the melting point of solutions containing ethylene glycol (EG) + NaCl + water [44; 45].

The constants in equation (12) are specific for each solution and cannot be applied to different combinations of solutes. When multisolute solution data is available, this approach results in accurate predictions. However, it is limited to solutions for which multisolute solution data are available. In addition, data for each new combination of solutes must be fit to obtain new coefficients.

Fahy fit functions to data for freezing point as a function of concentration for the ternary systems of glycerol + NaCl + water and Me_2SO_4 + NaCl + water [10], of the form:

$$T = \frac{-\left[0.7451 - \left(0.56865 - f\left(\sum_i x_i\right)\right)^{1/2}\right]}{6.405 \times 10^{-3}} \qquad (13)$$

where T is the temperature (^{o}C), f is an empirical function that is obtained from the fit, $\sum_i x_i$ is the total solute mole fraction (moles solute/total moles) where *i* refers to each solute, and x_i is the mole fraction of solute *i* (moles solute *i*/total moles).

Fahy used equation (13), along with other relationships, to calculate the composition, water content, salt concentration, and unfrozen fraction as a function of temperature. As with Pegg's equations, this approach results in highly accurate predictions, but is limited to solutions for which multisolute solution data are available and each new multisolute solution must be fit with a new function, f.

Multisolute solution theories derived from thermodynamic principles

Levin *et al*. proposed several models for the cytoplasm of an erythrocyte [22; 23; 24]. In two of these models, they assumed that the cytoplasm is an ideal solution with a certain amount of water bound to each solute. They referred to the solutes with water bound to them as 'hydrated'. In one study, Levin *et al.* modelled the cytoplasm of an erythrocyte as a non-ideal, non-dilute, hydrated, pseudo binary solution of water and a fictitious solute [23]. The fictitious solute represents all the solutes which are in the cytoplasm of a red blood cell. Levin *et al*. used van Laar type equations for the activity coefficients of the two solution species (solute and solvent). Since the van Laar equation uses van der Waal's mixing rules, which are not accurate for many solutions, Levin *et al*. replaced the

mixing rules with empirical constants. The resulting equations for the solvent and solute activities, a_w^h and a_m^h respectively, are:

$$\ln a_w^h = \frac{\alpha}{\left(1+\frac{\alpha}{\beta}\left(x_w^h / x_m^h\right)\right)^2} \tag{14}$$

$$\ln a_m^h = \frac{\beta}{\left(1+\frac{\beta}{\alpha}\left(x_m^h / x_w^h\right)\right)^2} - \beta \tag{15}$$

where a_w^h is the water activity on a hydrated basis, a_m^h is the solute activity on a hydrated basis, x_w^h is the water mole fraction on a hydrated basis, x_m^h is the solute mole fraction on a hydrated basis, and α and β are empirical constants. To determine the van Laar coefficients, α and β, the water activity of the cytoplasm as a function of concentration is required. This necessitates additional simplifying assumptions about the composition of the cytoplasm in order to determine the water activity. In addition, due to the use of empirical parameters, this approach is limited to the solutions for which multisolute solution data are available.

Multisolute solution theories in the absence of multisolute data

In order to predict multisolute solution behaviour, all of the previous solution theories describing non-ideal solutions require empirical parameters obtained by fitting the multisolute data of the solution of interest. Although these solution theories are accurate for the particular subset of solutions for which the empirical parameters can be determined, they cannot be applied to solutions for which there are no multisolute

solution data. In order to address this disadvantage, many investigators have used the approach of adding single-solute solution osmolalities to predict multisolute solution osmolalities (or freezing point depressions) [19; 26]. Most recently, Kleinhans and Mazur used this approach to predict the freezing point depressions of four different mixtures of a CPA and sodium chloride (NaCl) in water [20]. They fit the data for freezing point as a function of concentration of single-solute solutions containing water plus either Me_2SO_4, glycerol, EG, or NaCl with cubic polynomials as functions of solute molality.

$$T_{FP} = C_1 m + C_2 m^2 + C_3 m^3 \qquad (16)$$

where T_{FP} is the freezing point of the solution (°C), C_1, C_2, and C_3 are fitting parameters, and m is the solute molality.

These coefficients were then used to predict the freezing point depressions of solutions containing water with two solutes. For a two-solute solution, with a solute-A molality of m_A and a solute-B molality of m_B, the predicted freezing point is:

$$\begin{aligned} T_{FP}^{S} &= T_{FP}^{A} + T_{FP}^{B} \\ &= \left(C_{1A} m_A + C_{2A} m_A^2 + C_{3A} m_A^3\right) + \left(C_{1B} m_B + C_{2B} m_B^2 + C_{3B} m_B^3\right) \end{aligned} \qquad (17)$$

where T_{FP}^{S} is the freezing point of the two-solute solution, T_{FP}^{A} is the freezing point of a single-solute solution of solute A, and T_{FP}^{B} is the freezing point of a single-solute solution of solute B.

The summation of the freezing point depressions (or osmolalities) approach does allow prediction of multisolute solutions using only single-solute data. The fitting parameters (C_1, C_2, and C_3) account for the interactions between solute molecules of the same type. However this approach does not take into account the interactions between the different types of solute molecules (i.e. interactions between solute A and solute B). Nonetheless, this approach has been shown to work well in practice for the particular set of multisolute solutions in the Kleinhans and Mazur study.

The multisolute osmotic virial equation

The multisolute osmotic virial equation can be used to address the limitations of the previous solution theories. It is derived from thermodynamic principles and can be applied to multisolute solutions in the absence of multisolute solution data [9]. The mixing rules for the multisolute osmotic virial equation can be derived from regular solution theory. Regular solution theory, defined by Scatchard and Hildebrandt, is applicable to a wide range of solutes [36]. A regular solution is defined as a solution with zero excess volume ($v^E = 0$) and zero excess entropy of mixing ($s_{mixing}^E = 0$), i.e. a solution for which the non-idealities can be captured by corrections to the energetic terms alone. When deriving the mixing rules for the multisolute osmotic virial equation, if the additional assumption of a semi-dilute solution is made (i.e. the solute-solute excess interaction energy is less than at least one of the solvent-solute excess interaction energies), the resulting equation allows predictions of multisolute solutions using only single-solute data [9]. The multisolute osmotic virial equation takes into account interactions between all solute molecules. Both regular solution theory and the

multisolute osmotic virial equation previously proposed [9] contain assumptions that cannot be expected to accurately predict all solutions, especially those which are exceedingly non-ideal. However, the multisolute osmotic virial equation has been shown to work for a wide range of solutions [8; 9; 11; 13; 17; 25; 29; 38; 44; 48; 49; 50].

In order to make predictions of multisolute solution behaviour, the solute specific osmotic virial coefficients are required. The coefficients in the virial equation can be obtained from knowledge of the forces between molecules [36]. In the osmotic virial equation, the coefficients account for the interactions between the solute molecules in the solutions. In the absence of information regarding the interactions between the solute molecules, the osmotic virial coefficients can also be obtained by fitting data for osmolality of the single-solute solutions as a function of concentration to the single-solute osmotic virial equation. The osmotic virial coefficients can then be used in the multisolute osmotic virial equation to predict multisolute solution behaviour in the absence of multisolute data.

The single-solute osmotic virial equation is applicable to a range of solutions containing water plus a single solute (Figures 2-6). The osmolalities of single-solute solutions are represented as truncated polynomials in concentration, where each solute has unique coefficients for terms of second or higher order in concentration.

$$\pi = m_i + B_i m_i^{\,2} + C_i m_i^{\,3} + \ldots \qquad (18)$$

where π is the osmolality of the solution (osmoles/kg solvent), m_i is the molal concentration of the solute (moles solute/kg solvent), B_i [(moles solute/kg solvent)$^{-1}$] and C_i [(moles solute/kg solvent)$^{-2}$] are the second and third osmotic virial coefficients for use with molality, respectively.

The single-solute osmotic virial equation can also be written in terms of mole fraction:

$$\pi = A^*\left(x_i + B_i^* x_i^{\,2} + C_i^* x_i^{\,3} + ...\right) \qquad (19)$$

where x_i is the mole fraction of the solute (moles solute/total moles in solution), B_i^* [(moles solute/total moles)$^{-1}$] and C_i^* [(moles solute/total moles)$^{-2}$] are the second and third osmotic virial coefficient for use in mole fraction, respectively. The quantity in the parenthesis in equation (19) is osmole fraction ($\tilde{\pi}$) so an additional conversion factor, A^*, between osmole fraction and osmolality is needed. The conversion factor is $A^* = 1/(W_1 x_1)$, where W_1 is the molecular weight of the solvent (kg/mole) and x_1 is the mole fraction of the solvent (moles solvent/total moles).

Equations (18) and (19) are valid for non-electrolytes in solution. When the solute is an electrolyte, there is additional complexity due to the dissociation of the electrolytes into ions, screening of charges, etc. To account for this, an additional fitting parameter is used in the single-solute osmotic virial equation, the dissociation constant, k_{diss}.

$$\pi = k_{diss} m_i + B_i\left(k_{diss} m_i\right)^2 + C_i\left(k_{diss} m_i\right)^3 + ... \qquad (20)$$

This parameter accounts for the additional non-ideality of the solution behaviour from several electrolyte effects meaning that the “dissociation constant” may not be exactly equal to two, even for electrolytes known to completely dissociate [14]. For electrolytes, the single-solute osmotic virial equation is written in terms of mole fraction as:

$$\pi = A^*\left(k^*_{diss}x_i + B^*_i\left(k^*_{diss}x_i\right)^2 + C^*_i\left(k^*_{diss}x_i\right)^3 + ...\right) \qquad (21)$$

where k^*_{diss} is the dissociation constant for use with mole fraction.

Others have suggested that using the dissociation constant to account for the electrolyte solution behaviour is not adequate and that a more complicated solution theory, such as the Pitzer equation for electrolytes [34; 35], is required to describe electrolyte solutions. However, Prickett *et al*. [38] have recently shown that using the dissociation constant in the single-solute osmotic virial equation works as well as using the Pitzer equation for single solutions of NaCl, as well as in the multisolute osmotic virial equation for multisolute solutions containing a CPA and NaCl.

Mixing rules for the multisolute osmotic virial equation have been derived from thermodynamic first principles, allowing for predictions of multisolute solution behaviour using only single-solute data [9]. Any solution theory can be used to predict osmolality of solutions for which multisolute data is available. However, in the absence of multisolute data, the multisolute osmotic virial equation is the only solution theory based on thermodynamic principles that can make accurate predictions of non-ideal multisolute solution behaviour using only single-solute data.

The multisolute osmotic virial equation was shown to be accurate over a wide range of concentrations for a variety of aqueous solutions, including two small molecules, a protein plus an ideal solute, two proteins, and a small molecule plus an electrolyte [9; 38; 44]. The form of the multisolute osmotic virial equation we proposed is [9]:

$$\pi = \sum_i m_i + \sum_i \sum_j \frac{\left(B_i + B_j\right)}{2} m_i m_j + \sum_i \sum_j \sum_k \left(C_i C_j C_k\right)^{1/3} m_i m_j m_k \qquad (22)$$

where the subscripts *i, j,* and *k* refer to the individual solutes.

It should be noted that for electrolytes, the molality of the electrolyte should be multiplied by the dissociation constant in equation (22). Equation (22) can be used to predict multisolute solution behaviour based only on single-solute solution information.

For two non-electrolyte solutes, equation (22) is:

$$\begin{aligned}\pi = {} & m_2 + m_3 + B_2 m_2^2 + B_3 m_3^2 + \left(B_2 + B_3\right) m_2 m_3 + C_2 m_2^3 + C_3 m_3^3 \\ & + \left(C_2^2 C_3\right)^{1/3} m_2^2 m_3 + \left(C_2 C_3^2\right)^{1/3} m_2 m_3^2\end{aligned} \qquad (23)$$

where subscript 2 refers to the first solute and subscript 3 refers to the second solute (the subscript 1 is usually reserved for the solvent).

With one electrolyte solute and one non-electrolyte solute, equation (22) is:

$$\begin{aligned}\pi = {} & k_{diss} m_2 + m_3 + B_2 \left(k_{diss} m_2\right)^2 + B_3 m_3^2 + \left(B_2 + B_3\right) k_{diss} m_2 m_3 \\ & + C_2 \left(k_{diss} m_2\right)^3 + C_3 m_3^3 + \left(C_2^2 C_3\right)^{1/3} \left(k_{diss} m_2\right)^2 m_3 + \left(C_2 C_3^2\right)^{1/3} k_{diss} m_2 m_3^2\end{aligned} \qquad (24)$$

The multisolute osmotic virial equation can be used to make predictions of solution behaviour for a wide range of solutes using only single-solute data. Depending on the type of solute and the units of concentration, a form of the single-solute osmotic virial equation, equations (18) through (21), should be fit to the single-solute data to obtain the osmotic virial coefficients. Using those coefficients, the multisolute osmotic virial equation, equation 22, can be used to predict the solution behaviour for any combination of solutes.

Fitting the single-solute osmotic virial equation to data

Phase diagrams were obtained from the literature for various single-solute solutions. The phase diagrams were either given as freezing point depression as a function of solute concentration or as osmolality as a function of solute concentration. Various experimental methods were used to measure the phase diagrams, including freezing point depression measurements [9; 28], differential thermal analysis (DTA) or differential scanning calorimetry (DSC) [3; 4; 12; 15; 39], or membrane osmometry [5; 41; 46]. It should be noted that measuring the phase diagram of viscous solutions using freezing point depression measurements, DTA, or DSC can result in inaccurate results due to the viscosity of the solution slowing the ice crystal growth and the release of latent heat, particularly at high concentrations.

