# RETENTION OF THE AMORPHOUS STATE OF TREHALOSE AT HIGH RELATIVE HUMIDITY USING ORGANIC SALT ADDITIVES: A MECHANISTIC UNDERSTANDING

by

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# ABSTRACT

SHIMA ZIAEI. Retention of the Amorphous State of Trehalose at High Relative Humidity using Organic Salt Additives:
A Mechanistic Understanding. (Under the direction of DR. GLORIA ELLIOTT)

The glassy or amorphous solid state of trehalose has been utilized in the food and pharmaceutical industry to immobilize and stabilize sensitive molecules and it is increasingly being explored for the preservation of biologics. Retention of the glassy state is very dependent on the local humidity, as water is a known plasticizer that will decrease the glass transition temperature ($T_g$) of the solution, resulting in increased molecular mobility. If the storage temperature exceeds the $T_g$, the sample will generally crystallize at a rate that is dependent on the temperature and composition of the mixture. In order to preserve biologics during storage for extended times, delaying crystallization and retaining the amorphous state under adverse moisture excursions is desirable. Additives, such as polymers, other sugars, and salts have been used to modify the physical properties to increase the stability of trehalose glasses. Salts are of special interest due to the presence of pH buffering electrolytes in many formulations designed for biological systems. In this work the $T_g$ of trehalose and salt compositions were determined using a dynamic mechanical analyzer (DMA) and the crystallization kinetics and sorption isotherms were studied by dynamic vapor sorption (DVS) method. Sorption isotherms were fitted to the Brunauer–Emmett–Teller (BET) equation to gain insight into the microstructure and localized interactions in order to develop an improved understanding of metastability above $T_g$. With this knowledge, we were able to identify and

confirm a superior class of organic salt additives for stabilizing the amorphous state of trehalose.

# ACKNOWLEDGMENTS

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| FDA | food and drug administration |
| DSC | differential scanning calorimetry |
| DMA | dynamic mechanical analysis |
| BET | Brunauer, Emmett and Teller |
| EMC | equilibrium moisture content |
| RH | relative humidity |
| ERH | equilibrium relative humidity |
| MD | maltodextrin |
| PVP | polyvinylpyrrolidone |
| FTIR | fourier transform infrared spectroscopy |
| $T_g$ | glass transition temperature |
| $T_m$ | melting temperature |
| CMHP | choline monohydrogen phosphate |
| CDHP | choline dihydrogen phosphate |
| ChCl | choline chloride |
| TMAA | tetramethylammonium acetate |

# CHAPTER 1: TREHALOSE GLASS AND ITS IMPORTANCE IN PRESERVATION

## 1.1 Sugars in the pharmaceutical and food industry and their role in biopreservation

Over the past few decades the importance of stabilizing protein therapeutics has grown steadily. In 2015 nearly 30% of newly registered drugs at the FDA were protein drugs[1-3]. Sugars have been widely used in the pharmaceutical industry to stabilize proteins in the dry state in order to minimize refrigeration and thus facilitate easier storage and transportation [4, 5]. Sugars also have been utilized in the food industry to produce thousands of shelf-stable food products[6].

The prevalence of the amorphous states of sugars in nature has been an inspiration for the preservation of biologics[7, 8]. In nature, some species, such as the brine shrimp, survive very dry conditions, as low as 0.007 grams of water per gram of dry mass, in part by producing a trehalose 'glass' upon dehydration. Upon rehydration, active metabolism resumes, a phenomenon known as "anhydrobiosis"[8]. Researchers have been using a range of sugars, such as trehalose, sucrose, and lactose to stabilize biologics[9] due to the very low molecular mobility and very high viscosity ($10^{12}$ Pa. s) in the glassy amorphous state[10, 11]. A range of processing methods have been utilized to dry biologics, including air drying, microwave assisted drying, spray drying and lyophilization [14]. Retaining the glassy state is desirable for long-term storage of biologics and sensitive molecules, as the crystallization of trehalose can lead to the loss of its protective effect as a result of changes in the interactions between trehalose and the biologic [1].

## 1.2 Sugar Glasses

A glass is a disordered substance that conforms to the shape of a container like a liquid, but mechanically behaves like a solid. When an amorphous material transitions from the soft rubbery fluid-like state with a viscosity of $10^6 – 10^8$ Pa. s to hard brittle glassy state with the viscosity of $10^{12}$ Pa. s this transition is known as glass transition ($T_g$). Different techniques are used to make glasses but the most common approach is to quickly cool a liquid below the phase change temperature to avoid crystallization (supercooling) and preserve the amorphous structure[12, 13]. Other common methods used to produce a glassy state are evaporative drying (microwave assisted drying), freeze-drying, and spray drying[14]. The process by which the glass is formed can affect the glass properties and the glass transition temperature ($T_g$). For example, Kovacs, in his classical study of polyvinyl acetate in 1958 showed how different cooling rates can change the location of the glass transition [15, 16]. The transition into the glass state happens gradually as the material changes from a soft molten rubbery state to a hard, brittle glass[15]. The glass transition usually happens at approximately 2/3 of the melting temperature as the shear viscosity reaches to $10^{13}$ poise[14, 15].

## 1.3 Experimental Techniques for $T_g$ Measurements

Different experimental techniques can be used to measure the $T_g$, and each can lead to different values. The two most common experimental techniques to measure $T_g$ are: differential scanning calorimetry (DSC) and dynamic mechanical analysis (DMA).

To determine $T_g$ by DSC, the amount of heat required to increase the temperature of a known mass of sample and a reference (i.e. the heat capacity) is determined as a function of temperature. Both sample and reference (usually an empty pan) are maintained at the same temperature but as sample goes through changes in structure, the amount of energy required to

maintain the temperature will change while the reference has a well-defined baseline heat capacity over the range of temperature scan. In the case of a glass transition, the heat capacity of the sample will have different values before and after the transition, attributed to differences in the mechanical properties and viscosity of the sample in the hard rigid solid state compared to the liquid and rubbery state[15, 17]. Figure 1 represents several common features of a DSC curve.

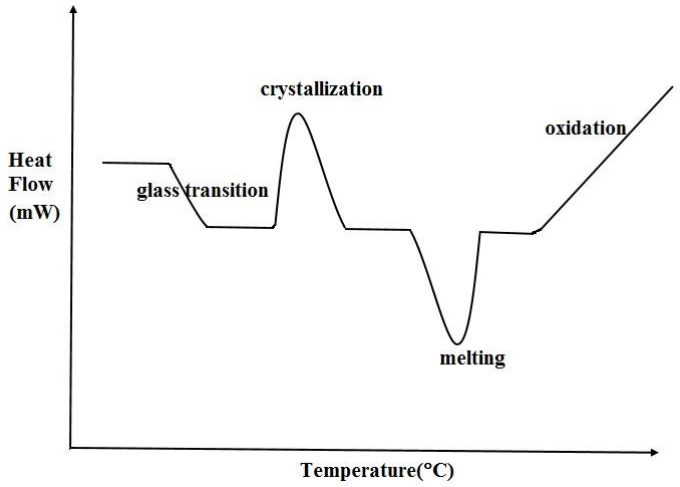


Figure 1. A schematic DSC curve

DMA enables the determination of $T_g$ from the viscoelastic behavior of a material. The $T_g$ can be determined by changes in the storage modulus(E'), loss modulus(E") and tan delta (tanδ = E"/E'). The ability of the material to store energy, (E'), decreases dramatically during the glassy transition as the sample transitions from solid to rubbery state. Conversely, the ability of the sample to lose energy, (E"), approaches a maximum value during the glassy transition. Tan delta which is the ratio of loss modulus to storage modulus, also attains a maximum value,

like loss modulus, during the glass transition[15, 18]. Figure 2 shows a DMA curve for a material exhibiting a glass transition.

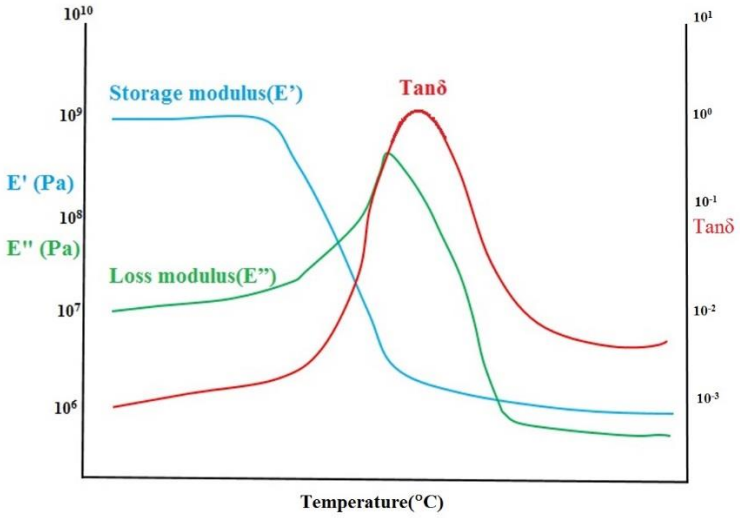


Figure 2. A schematic DMA curve

### 1.4 Trehalose

Trehalose is a non-reducing disaccharide with molecular formula $C_{12}H_{22}O_{11}$. It is composed of two glucose molecules and has a molar mass of 342.31g/mol[21]. Among all the disaccharides trehalose has the highest glass transition temperature (120°C) and is considered a good glass former. Trehalose has higher solubility in water compared to other disaccharide sugars[22]. Trehalose is a superior excipient not just because of its good glass-forming tendency or good solubility in water but it has also been found to preserve liposomes during in the anhydrous state by directly interacting with polar head groups [21]. Trehalose has also been widely used as a protective agent for lyophilized foods due to its superior outcomes compared to other sugars [9].

The glassy amorphous state, while desirable for protecting biomolecules and biological structures, is not stable and tends to crystallize with a rate dependent on moisture content and

temperature[23]. Water is a plasticizer that decreases the glass transition temperature and viscosity of the glassy matrix and increases the molecular mobility. When the $T_g$ of the mixture becomes lower than room temperature, the associated increase in molecular mobility enables rapid crystallization. Therefore, a sugar like trehalose with a high glass transition is more effective and more stable compared to other disaccharides with lower $T_g$ values[10, 24].

Table 1 compares the $T_g$ of some disaccharides. As is shown, sucrose has the lowest and trehalose has the highest $T_g$ value[25].

Table 1. The glass transition temperature of disaccharides

| Sugar | $T_g$(°C) |
|---|---|
| Sucrose | 75 |
| Maltose | 100 |
| Lactose | 114 |
| Trehalose | 120 |

Studies demonstrate that pure trehalose glass crystallizes at and above 44% RH[26, 27]. When the moisture content reaches approximately 10%, which is the water content required to form trehalose dihydrate[21], trehalose crystallizes and it loses its protective effect because of the disruption of interactions between trehalose and the biologic. Even though retention of the amorphous state is required for the long-term storage of biologics in sugar glasses, due to adverse environmental conditions such as humidity, the water content of the glass can change and this water uptake can lead to crystallization. Different additives have been added to trehalose in order to increase the stability of trehalose glass such as polymers, sugars and salts[28].

### 1.5 Glass Transition Temperature of Binary Blends

The glass transition temperature of binary blends and copolymers can be predicted with varying degrees of accuracy using different models [15]. One of the most simple of the $T_g$ equations is the Fox equation, which is shown in equation (1) for a binary system:

$$\frac{1}{T_g} = \frac{x_1}{T_{g,1}} + \frac{1-x_1}{T_{g,2}} \qquad (1)$$

where $x_1$ is the weight fraction of component 1 and $1 - x_1$ is the weight fraction of component 2. This model is based on a simple weight fraction of components.

The Gordon and Taylor model is shown in equation (2) and includes an additional parameter. Index 2 in this equation refers to the component with higher $T_g$ value and $k_{GT}$ is the Gordon and Taylor fitting parameter, which accounts for the components in the blend having unequal contributions[19].

$$T_g = \frac{x_1\ T_{g,1} + k_{GT}(1-x_1)\,T_{g,2}}{x_1 + k_{GT}(1-x_1)} \qquad (2)$$

Kwei identifed several mixtures of resins with $T_g$ behaviors that deviated from the Gordan and Taylor equation, which he interpreted as arising from the different hydrogen bonding contribution of components in the mixture and he resolved the deviation by adding another parameter $q$ to the original Gordon and Taylor equation. Equation (3) shows Kwei equation[20]:

$$T_g = \frac{x_1\ T_{g,1} + k_{KW}(1-x_1)\,T_{g,2}}{x_1 + k_{KW}(1-x_1)} + q x_1\,(1 - x_1) \qquad (3)$$

As in equation 2, index 2 refers to the component with higher $T_g$ value. The $T_g$ value of sugar-water mixtures can be predicted by both the Gordon and Taylor equation and the Kwei equation.

### 1.6 Water Sorption Isotherm

Changes in compositions can lead to different moisture sorption characteristics, which can also affect the stability of sugar glasses. Equilibrium moisture content (EMC) is the water content of the sample in equilibrium with the surrounding environment's relative humidity (RH). The quantitative relationship between the sample EMC and the environmental RH in constant temperature is called a "moisture sorption isotherm". In order to determine the sorption isotherm for a composition, the mixture is placed in different relative humidity environments until the sample is in equilibrium with the surrounding equilibrium relative humidity (ERH). Water activity ($a_W$) is a thermophysical property of water molecules the describes its ability to participate in chemical and physical reactions and is defined as the percentage of ERH or the ratio of water vapor pressure in the composition ($p_{sys}$) to water vapor pressure of pure water ($p_{sat}$).

$$a_W = \frac{p_{sys}}{p_{sat}} = \frac{ERH}{100} \qquad (4)$$

Increasing the environment temperature decreases the EMC, while increasing the ERH increases EMC. Sometimes the EMC while taking on water (adsorption) is different from EMC while losing water (desorption) and leads to different adsorption and desorption trends. This phenomenon is called hysteresis and is shown in figure 1[29].

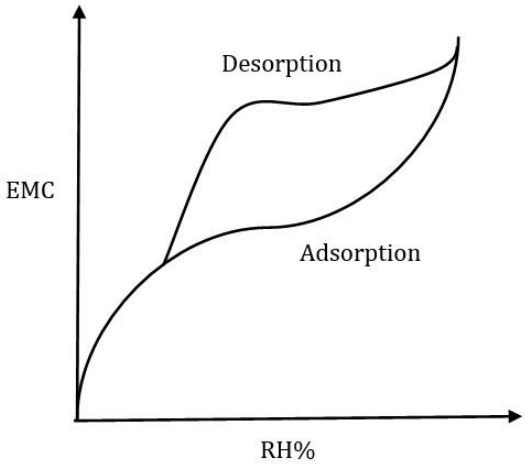


Figure 3. The occurrence of hysteresis in a water sorption isotherm

Brunauer, Emmett and Teller (BET) described five different sorption isotherms[29-31]. Type 1 represents monolayer adsorption and is known as Langmuir. In this sorption isotherm water uptake is quick initially but since there are limited sites on the surface of the sample, the water uptake slows down and levels off[29, 32]. Type 2 is a sigmoid isotherm, which initially is similar to type 1 but the difference is moisture penetrates and diffuses throughout the sample. Type 3 is known as Flory Huggins and represents multilayer adsorption[30]. Type 2 isotherms are more common among hydrophilic polymers and less hydrophilic polymers sorption isotherms are characterized with type 3. The well known BET equation models isotherms type 2 and type 3 and is given as:

$$W(a_0) = \frac{W_B\, C_B\, a_0}{(1-a_0)(1+(C_B-1)a_0)} \qquad (5)$$

In this equation $a_0$ is water activity, $\mathrm{W}(\mathrm{a}_0)$ is the water content of the sample at water activity $a_0$, $\mathrm{W_B}$ and $\mathrm{C_B}$ are BET equation fitting parameters. $\mathrm{W_B}$ represents the hydrophilic sites on the surface and reflects the amount of water sitting on the surface of the sample and $\mathrm{C_B}$ reflects

the overall free energy of water sorption and the tendancy for interaction between water and the solid components of the sample[33, 34]. Figure 4 provides a visual interpreation of the information that the BET equation fitting parameters represent. The sample is shown as the white rectangular region, while the water molecules are in blue. For example in figure 4 (a) both $W_B$ and $C_B$ values are low, suggesting that the amount of water sitting on the surface and the amount of water interacting with samples components are both low.

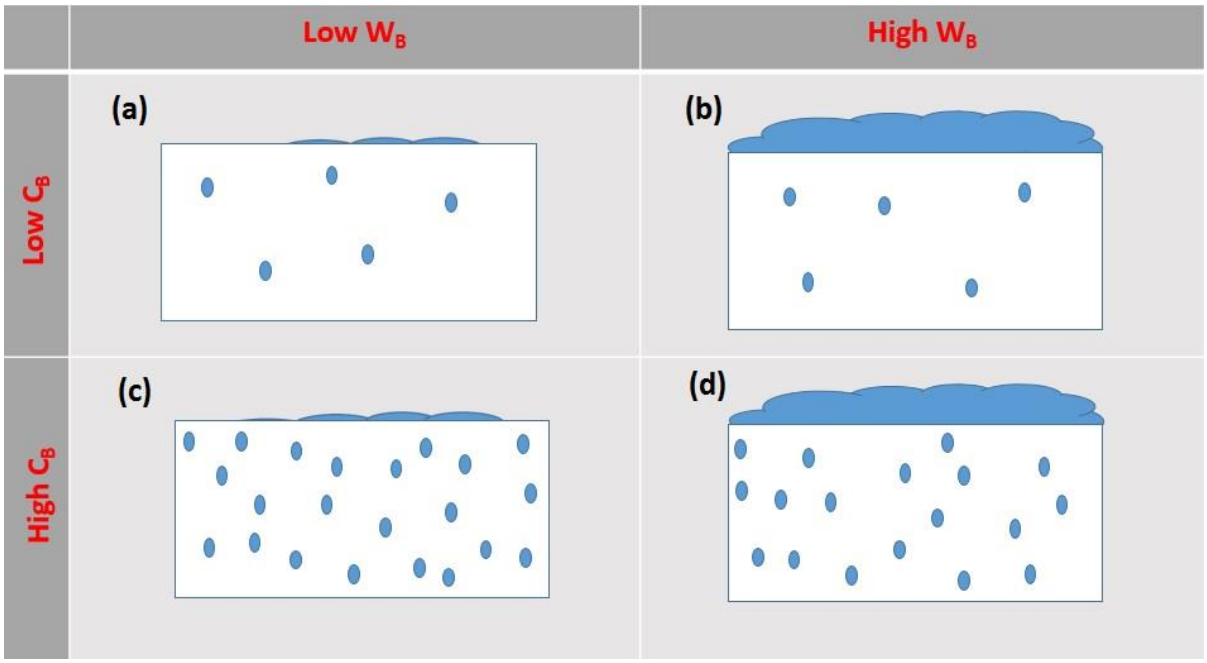


Figure 4. Schematic of the information, BET fitting parameters represent

Isotherm type 4 is initially similar to type 2 but reaches a plateau at an intermediate RH value. Isotherm type 5 is also similar to type 3 but reaches a limit toward the end of the isotherm[29, 31]. Figure 5 shows the five different types of sorption isotherms described by Brunauer, Emmett and Teller[31].

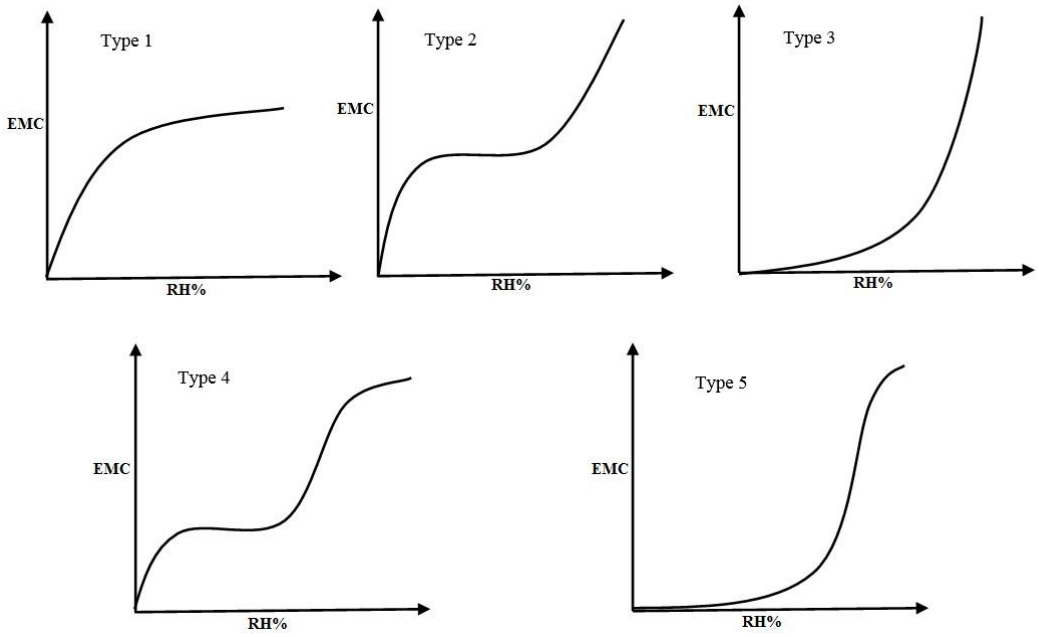


Figure 5. Sorption isotherms graphs by Brunauer, Emmett and Teller

Sorption isotherms can be divided into three different stages as shown in figure 3. At low water activity ($a_W$<0.2) the moisture content in the sample represents bound water, the water molecules that chemically interact with the sample. In the second region (0.2<$a_W$<0.6) water is loosely bond, and in the last region ($a_W$>0.6) the water is considered free water. The main difference between isotherm type 2 and type 3 lies in the interactions between water and the solid components of the sample. As is shown in figure 6, the EMC of region A, which represents bound water, is lower in isotherm type 3 compared to isotherm type 2. The $C_B$ fitting parameter value, which reflects the free energy of water sorption and the amount of chemically interacted water from the BET equation, has higher values in isotherm type 2 compared to type 3[29, 35]. The relevance of these isotherm types to sugar glass stability will be discussed in greater detail in later chapters.

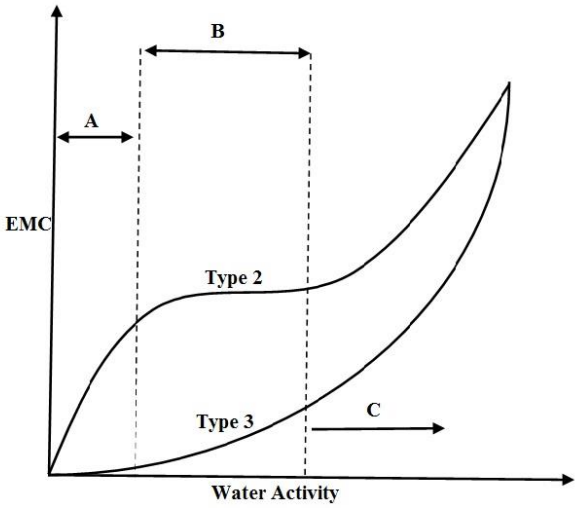


Figure 6. The three regions of sorption isotherm graph

## 1.7 Trehalose Additives

### 1.7.1 Polymers

For many years the food and pharmaceutical industries have used different additives to achieve the stability of products that are vulnerable to moisture sorption and eventually crystallization during transportation and storage. Trehalose additives can be divided into three different categories, each with different mechanisms of action. The first group is polymers. Studies have shown that the addition of maltodextrin to trehalose in a weight ratio of (50:50) can suppress crystallization at 44% RH and delay crystallization above 44% RH[36]. Maltodextrin (MD) is a polysaccharide with a high glass transition temperature of 160°C. MD has a high molecular weight and the addition of MD to trehalose increases the $T_g$ value of sugar systems. As shown in figure 7 by increasing the weight ratio of MD to trehalose the $T_g$ of the anhydrous sugar system increases.

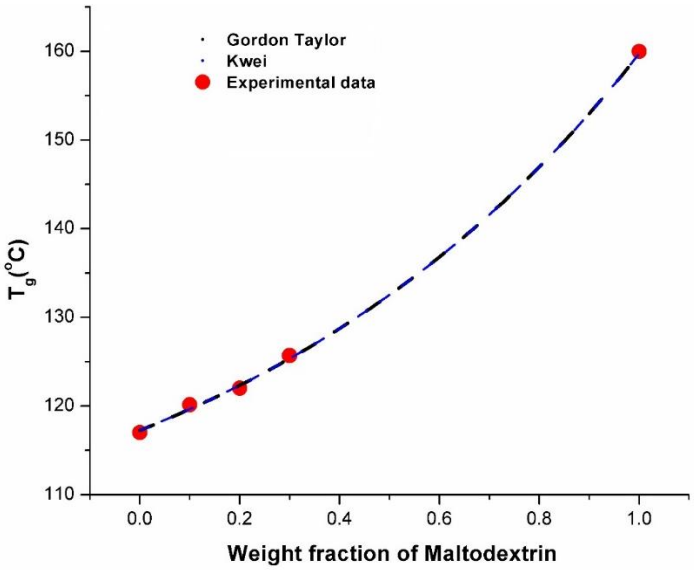


Figure 7. The $T_g$ of trehalose:MD systems in the dry state with different weight ratios of MD [37]

Experimental data obtained by Sillick and Gregson [37] was fitted to the Gordon-Taylor and Kwei equations in order to evaluate the $T_g$ of trehalose:MD with different weight fractions. Fitting parameters are shown in table 2.

Table 2. Gordon-Taylor and Kewi equations fitting parameters of trehalose:MD compositions

| | Gordon- Taylor | Kwei |
|---|---|---|
| K value | 0.579 | 0.538 |
| q Value | _____ | 2.36 |
| R square | 0.969 | 0.989 |

The glass transition temperature of tertiary blends of trehalose-MD-water with increasing weight fraction of solutes to water is shown in figure 8 with the weight ratio of trehalose:MD fixed at 50:50. The experimental data was obtained from the study by Gorska and et al. [38]

and then fit to the Gordon-Taylor equation. For this fit $T_{g1}$ is the $T_g$ of water and $T_{g2}$ is the $T_g$ of trehalose:MD at the weight ratio of 50:50. The Gordon-Taylor fitting parameter yielded k=19.54 and R squared is 0.980.

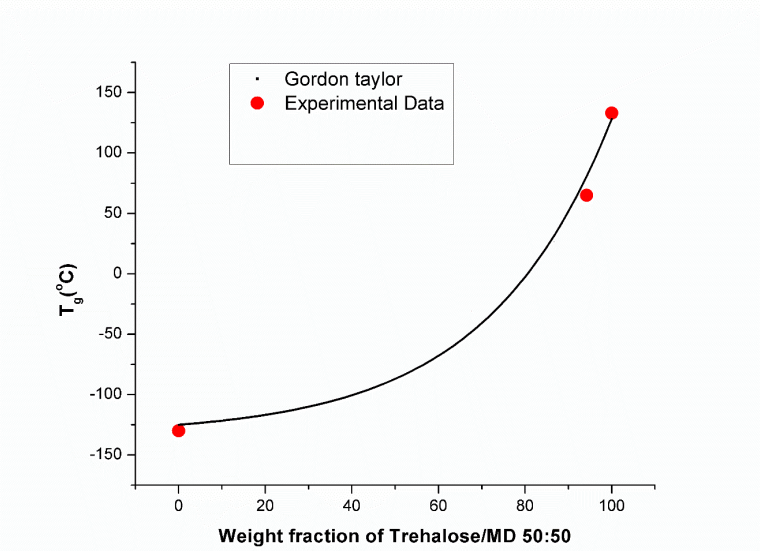


Figure 8. The $T_g$ of aqueous trehalose:MD with the weight fraction of trehalose:MD 50:50 in different weight ratios of solutes [38]

As mentioned previously, the water content required to form trehalose dihydrate is approximately 10% and the $T_g$ of binary blends of trehalose-water at this water content is approximately 14°C, which is lower than room temperature and leads to crystallization. While the $T_g$ of the tertiary blends of trehalose-MD-water with 10% water content when the weight ratio of trehalose:MD 50:50 is approximately 43°C and is above room temperature. The stabilizing effect of MD addition to trehalose is due to the high molecular weight of MD which increases the $T_g$ of sugar system, an effect that persists in the presence of water[36].

Polyvinylpyrrolidone (PVP) is another effective polymeric additive to trehalose that inhibits crystallization at elevated humidities[39]. FTIR analysis has shown that when PVP is added to trehalose with a weight ratio of 3:1, 80% of the C=O groups of PVP hydrogen bond with

trehalose. CNMR relaxation studies revealed that PVP addition to sucrose decreased the side chain movements and molecular mobility of sucrose when the relative humidity increased compared to pure sucrose matrix[39, 40].

The addition of PVP to trehalose increases the $T_g$ of the anhydrous sugar system, in proportion to the ratio of PVP to trehalose. The experimental data shown in figure 9 is from the study by Imamura and et al [40], which was fit to the Gordon-Taylor equation, yielding k=0.331 and an R squared value of 0.996.

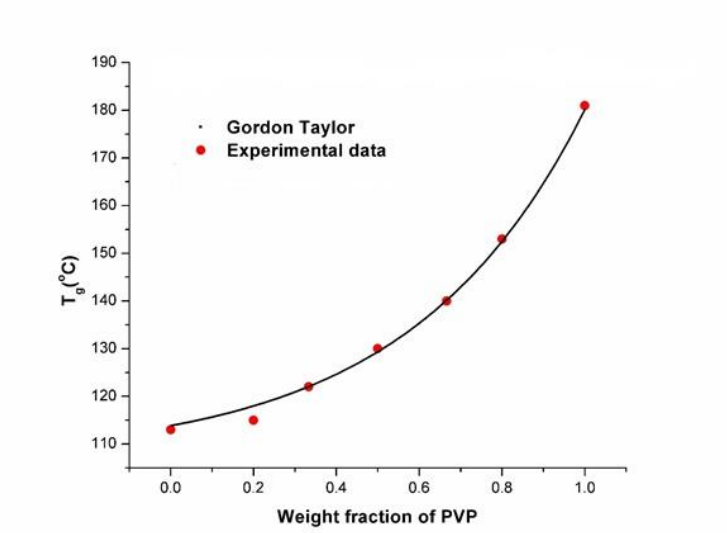


Figure 9. The $T_g$ of trehalose-PVP mixtures at different weight ratios of PVP[40]

The tertiary blends of trehalose-PVP-water shown in figure 10 exhibit higher $T_g$ values than trehalose-water system with the same water content. For instance the $T_g$ of tertiary blends of trehalose-PVP-water with 10% water is approximately 53.2°C and is well above the room temperature. The Gordon-Taylor fitting parameter was determined to be 0.236 and the R squared value was 0.985.

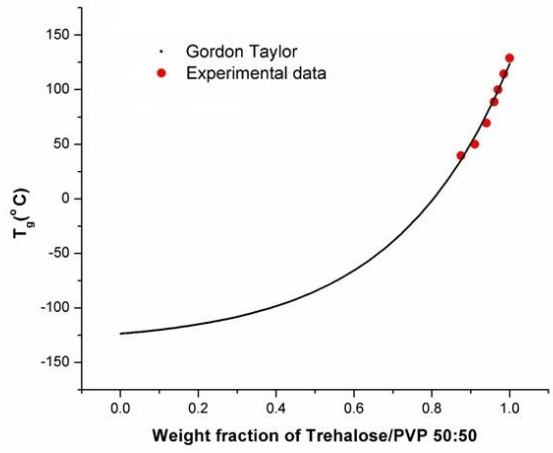


Figure 10. The $T_g$ of aqueous trehalose-PVP with different water contents. Trehalose:PVP weight ratio is 50:50 [40]

Pullulan is also polysaccharide and a good stabilizing additive to trehalose. The $T_g$ of pullulan is 261°C and the addition of pullulan to trehalose with the weight ratio of 50:50 prevented crystallization at 30°C and 56%RH. The stabilizing mechanism, like other polymers, is considered to be the increase in the $T_g$ of the sugar system in the dry state and in the presence of water[41].

### 1.7.2 Other Sugars

The second group of stabilizing additives that can be used to stabilize trehalose is other sugars. For example the addition of trehalose to sucrose (weight fraction 25:75) inhibits crystallization of sucrose at 33%RH and delays crystallization of trehalose and sucrose at 54% RH for 5 days[42].

Raffinose is a trisaccharide that is composed of glucose, fructose and galactose. The addition of 5% raffinose to sucrose delays crystallization while the $T_g$ of the sugar system does not increase significantly[43]. Therefore the addition of a sugar to another sugar delays crystallization regardless of the changes in the $T_g$. This glass stabilizing mechanism is

associated with steric hindrance as raffinose is thought to block the major crystallization growing face of sucrose that leads to delayed crystallization. The delay in lactose crystallization when trehalose is added to lactose (weight fraction 40:60) is also not attributed to the $T_g$ effect and is considered as lattice interference that impacts crystal growth [9, 44]. The underlying mechanism in delaying crystallization by adding another sugar is considered to be steric hindrance.

### 1.7.3 Salts

Studies have shown that some salts can help delay crystallization and can extend the shelf life of sugar systems[45-48]. It should be noted that electrolytes are essential for biological systems, hence their inclusion in a wide range of biological formulations. A salt is an ionic compound composed of cations (positive charge) and anions (negative charge) that makes the salt electrically neutral. Ions can be either organic or inorganic. Example organic ions include acetate ($C_2H_3O^{2-}$), citrate ($C_6H_5O_7^{3-}$), choline ($C_5H_{14}NO^{+}$) and common inorganic ions include chloride ($Cl^{-}$), sodium ($Na^{+}$), calcium ($Ca^{2+}$), magnesium ($Mg^{2+}$).

Various studies have focused on the effect of anions on the stability of amorphous sugar compositions. For example, it has been reported that anions with hydrogen bonding ability can crosslink with sugar and increase the $T_g$ of the mixture, delaying crystallization of the amorphous system[48]. Sodium and potassium phosphate can increase the $T_g$ of sucrose in all pH ranges while adding phosphate salts increased the $T_g$ of trehalose just in basic pH ranges. In acidic pH ranges phosphate ions mostly appear as the dihydrogen phosphate ($H_2PO4^{1-}$) form, which is unable to effectively interact with trehalose molecules whereas the

monohydrogen phosphate ($HPO4^{2-}$) form yields a strong hydrogen bonding network with trehalose [78].

Buera and et al. also showed for salts with the same cation but different anions, salts with bigger anions led to better delay of crystallization[47]. For example between potassium citrate and potassium chloride, potassium citrate was more effective in delaying crystallization. Magnesium acetate was also shown to be a better additive than magnesium chloride for crystallization suppression, suggesting that the bigger size of the anion contributed to steric hindrance of crystal formation and growth. Strong interactions of ions with water that can prevent formation of trehalose dihydrate can also be another underlying mechanism contributing to this effect. Among the salts mentioned earlier in this study both citrate and acetate anions have higher hydration free energy (higher tendency to interact with water) than chloride ion [49, 50].

Mazzobre et al evaluated the effect of cations and studied a chloride family of salts with different metal cations[45, 46]. $MgCl_2$, NaCl, KCl and $CaCl_2$ do not contain either a crosslinking or a big anion. They observed that the higher the ratio of $\frac{\text{charge}}{size}$ of the cations the longer crystallization was suppressed. In terms of effectiveness of the cation, magnesium was the most effective and potassium was the least effective $Mg^{2+}$>$Ca^{2+}$>$Na^{+}$>$K^{+}$. Adding salts to trehalose increased the uptake of water while delaying crystallization, suggesting that effects were due to water-cation interactions. Aqvist, in a different study, showed that among the cations just mentioned, magnesium had the highest hydration free energy (tendency to interact with water) and potassium had the lowest[51]. The ranking, in terms of hydration free energy of cations is: $Mg^{2+}$>$Ca^{2+}$>$Sr^{2+} > Ba^{2+} > Li^{+} > Na^{+}$>$K^{+} > Rb^{+} > Cs^{+}$.

It can be observed that among the inorganic salts with metal cations, the stabilizing effect of cations followed the same trend as water-cation interaction tendency.