In order to fit the data to the single-solute osmotic virial equation, the freezing point data were converted to osmolality using the nonlinear conversion, equation (2). To obtain the osmotic virial coefficients for each solute, the single-solute osmotic virial equation using

concentration units of molality (equation (18) or (20)) or mole fraction (equation (19) or (21)) was fit to the single-solute osmolality as a function of concentration data by minimizing the sum of squared errors (SSE).

$$SSE = \sum_{m} (y_i - f_i)^2 \qquad (25)$$

where y_i is the value of the ith data point, f_i is the value calculated from the osmotic virial equation at the ith data point, and *m* is the number of data points. The sum of squared errors was minimized using the SOLVER function in Excel (Microsoft, Redmond, WA, USA). The sum of squared errors can also be minimized using a matrix approach (see Appendix B), but the results are the same as using SOLVER in Excel.

The coefficients in the single-solute osmotic virial equation can be derived directly from knowledge of the interactions between solute molecules [36]. The second virial coefficient comes from interactions between two solute molecules; the third virial coefficient comes from interactions between three solute molecules, and so on. Because of this physical basis, only a small number of terms are needed in the single-solute osmotic virial equation to accurately capture the solution behaviour of a wide range of solutes. To determine which order of polynomial adequately fits the single-solute data, increasing orders of the single-solute osmotic virial equation (starting with linear) were used for each solute and the adjusted R^2 parameter was calculated for each order of polynomial. The adjusted R^2 is a measure of the goodness of fit of an equation to a data set, which also takes into account the number of parameters in the

fitted equation. The standard R^2, often used to determine goodness of fit, does not take into account the number of parameters in the model and may erroneously increase with increasing number of parameters in the equation. The adjusted R^2 was used to assess the necessity of adding additional parameters to the model [6].

$$adjusted\ R^2 = 1 - \frac{VAR_E}{VAR_T} \tag{26}$$

where estimates of the variances of the errors, VAR_E, and the observations, VAR_T, are defined as:

$$VAR_E = \frac{SSE}{m-n} = \frac{\sum_m (y_i - f_i)^2}{m-n}$$
$$VAR_T = \frac{TSS}{m-1} = \frac{\sum_m (y_i - \bar{y})^2}{m-1} \tag{27}$$

where $\bar{y}$ is the average of all of the data points and *n* is the number of parameters in the model. SSE is the sum of squared errors, also called the residual sum of squares, and TSS is the total sum of squares.

Increasing orders of polynomial were used until the adjusted R^2 parameter either decreased or remained constant to the third significant figure (i.e. less than a 1% improvement was achieved by adding another parameter). For the fitted equations from which the osmotic virial coefficients were calculated, the adjusted R^2 values were ≥0.99 for the fits in molality and ≥0.96 for the fits in mole fraction.

The 95% confidence intervals (α = 0.05) were also calculated for the osmotic virial coefficients. In the osmotic virial equation, there is no linear coefficient obtained when the data is fit to the equation, so the quadratic coefficient (the second virial coefficient) is the first regression coefficient , the cubic coefficient is the second regression coefficient, etc. The confidence intervals were calculated using the following formula:

$$100(1-\alpha)\%CI = t_{\alpha/2,m-n}\hat{\sigma}\sqrt{\left(J^T J\right)_{jj}^{-1}} \tag{28}$$

where $t_{\alpha/2,m-n}$ is the Student's t-test value at a significance of α/2 and m-n degrees of freedom; $\hat{\sigma}$ is the model standard deviation, and J is the Jacobian matrix. The subscript *j* refers to the order of the regression coefficient (i.e. for the second virial coefficient (first regression coefficient), *j* = 1). The model standard deviation is calculated by:

$$\hat{\sigma} = \sqrt{\frac{SSE}{m-n}} \tag{29}$$

The Jacobian matrix is the derivative of the regression equation (i.e. the single-solute osmotic virial equation) with respect to each regression coefficient (i.e. each osmotic virial coefficient). When the single-solute osmotic virial equation, for a non-electrolyte solute (solute *i*), is truncated after the cubic term, the Jacobian matrix would be:

$$J = \left[\frac{d\pi}{dB_i}; \frac{d\pi}{dC_i}\right] \tag{30}$$

where $\frac{d\pi}{dB_i} = m_i^2$

$\frac{d\pi}{dC_i} = m_i^3$

The m_i is the molality of the solute *i*. These derivatives would be evaluated at each data point to generate the Jacobian matrix.

Results

Single-solute phase diagrams for many solutes were obtained from the literature. The data for freezing point depression as a function of concentration were converted to osmolality as a function of concentration using equation (3). For non-electrolyte solutions, the data were fit to the single-solute osmotic virial equation in concentration units of molality (equation (18)) and mole fraction (equation (19)). The data for electrolytes were fit to equations (20) and (21). The single-solute osmotic virial equation fits are shown on Figures 2 – 6. The solutes have been grouped by type of molecules (i.e. electrolytes, common CPAs, sugars, alcohols, and macromolecules). The osmotic virial coefficients for use with solute molality are listed in Table 2a and for use with solute mole fraction in Table 2b. Tables 2a and 2b also contain the concentration ranges that were used to fit for the coefficients of each solute and the solubility limits for the electrolytes and sugars [28; 42].

The previously reported osmotic virial coefficients for Me_2SO_4, glycerol, BSA, and OVL [9] have been updated since the freezing point depression data have now been converted to osmolality using the nonlinear conversion, equation (2). In addition, the

adjusted R^2 criterion (equation (26)) was applied in order to determine the lowest order polynomial order that adequately fits the single-solute data. These new coefficients were used to predict the ternary solutions of Me_2SO_4 + glycerol + water and BSA + OVL + water. Results in Figures 7 and 8 demonstrate that the osmotic virial equation with the updated coefficients results in accurate predictions for both ternary solutions. Using the adjusted R^2 criterion, the single-solute OVL data are adequately represented using a quadratic polynomial, rather than the cubic polynomial previously used [9], and these new coefficients result in improved predictions from the multisolute osmotic virial equation.

Solution theories that allow predictions of multisolute solution behaviour using single-solute solution data are (i) ideal, dilute solution theory, (ii) adding osmolalities (or freezing point depressions), and (iii) the osmotic virial equation. In order to assess which solution theory provides the most accurate predictions of solution osmolality, the errors in the predictions from each solution theory were quantified. The percent error was calculated using:

$$\%error = \frac{|Prediction - Measured|}{Measured} \times 100 \qquad (31)$$

The errors in the predictions of osmolality for each solution theory for a range of solutions, calculated for the maximum measured total solute molality, are listed in Table 3. In addition to determining the percent error at the maximum molality, the sum of squared errors (SSE) was calculated using equation (25) to assess how accurately each solution theory predicted the measured data points over the entire range of the

solute concentration. Since the SSE is a summation over all of the data points, the value obtained depends on the number of data points. Since each multisolute solution has a different number of data points, the values of the SSE should only be used to compare between solution theories for a specific solution. The SSE for each solution theory are also listed in Table 3. For these comparisons, the coefficients from Table 2a were used for both the adding osmolalities approach and the osmotic virial equation approach. From Table 3, it can be seen that the predictions of the multisolute solution osmolality from the multisolute osmotic virial equation result in smaller errors than the practice of adding osmolalities or assuming ideal, dilute solution, with the exception of the glycerol + NaCl + water solution. The predictions from adding osmolalities of the glycerol + NaCl + water solution osmolality resulted in the smallest error. The errors in the predictions of multisolute solution osmolality from multisolute osmotic virial equation are significantly smaller for the very non-ideal solutions which contain two CPA molecules (glycerol + Me_2SO_4 + water) or macromolecules (Hb + ideal + water and BSA + OVL + water).

When utilizing the practice of adding freezing point depressions, Kleinhans and Mazur [20] used a slightly different approach for fitting for the single-solute coefficients and they compared their predictions to the predictions from Pegg's fitting equations [31; 32]. It is important to compare the errors in the predictions from the multisolute osmotic virial equation (utilizing the coefficients in Table 2a) with the errors in the predictions from the Kleinhans and Mazur approach (utilizing the coefficients in their study). The calculated percent error, calculated at the approximate maximum solute molality shown for each

solution in the Kleinhans and Mazur study [20], and the SSE are shown in Table 4 for solutions containing Me_2SO_4 + NaCl + water and glycerol + NaCl + water. The percent errors in Table 4 are calculated using equation (31), substituting the values from Pegg's fitting equations for measured data. The SSE shown in Table 4 are calculated using equation (25), substituting values from Pegg's fitting equations for measured data. The squared error (i.e. squaring the difference between the value from Pegg's fitting equation and the prediction) was calculated at 5% increments of total solute weight percent over the concentration range shown in Kleinhans and Mazur's paper for each solution [20]. As mentioned previously, the SSE should only be used to compare the accuracy of each prediction for a specific solution and should not be used to compare different solutions. For solutions containing glycerol + NaCl + water, it can be seen in Table 4 that the predictions from the Kleinhans and Mazur approach results in smaller errors than the predictions from the multisolute osmotic virial equation, although the errors in the predictions are large for both approaches. For the solutions containing Me_2SO_4 + NaCl + water, the errors in the predictions of the solution osmolality from multisolute osmotic virial equation are smaller than the errors in the predictions using the Kleinhans and Mazur approach.

From Tables 3 and 4, it can be seen that, of the three predictive multisolute solution theories, the multisolute osmotic virial equation provides the most accurate predictions for all of the multisolute solutions investigated, except for solutions which contain high concentrations of glycerol. This may be due to the difficulty of accurately measuring the freezing point of the highly viscous glycerol solutions.

Conclusions

The single-solute osmotic virial equation can be fit to a wide range of solute data in molality and mole fraction to obtain the osmotic virial coefficients. One additional fitting parameter, the dissociation constant, is required to capture the solution behaviour of electrolytes. Using only the single-solute osmotic virial coefficients, the osmolality of many multisolute solutions can be predicted using the multisolute osmotic virial equation. The osmotic virial coefficients are provided herein for a range of solutes in water including many common CPAs, electrolytes, sugars, alcohols, and macromolecules for use with solution concentration units of both molality and mole fraction.

The osmotic virial coefficients provided in this study can be used in the multisolute osmotic virial equation to predict the solution behaviour of any combination of the solutes. Thus, in the absence of multisolute solution data, the multisolute osmotic virial equation should be used to predict multisolute solution behaviour. In addition to only requiring single-solute information to make predictions of multisolute solution behaviour, the mixing rules for the multisolute osmotic virial equation can be derived from thermodynamic first principles. The multisolute osmotic virial equation has been shown to be accurate for a wide range of multisolute solutions [9; 38; 44]. When compared to other solution theories which only require single-solute information, such as assuming an ideal and dilute solution or the practice of adding osmolalities, the multisolute osmotic virial equation provides more accurate predictions for all of the solutions

studied, except for solutions of glycerol + NaCl + water containing high concentrations of glycerol (see Tables 3 and 4). Since accurate measurement of the freezing point depression of highly viscous solutions is difficult, this may account for the differences between the predicted and measured values of multisolute solutions containing high concentrations of glycerol. In addition to being less accurate for most non-ideal solutions, the ideal and dilute solution theory and the practice of adding osmolalities contain simplifying assumptions regarding the interactions between the solute molecules which are not thermodynamically correct for non-ideal solutions.

Many other solution theories have been proposed for multisolute solutions of interest in cryobiology. These solution theories have resulted in accurate predictions of multisolute solution behaviour, but only for very specific combinations of solutes. The approach of adding osmolalities, recently utilized by Kleinhans and Mazur [20], has been shown to be accurate for three CPA + NaCl + water solutions, but does not work well for other multisolute solutions, such as aqueous mixtures of two CPAs, a protein and an ideal solute, or two proteins [9]. The osmotic virial equation is more accurate for the more non-ideal mixtures, because all of the solute-solute interactions are taken into account.

We have reviewed the conversions between freezing point depression, osmolality, osmotic coefficient, and activity. Using the linear conversion between freezing point depression and osmolality, equation (4), introduces significant error as the freezing point depression increases as compared to the nonlinear conversion, equation (2).

Specifically, errors of over 7% error at 20 ^{o}C and over 18% error at 50 ^{o}C are introduced by using the linear conversion instead of the nonlinear conversion.

Predictions of multisolute solution behaviour are needed in cryobiology, since both osmolality and freezing point depression play such crucial roles in the cryopreservation process. In addition, since there is such a wide range of solutes present in cryobiological solutions, from proteins to electrolytes to CPAs, the solution behaviour of all of the different solutions of interest cannot be measured. The multisolute osmotic virial equation with the proposed mixing rules is an accurate solution theory based on thermodynamic principles that allows for predictions of multisolute solution behaviour using only single-solute information.

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Appendix A: Relationship between freezing point depression and osmolality

Pure component equations

The Gibbs-Duhem relation for a pure component is [36]:

$$SdT - VdP + nd\mu = 0 \quad \text{(A1)}$$

where S is entropy, T is temperature, V is volume, P is pressure, n is the number of moles, and μ is the chemical potential of the pure component.