In summary, the stabilizing effect of salts can be attributed to the presence of hydrogen bonding anions that can increase the $T_g$ of the sugar system, steric hindrance due to the presence of big ions, and water-ion interactions that prevent the formation of trehalose dihydrate.

### 1.7.4 Summary of the Underlying Stabilizing Mechanisms of Trehalose Additives

Polymers are large molecules with a high molecular weight. Stabilization of trehalose by polymers has been attributed to the increase in the $T_g$ of the sugar composition, an effect that persists in the presence of water. The delay in crystallization achieved by adding another sugar is considered to be linked to steric hindrance, where the physical presence of secondary sugar molecules of a different type interferes with the association or clustering of the predominant sugar molecules and thus delays crystallization. Salts can delay crystallization of sugar systems for variety of reasons. Some salt anions can make hydrogen bonds and increase the $T_g$ of the sugar system. Steric hindrance due to the presence of big ions have the potential to also suppress crystallization, and finally water-ion interactions that prevent the formation of trehalose dihydrate is another potential stabilizing mechanism when salts are added to sugar systems. The stabilization potential of different salt classes will be explored in subsequent chapters.

# CHAPTER 2: OVERVIEW OF EXPERIMENTAL STUDIES

## 2.1 Rationale

The amorphous state of sugars has been utilized in the bio-preservation field, food industry, and pharmaceutical industry to preserve sensitive structures [7-9, 21, 24, 52-63]. In the glassy amorphous state, sugars have high viscosity and extremely low molecular mobility, which makes them ideal for the preservation of different biologics such as proteins and cells membranes [58, 60-62, 64]. Even though in the glassy state the molecular mobility is low, the system is not in thermodynamic equilibrium and will tend to crystallize when the temperature exceeds the glass transition temperature ($T_g$) of the solution. The prediction of a suitable storage temperature of dry preserved samples thus requires knowledge of the glass transition. As the temperature increases above $T_g$ the molecular mobility increases, enabling molecules to rearrange into a crystalline structure. In order to preserve biologics during extended storage times, delaying crystallization and retaining the glassy state is desirable[1, 28] The rate of crystallization will be dependent on the temperature, moisture content, as well as the composition of the mixture. Water acts as a strong plasticizer of sugar solutions by decreasing the $T_g$, yet water cannot be completely eliminated from biological systems without injury[65-71]. Sugar glasses will take up moisture from their stored environment to varying degrees, depending on the composition of the glass and the relative humidity (RH) of the environment. The moisture update can be significant enough to decrease the $T_g$ of the system to below the storage temperature, which renders the system amenable to crystallization on a short time scale,

but depending on the components present, this can be delayed for practical periods of time[9, 38, 44, 46, 72-75].

Pure trehalose glass crystallizes above 44% RH in less than one day therefore maintenance of a low relative humidity in the environment of the sample is critical for maintaining the desirable features of the glass. The crystallization of amorphous trehalose leads to the loss of random structure and eventually loss of the protective effect of trehalose[28, 61, 76]. Maintenance of low humidity can be achieved with specialized packaging, but additives that delay crystallization at high relative humidity can also be beneficial, reducing the packaging and storage demands.

Different additives have been used to modify the physical properties of trehalose, and to increase the stability and retention of trehalose glass, including polymers, sugars, and salts as described previously in chapter 1. The presence of salts in sugar systems is of special interest due to the presence of electrolytes in many biological systems, and the frequent inclusion of salts in formulations for pH buffering purposes. When designing formulations for the preservation of biologics, specifically proteins for therapeutic purposes, components should be biocompatible and those that are naturally found in the human body such as choline and phosphate based salts can be good options.

Previous work in our lab showed the addition of sodium mono hydrogen phosphate can delay crystallization of trehalose glass at 61% RH while sodium dihydration phosphate as an additive to trehalose was not a crystallization suppressant[77]. Weng and et al. reported [78] that trehalose-dihydrogen phosphate mixture differed from trehalose-monohydrogen phosphate in the strength of the hydrogen bonding network. Monohydrogen phosphate anion made stronger, shorter, and more linear hydrogen bonds with trehalose, while the strength of hydrogen bonds

between dihydrogen phosphate anion and trehalose were comparable to trehalose-trehalose hydrogen bonds. This suggests that the addition of salts with crosslinking anions may lead to more stable compostions due to the stronger hydrogen bonding network of the sugar-salt mixture that can resist against the water penetration for a longer time compared to the weaker trehalose-trehalose network. As mentioned earlier in chapter 1, other hydrogen bonding anions like acetate and citrate (both of them are organic) [47] can delay crystallization of the sugar system when the mixture is exposed to high RH compared to chloride anion. The addition of organic salts with either an organic cation or organic anion to sugar systems and their underlying stability mechanism have not been well studied to date.

## 2.2 Overall Goals

Hypotheses have been put forward by others to explain the stabilizing mechanism of trehalose by salts, but a comprehensive picture is still lacking. Specifically there are gaps in understanding the specific interactions between water, salts, and trehalose that play an essential role in persistence of the amorphous structure and stabilization of biomolecules. The objective of this work is thus to understand the underlying mechanisms that lead to longer shelf life of trehalose based glasses in extreme environmental conditions (elevated humidities) and to determine the effects of different salts including organic salts on the water sorption characteristics and crystallization kinetics of trehalose glass. Water sorption studies can provide insight into the microstructure of the system and the interaction of water with the solid components, and thus can yield information that improves our understanding of stabilizing characteristics.

## 2.3 Specific Aims

In order to achieve the research goals discussed above, the following aims were proposed:

AIM 1: To determine if the delay of crystallization in trehalose glasses held at 61% relative humidity correlated with changes in $T_g$.

Crystallization of a glass occurs when the amorphous matrix is held above its glass transition temperature for a finite period of time. The proposed stabilizing effects of some additives like maltodextrin and PVP have been attributed to the increase in $T_g$ of the tertiary blends of trehalose/polymer/water compared to pure trehalose solution at the same moisture content. Previous work in our lab [77] has shown that the addition of certain phosphate salts (i.e. sodium monohydrogen phosphate and choline monohydrogen phosphate can suppress crystallization of trehalose glass in 61% relative humidity for a longer period than is possible with pure trehalose. The specific aim of this experiment was to test the hypothesis that the crystallization delay was due to an increase in $T_g$ attributed to cross-linking between trehalose and the phosphate anion[78]. The $T_g$ of trehalose and trehalose salt compositions with moisture contents in the range of 0-0.4 $gH_2O/gdw$ were determined using a dynamic mechanical analyzer (DMA). The salts sodium monohydrogen phosphate and choline monohydrogen phosphate were investigated with a salt:trehalose ratios of 1:4.

AIM 2: To determine if the delay of crystallization in trehalose based systems correlated with water sorption characteristics. Aim 2a) To determine the effect of composition (crosslinking VS noncrosslinking anions) on the equilibrium moisture content and crystallization behavior of sugar based systems at different relative humidities. Aim 2b) To determine the sorption isotherm and water sorption constants of these same compositions to gain insight about the microstructure of that system and water solid components interactions.

The amount of water absorbed by the system depends on the relative humidity of the environment. Plotting the equilibrium moisture content of the sample together with its the

corresponding relative humidity, i.e. creating a 'sorption isotherm', can yield an improved physical understanding of the nature of water sorption the interaction of water with the components.

In order to further explore the role of cross-linking anion on the delay of crystallization we studied sorption isotherms of different trehalose/salt mixtures with either a crosslinking anion or a non-crosslinking anion. Choline is a big organic cation, so in order to evaluate the effect of cation size, choline salts results were compared with sodium cation salts with the same anion. Mixtures of salt/trehalose glasses were exposed to humidity levels ranging from 7% to 76%. The salts sodium monohydrogen phosphate (small cation/crosslinking anion), sodium dihydrogen phosphate (small cation/noncrosslinking anion), choline monohydrogen phosphate (big cation/crosslinking anion), choline dihydrogen phosphate (big cation/noncrosslinking anion) were evaluated in salt: trehalose ratios of 1:2. The previous sorption study in our lab showed that the crystallization delay effect was not observed for low ratios of salt:trehalose such as 1:4.8 but retention of the glass happened in higher ratios of salt to trehalose (1:2)[77]. The trehalose:salt sorption isotherms were compared to pure trehalose.

AIM 3: To determine if the design rules emerging from Aim 2 could be generalized to other salts.

The equilibrium moisture content (EMC) and BET sorption isotherms were determined for choline citrate, choline acetate, tetramethyl ammonium acetate, choline chloride, sodium acetate, sodium citrate, calcium chloride, magnesium chloride, sodium chloride mixed with trehalose at a molar ratio of 1:2 of salt:trehalose. Different salts were chosen in order to confirm and/or further refine the chemical characteristics of salts that cause delay of crystallization. These categories are summarized below:

Category 1: Salts with a small metal cation and a non-crosslinking anion such as magnesium chloride, calcium chloride, and sodium chloride were evaluated in order to determine the effect of salt hygroscopicity on the delay of crystallization.

Category 2: Salts with the same anion with either a metal or an organic cation such as choline acetate, tetramethylammonium acetate and sodium acetate were selected in order to determine the effect of cation size (steric hindrance) on crystallization suppression.

Category 3: Salts with the same cation but with either a cross-linking or non-crosslinking anion such as sodium citrate, sodium phosphate salts, and sodium chloride were selected in order to determine the role of hydrogen bonding ability of anions on the delay of crystallization.

# CHAPTER 3: GLASS TRANSITION TEMPERATURE STUDIES OF PURE TREHALOSE AND TREHALOSE-SALT MIXTURES WITH DIFFERENT WATER CONTENTS

## 3.1 MATERIALS AND METHODS

### 3.1.1 Glass Transition Temperature measurements

High purity α-α-trehalose dehydrate and sodium monohydrogen phosphate were purchased from Ferro Pfanstieh (IL, USA) and Sigma Aldrich (MO, USA), respectively. Choline monohydrogen phosphate was made by mixing 2 moles of choline hydroxide solution (20 wt% water) and 1 mole of o-phosphoric acid solution (85 wt% water). Choline hydrogen solution and o-phosphoric acid were purchased respectively from Sigma Aldrich (MO, USA) and Fisher Scientific (MA, USA). These salts were used to prepare solutions of 20% (w/v) trehalose/salt mixtures (molar ratio 4:1) by adding the appropriate masses of trehalose or salt to 18.2 MΩ water. In order determine the effect of salt addition on the glass transition temperature of trehalose in the dry state, both trehalose:salt solutions and pure trehalose solution were lyophilized using a Virtis Bench top lyophilizer (SP Scientific, USA). Samples were moved to a $P_2O_5$ desiccator right after lyophilization.

In order to measure the $T_g$ of wet samples and evaluate the effect of salt addition on the $T_g$ of trehalose in the presence of water, aqueous solutions were pipetted onto rectangular filter papers and then dried to different water contents using a SAM microwave at 11% power.

### 3.1.2 Dynamic Mechanical Analysis of Dehydrated Samples

A Q800 Dynamic Mechanical Analyzer (TA Instrument, USA) was used to determine the glass transition temperature ($T_g$) of the samples. The DMA was calibrated with a polycarbonate bar in the single cantilever mode at 1 Hz frequency and nitrogen gas was used as the purge gas. In

order to load the sample into the single cantilever clamp a stainless steel material pocket purchased from Perkin Elmer (CT, USA) was used. Lyophilized samples were melted on a hot plate at 200°C<T<230°C and immediately quenched at room temperature in order to attain the glassy state. Samples were then loaded into the DMA and subjected to the heating scan of 5°C /min to 220°C at frequency of 1 Hz and amplitude of 30 μm.[79] Samples were dried by melting the lyophilized samples on the material pocket on a hot plate at 220°C and quenching at room temperature. The water content of the equivalently dried samples was determined to be 0.0032 $\pm$ 0.0008 $gH_2O/gdw$ using a Karl Fisher titrator. The $T_g$ was determined by the study of viscoelastic properties of glasses that are temperature dependent. The viscoelastic properties [storage Modulus(E'), Loss modulus(E"), and Tanδ changed drastically at the glass transition temperature. For each sample 3 replicates were tested. In this work all the reported $T_g$ values are by storage modulus(E') by taking the midpoint of intersecting slopes.

### 3.1.3 Dynamic Mechanical Analysis of Humidified Samples

A 50 μL droplet of each solution was pipetted onto a rectangular (30mm*5.5mm) borosilicate glass microfiber paper (Whatman, UK) and then processed in a microwave (SAM 225, CEM Corp.; USA) for different time periods from 5 minutes to 30 minutes at 11% relative humidity. Two samples were prepared and dried each time, one for measuring the end moisture content by a Karl Fisher titrator (Mettler Toledo; Germany) and the other one was put immediately into a stainless steel material pocket (Perkin Elmer, USA) and loaded into a Q800 dynamic mechanical analyzer (TA Instruments) in the single cantilever mode to determine the $T_g$ of the compositions at 1 Hz frequency and amplitude of 30μm as a function of temperature. Samples were quenched to -100°C, stabilized for 15 minutes then heated to 220°C at 5°C /min to generate data for thermal analysis. Quenching was achieved by controlled delivery of cold

nitrogen gas into the DMA chamber. For each microwave processing time 3 replicates were tested.

### 3.2 Results and Discussions

Analysis of DMA data demonstrated that the addition of sodium monohydrogen phosphate and choline monohydrogen phosphate even at a small molar ratio of 1:4 salt to trehalose increased the $T_g$ in the dry state by almost 10°C, as shown in Table 3. As discussed previously, the increase in $T_g$ in the dry state can be attributed to stronger hydrogen bonds resulting from cross-linking of the monohydrogen phosphate with trehalose compared to a pure trehalose-trehalose network[78].

Table 3. The DMA Results for dehydrated samples

| | Pure Trehalose | Trehalose-sodium monohydrogen phosphate | Trehalose- choline monohydrogen phosphate |
|---|---|---|---|
| Average $T_g$ | 121.0 | 129.8 | 131.0 |
| STDEV | ±2.0 | ±2.0 | ±3.2 |

DMA measurements of wet compositions also demonstrated that increasing the water content of the system caused $T_g$ to be depressed. Figure 10 represents two DMA traces of trehalose:sodium monohydrogen samples with different processing times. As it was mentioned earlier, at the glass transition temperature the storage modulus decreased suddenly and loss modulus and Tanδ attained their highest value. In figure 11, the glass transition of the drier sample that had been processed in the microwave for a longer period of time(figure 10 (b)), can be seen to occur at a higher temperature value.

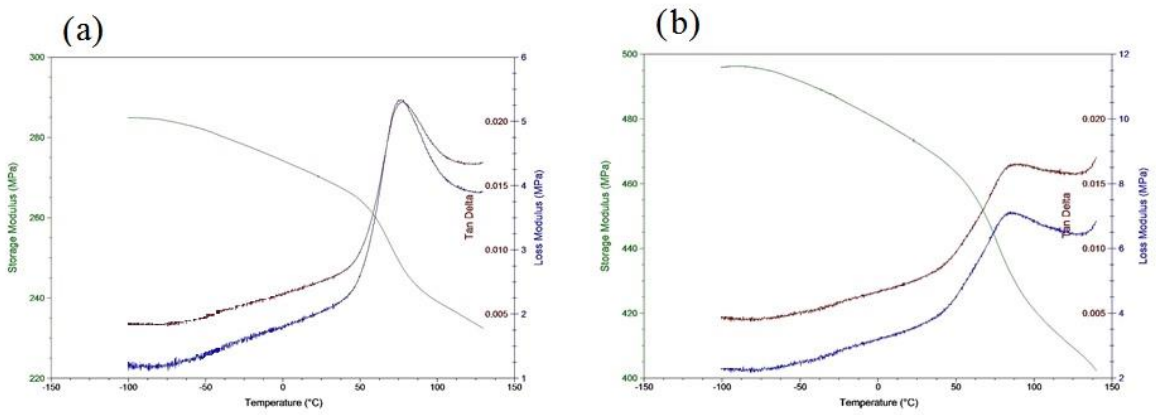


Figure 11. DMA traces of (a) trehalose:sodium monohydrogen phosphate sample after 25 minutes microwave and (b) trehalose:sodium monohydrogen phosphate sample after 30 minutes microwave

The glass transition temperature values of trehalose and trehalose-salt samples as a function of water contents were fitted to Gordon and Taylor equation and are shown in figure 12. The analysis of humidified compositions revealed that increasing the water content of the system depressed the $T_g$ of pure trehalose, as expected. The same was true of the trehalose-salt compositions, with no apparent retention of the $T_g$ increase effect from combining trehalose with salt. This would suggest that water caused dissociation of trehalose-$HPO_4^{2-}$ interactions and thus loss of the hydrogen bonding between trehalose and salt as water was added into the system. The higher stability of trehalose-choline monohydrogen phosphate and trehalose-sodium monohydrogen phosphate in high relative humidity environments cannot be attributed

to an increase of $T_g$ of the tertiary blends of trehalose-salt- water compared to binary blend, at the same ratio of water

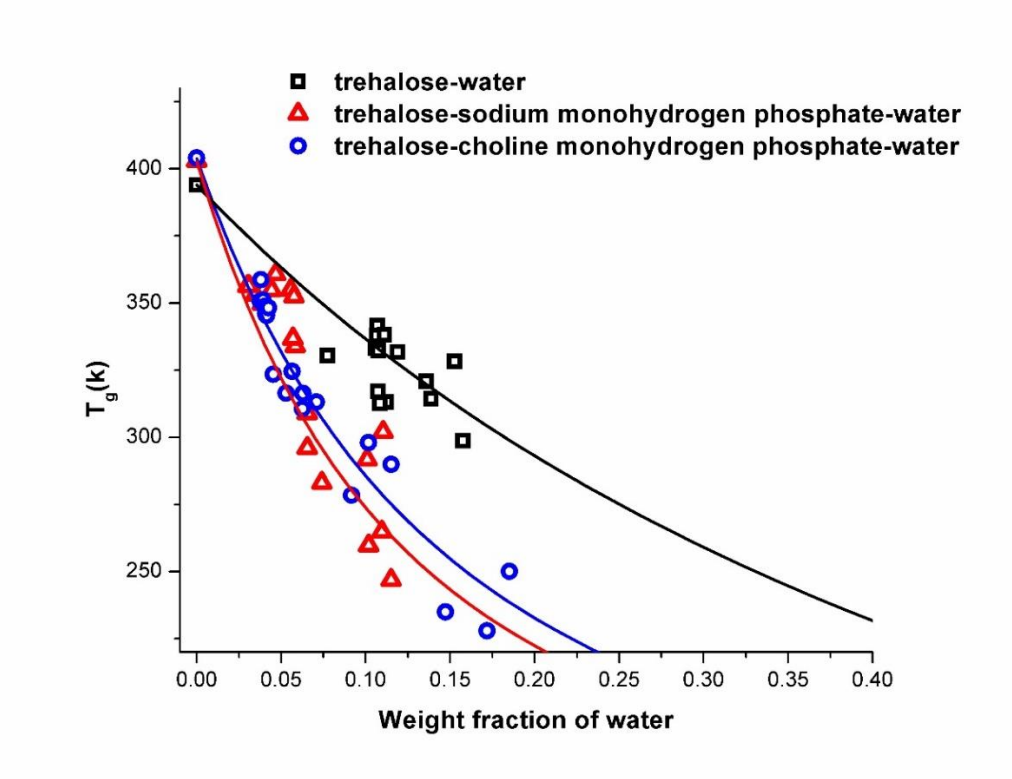


Figure 12. Glass transition temperature of trehalose-salt mixtures in different water contents

Gordon and Taylor Equation fitting parameters of the compositions are shown in table 4.

Table 4. Gordon and Taylor fitting parameters for different aqueous compositions

| | K value | R square |
|---|---|---|
| Trehalose-water | 2.602 | 0.957 |
| Trehalose-water-sodium monohydrogen phosphate | 8.517 | 0.905 |
| Trehalose-water-choline monohydrogen phosphate | 7.196 | 0.94 |

# CHAPTER 4: CRYSTALLIZATION KINETICS AND WATER SORPTION CHARACTERISTICS OF TREHALOSE MIXED WITH ORGANIC AND INORGANIC PHOSPHATE SALTS

## 4.1 MATERIALS AND METHODS

### 4.1.1 Materials

Trehalose, sodium monohydrogen phosphate and choline monohydrogen phosphate were acquired or prepared as mentioned in the previous chapter. Sodium dihydrogen phosphate ($NaH_2PO_4$) was purchased from Sigma Aldrich (MO, USA). Choline dihydrogen phosphate ($ChH_2PO_4$) was also prepared by mixing Phosphoric acid 85 wt% in $H_2O$ and Choline hydroxide solution 45 wt% in methanol. Phosphoric and acid and choline hydroxide were purchased from Sigma Aldrich (MO, USA).These salts were used to prepare solutions of 20% (w/v) trehalose/salt mixtures with the molar ratio of 2:1 trehalose:salt.

Seven different salts were used to make saturated salt solutions[80]. Lithium bromide (LiBr), lithium chloride (LiCl), and magnesium chloride ($MgCl_2$) were purchased from Fisher Scientific (NH, USA). Sodium bromide (NaBr) and potassium acetate ($KCH_3CO_2$) were purchased from Alfa Aesar (MA, USA). Potassium carbonate ($K_2CO_3$) and sodium chloride (NaCl) were purchased from Sigma Aldrich (MO, USA) and Amresco Inc (OH, USA), respectively.

### 4.1.1 Constant Relative Humidity Jars

Saturated salt solutions were used to provide standard constant relative humidity in jars. LiBr, LiCl, $KCH_3CO_2$, $MgCl_2$, $K_2CO_3$, NaBr and NaCl provide 7%, 12%, 23%, 33%, 43%, 59% and 76%RH, respectively[80]. Each salt was added to a Mason glass jar with 50 ml water and was

stirred until a thick saturated mixture was formed. These jars are shown in figure 13. The mixtures were adjusted to ensure that they contained some free water. The jars were sealed and put aside for 24 hours to allow them to come to equilibrium.

Figure 13. Constant relative humidity jars, using different saturated salt solutions in mason jars

A relative humidity and temperature meter, part number: HH314A was purchased from Omega and was used to check the relative humidity of each jar. All experiments were conducted at room temperature.

### 4.1.2 Sample preparation for sorption studies

Sodium dihydrogen phosphate, sodium monohydrogen phosphate, choline dihydrogenphosphate, and choline monohydrogen phosphate were used to prepare solutions of 20% (w/v) of 2:1 trehalose:salt mixtures, molar basis, by adding the appropriate masses of trehalose and salt to 18.2 MΩ water. Also pure trehalose solution of 20% (w/v) was prepared as a control. Aliquots of 100 µl of solution were pipetted onto a glass coverslip purchased

from Fisher Scientific (PA, USA) and dried for 35 minutes using a SAM microwave at a power setting of 19% (nominally 146 W) at 11% relative humidity. Samples were then transferred to constant relative humidity jars for sorption studies. A gravimetric method was used to determine the water content of samples after microwave processing and throughout the sorption studies, as described in the next section.

### 4.1.3 Bake out method and gravimetric determination of samples water content

In order to evaluate the water content of each sample after microwave processing, the mass of each sample was measured before and after processing using a AX105DR Mettler Toledo balance (Switzerland). The solid content of each solution was determined by the bake-out method, which involved drying each solution for 48 hours at 90 °C in a VWR gravity convention oven (PA, USA) followed by cooling of the samples in a phosphorus pentoxide dessicater for 5 hours. The final measured mass represented the solid content of each solution[81, 82]. Moisture sorption characteristics of samples were investigated by measuring the changes in mass of each sample every day. Throughout the experiments samples were also observed for crystal formation.

### 4.1.4 Data Analysis

The initial water content of each sample after microwave processing ranged from 0.03 to less than 0.1 grams water per gram dry weight ($gH_2O/gdw$) after 35 minutes of rapid evaporative drying using the SAM microwave inside the relative humidity chamber at 11% RH. This range of water contents at room temperature yields samples that are in the glassy state. The glassy samples were then moved to constant relative humidity jars and the mass changes of samples (changes in water content) in each relative humidity jar and their crystallization kinetics were studied. The moisture sorption isotherms (water content as a function of relative pressure)

were also plotted and fit to BET model using OriginPro 8. Sorption data is reported as the average of at least three replicates.

### 4.2 Results and Discussions

This study revealed that the samples weights changed the most over the first day and then reached an equilibrium value within 9-10 days. Figure 14 shows the sorption kinetics of trehalose- sodium dihydrogen phosphate samples.

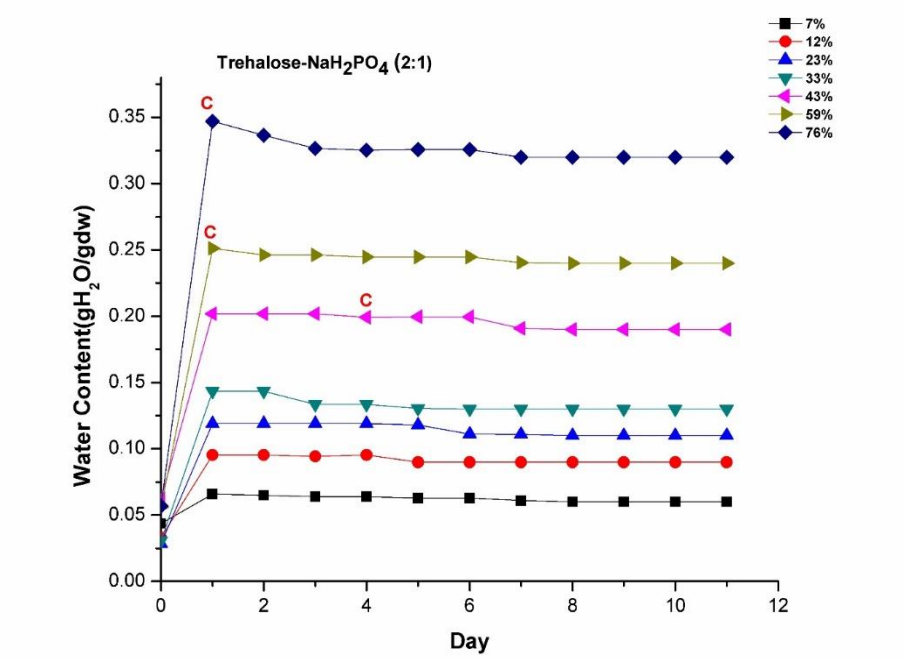


Figure 14. Sorption kinetics of trehalose-sodium dihydrogen phosphate with molar ratio of 2:1 at room temperature

The addition of sodium dihydrogen phosphate to trehalose delayed crystallization at 44% RH up to 4 days but all samples above 44% RH crystallized in the first day just like pure trehalose samples. Figure 15 shows sorption kinetics of trehalose- sodium monohydrogen phosphate samples. The addition of sodium mono hydrogen phosphate suppressed crystallization at 44% RH and delayed the crystallization above 44% RH up to 3 days. Sodium monohydrogen

phosphate was more effective in delaying crystallization compared to sodium dihydrogen phosphate, which may be the result of the hydrogen bonding ability of the anion. Using molecular dynamics simulation Weng and et al. showed that $HPO_4^{2-}$ can crosslink trehalose while $H_2PO_4^{1-}$ anions do not make hydrogen bonds with trehalose.

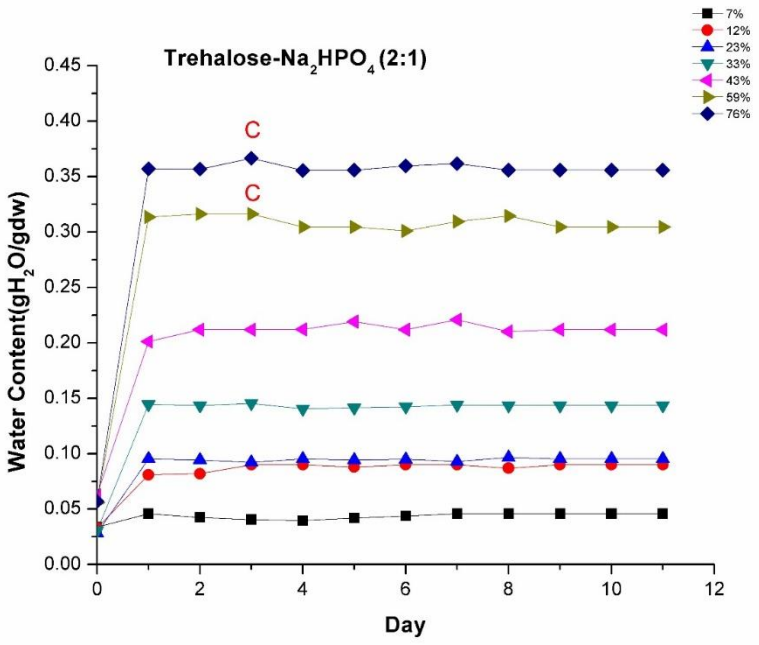


Figure 15. Sorption kinetics of trehalose-sodium monohydrogen phosphate with molar ratio of 2:1 at room temperature

Figure 16 shows the sorption kinetics of trehalose-choline dihydrogen phosphate at the molar ratio of 2:1 at room temperature. The addition of choline dihydrogen phosphate was able to suppress crystallization at 44%RH and delay crystallization above 44% RH for up to 3 days. Choline dihydrogen phosphate is an organic salt consisting of a big cation and a non-crosslinking anion.

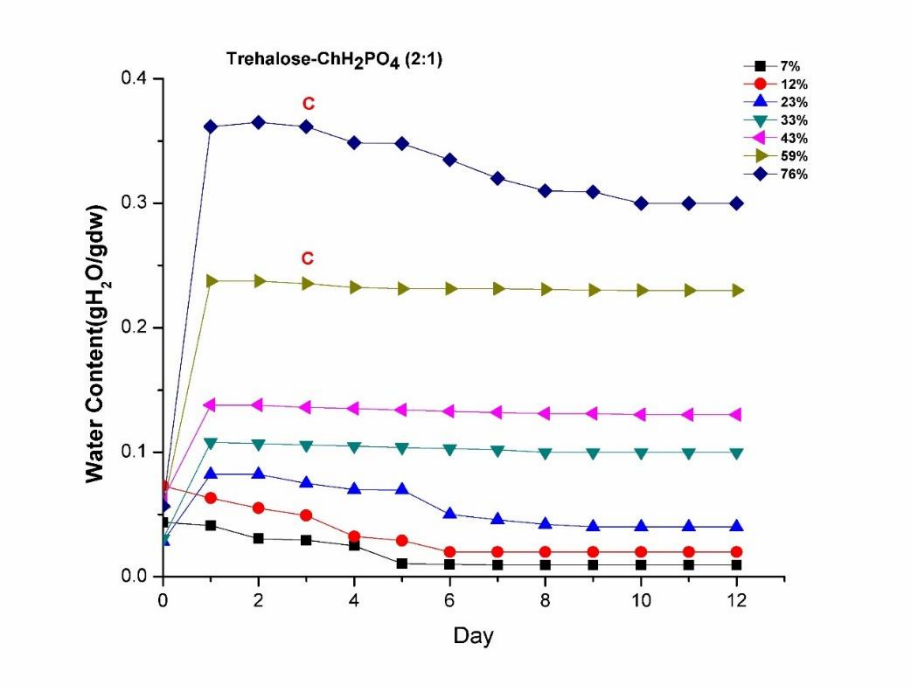


Figure 16. Sorption kinetics of trehalose-choline dihydrogen phosphate (CDHP) with molar ratio of trehalose:salt 2:1 at room temperature

Figure 17 shows the sorption kinetics of trehalose-choline monohydrogen phosphate for the molar ratio of trehalose:salt 2:1 at room temperature. Pure trehalose samples crystallized at 44% RH and above in the first 24 hours while in contrast with pure trehalose, trehalose-choline monohydrogen phosphate did not crystallize at 44% RH and delayed crystallization above 44% RH for up to 6 days, making choline monohydrogen phosphate the most effective salt among the salts tested so far. The amount of crystals observed in trehalose-choline monohydrogen phosphate samples as shown in figure 18 comprised only a small portion of the sample.

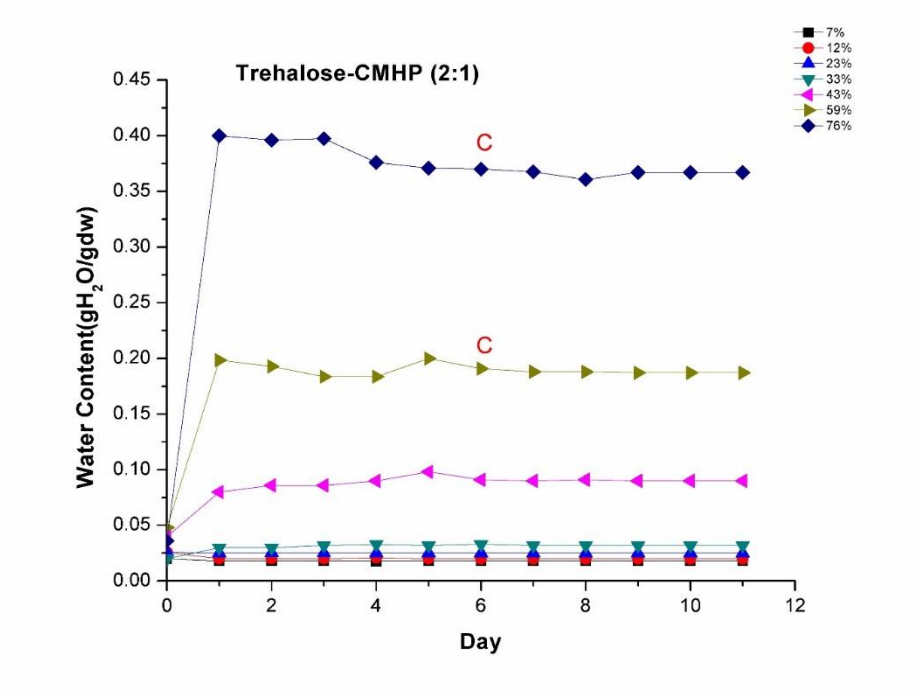


Figure 17. Sorption Kinetics of trehalose-choline monohydrogen phosphate(CMHP) at molar ratio of 2:1 at room temperature

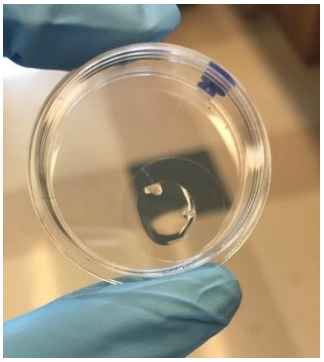

Figure 18. Crystallized sample of trehalose-choline monohydrogen phosphate (2:1) exposed to76% RH

Choline monohydrogen phosphate is an organic salt, consisting of a big organic cation and a crosslinking anion. The stability difference between trehalose-choline monohydrogen phosphate and trehalose-choline dihydrogen phosphate could be attributed to the anion hydrogen bonding ability with trehalose. Choline monohydrogen phosphate was also a better

additive than sodium monohydrogen phosphate even though both contained a crosslinking anion. Choline is a big cation while sodium is a small metal cation, suggesting a role for the cation size.

Sorption isotherms and BET equation fitting parameters $C_B$ and $W_B$ can also provide insights about stabilizing mechanisms, as mentioned in chapter 1. The $C_B$ constant represents the interactions between water and the solid components during moisture uptake process. The $W_B$ parameter represents the amount of adsorbed water accumulating on the surface of the sample. Figure 19 shows the sorption isotherm of trehalose-sodium dihydrogen phosphate (2:1) at room temperature. Figure 19 was obtained in Origin pro 8 and the sorption data is fit to the BET model. The sorption isotherm for trehalose-sodium dihydrogen phosphate is characterized as a type 2 and table 5 shows the BET model fit parameters regarding to these samples.