The pressure and temperature dependence of the chemical potential are needed. To find the pressure dependence of the chemical potential, the temperature is set to be constant so that equation (A1) gives:

$$-VdP + nd\mu = 0$$
$$d\mu = \frac{V}{n} dP \quad \text{(A2)}$$

where $V/n = \upsilon$, the molar volume. Assuming that the substance is incompressible (υ = constant) equation (A2) can be integrated to give:

$$\mu(T,P) = \mu(T,P_{ref}) + \upsilon(P - P_{ref}) \quad \text{(A3)}$$

To determine the temperature dependence, set the pressure to be constant so that equation (A1) gives:

$$SdT + nd\mu = 0$$
$$d\mu = \frac{-S}{n} dT \quad \text{(A4)}$$

where $S/n = s$, the molar entropy. Assuming that the molar entropy, s, does not depend on temperature, equation (A4) can be integrated to give:

$$\mu(T,P)=\mu(T_{ref},P)+s(T_{ref}-T) \quad \text{(A5)}$$

Substituting equation (A3), evaluated at T_{ref}, for $\mu(T_{ref},P)$ in equation (A5) gives:

$$\mu(T,P)=\mu(T_{ref},P_{ref})+\upsilon(P-P_{ref})+s(T_{ref}-T) \quad \text{(A6)}$$

Realizing that the above derivation was for a pure component, equation (A6) can be written for the pure solvent, water, denoted with subscript 1, in a multicomponent solution.

$$\mu_1^o(T,P)=\mu_1^o(T_{ref},P_{ref})+\overline{\upsilon_1^o}(P-P_{ref})+\overline{s_1^o}(T_{ref}-T) \quad \text{(A7)}$$

where $\overline{\upsilon_1^o}$ is the partial molar volume of water and $\overline{s_1^o}$ is the partial molar entropy of water. The superscript *o* refers to the pure component.

Multicomponent equations

For a multicomponent solution of solvent (subscript 1) and solute (subscript 2):

$$\mu_1(T,P,x_2)=\mu_1^o(T,P)-\overline{\upsilon_1^o}\Pi \quad \text{(A8)}$$

where x_2 is the mole fraction of the solute and Π is the osmotic pressure.

Substituting equation (A7) into (A8) gives:

$$\mu_1(T,P,x_2)=\mu_1^o(T_{ref},P_{ref})+\overline{\upsilon_1^o}(P-P_{ref})+\overline{s_1^o}(T_{ref}-T)-\overline{\upsilon_1^o}\Pi \quad \text{(A9)}$$

At equilibrium (i.e. the freezing point), assuming that curvature effects can be neglected:

$$T^L=T^S=T_{FP} \quad \text{(A10)}$$

$$P^L = P^S = P^R \quad \text{(A11)}$$

$$\mu_1^L = \mu_1^S \quad \text{(A12)}$$

where T^L is the temperature of the liquid, T^S is the temperature of the solid and T_{FP} is the freezing point temperature, P^L is the pressure of the liquid, P^S is the pressure of the solid, P^R is the pressure at which the freezing process is occurring, μ_1^L is the chemical potential of the water in the liquid solution, and μ_1^S is the chemical potential of the pure water in the solid ice.

Substituting equation (A9) into the equilibrium equation (A12) gives:

$$\mu_1^{L^O}\left(T_{ref}, P_{ref}\right) + \overline{\upsilon_1^{L^O}}\left(P^L - P_{ref}\right) + \overline{s_1^{L^O}}\left(T_{ref} - T^L\right) - \overline{\upsilon_1^{L^O}}\Pi = \mu_1^{S^O}\left(T_{ref}, P_{ref}\right) + \overline{\upsilon_1^{S^O}}\left(P^S - P_{ref}\right) + \overline{s_1^{S^O}}\left(T_{ref} - T^S\right) \quad \text{(A13)}$$

Since the freezing process is occurring at constant pressure, set the reference pressure to be P^R and the reference temperature to be the freezing point of the pure solvent, T_{FP}^o. Using this reference point and the other two equilibrium conditions, equations (A10) and (A11), gives:

$$\overline{s_1^{L^O}}\left(T_{FP}^o - T_{FP}\right) - \overline{\upsilon_1^{L^O}}\Pi = \overline{s_1^{S^O}}\left(T_{FP}^o - T_{FP}\right) \quad \text{(A14)}$$

Rearranging and substituting $\Pi = RT\rho_1\pi$ (where $T = T_{FP}$ in this case) into equation (A14) gives:

$$\Delta T_{FP} = T_{FP}^o - T_{FP} = \left[\frac{\overline{\upsilon_1^{L^O}}}{\overline{s_1^{L^O}} - \overline{s_1^{S^O}}}\right] RT_{FP}\rho_1\pi = \left[\frac{W_1}{\overline{s_1^{L^O}} - \overline{s_1^{S^O}}}\right] RT_{FP}\pi \quad \text{(A15)}$$

Appendix B: Different methods to minimize SSE

Linear regression, which is done by minimizing the sum of squared errors (SSE) (equation (25)), can be done multiple ways. The Excel SOLVER approach is perhaps the most straightforward method, but can be time-consuming. Using a matrix approach to solve the linear regression equation allows for quicker determination of coefficients for multiple models (i.e. quadratic, cubic, etc). It also allows for easy determination of the confidence intervals of the coefficients.

The linear regression equation can be generalized as:

$$\vec{y} = \sum_{i=1}^{n} \vec{\beta}_i f_i(\vec{x}) + \varepsilon \qquad \text{(B1)}$$

where $\vec{\beta}_i$ is a vector that contains the regression coefficients, $f_i(\vec{x})$ are the functions of the variable x that are multiplied by the regression coefficients, $\underline{\varepsilon}$ is a vector of the errors in the prediction, and n is the number of regression coefficients. The vector, $\vec{y}$, contains the values of the dependent variable for each data point.

$$\vec{y} = \begin{pmatrix} y_1 \\ y_2 \\ \vdots \\ y_m \end{pmatrix}$$

The individual functions, f_i, are known and $\vec{\beta}$ are the unknown coefficients. The $\underline{\underline{A}}$ matrix is defined as follows:

$$\underline{\underline{A}} = \begin{bmatrix} f_1(\vec{x}_1) & f_2(\vec{x}_1) & f_3(\vec{x}_1) & \cdots & f_n(\vec{x}_1) \\ f_1(\vec{x}_2) & f_2(\vec{x}_2) & f_3(\vec{x}_2) & \cdots & f_n(\vec{x}_2) \\ \vdots & & & \ddots & \\ f_1(\vec{x}_m) & f_2(\vec{x}_m) & f_3(\vec{x}_m) & \cdots & f_n(\vec{x}_m) \end{bmatrix}$$

where m is the number of data points.

To solve for $\vec{\beta}$, the following equation can be used [6]:

$$\vec{\beta} = \left(\underline{\underline{A^T}}\,\underline{\underline{A}}\right)^{-1} \underline{\underline{A^T}}\,\vec{y} \qquad \text{(B2)}$$

where $\underline{\underline{A^T}}$ is the transpose of the $\underline{\underline{A}}$ matrix and the superscript *-1* indicates the inverse of the matrix. This gives a vector of the form:

$\vec{\beta} =$

β_1
β_2
β_3
...
β_n

where β_1 is the first regression coefficient, β_2 is the second regression coefficient, β_3 is the third regression coefficient, etc.

To determine the confidence intervals of the regression coefficients, the model standard deviation, $\hat{\sigma}$, is needed:

$$\hat{\sigma} = \sqrt{\frac{SSE}{m-n}} = \sqrt{\frac{\vec{y}^T\vec{y} - \vec{\beta}A^T\vec{y}}{m-n}} \qquad \text{(B3)}$$

The confidence intervals can be found using the following formula:

$$\beta_i \pm t_{\alpha/2,m-n}\hat{\sigma}\sqrt{\left(\underline{\underline{A}}^T\underline{\underline{A}}\right)^{-1}_{ii}} \quad \text{(B4)}$$

where β_i is the ith regression coefficient (i = 1 to n) and $t_{\alpha/2,m-n}$ is the Student's t-test value at a significance of α/2 and (m-n) degrees of freedom.

The single-solute osmotic virial equation does not have a linear coefficient, so the equation can be re-arranged so that it is the form: $y=\beta_1 x+\beta_2 x^2+\beta_3 x^3+....$ This can be done by dividing both sides of the equation by the concentration, and subtracting one. Thus, the osmotic virial equation for non-electrolytes in molality (18) becomes:

$$\frac{\pi}{m_i}-1=B_i m_i+C_i m_i^2+... \quad \text{(B5)}$$

Using MatLab (MathWorks, Natick, MA), the osmotic virial coefficients were determined for each solute. To obtain the coefficients for use in molality for non-electrolyte solutes matrices were set up in the following manner:

$\vec{y}=$

π/m_i-1(1)
π/m_i-1(2)
π/m_i-1 (3)
...
π/m_i-1 (m)

where the number in parenthesis indicates the rank order of the data point (i.e. (1) indicates that this is the first data point, (2) is the second data point, etc).

$\underline{\underline{A}}$ =

m_i (1)	m_i^2 (1)	m_i^3 (1)	…	m_i^n (1)
m_i (2)	m_i^2 (2)	m_i^3 (2)	…	m_i^n (2)
m_i (3)	m_i^2 (3)	m_i^3 (3)	…	m_i^n (3)
...	…	…	…	…
m_i (m)	m_i^2 (m)	m_i^3 (m)	…	m_i^n (m)

where m_i is the molality of the solute (to solve for the osmotic virial coefficients in mole fraction, the $\underline{\underline{A}}$ matrix would contain mole fraction (x_i) instead of molality). The number of columns in the $\underline{\underline{A}}$-matrix is determined by the number of regression coefficients that are being fit to the data (i.e. number of columns = n).

For electrolytes, there is a linear term in the osmotic virial equation (k_{diss}), so to solve for the osmotic virial coefficients, the $\vec{y}$ vector contains the osmolality (not $\pi/m_i - 1$). Table B1 contains a summary of the matrix approach for each type of solute.

Using the matrix approach, the coefficients for increasing orders of the osmotic virial equation can be quickly determined by simply adding additional columns to the $\underline{\underline{A}}$ matrix (containing increasing orders of the solute concentration) and using equation (B2) to

obtain the $\underline{\underline{A}}$ matrix. The osmotic virial coefficients can be determined from the values in the $\underline{\underline{A}}$ matrix, depending on the type of solute and concentration units, using the methods outlined in Table B1.

It was found that using the matrix method or the SOLVER function in Excel to minimize the sum of squared errors gave the same results for the osmotic virial coefficients.

Figure Captions

Figure 1. Osmolality determined from the freezing point using either the linear conversion (Eq. (4)) or the nonlinear conversion (Eq. (2)).

Figure 2. Osmolality of single-solute aqueous CPA solutions as a function of (a) solute molality and (b) solute mole fraction. The Me_2SO_4 data are from Rasmussen and Mackenzie [39], Hildebrandt [15], Boutron [3], and our lab [9]. The glycerol data are from the CRC tables [42], Boutron [3], Melinder [27], and our lab [9]. The propylene glycol (PG) data are from the CRC tables [42], Boutron [4], and Melinder [27]. The ethylene glycol (EG) data are from the CRC tables [42]. Eq. (18) was fit to the data in molality and eq. (19) was fit to the data in mole fraction in order to obtain the osmotic virial coefficients for each solute. The dashed line is for an ideal, dilute solute (π = m). The ideal, dilute line is not linear in the mole fraction graphs due to the nonlinear conversion between mole fraction and molality.

Figure 3. Osmolality of single-solute aqueous electrolyte solutions as a function of (a) solute molality and (b) solute mole fraction. The NaCl and KCl data are from the CRC tables [42]. Eq. (20) was fit to the data in molality and eq. (21) was fit to the data in mole fraction in order to obtain the dissociation constant and the osmotic virial coefficients for each solute. The dashed line is for an ideal, dilute solute (π = m). The ideal, dilute line is not linear in the mole fraction graphs due to the nonlinear conversion between mole fraction and molality.

Figure 4. Osmolality of single-solute aqueous alcohol solutions as a function of (a) solute molality and (b) solute mole fraction. The methanol and ethanol data are from the CRC tables [42]. Eq. (18) was fit to the data in molality and eq. (19) was fit to the data in mole fraction in order to obtain the osmotic virial coefficients for each solute. The dashed line is for an ideal, dilute solute ($\pi = m$). The ideal, dilute line is not linear in the mole fraction graphs due to the nonlinear conversion between mole fraction and molality.

Figure 5. Osmolality of single-solute aqueous sugar solutions as a function of (a) solute molality and (b) solute mole fraction. The sucrose, dextrose, and mannitol data are from the CRC tables [42]. The data for trehalose is from Miller et al. [28]. Eq. (18) was fit to the data in molality and eq. (19) was fit to the data in mole fraction in order to obtain the osmotic virial coefficients for each solute. The dashed line is for an ideal, dilute solute ($\pi = m$). The ideal, dilute line is not linear in the mole fraction graphs due to the nonlinear conversion between mole fraction and molality.