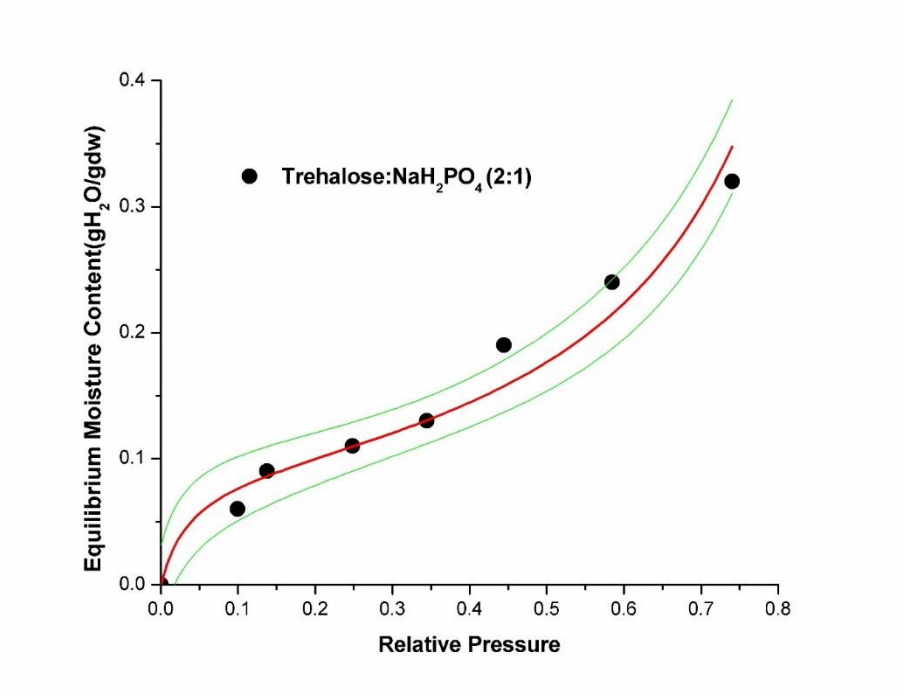


Figure 19. Moisture sorption isotherm for trehalose-sodium dihydrogen phosphate molar ratio (2:1)

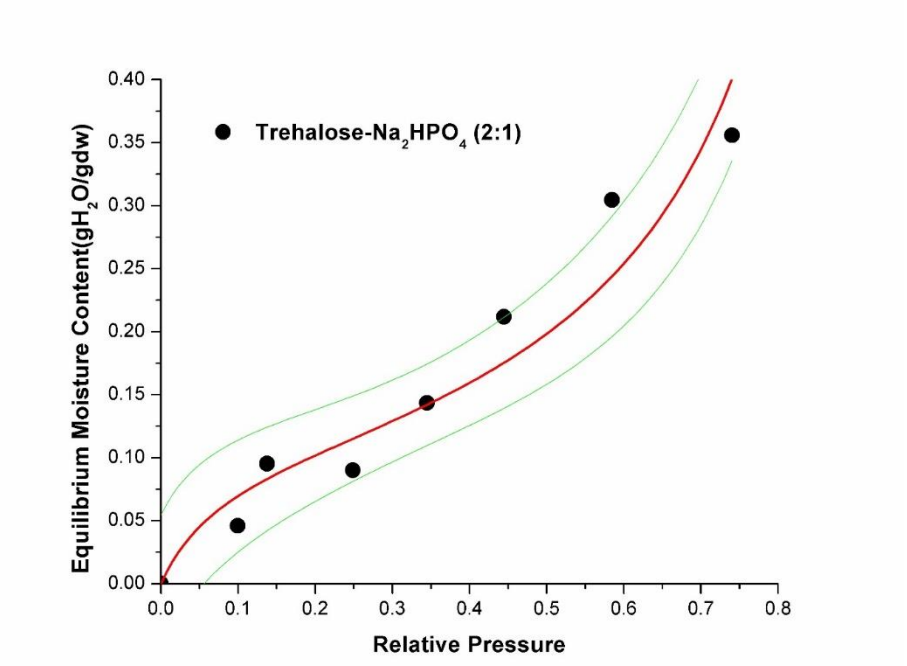


Figure 20. Moisture sorption isotherm for trehalose- sodium monohydrogen phosphate (2:1)

The sorption isotherm for trehalose-sodium monohydrogen phosphate is characterized as a type 2 as it is shown in figure 20 and table 5 shows the BET model fit parameters regarding to these samples.

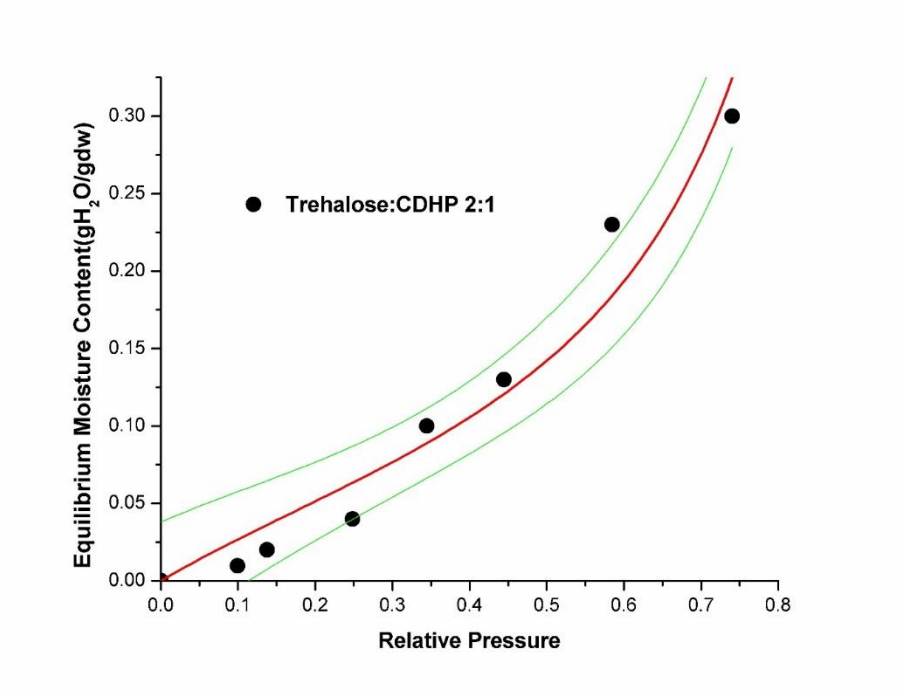


Figure 21. Moisture sorption isotherm for trehalose-choline dihydrogen phosphate (CDHP) molar ratio (2:1)

The sorption isotherm of trehalose-choline dihydrogen phosphate is shown in figure 21. The addition of choline dihydrogen phosphate ($ChH_2PO_4$ or CDHP) to trehalose did not change the isotherm type of trehalose, the same is sodium mono hydrogen phosphate and sodium dihydrogen phosphate and their sorption isotherms are all recognized as isotherm type 2. Table 5 shows the BET model fitting parameters of trehalose-choline dihydrogen phosphate samples.

Figure 22 compares the sorption isotherm of pure trehalose and trehalose-choline monohydrogen phosphate. It has been reported repeatedly in the literature and also this work shows pure trehalose sorption isotherm is characterized as a type 2 while the addition of choline monohydrogen phosphate to trehalose changed the sorption isotherm from type 2 to type 3. As discussed earlier in isotherm type 3 comparing to type 2, samples equilibrate to lower moisture contents in low relative pressure, which represents lower chemical reactions of water with the sugar-salt system.

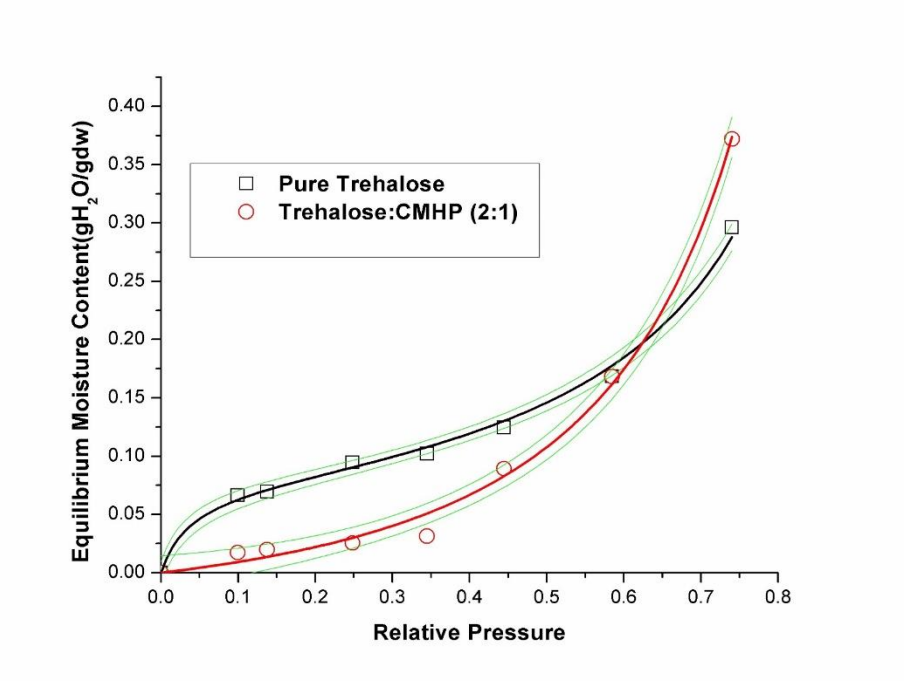


Figure 22. Sorption isotherm of pure trehalose VS trehalose-choline monohydrogen phosphate(CMHP) mixture

Figure 22 shows trehalose-choline monohydrogen samples equilibrate to higher moisture contents in elevated humidities (above 60% RH) compared to pure trehalose which represents higher amount of free water in this system. Table 5 shows the fit parameters regarding to pure trehalose and trehalose-choline monohydrogen phosphate samples.

The $C_B$ value for pure trehalose sample that also shows the amount of water that chemically react with trehalose molecules in this system is significantly higher than the $C_B$ constant for trehalose-choline mono hydrogen phosphate. The $W_B$ value for trehalose-choline monohydrogen phosphate is significantly higher than pure trehalose. When more water sits on the surface of a sample it means that smaller amount of equilibrium moisture content has penetrated into the sample. Table 5 summarizes the information from sorption kinetics and sorption isotherms of pure trehalose and trehalose-salts mixtures with the molar ratio of 2:1. The table is arranged in descending order of stability.

Table 5. Data from sorption studies of trehalose-salt mixture with the molar ratio of 2:1 at room temperature at 76% RH

| | EMC($gH_2O$/gdw) At 76% RH | Crystallization At 76% RH | Isotherm | $W_B$ | $C_B$ | R-Square |
|---|---|---|---|---|---|---|
| Trehalose-$Ch_2HPO_4$ | 0.371 | Day 6 | Type 3 | 0.171 ± 0.02 | 0.458 ± 0.11 | 0.993 |
| Trehalose-$ChH_2PO_4$ | 0.311 | Day 3 | Type 2 | 0.093 ± 0.01 | 3.101 ± 1.79 | 0.945 |
| Trehalose-$Na_2HPO_4$ | 0.355 | Day 3 | Type 2 | 0.106 ± 0.009 | 12.7 ± 11.7 | 0.911 |
| Trehalose-$NaH_2PO_4$ | 0.326 | Day 1 | Type 2 | 0.093 ± 0.005 | 26.8 ± 23.9 | 0.957 |
| Pure Trehalose | 0.296 | Day 1 | Type 2 | 0.069 ± 0.001 | 26.04 ± 8.42 | 0.994 |

Among all tested compositions, pure trehalose was the least stable and trehalose-choline mono hydrogen phosphate was the most stable composition at elevated humidities (above 44% RH). Trehalose-sodium dihydrogen phosphate also crystallized at elevated humidities in the first 24 hours like pure trehalose but the addition of sodium dihydrogen phosphate could delay crystallization at 44% RH up to 4 days while pure trehalose crystallizes at 44% RH in the first day. Sodium monohydrogen phosphate was a better additive to trehalose than sodium dihydrogen phosphate and could delay crystallization at elevated humidities up to 3 days. What

makes sodium monohydrogen phosphate a better additive to trehalose than sodium dihydrogen phosphate might be, the stronger hydrogen bonding network. By comparing the $W_B$ value of these two compositions from table 5 it was hypothesized that the stronger hydrogen bonding network could lead to higher accumulation of water on the surface of the sample.

The stability of trehalose-choline dihydrogen phosphate and trehalose-sodium mono hydrogen phosphate are comparable at elevated humidities. Both compositions delayed crystallization for up to 3 days. These salts consisted of either a big organic cation (choline) or a crosslinking anion ($HPO_4^{-2}$).

The best stabilizing result was from a choline monohydrogen phosphate salt with a big organic cation and a crosslinking anion. The sorption isotherm of trehalose-choline monohydrogen phosphate was a type 3, suggesting a high amount of water sitting on the surface of the sample, which might be due to the stronger hydrogen bonding network. This would make penetration of water into to the sample harder, and could also account for minimal water chemically reacting with solid components.

The addition of salts to trehalose increases the equilibrium moisture content of trehalose at elevated humidities. This study showed that the compositions with longer stability also equilibrated to higher moisture levels. This can be interpreted, as the water loving characteristics of ions may be also a factor in preventing trehalose molecules to satisfy their water level requirement to form trehalose dihydrate (crystallized trehalose).

Based on these observations and results from previous studies by others we hypothesized that there would be classes of organic salts that have the potential to suppress crystallization due to multiple complementary mechanism, including: a) the presence of crosslinking anion that can make a strong hydrogen bonding network with trehalose, b) steric hindrance due to the

presence of a big organic cation, and, c) interactions of water with hydrophilic ions that prevent the formation of trehalose dihydrate. As described in subsequent chapters, salts from different families were studied to better understand these various contributions to crystallization suppression.

# CHAPTER 5: BROAD CRYSTALLIZATION AND SORPTION STUDIES OF TREHALOSE MIXED WITH DIFFERENT SALT FAMILIES

## 5.1 Introduction

In the previous chapter after studying the sorption kinetics, sorption isotherms, and BET equation fitting parameters of trehalose mixed with some phosphate salts with organic or inorganic cations it was possible to generate some hypotheses with respect to the underlying stability mechanisms, such as the role of cation size, the crosslinking ability of the anion, and the high equilibrium moisture content of the sample in elevated humidity that can be interpreted as the tendency of salt ions to interact with water and prevent trehalose from forming the dihydrate. In order to evaluate if the results from the initial study could be generalized to other salts, a wider range of salts with specific characteristics were studied.

## 5.2 Materials and Methods

### 5.1.1 Materials

Choline citrate, tetramethylammonium acetate, calcium chloride, and sodium chloride were purchased from Tokyo Chemical Industry (Tokyo, Japan), OXChem Corpration (IL, USA), Acros Organics (NJ, USA), and Amresco (OH, USA) respectively. Choline chloride, choline acetate, sodium acetate, sodium citrate, and magnesium chloride were all purchased from Sigma Aldrich (MO, USA)

### 5.1.2 Sample preparation for sorption studies

Salts were used to prepare solutions of 20% (w/v) of trehalose/salt mixtures (molar ratio 2:1) and samples were dried for 35 minutes by microwave as described in the previous chapter. Samples were then transferred to constant relative humidity jars for sorption studies. The

constant relative humidity of jars varied from 7% RH to 76% RH. The gravimetric method was used to measure the water content of samples after microwave processing and during the sorption studies and all experiments occurred at room temperature. For each trehalose:salt composition three replicates were tested.

### 5.2 Results and Discussion

Moisture sorption kinetics of trehalose-sodium chloride, trehalose-calcium chloride, and trehalose-magnesium chloride were investigated by measuring the changes in mass of each sample every day and evaluating the water content. Throughout the experiments samples were also observed for crystal formation. Sorption kinetics of trehalose-sodium chloride with the molar ratio of 2:1 at room temperature is shown in figure 23. The addition of sodium chloride to trehalose did not delay crystallization at 76% RH but at 59% and 44% RH it delayed the crystallization of trehalose from the first day to 2 and 4 days, respectively.

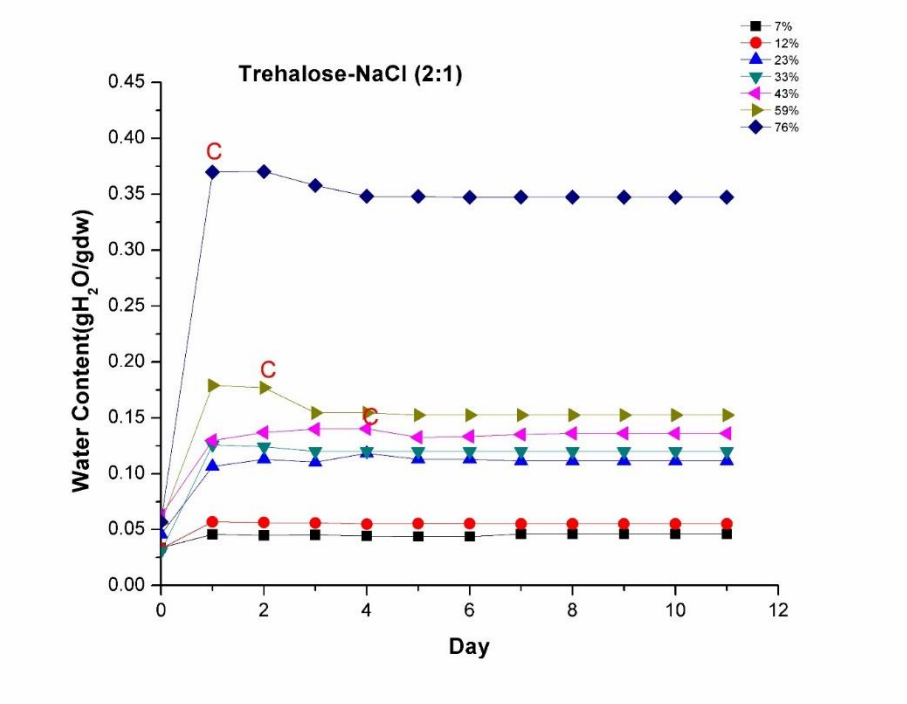


Figure 23. Sorption kinetics of trehalose-sodium chloride with molar ratio of 2:1 at room temperature

The sorption kinetics of trehalose-calcium chloride (2:1) at room temperature is shown in figure 24. The addition of calcium chloride to trehalose was able to delay the crystallization for 2 days at elevated humidity which made it a better additive for trehalose than sodium chloride and sodium dihydrogen phosphate but still not as effective as sodium monohydrogen phosphate, choline dihydrogen phosphate ,and choline monohydrogen phosphate.