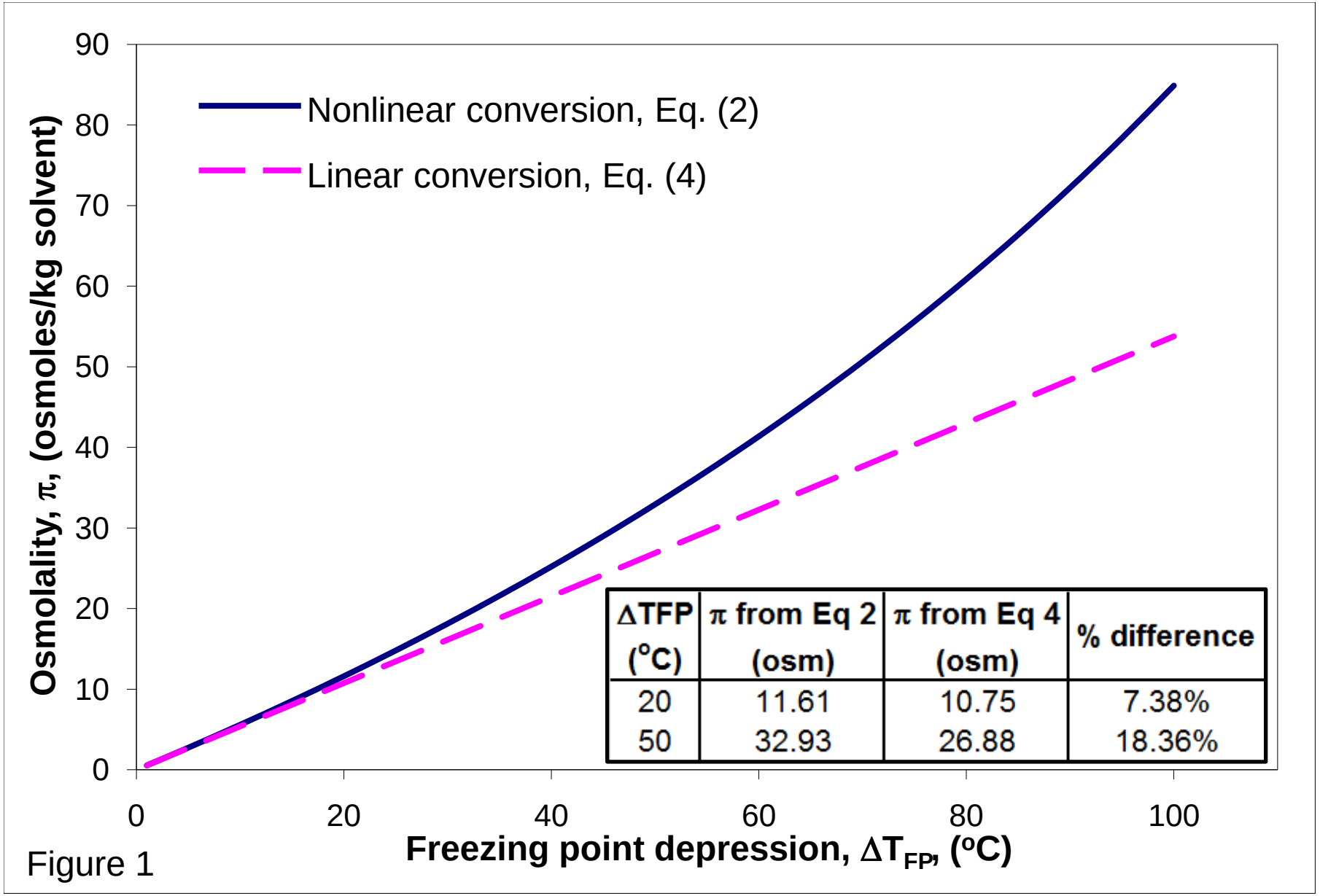
Figure 6. Osmolality of single-solute aqueous macromolecule solutions as a function of (a) solute molality and (b) solute mole fraction. The hemoglobin (Hb) data are Adair's data published by Dick [5]. The bovine serum albumin (BSA) data are from Vilker et al. [41]. The ovalbumin (OVL) data are from Yousef et al. [46]. Eq. (18) was fit to the data in molality and eq. (19) was fit to the data in mole fraction in order to obtain the osmotic virial coefficients for each solute. The dashed line is for an ideal, dilute solute ($\pi = m$).

The ideal, dilute line is not linear in the mole fraction graphs due to the nonlinear conversion between mole fraction and molality.

Figure 7. Osmolality of a Me_2SO_4 + glycerol + water solution as a function of total solute molality for (a) R = 0.5 and (b) R = 2.0; where R = mass glycerol/mass Me_2SO_4. The diamonds are our experimental measurements [9], which have now been converted from freezing point depression to osmolality using the nonlinear conversion, equation (2). The solid line is the prediction from the multisolute osmotic virial equation (Eq. (23)). The long-dashed line is the prediction from adding osmolalities and the short-dashed line is the prediction from assuming an ideal, dilute solution ($\pi = m_{Me2SO4} + m_{glycerol}$)

Figure 8. Osmolality of a BSA + OVL + water solution as a function of total solute molality for R = 1.5, where R = mass BSA/mass OVL. The diamonds are experimental measurements from Yousef et al. [47]. The solid line is the prediction from the multisolute osmotic virial equation (Eq. (23)). The long-dashed line is the prediction from adding osmolalities and the short-dashed line is the prediction from assuming an ideal, dilute solution ($\pi = m_{BSA} + m_{OVL}$). The long-and-short-dashed line is a model from the literature [47].

Figure(s)



ΔTFP (ºC)	π from Eq 2 (osm)	π from Eq 4 (osm)	% difference
20	11.61	10.75	7.38%
50	32.93	26.88	18.36%

Figure 1

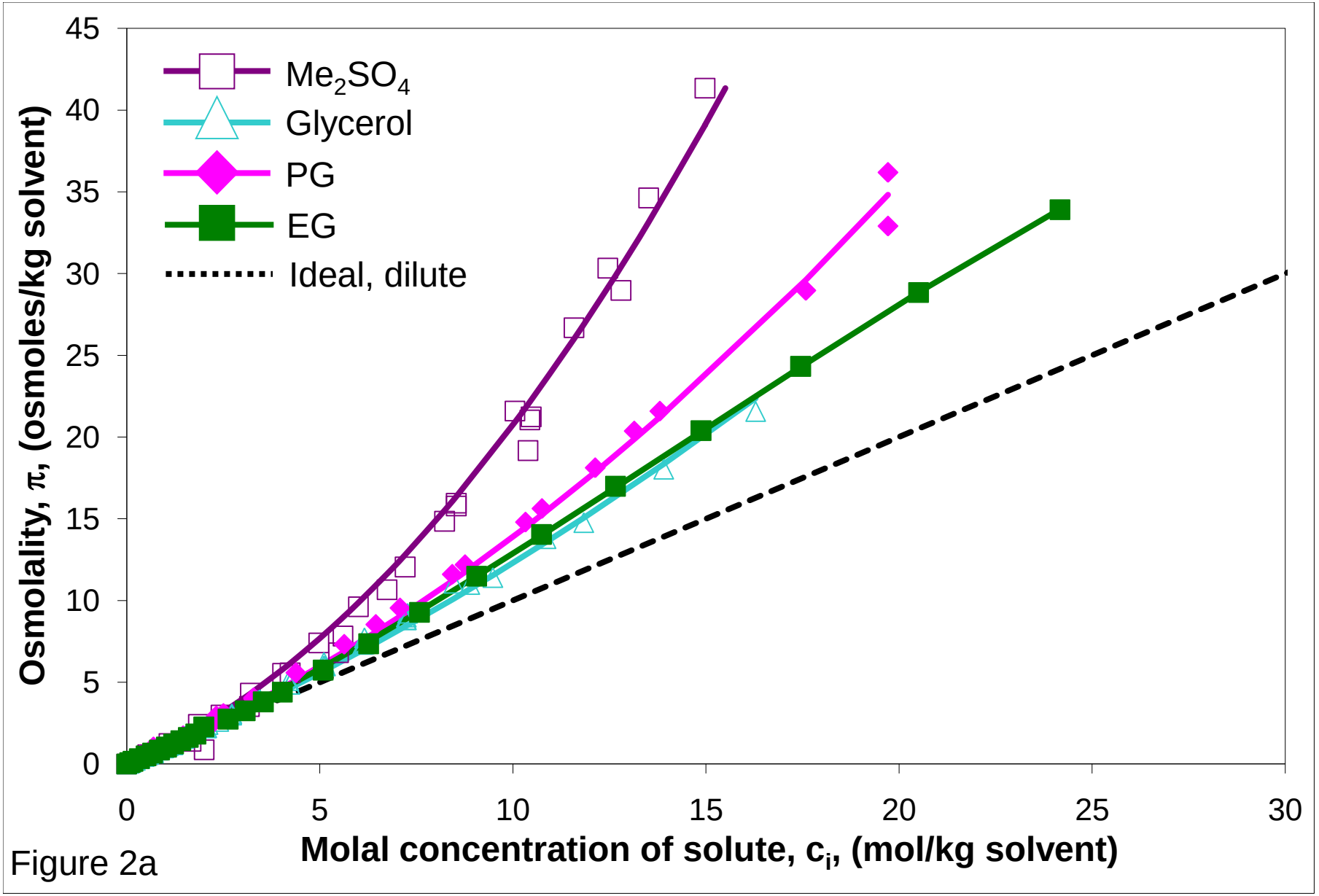

45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
Osmolality, π, (osmoles/kg solvent)
Molal concentration of solute, c_i, (mol/kg solvent)
Me_2SO_4
Glycerol
PG
EG
Ideal, dilute

Figure 2a

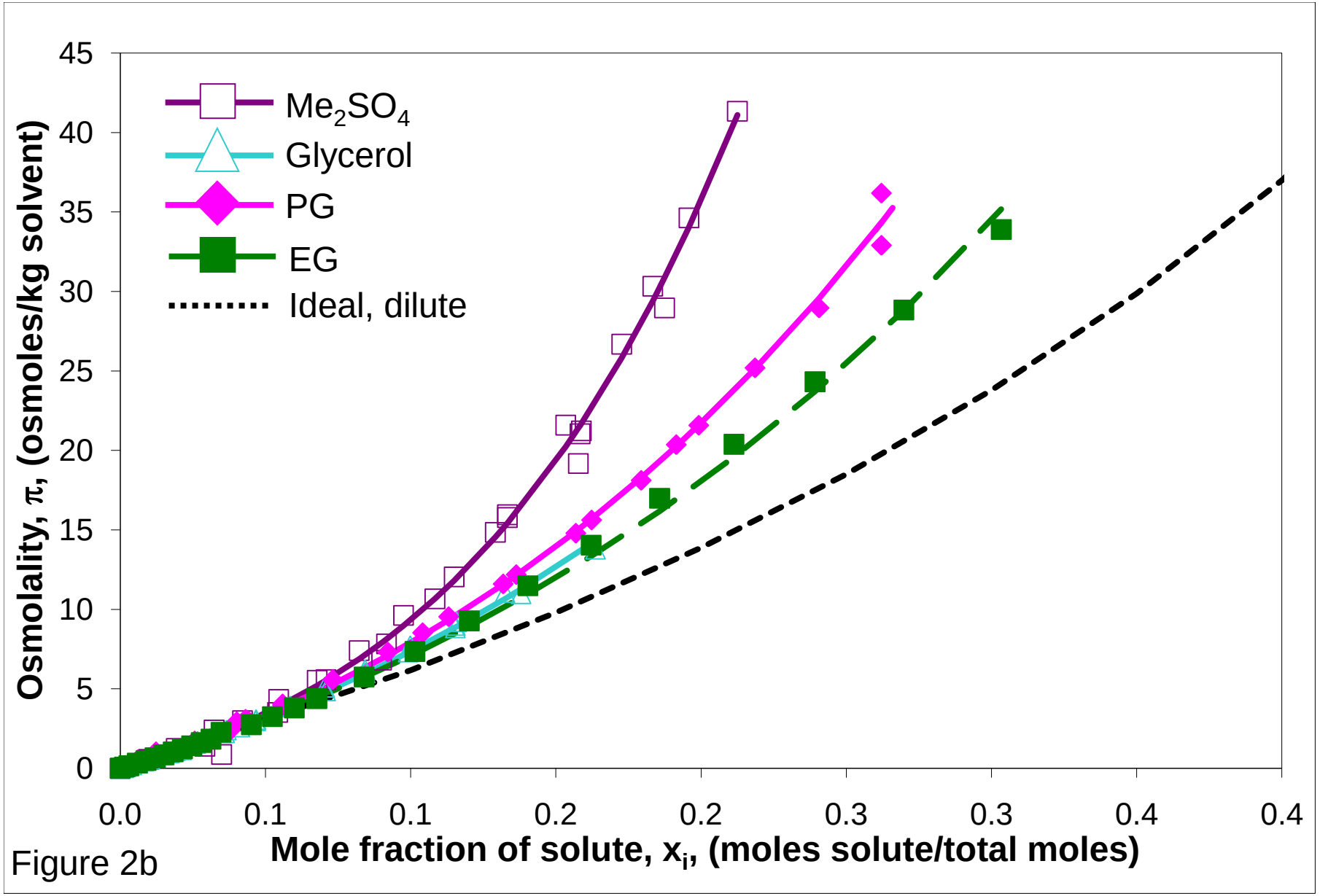

45
40
35
30
25
20
15
10
5
0
Osmolality, π, (osmoles/kg solvent)
Me2SO4
Glycerol
PG
EG
Ideal, dilute
0.0
0.1
0.1
0.2
0.2
0.3
0.3
0.4
0.4
Mole fraction of solute, xi, (moles solute/total moles)

Figure 2b

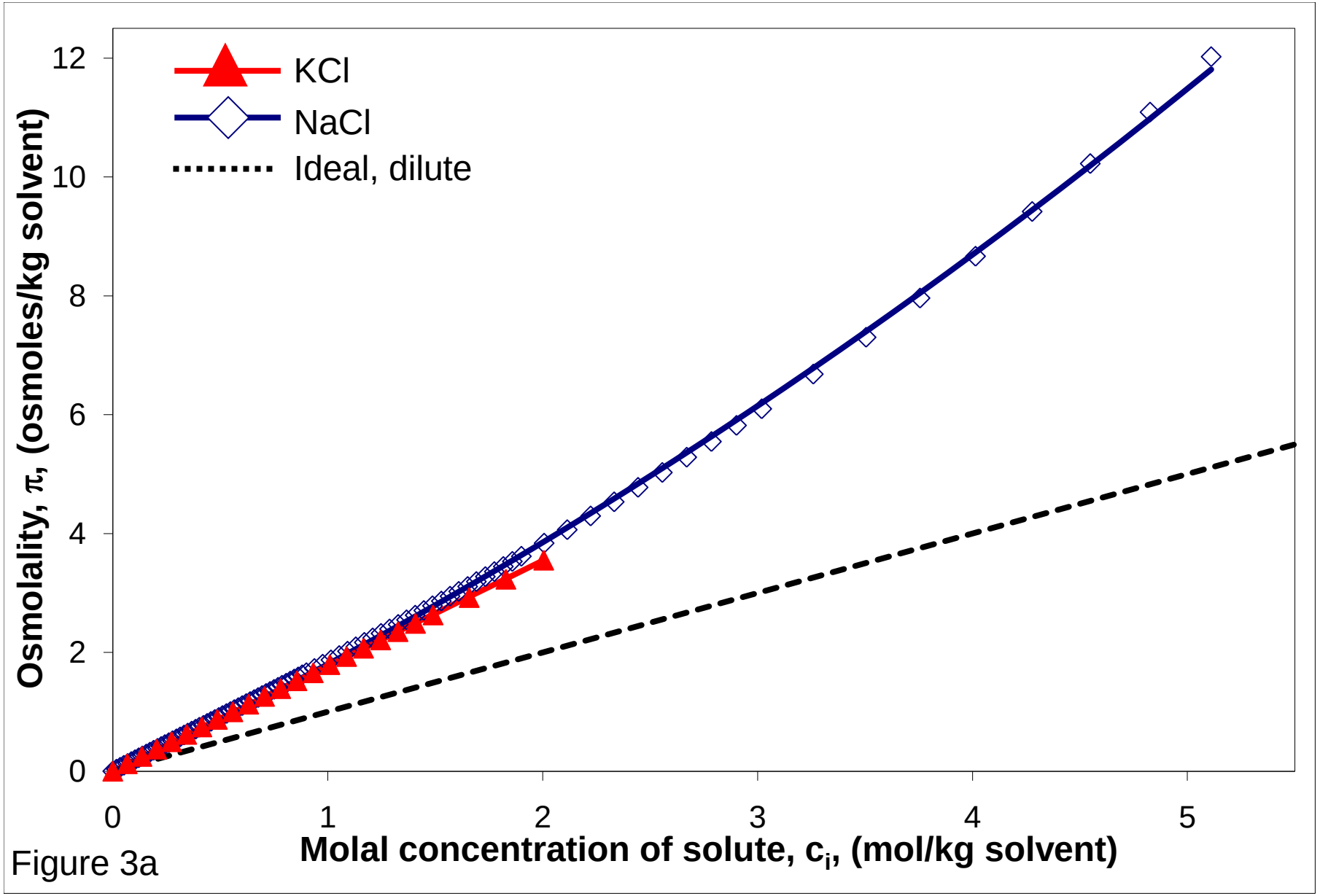

KCl
NaCl
Ideal, dilute
Osmolality, π, (osmoles/kg solvent)
Molal concentration of solute, c_i, (mol/kg solvent)
0
2
4
6
8
10
12
0
1
2
3
4
5

Figure 3a

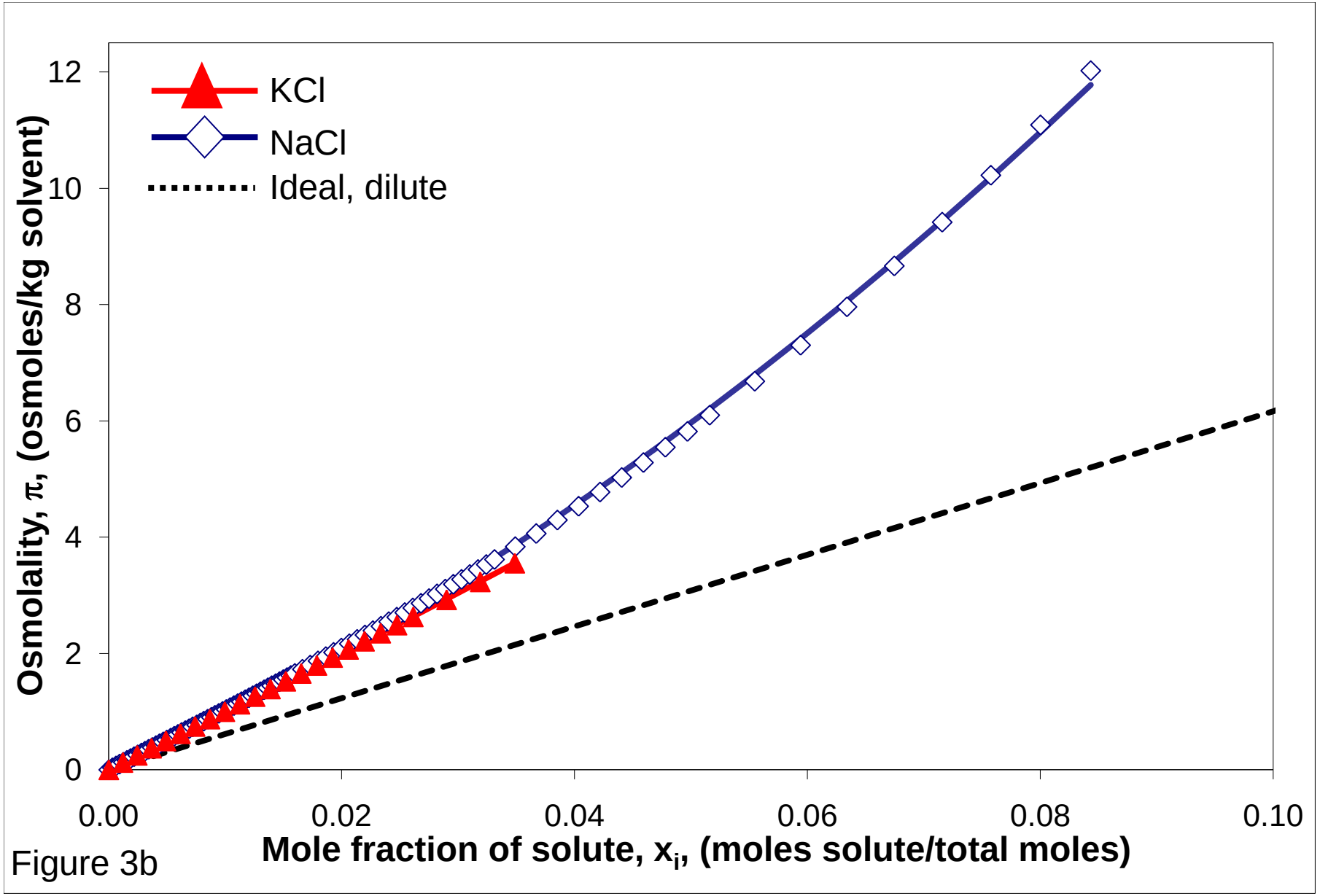

KCl
NaCl
Ideal, dilute
Osmolality, π, (osmoles/kg solvent)
0
2
4
6
8
10
12
0.00
0.02
0.04
0.06
0.08
0.10
Mole fraction of solute, x_i, (moles solute/total moles)

Figure 3b

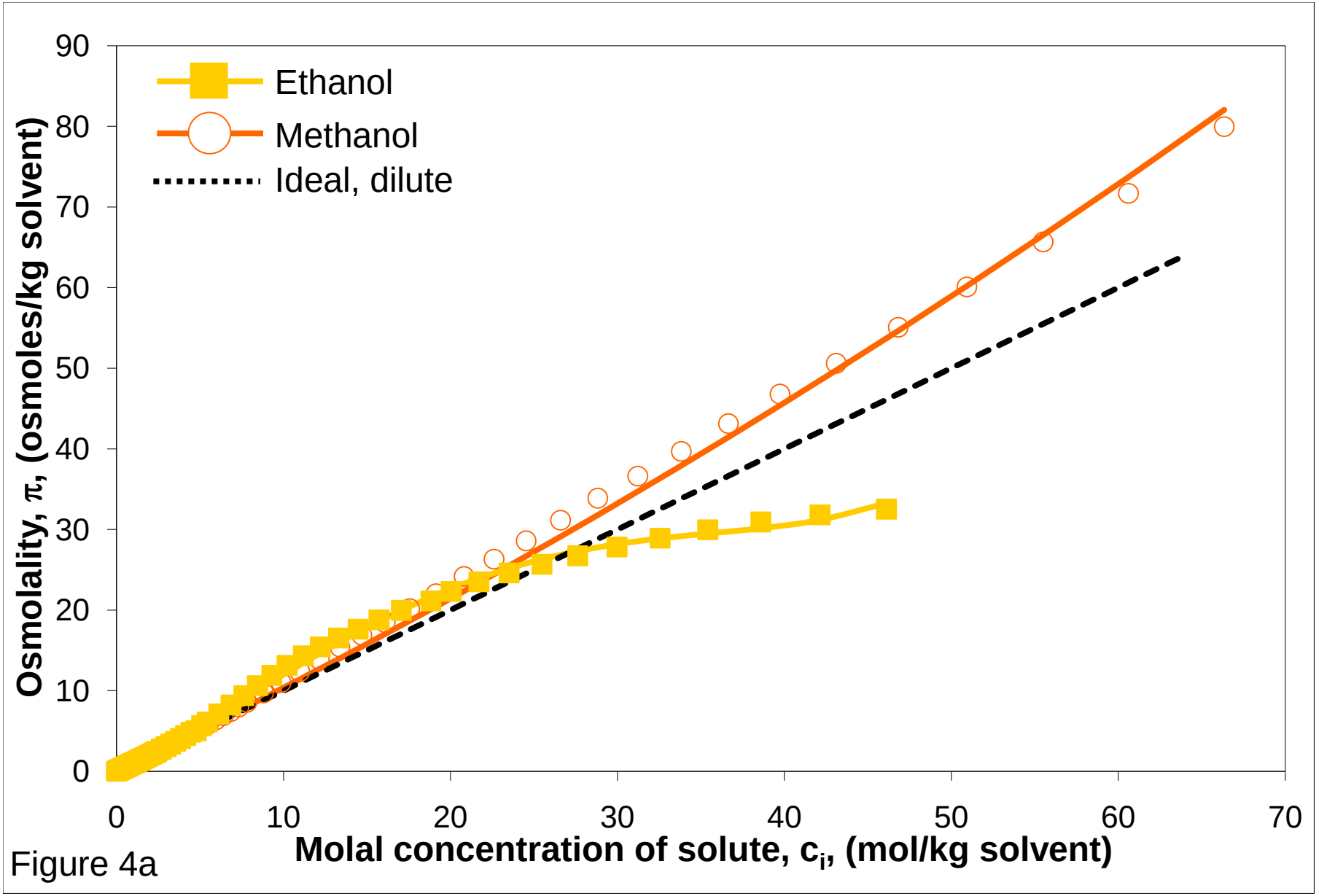

Ethanol
Methanol
Ideal, dilute
Osmolality, π, (osmoles/kg solvent)
Molal concentration of solute, c_i, (mol/kg solvent)
0
10
20
30
40
50
60
70
80
90

Figure 4a

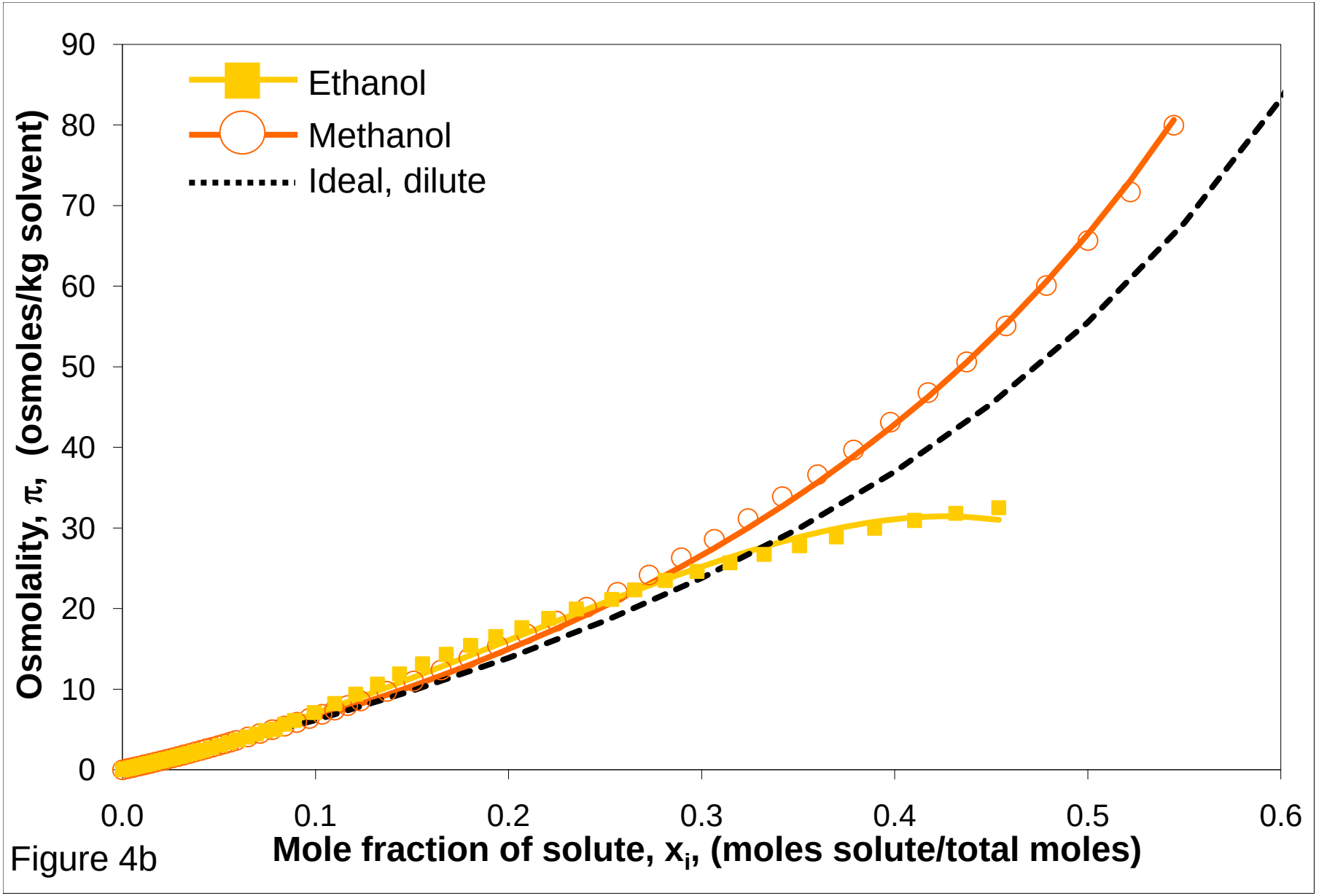

Ethanol
Methanol
Ideal, dilute
Osmolality, π, (osmoles/kg solvent)
0
10
20
30
40
50
60
70
80
90
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Mole fraction of solute, x_i, (moles solute/total moles)