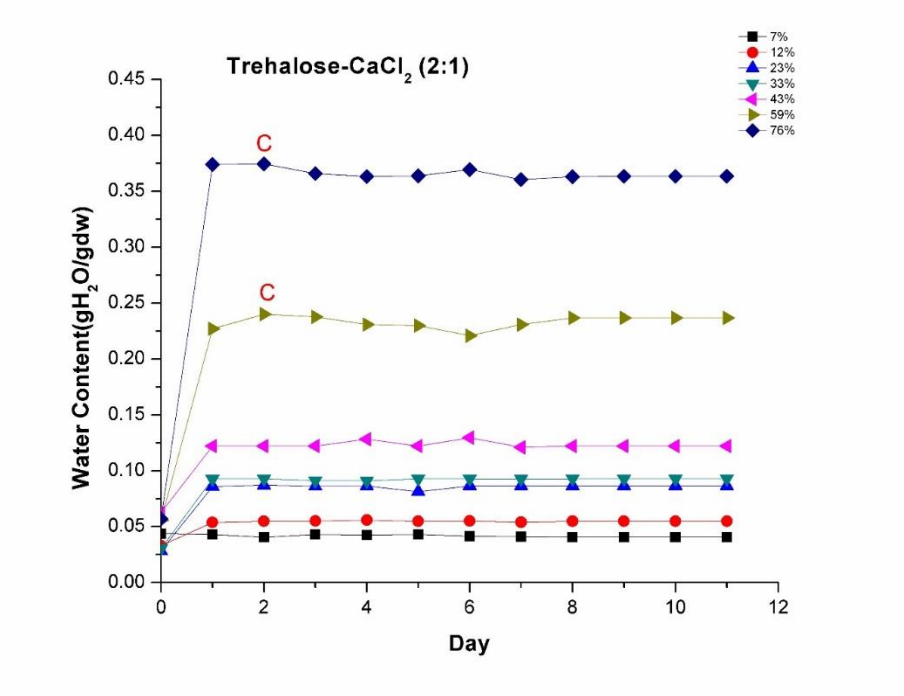


Figure 24. Sorption kinetics of trehalose-calcium chloride with molar ratio of 2:1 at room temperature

The sorption kinetics of trehalose-magnesium chloride (molar ratio 2:1) at room temperature is represented in figure 25. Trehalose- magnesium chloride samples crystallized after 3 days at 76% RH. The stability outcome of $MgCl_2$ as an additive to trehalose is comparable to sodium monohydrogen phosphate and both of these components delayed crystallization for a longer time than sodium chloride, sodium dihydrogen phosphate and calcium chloride.

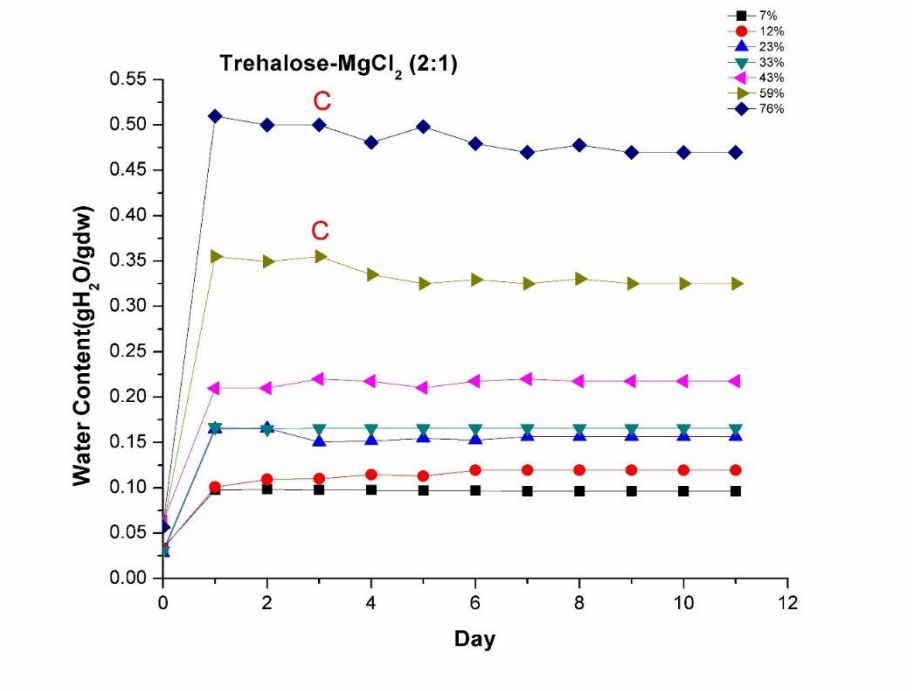


Figure 25. Sorption kinetics of trehalose-magnesium chloride with molar ratio of 2:1 at room temperature

The Sorption kinetics of trehalose-sodium acetate (2:1) at room temperature is shown in figure 26. These samples crystallized after 4 days at elevated humidities, making sodium acetate a better additive to trehalose than sodium chloride, sodium dihydrogen phosphate, and sodium monohydrogen phosphate.

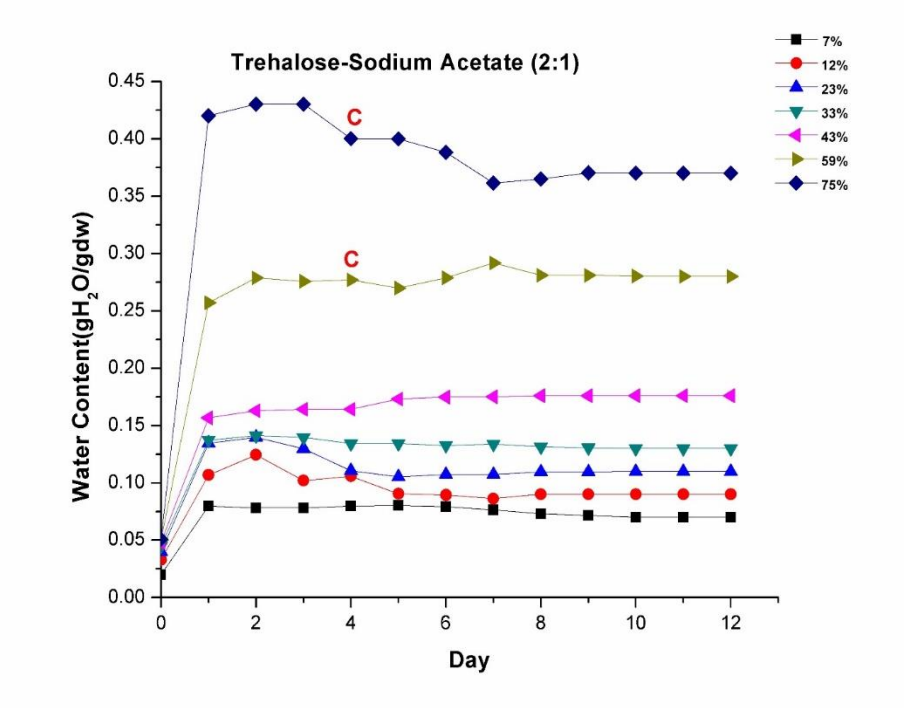


Figure 26. Sorption kinetics of trehalose-sodium acetate with molar ratio of 2:1 at room temperature

Sorption kinetics of the trehalose-sodium citrate composition with the molar ratio of trehalose:salt 2:1 at room temperature is shown in figure 37. Trehalose-sodium citrate samples crystallized at 76% RH at day 4 like trehalose-sodium acetate samples but sodium citrate delayed crystallization for a longer time at 59% RH compared to sodium acetate.

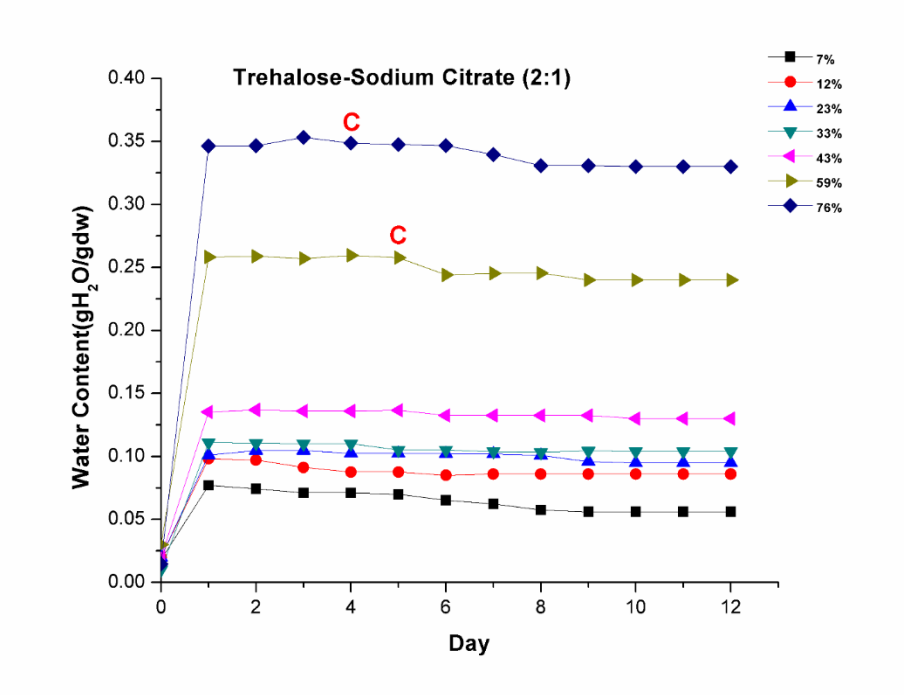


Figure 27. Sorption kinetics of trehalose-sodium citrate with molar ratio of 2:1 at room temperature

These sodium salts are ranked from the most stabilizing to the least stabilizing trehalose additive in elevated humidity:

Sodium citrate> sodium acetate> sodium monohydrogen phosphate> sodium dihydrogen phosphate> sodium chloride

In order evaluate the specific effect of large organic cations, addtional salts were chosen for study. The sorption kinetics of trehalose-choline chloride is shown in figure 28.

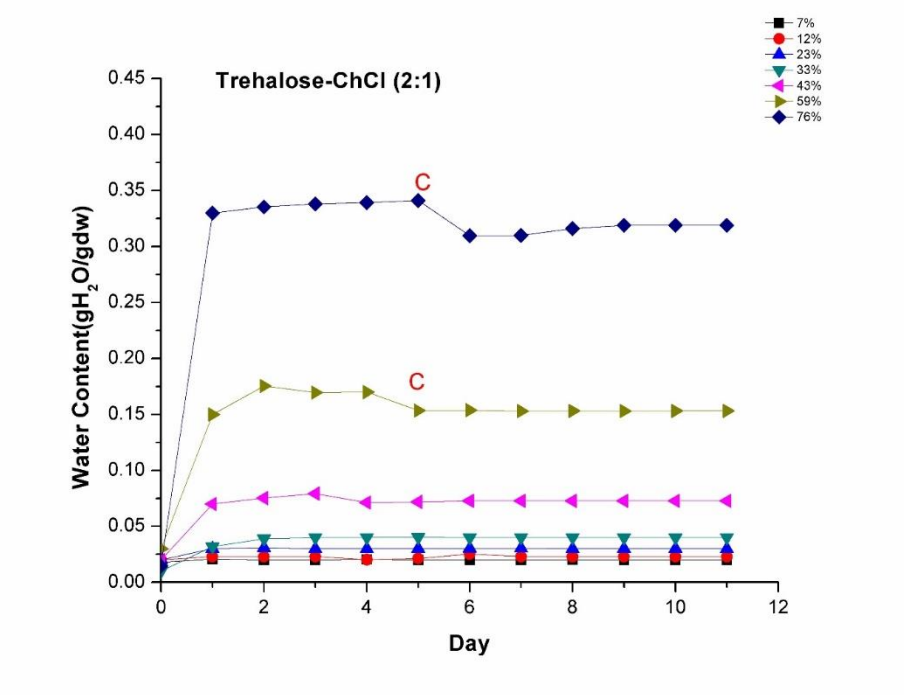


Figure 28. Sorption kinetics of trehalose-choline chloride with molar ratio of 2:1 at room temperature

Choline chloride delayed crystallization for 5 days, which was not quite as effective as choline monohydrogen phosphate as a stabilizing additive, but was still better than choline dihydrogen phosphate and the chloride family with metal cations. The sorption kinetics of trehalose-choline acetate (molar ratio of trehalose:salt 2:1) is represented in figure 29. The addition of choline acetate delayed the crystallization for 10 days at 76% RH but in contrast with previous trends, the samples of trehalose-choline acetate at 59% RH crystallized on day 4, faster than samples at 76% RH.

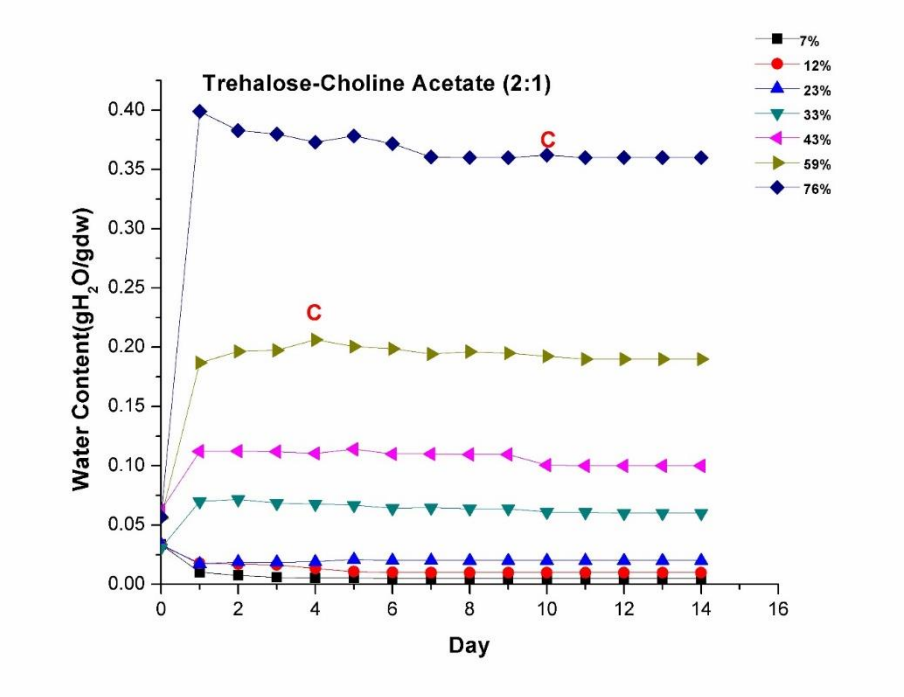


Figure 29. Sorption kinetics of trehalose-choline acetate with molar ratio of 2:1 at room temperature

The sorption kinetics of trehalose-tetramethylammonium acetate at the molar ratio of trehalose:salt 2:1 at room temperature is shown in figure 30. Tetramethylammonium acetate was a better additive to trehalose than choline acetate and delayed crystallization at 76% and 59% RH for 11 days and 13 days, respectively.

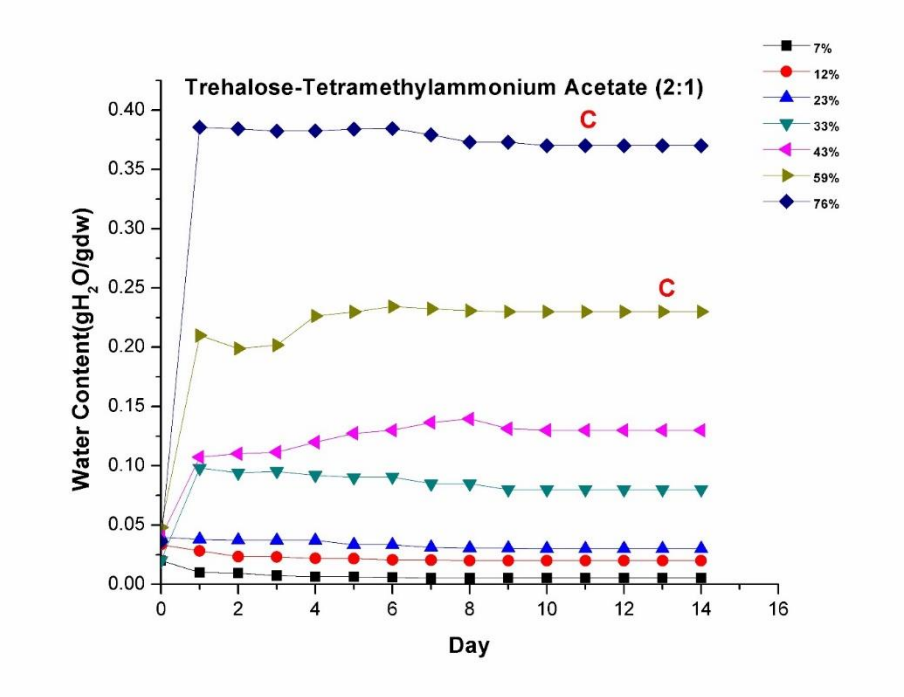


Figure 30. Sorption kinetics of trehalose-tetramethylammonium acetate with molar ratio of 2:1 at room temperature

The sorption kinetics of trehalose-choline citrate with the molar ratio of trehalose:salt 2:1 at room temperature is shown in figure 31. These samples never crystallized. Four months after starting the experiment trehalose-choline citrate samples were checked and they were still stable. As it is shown in figure 31 these samples equilibrate to high moisture content of approximately 0.58 $gH_2O/gdw$.