Figure 4b

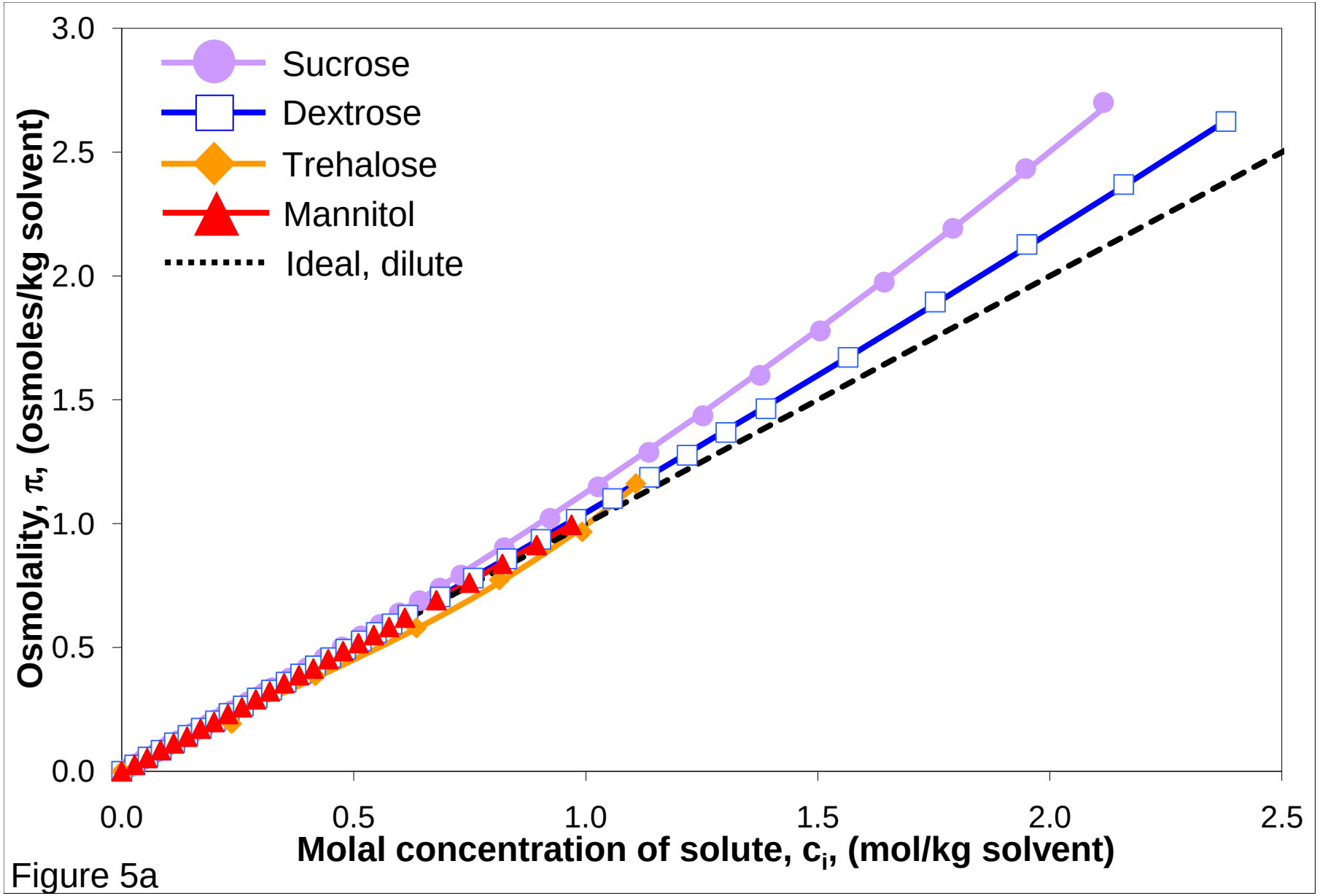

Sucrose
Dextrose
Trehalose
Mannitol
Ideal, dilute
Osmolality, π, (osmoles/kg solvent)
Molal concentration of solute, c_i, (mol/kg solvent)
0.0
0.5
1.0
1.5
2.0
2.5
3.0

Figure 5a

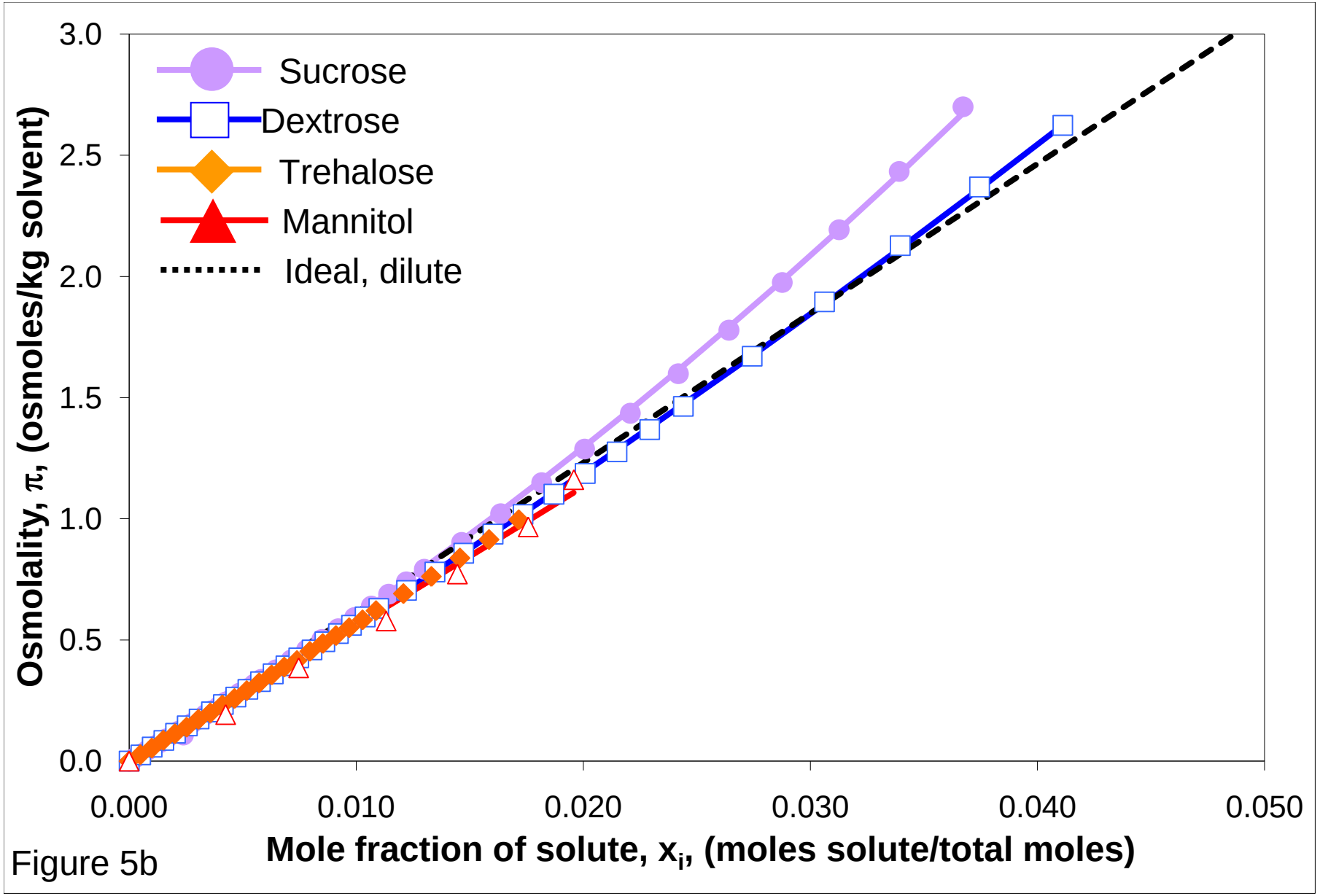

Sucrose
Dextrose
Trehalose
Mannitol
Ideal, dilute
Osmolality, π, (osmoles/kg solvent)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.000
0.010
0.020
0.030
0.040
0.050
Mole fraction of solute, x_i, (moles solute/total moles)

Figure 5b

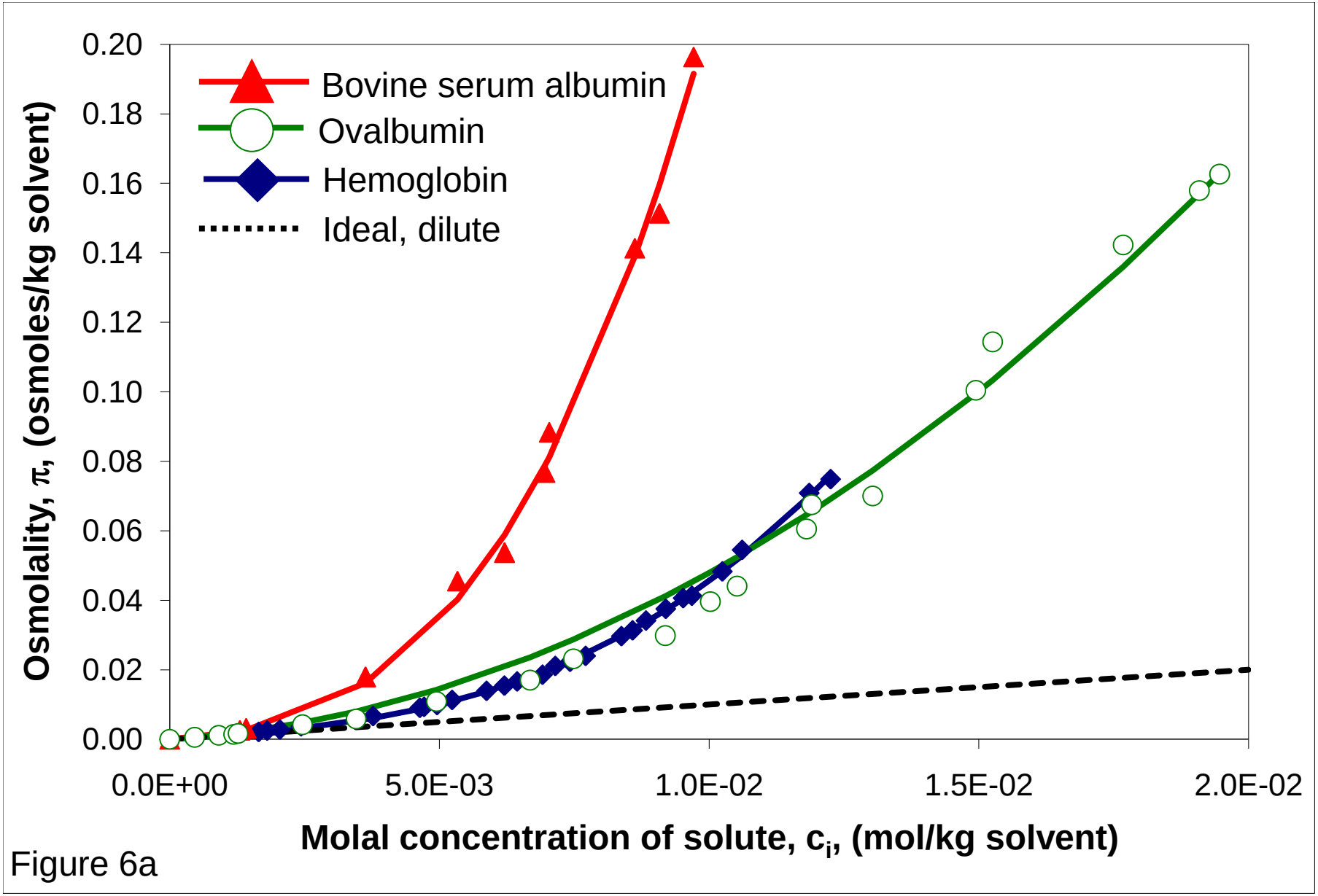

Bovine serum albumin
Ovalbumin
Hemoglobin
Ideal, dilute
Osmolality, π, (osmoles/kg solvent)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.0E+00
5.0E-03
1.0E-02
1.5E-02
2.0E-02
Molal concentration of solute, c_i, (mol/kg solvent)

Figure 6a

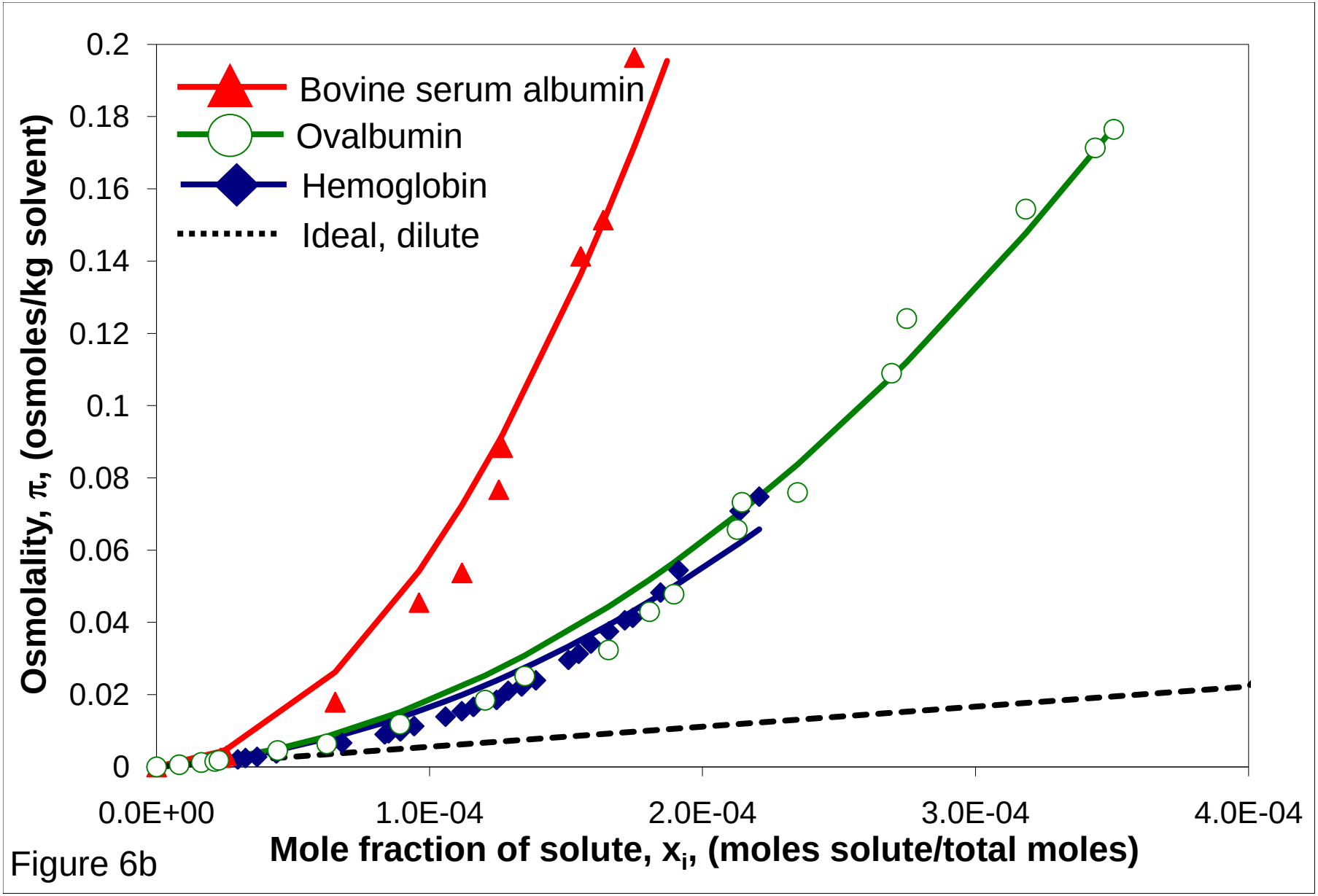

Bovine serum albumin
Ovalbumin
Hemoglobin
Ideal, dilute
Osmolality, π, (osmoles/kg solvent)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0.0E+00
1.0E-04
2.0E-04
3.0E-04
4.0E-04
Mole fraction of solute, x_i, (moles solute/total moles)

Figure 6b

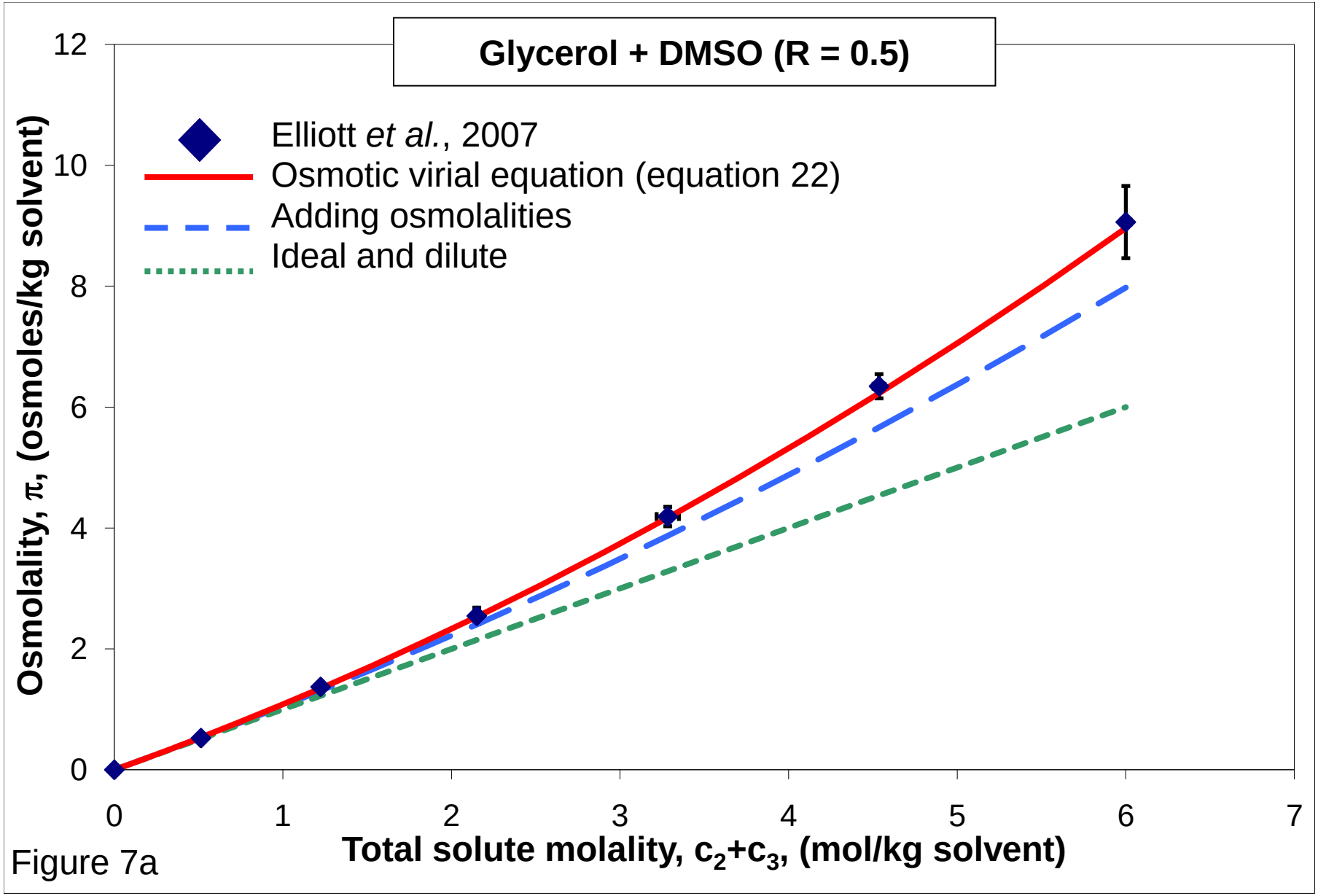

Glycerol + DMSO (R = 0.5)
Elliott et al., 2007
Osmotic virial equation (equation 22)
Adding osmolalities
Ideal and dilute
Osmolality, π, (osmoles/kg solvent)
Total solute molality, c2+c3, (mol/kg solvent)
0
2
4
6
8
10
12
0
1
2
3
4
5
6
7

Figure 7a

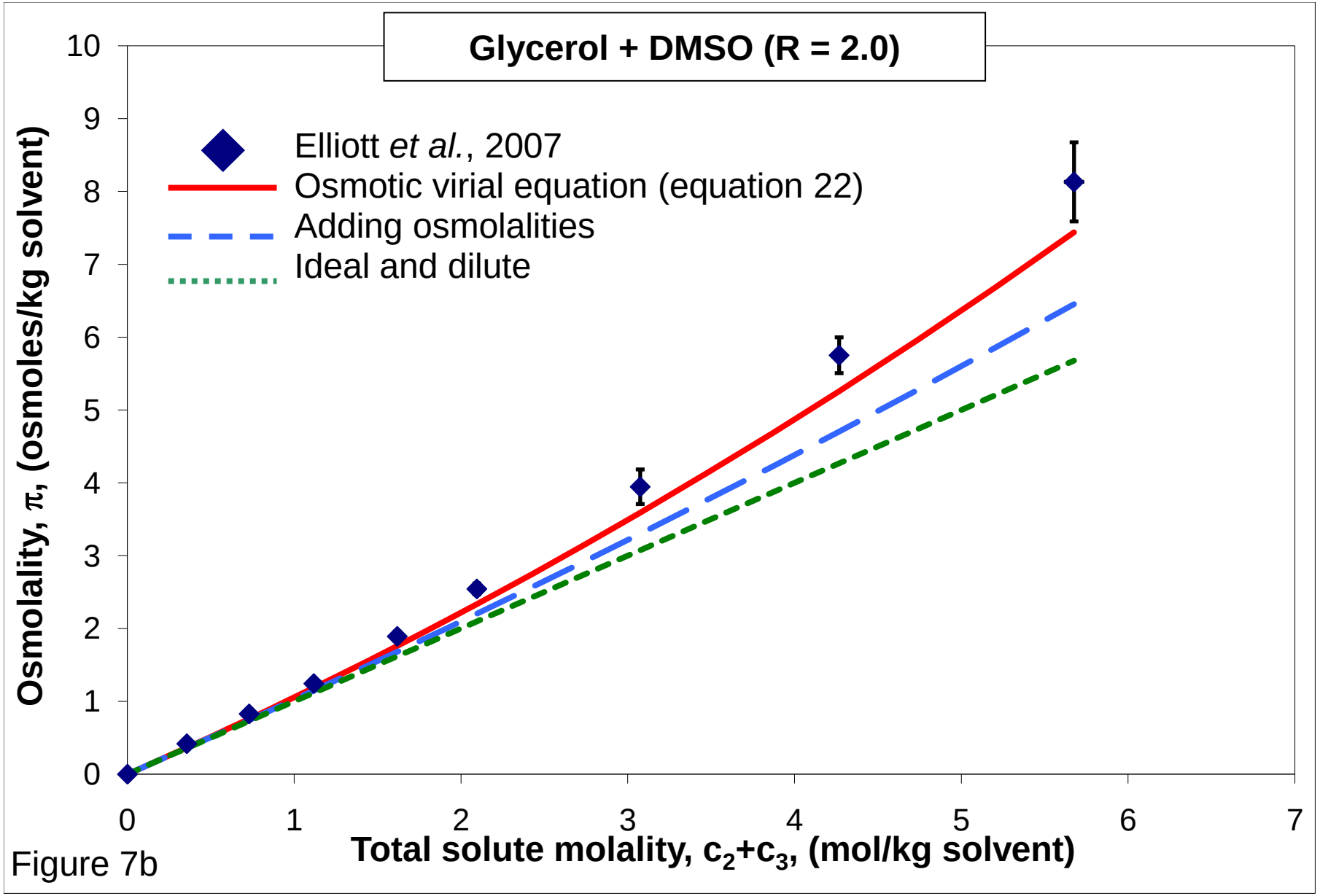

Glycerol + DMSO (R = 2.0)
Elliott *et al.*, 2007
Osmotic virial equation (equation 22)
Adding osmolalities
Ideal and dilute
Osmolality, π, (osmoles/kg solvent)
Total solute molality, c_2+c_3, (mol/kg solvent)
0
1
2
3
4
5
6
7
8
9
10