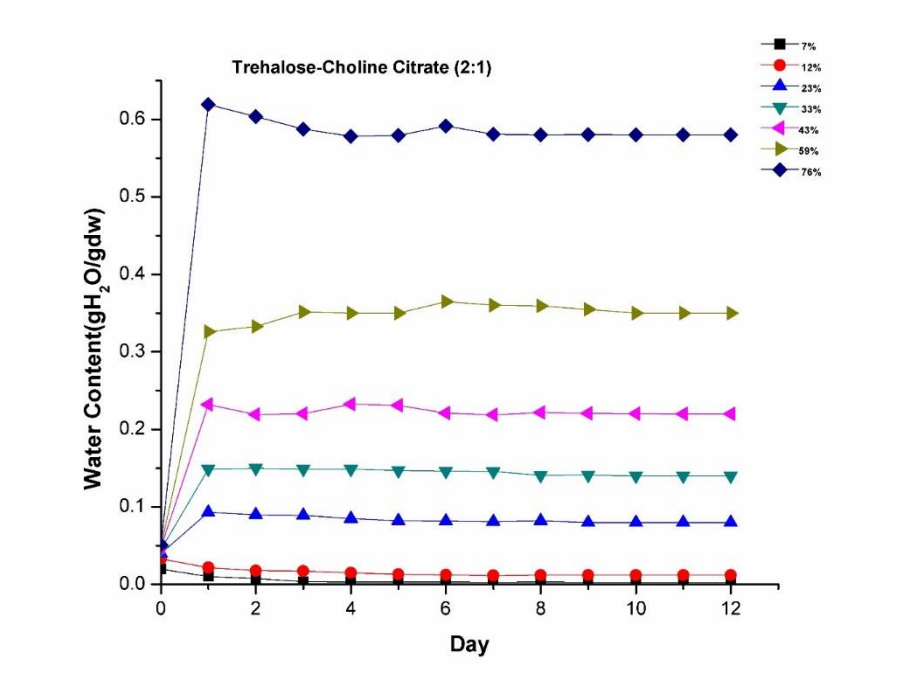


Figure 31. Sorption kinetics of trehalose-choline citrate with molar ratio of 2:1 at room temperature

Sorption kinetics studies in this chapter confirmed our studies and hypotheses in the previous chapter that bigger organic cations as well as crosslinking anions lead to a longer delay of crystallization. It was also observed that the most stable trehalose:salt compostions equilibrated to higher moisture contents in elevated humidity. By plotting the sorption isotherms and fitting them to BET equation, the BET fitting constants can provide insights about the microstructure and the localized water-components interactions. The sorption isotherm of trehalose-sodium chloride (2:1) at room temperature is shown in figure 32 and is characterized as type 2, the same as pure trehalose but the BET fitting parameters reveal differences, as shown in table 6.

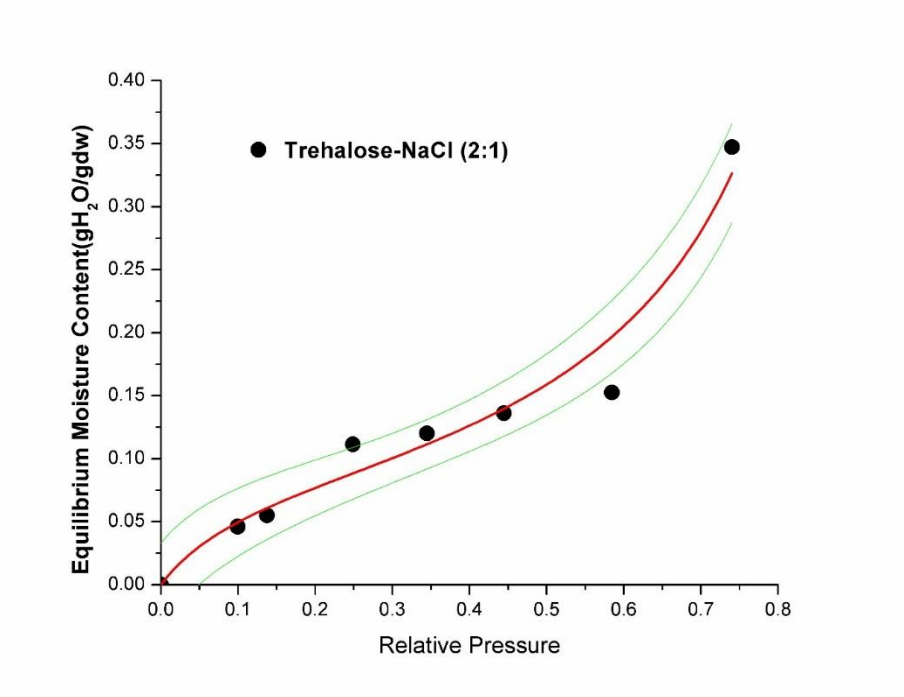


Figure 32. Moisture sorption isotherm for trehalose- sodium chloride with molar ratio of 2:1

Comparing the trehalose-sodium chloride BET fitting parameters from table 6 to pure trehalose (table 5) suggests that more water is adsorbed on the surface of trehalose-sodium chloride samples (higher $W_B$ value) and there are less interactions between water and solid components when sodium chloride is added to trehalose (lower $C_B$ value). Compared to all of the phosphate salts tested, sodium chloride was inferior at delaying crystallization at elevated humidity. Also trehalose-sodium chloride samples have lower $W_B$ values compared to the tested phosphate salts. The sorption isotherm of trehalose-calcium chloride (molar ratio 2:1) is shown in figure 33 and is a type 2 with higher $W_B$ and lower $C_B$ values than pure trehalose, trehalose-sodium chloride and trehalose-sodium dihydrogen phosphate. The BET fitting parameters of trehalose-calcium chloride are shown in table 6.

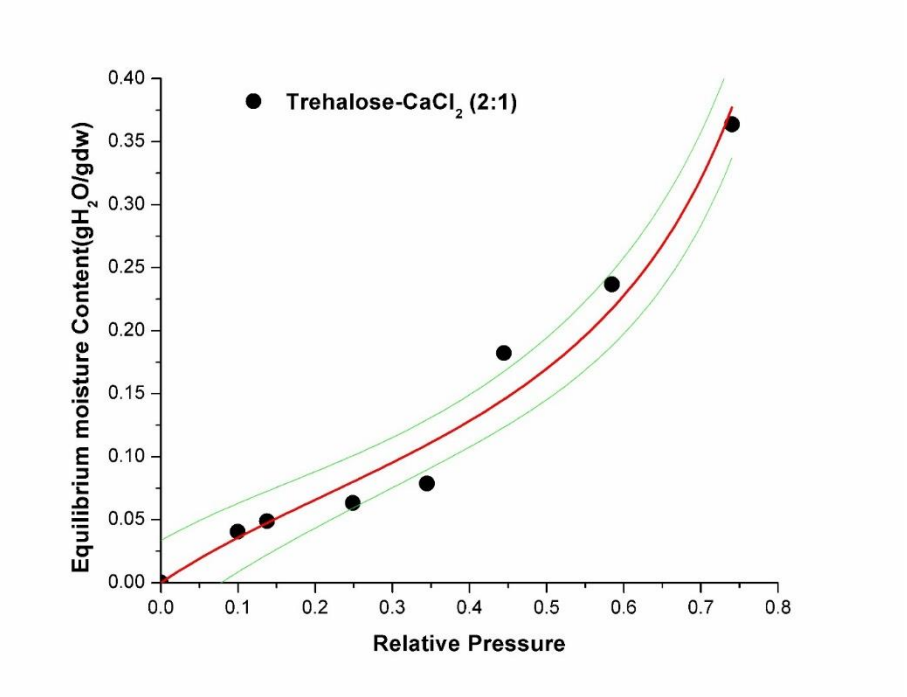


Figure 33. Moisture sorption isotherm for trehalose-calcium chloride molar ratio (2:1)

The trehalose-magnesium chloride sorption isotherm was determined to be type 2 and is shown in figure 34.

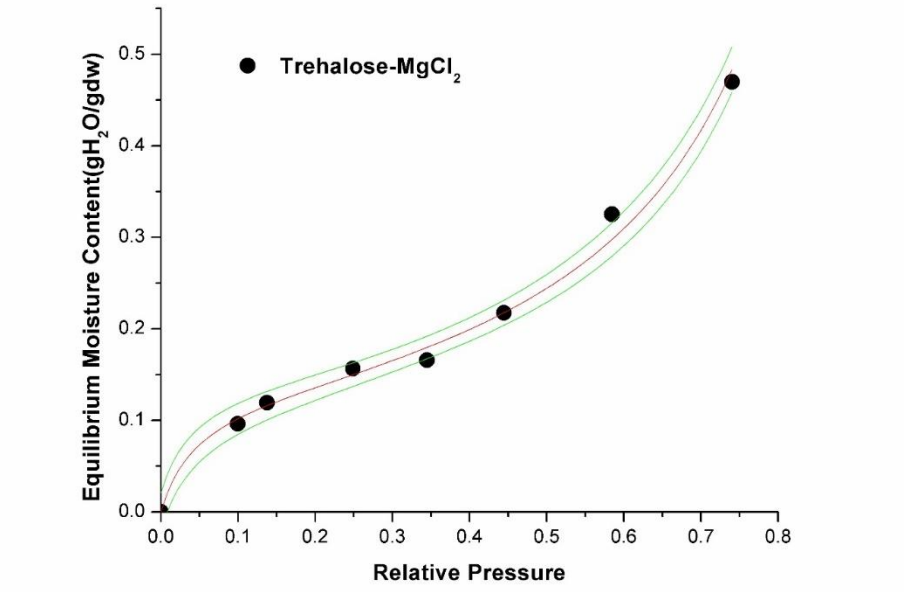


Figure 34. Moisture sorption isotherm for trehalose-magnesium chloride molar ratio (2:1)

Comparing the BET fitting parameters from table 6 to pure trehalose, trehalose-magnesium chloride samples have a higher $W_B$ value and lower $C_B$ value. So far it's been observed that all trehalose-salt mixtures have higher $W_B$ and lower $C_B$ values compared to pure trehalose which physically can be interpreted as more accumulation of water on the surface of the sample and less water chemically associated with solid components, leading to a delay of crystallization.

The sorption isotherm of trehalose-sodium acetate is shown in figure 35 and is characterized as a type 2, the same as pure trehalose and other trehalose:salt compounds with metal ion salts that were not superior stabilizing additives. BET model fitting parameters of trehalose-sodium acetate are shown in table 6.

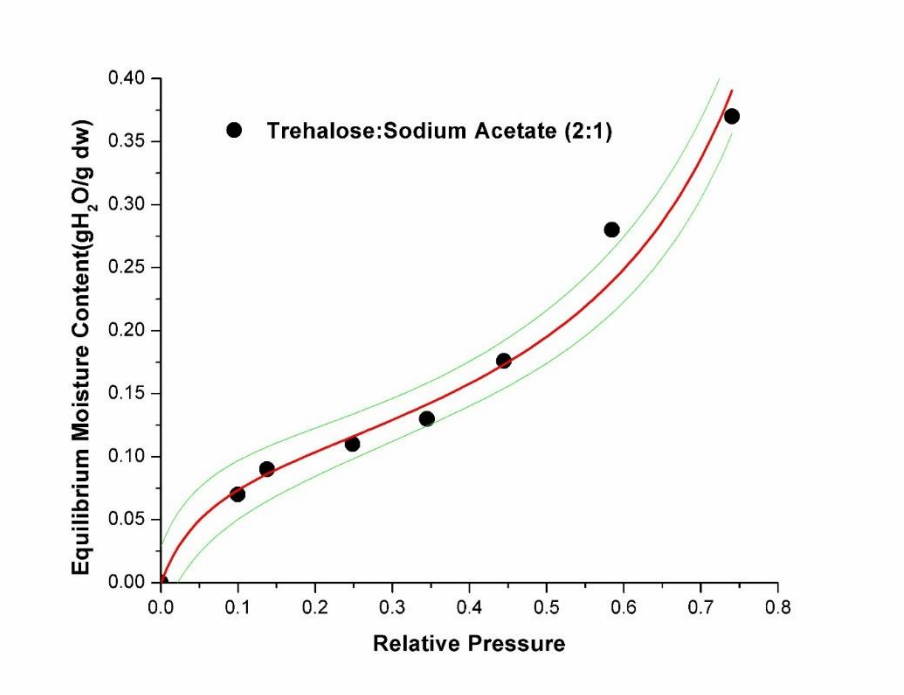


Figure 35. Moisture sorption isotherm for trehalose-sodium acetate molar ratio (2:1)

In order to evaluate the anion effect when paired with an organic cation like choline, the salt choline citrate was also added to the panel. Trehalose-sodium citrate compositions were slightly more stable compared to trehalose-sodium acetate compositions. The sorption isotherm and BET fitting parameters of trehalose-sodium citrate are shown in figure 36 and table 6, respectively. The sorption isotherm of trehalose-sodium citrate is characterized as a type 2 with higher $W_B$ and lower $C_B$ compared to trehalose. The addition of sodium citrate to trehalose was shown to delay crystallization at 59% RH for an extra day compared to sodium acetate. The $C_B$ value of trehalose-sodium citrate system is lower than the $C_B$ value of trehalose-sodium acetate samples, indicative of less chemical interaction of water with solid components of this system.

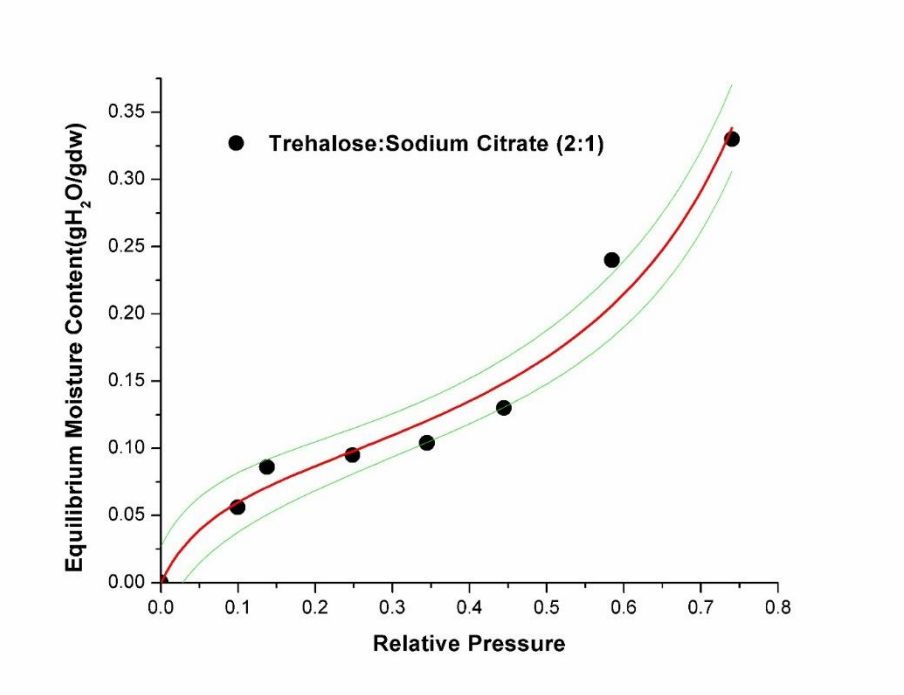


Figure 36. Moisture sorption isotherm for trehalose-sodium citrate molar ratio (2:1)

The sorption isotherm of trehalose-choline chloride is characterized as a type 3 and is shown in figure 37, similar to trehalose-choline monohydrogen phosphate. The associated BET fitting parameters are shown in table 6. This composition has slightly lower $W_B$ and slightly higher $C_B$ values than trehalose-choline monohydrogen phosphate and has significantly higher $W_B$ and lower $C_B$ values compared to trehalose.

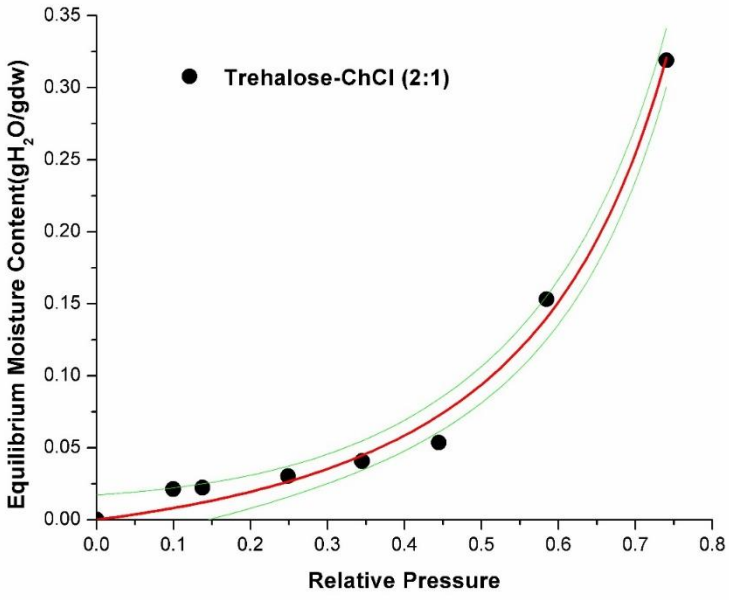


Figure 37. Moisture sorption isotherm for trehalose-choline chloride molar ratio (2:1)

The sorption isotherm of trehalose-choline acetate was observed to be a type 3 and is shown in figure 38. This composition had significantly higher $W_B$ and lower $C_B$ values compared to trehalose. Type 3 sorption isotherms have lowers amount of bound water in the system compared to compounds with sorption isotherm type 2. All superior compounds of trehalose that were highly stable in elevated humidity such as trehalose-choline monohydrogen phosphate, trehalose-choline acetate, and trehalose-choline chloride sorption isotherm were observed to be type 3.

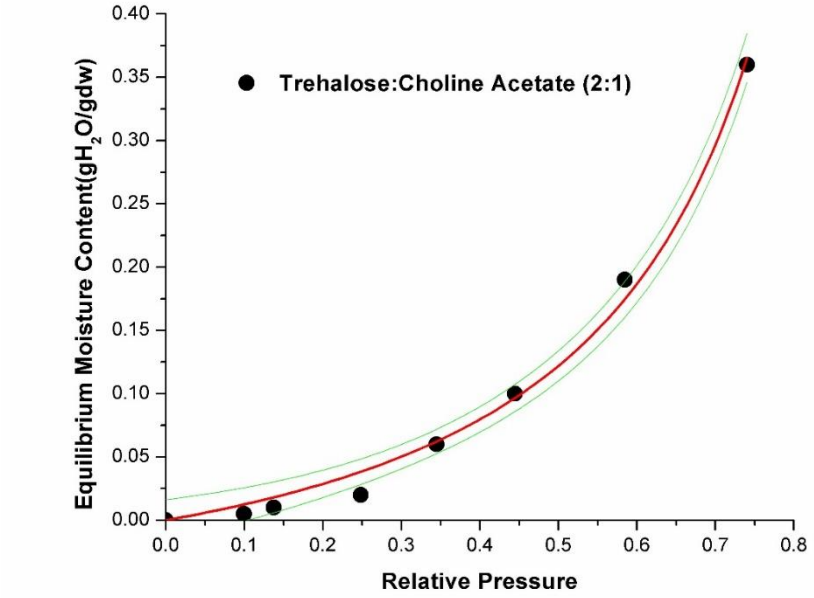


Figure 38. Moisture sorption isotherm for trehalose-choline acetate molar ratio (2:1)

In order to evaluate the effect of cation on the stability of trehalose glass in the presence of acetate salts the choline acetate salt cation was substituted with tetramethylammonium (TMAA). The sorption isotherm of trehalose-tetramethylammonium acetate is shown in figure 39 and is characterized as a type 3 with significantly higher $W_B$ and lower $C_B$ values compared to trehalose.