Figure 7b

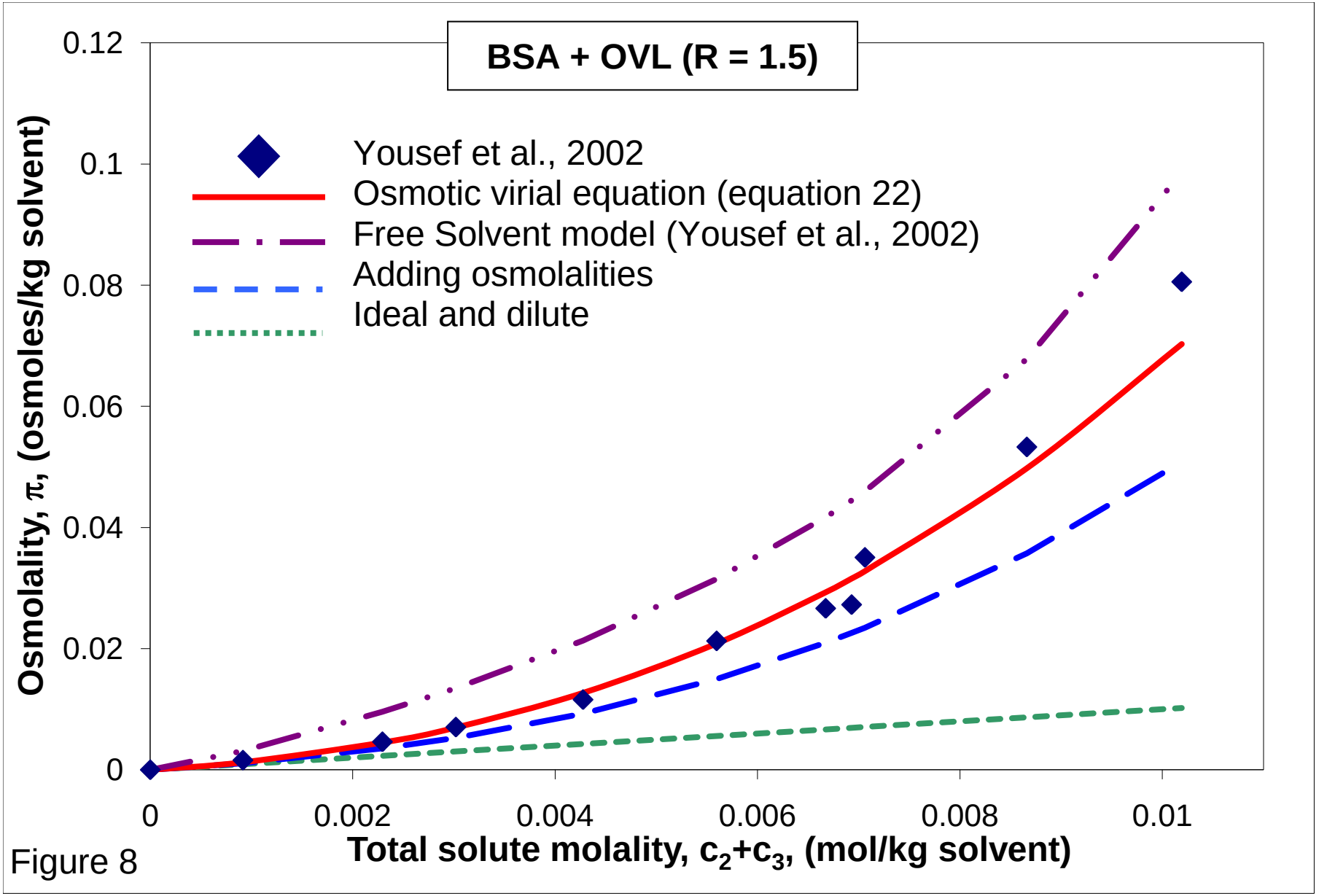

BSA + OVL (R = 1.5)
Yousef et al., 2002
Osmotic virial equation (equation 22)
Free Solvent model (Yousef et al., 2002)
Adding osmolalities
Ideal and dilute
Osmolality, π, (osmoles/kg solvent)
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.002
0.004
0.006
0.008
0.01
Total solute molality, c2+c3, (mol/kg solvent)

Figure 8

Table(s)

Table 1. Values for constants in the freezing point to osmolality conversion (equations 1 - 3).

Constant	Value
T_{FP}^{o}	273.15 K
W_1	1.802x10^{-2} kg/mole
$\overline{s_1^{o^L}} - \overline{s_1^{o^S}}$	22.00 J/moleK
R	8.314 J/moleK

Table 2a. Osmotic virial coefficients for use with solution molality.

Solute † [Reference]	Kdiss [±95% CI‡]	B molal^{-1} [±95% CI‡]	C molal^{-2} [±95% CI‡]	Adj. R^2	Max Molality	Solubility limit § (molal) [Temp]
NaCl [42]	1.678 [±0.02]	0.044 [±0.002]	0*	1.000	5.111	6.100 [0 ^{o}C]
KCl [42]	1.772 [±0.003]	0	0	1.000	2.005	3.726 [0 ^{o}C]
Me_2SO_4 [3; 9; 15; 39]	1	0.108 [±0.005]	0	0.990	14.975	
Glycerol [3; 9; 27; 42]	1	0.023 [±0.001]	0	0.996	10.859	
PG [4; 27; 42]	1	0.039 [±0.001]	0	0.997	19.713	
EG [42]	1	0.037 [±0.001]	-0.001 [0.0001]	1.000	24.166	
Methanol [42]	1	0.004 [±0.0003]	0	0.998	66.345	
Mannitol [42]	1	0	0	1.000	0.999	1.181 [25 ^{o}C]
Sucrose [42]	1	0.125 [±0.002]	0	1.000	2.115	5.958 [20 ^{o}C]

Dextrose [42]	1	0.044 [±0.001]	0	1.000	2.379	4.542 [15 ^{o}C]
Trehalose [28]	1	-0.394 [±0.2]	0.388 [±0.2]	0.998	1.108	1.325 [-1.2 ^{o}C]
Hemoglobin [5]	1	49.252 [±18.6]	3.07×10^{4} [$\pm 1.83 \times 10^{3}$]	0.999	1.23×10^{-2}	
BSA [41]	1	3.70×10^{2} [$\pm 3.62 \times 10^{2}$]	1.60×10^{5} [$\pm 4.25 \times 10^{4}$]	0.994	9.72×10^{-3}	
OVL [46]	1	3.78×10^{2} [±14.9]	0	0.990	1.95×10^{-2}	

[†]In addition to the solutes shown in the table, some very non-ideal solutes can be described using the osmotic virial equation. As an example, ethanol is a very non-ideal solute and requires three parameters to adequately fit the solution behaviour for use with molality. (B = 0.0376, C = -0.002, D = 0.000023, adj R^2 = 0.999) (see Figure 4a).

[‡]95% confidence intervals were calculated using equation (28).

[§] A blank indicates that there is either no solubility limit or the solubility limit is unknown.

*Where 0 appears in table, it indicates that the coefficient was not included in the fit (i.e. C = 0, indicates a quadratic fit was adequate).

Table 2b. Osmotic virial coefficients for use with solution mole fraction.

Solute † [Reference]	Kdiss [±95% CI‡]	B $\left(\frac{mole\ solute}{mole\ total}\right)^{-1}$ [±95% CI‡]	C $\left(\frac{mole\ solute}{mole\ total}\right)^{-2}$ [±95% CI‡]	Adj. R^2	Max Mole Fraction	Solubility limit § $\left(\frac{mole\ solute}{mole\ total}\right)$ [Temp]
NaCl [42]	1.663 [±0.02]	2.749 [±0.1]	0*	1.000	0.084	0.099 [0 °C]
KCl [42]	1.772 [±0.003]	0	0	1.000	0.035	0.063 [0 °C]
Me_2SO_4 [3; 9; 15; 39]	1	2.423 [±1.4]	27.231 [±8.0]	0.995	0.213	
Glycerol [3; 9; 27; 42]	1	1.950 [±0.1]	0	0.998	0.164	
PG [4; 27; 42]	1	2.831 [±0.08]	0	0.999	0.262	
EG [42]	1	1.501 [±0.07]	0	0.999	0.303	
Methanol [42]	1	0.395 [±0.02]	0	0.999	0.545	
Mannitol	1	0	0	0.999	0.017	0.021

[42]						[25 ^{o}C]
Sucrose [42]	1	7.182 [±0.1]	0	1.000	0.037	0.097 [20 ^{o}C]
Dextrose [42]	1	2.513 [±0.05]	0	1.000	0.041	0.076 [15 ^{o}C]
Trehalose [28]	1	-22.418 [±9.3]	1.250×10^{3} [$\pm 5.2 \times 10^{2}$]	0.998	0.020	0.023 [-1.2 ^{o}C]
Hemoglobin [5]	1	1.978×10^{4} [$\pm 1.3 \times 10^{3}$]	0	0.960	2.21×10^{-4}	
BSA [41]	1	9.535×10^{4} [$\pm 8.4 \times 10^{3}$]	0	0.961	1.75×10^{-4}	
OVL [46]	1	2.310×10^{4} [$\pm 8.8 \times 10^{2}$]	0	0.990	3.51×10^{-4}	

[†]In addition to the solutes shown in the table, some very non-ideal solutes can be described using the osmotic virial equation. As an example, ethanol is a very non-ideal solute and requires two parameters to adequately fit the solution behaviour for use with mole fraction. (B = 1.9949, C = -5.9843, adj R^2 = 0.999) (see Figure 4b).

[‡]95% confidence intervals were calculated using equation (28).

[§] A blank indicates that there is either no solubility limit or the solubility limit is unknown.

*Where 0 appears in table, it indicates that the coefficient was not included in the fit (i.e. C = 0, indicates a quadratic fit was adequate).

Table 3. Percent error and sum of squared errors in using (i) ideal and dilute, (ii) adding osmolalities and (iii) multisolute OVE to predict each multisolute solution as compared to measured data.

Solutes [R-value] †	Maximum total solute molality	Measured data source	Ideal, dilute		Adding Osmolalities		Multisolute OVE	
			% error at max. molality ‡	SSE §	% error at max. molality ‡	SSE §	% error at max. molality ‡	SSE §
Glycerol + Me_2SO_4 [R = 0.5]	6.0	[9]	33.8%	13.63	12.0%	1.76	1.1%	0.02
Glycerol + Me_2SO_4 [R = 2.0]	5.7	[9]	30.2%	9.30	20.6%	4.52	8.5%	0.92
Me_2SO_4 +NaCl [R=0.2]	5.1	[15]	30.7%	$1.32x10^2$	9.4%	2.52	2.9%	0.80
Me_2SO_4	16.1	[15]	62.5%	$2.07x10^3$	8.0%	29.08	1.1%	5.28

+NaCl [R=19.0]								
Glycerol + NaCl [R=0.67]	6.3	[40]	28.3%	14.23	9.1%	1.42	2.0%	0.15
Glycerol + NaCl [R=9.0]	17.2	[40]	16.0%	23.31	9.4%	5.01	28.1%	43.97
Hb + ideal*	$c/c_o = 2.8$	[7; 30]	46.5%	0.59	30.2%	0.24	6.4%	0.013
BSA + OVL [R=1.5]	0.01	[47]	87.4%	0.0089	36.9%	0.0014	12.7%	0.00015

† R values are the mass ratios: $R = \frac{Mass\,of\ solute\,1}{Mass\,of\ solute\,2}$

‡ Percent error calculated using eq. (31) at the maximum total solute molality at which osmolality was measured for each solution.

[§]SSE calculated using eq. (25). The values of the SSE should only be compared for the different predictions for each specific solution, not between solutions.

*Predictions of the RBC cytoplasm using the Hb + ideal osmotic virial equation model were done in relative concentration (c/c_o) [9].

Table 4. Percent error and sum of squared errors in using (i) the Kleinhans and Mazur approach (adding osmolalities) [20] and (ii) the multisolute OVE to predict each multisolute solution osmolality as compared to Pegg's fitting equations [31; 32].

Solutes [R-value] †	Maximum total solute molality	Kleinhans and Mazur [20]		Multisolute OVE	
		% error at max. molality ‡	SSE §	% error at max. molality ‡	SSE §
Me_2SO_4 + NaCl [R=4.60]	11.1	23.1%	60.4	1.6%	7.0
Me_2SO_4 + NaCl [R=9.55]	16.1	11.6%	72.0	0.1%	12.7
Me_2SO_4 + NaCl [R=14.87]	16.0	6.7%	29.6	0.6%	14.7
Glycerol + NaCl [R=5.43]	22.0	27.8%	89.1	48.1%	3.01×10^2
Glycerol + NaCl [R=11.28]	26.5	28.1%	1.13×10^2	53.1%	4.24×10^2

Glycerol + NaCl [R=17.6]	26.1	22.4%	68.8	44.3%	2.72×10^{2}

[†]R values are the mass ratios: $R = \frac{Mass\ of\ solute\ 1}{Mass\ of\ solute\ 2}$

[‡]Percent error calculated using eq. (31) at the maximum total solute molality, substituting values from Pegg's fitting equations [31; 32] for the experimental data.

[§]SSE calculated using eq. (25), substituting values from Pegg's fitting equations [31; 32] at 5% total solute weight percent increments [31; 32] for the experimental data. The values of the SSE should only be compared for the different predictions for each specific solution, not between solutions.

Table B1: Summary of matrix approach to determine osmotic virial coefficients

Type of solute [concentration units]	$\vec{y}$ vector	Conversion of coefficients in $\vec{\beta}$ to osmotic virial coefficients
Non-electrolytes [molality]	$\pi/m_i - 1$	$\beta_1 = B$ $\beta_2 = C$ …
Electrolytes [molality]	π	$\beta_1 = k_{diss}$ $\beta_2 = B(k_{diss})^2$ $\beta_3 = C(k_{diss})^3$ …
Non-electrolytes [mole fraction]	$\pi/A^* x_i - 1$	$\beta_1 = B^*$ $\beta_2 = C^*$ …
Electrolytes [mole fraction]	π/A^*	$\beta_1 = k_{diss}^*$ $\beta_2 = B^*(k_{diss}^*)^2$ $\beta_3 = C^*(k_{diss}^*)^3$ …

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Conflict of Interest

There is no conflict of interest declared by the authors.