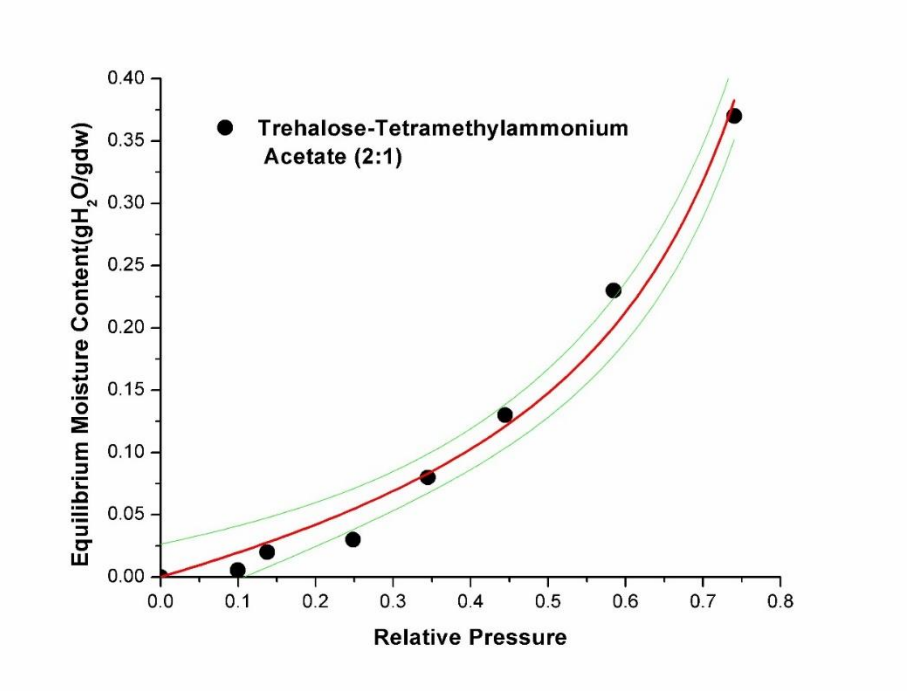


Figure 39. Moisture sorption isotherm for trehalose-tetramethylammonium acetate molar ratio (2:1)

Finally, the sorption isotherm of trehalose-choline citrate, also with the molar ratio of 2:1 at room temperature, is represented in figure 40. The trehalose-choline citrate composition was the most stable composition of all that were tested and never crystallized. Choline citrate is composed of choline, which is a big organic cation and citrate, which is a big organic anion. The large size of these ions may help suppress crystallization by blocking trehalose molecules from organizing into a shape that enables crystals to form. As mentioned in chapter 1 this mechanism of delaying crystallization is called steric hindrance. Citrate also crosslinks with trehalose. The sorption isotherm fitting parameters are shown in table 6. Trehalose-choline citrate had the highest $W_B$ value among all compositions tested. The higher amount of adsorbed water on the surface of the sample, which this parameter represents, may be suggestive of the presence of crosslinking ions that form strong hydrogen bonding networks that resist the penetration of water. Choline citrate salt is hygroscopic and trehalose-choline

citrate samples equilibrate to the highest moisture contents compared to other trehalose-salt compositions at elevated humidity. Water-ion interactions that prevent formation of trehalose dihydrate can be another mechanism for suppressing crystallization. The addition of choline citrate, with three possible mechanisms of crystallization suppression (steric hindrance, crosslinking anion, and water-ion interaction) was observed to be the best stabilizing additive for trehalose at the molar ratio tested.

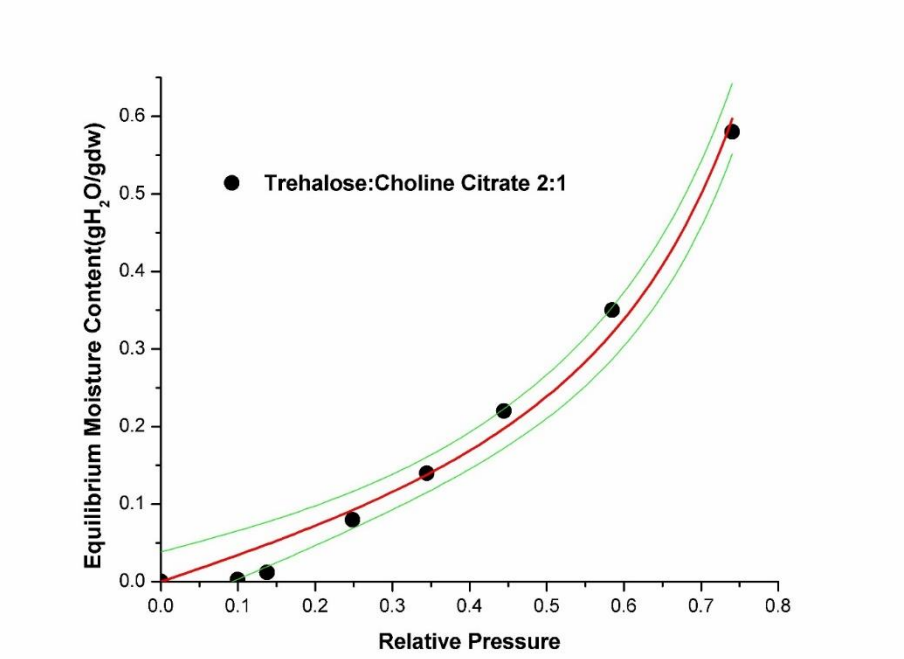


Figure 40. Moisture sorption isotherm for trehalose-sodium citrate molar ratio (2:1)

Table 6 summarizes the information from the sorption kinetics and sorption isotherms of pure trehalose and trehalose-salts mixtures with the molar ratio of 2:1. The table is arranged in descending order of stability. Compositions with type 3 sorption isotherms emerged as the superior formulations for delaying crystallization at elevated humidity. Among this superior

group the samples with higher EMC and higher $W_B$ values yielded better stability. All compositions had lower $C_B$ values compared to pure trehalose.

Table 6. Data from sorption studies of trehalose and trehalose-salt mixture with the molar ratio of 2:1 at room temperature at 76% RH

| | EMC($gH_2O$/gdw) At 76% RH | Crystallization At 76% RH | Isotherm | $W_B$ | $C_B$ | R-Square |
|---|---|---|---|---|---|---|
| **Trehalose-Choline Citrate** | 0.580 | Never Crystallized | Type 3 | 0.185 ± 0.014 | 1.81 ± 0.56 | 0.983 |
| **Trehalose-TMAA** | 0.375 | Day 11 | Type 3 | 0.122 ± 0.011 | 1.52 ± 0.51 | 0.981 |
| **Trehalose-Choline Acetate** | 0.363 | Day 10 | Type 3 | 0.135 ± 0.011 | 0.815 ± 0.19 | 0.992 |
| **Trehalose-ChCl** | 0.318 | Day 5 | Type 3 | 0.143 ± 0.02 | 0.484 ± 0.16 | 0.987 |
| **Trehalose-Na Citrate** | 0.335 | Day 4 | Type 2 | 0.090 ± 0.004 | 13.2± 7.3 | 0.969 |
| **Trehalose-Na Acetate** | 0.378 | Day 4 | Type 2 | 0.103 ± 0.004 | 15.8 ± 8.6 | 0.973 |
| **Trehalose-$MgCl_2$** | 0.469 | Day 3 | Type 2 | 0.127 ± 0.003 | 22.8± 8.8 | 0.991 |
| **Trehalose-$CaCl_2$** | 0.363 | Day 2 | Type 2 | 0.110 ± 0.008 | 3.88 ± 1.7 | 0.965 |
| **Trehalose-NaCl** | 0.347 | Day 1 | Type 2 | 0.088 ± 0.006 | 9.25 ± 5.6 | 0.953 |
| **Pure Trehalose** | 0.296 | Day 1 | Type 2 | 0.069 ± 0.001 | 26.04 ± 8.4 | 0.994 |

# CHAPTER 6: SUMMARY AND SUGGESTIONS FOR FUTURE WORK

## 6.1 Summary

The equilibrium moisture content for all trehalose:salt compositions at 76%RH at room temperature are summarized in table 7. The amorphous state stability increases diagonally from the least stable composition on top left of the table to the most stable composition on bottom right of the table, and an increase in EMC diagonally in this table can be seen.

Table 7. Equilibrium moisture content and number of delay of crystallization days at 76% RH of trehalose-salt mixture with the molar ratio of 2:1 at room temperature.

| | $Na^+$ | $Ca^{2+}$ | $Mg^{2+}$ | $C_4H_{12}N^+$ | $C_5H_{14}NO^+$ |
|---|---|---|---|---|---|
| | EMC ($gH_2O/gdw$) /Crystallization | EMC ($gH_2O/gdw$) /Crystallization | EMC ($gH_2O/gdw$) /Crystallization | EMC ($gH_2O/gdw$) /Crystallization | EMC ($gH_2O/gdw$) /Crystallization |
| $H_2PO_4^-$ | 0.347/day1 | | | | 0.311/day3 |
| $Cl^-$ | 0.326/day1 | 0.363/day2 | 0.469/day3 | | 0.318/day5 |
| $HPO_4^{2-}$ | 0.355/day3 | | | | 0.371/day6 |
| $C_2H_3O^{2-}$ | 0.378/day4 | | | 0.375/day11 | 0.363/day10 |
| $C_6H_5O_7^{3-}$ | 0.335/day4 | | | | 0.581/Never |

The anion effect on sodium salts is shown in figure 41 by comparing the effectiveness of different sodium salt additives to trehalose in delaying crystallization at 76% RH at room temperature when the salts were added to trehalose with a molar ratio of 2:1 trehalose:salt. The least effective sodium salts were sodium dihydrogen phosphate and sodium chloride, which could not delay crystallization of trehalose at 76% RH. Neither of these salts were composed of a big cation or a crosslinking anion. Of this family, the most effective salts

were sodium citrate and sodium acetate which delayed crystallization for 4 days at 76% RH and both these salts contained big organic anions.

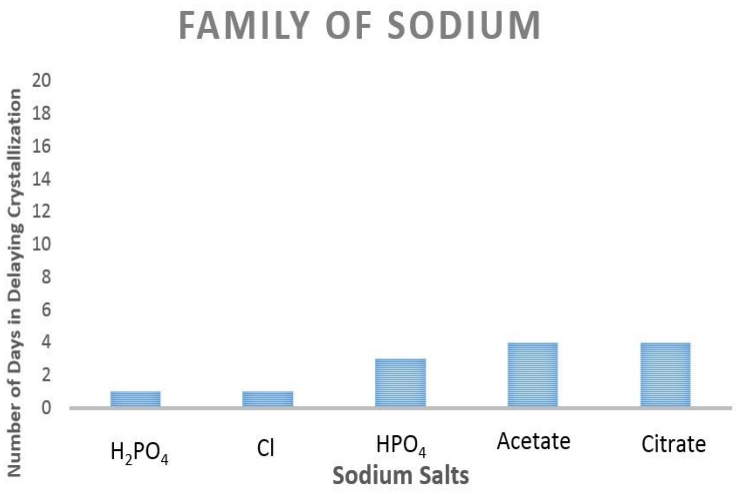


Figure 41. The effectiveness of different sodium salts as an additive to trehalose in delaying crystallization at 76% RH with the molar ratio of 2:1 trehalose:salt at room temperature

The anion effect on choline salts is shown in figure 42 by comparing the effectiveness of different choline salt additives, for the same conditions described in the previous section. The least effective choline salt was choline dihydrogen phosphate which delayed crystallization at 76% RH for 3 days. This salt is composed of a big organic cation therefore the delay of crystallization was likely due to steric hindrance. The most effective salt was choline citrate and the samples of trehalose-choline citrate never crystallized in 76% RH. The stabilizing effect of this salt could be attributed to the combination of steric hindrance, crosslinking anion, and water-ion interactions.

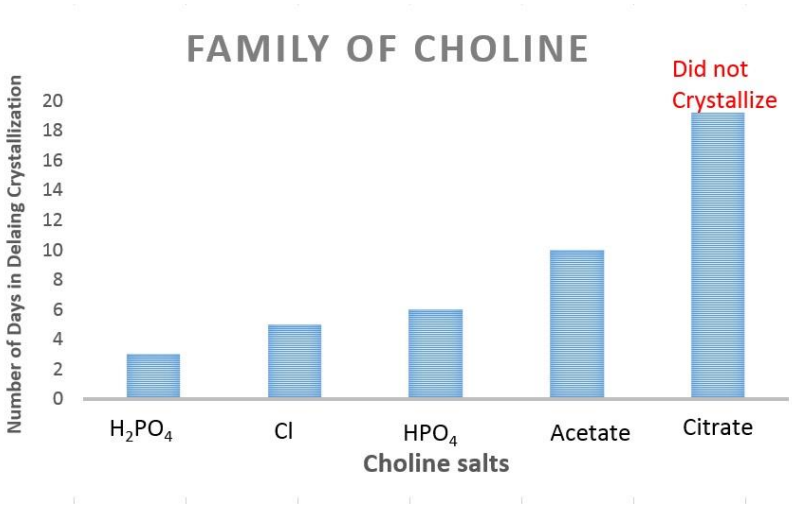


Figure 42. The effectiveness of different Choline salts as an additive to trehalose in delaying crystallization at 76% RH with the molar ratio of 2:1 trehalose:salt at room temperature

The effectiveness of different salts in delaying crystallization at 76% RH as a function of equilibrium moisture content is shown in figure 43. More effective salts delay crystallization for a longer number of days which is the vertical axis. The best trend line comes from the blue ellipse, the organic salts family, composed of either a big organic cation or a big organic anion and their effectiveness increases with the presence of a crosslinking anion. Samples that equilibrate to higher moisture levels in this family are the more stabilizing additives. This trend also holds up with the sodium family. The addition of sodium acetate or citrate with big organic anions lead to more stable compounds and trehalose-sodium acetate equilibrates to the highest moisture content at 76% RH. The moisture content trend also holds up with the metal cation-chloride salt family. This family of salts do not have a big organic cation or a crosslinking anion, but magnesium chloride salt, which had the highest equilibrium moisture content, was the most effective salt of this family.

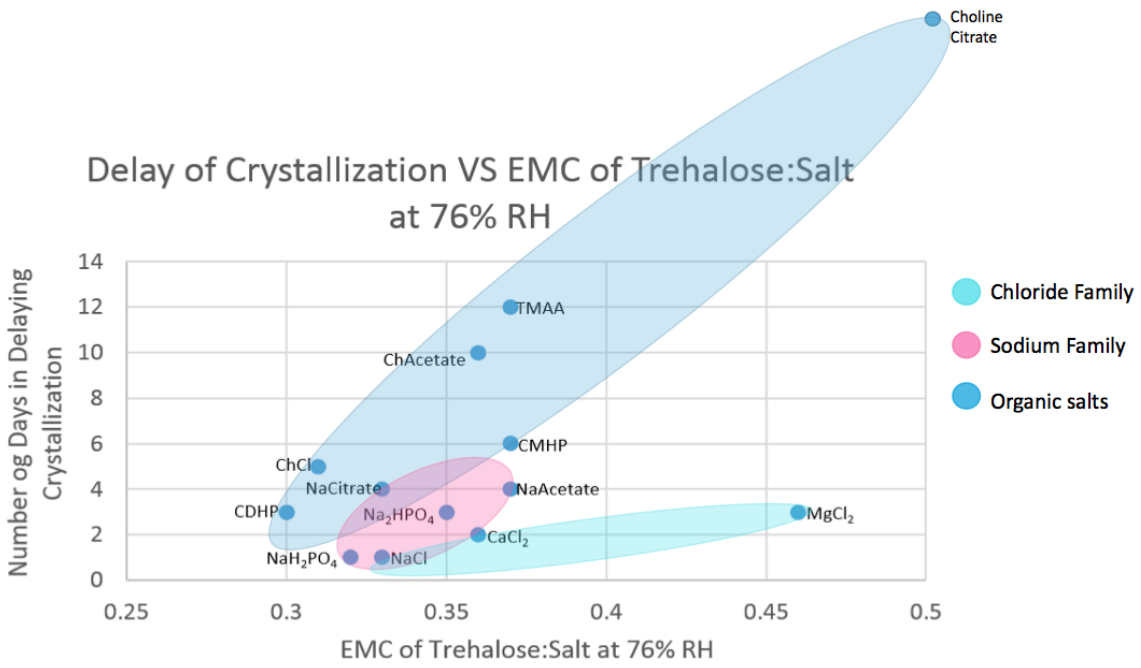


Figure 43. The effectiveness of different trehalose:salt compositions (2:1) as a function of equilibrium moisture content at 76%RH at room temperature

## 6.2 Future work

In this study choline citrate was the most effective trehalose additive for suppressing crystallization at elevated humidity. Choline citrate was added to trehalose with the molar ratio of 1:2, but lower ratios may also be effective and should be studied. Determination of the $T_g$ of trehalose-choline citrate, trehalose-tetramethylammonium acetate, and trehalose-choline acetate compositions in the dry state and in the presence of water would be beneficial for formulation design purposes. Molecular dynamics simulation of trehalose-organic salts could also be beneficial in order to further clarify how salts may affect molecular clustering phenomena and help the retention of amorphous state. Finally, this study was conducted

without the presence of biologics. Further studies are suggested in order to evaluate how these trehalose-organic compositions can affect biopreservation.

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