DYNAMIC WATER SORPTION IN TREHALOSE-SALT MIXTURES: EFFECT OF COMPOSITION ON RETENTION OF THE AMORPHOUS STATE

by

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A thesis submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Master of Science in
Mechanical Engineering

Charlotte

2014

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ABSTRACT

BABAK BAGHERI. Dynamic water Sorption in trehalose-salt mixtures: effect of composition on retention of the amorphous state (Under the direction of DR. GLORIA D. ELLIOTT)

The disaccharide trehalose has received considerable attention in the bio-preservation field due to its outstanding properties as a glass former, and it is widely used in the pharmaceutical and food industries to stabilize dried products. In part, due to the high viscosity and low molecular mobility in the amorphous glass state, trehalose can preserve the life activities of biomaterials in severe conditions, such as desiccation or freezing. The glassy amorphous state is a meta-stable state that will convert to a crystal eventually, with a rate dependent on the temperature and moisture content. It is well known that amorphous trehalose crystallizes at 44% RH and higher, yet sugar crystallization can have a detrimental effect on preserved materials. In this study, a series of phosphate salts were explored as additives to trehalose compositions in order to understand their effectiveness at suppressing crystallization and retaining the desired glassy amorphous form. To study these phenomena, a Dynamic Vapor Sorption (DVS) experimental set-up was established, which employed containers with constant relative humidity achieved using supersaturated salt solutions. Microwave-assisted processing was used to reach a dry amorphous state in a series of salt-trehalose compositions. These samples were then evaluated for water uptake characteristics and the visual onset of crystallization. Four salts were studied, with choline or sodium as the cation, and monohydrogen phosphate (HPO_4) or dihydrogen phosphate (H_2PO_4) as the anion.

It was shown that the crystal suppression efficacy increased with increasing concentration of salt in the mixture, with the exception of compositions containing

sodium dihydrogen phosphate, in which samples at all sugar:salt molar ratios crystallized within the same time period as pure trehalose. Of the salts evaluated, choline hydrogen phosphate was found to be most effective for suppressing crystallization. No crystals were observed in choline hydrogen phosphate:trehalose samples after 15 days (1:0.7 molar ratio) at 61% RH, whereas pure trehalose samples had crystallized at day 1. The magnitude of water sorption in salt-trehalose samples also increased with rising salt fraction for all combinations studied. Although avoidance of crystals is desirable, further studies are needed to understand the effect of increased water uptake on the glass transition temperature and the ability to preserve biomaterials in this matrix.

ACKNOWLEDGMENTS

I would like to express my special appreciation and thanks to my advisor Professor Dr. Gloria D. Elliott, you have been a tremendous mentor for me. I would like to thank you for encouraging my research and for allowing me to grow as a research person; your advice on research has been priceless. I would also like to thank my lab mates (BioStability Lab), Mr. Matthew Van Vorst, Dr. Sharonda LeBlanc (Post-doctoral Associate) for supporting me in performing experiments and collecting data and for your brilliant comments and suggestions, thanks to you. I would also mention this study was supported by grant #5RO1GM101796 from the National Institute of Health

A special thanks to my family. Words cannot express how grateful I am to my mother, my father and my sisters for all of the sacrifices that you've made on my behalf. Your prayer for me was what sustained me thus far. At the end I would like express appreciation to my brother-in-law and my best friend Mr. Sam Khatibinaz who supported me in writing, and incented me to strive towards my goal.

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LIST OF ABBREVIATIONS

ASTM	American society for testing and materials
BET	Brunauer Emmett Teller
CARS	coherent anti-stokes Raman spectroscopy
CCD	charge coupled devices
CDHP	choline dihydrogen phosphate
CMHP	choline monohydrogen phosphate
DSC	differential scanning calorimeter
DW	Dry Weight
DTA	differential thermal analysis
DVS	dynamic vapor sorption
EMC	Equilibrium Moisture Content
ERH	equilibrium relative humidity
FTIR	Fourier transform infrared spectroscopy
GAB	Guggenheim Anderson Deboer
GC	glass-to-crystal
HNB	homogeneous nucleation-based
KF	Karl Fischer titrator
MD	maltodextrin
NIF	nifedipine
PBS	Phosphate-buffered saline
PDA	photodiode arrays
PMT	photomultiplier tubes
PVP	poly vinyl pyrrolidone

SERRS	surface enhanced resonance spectroscopy
SERS	surface enhanced Raman spectroscopy
TMA	thermal mechanical analysis
VTF	Vogel Tamman Fulcher
WLF	Williams Landel Ferry
XRD	X-ray diffraction

CHAPTER 1: INTRODUCTION

1.1 Sugar Glasses and Their Role in the Preservation of Biologics

The amorphous state of sugars has been the subject of many thorough studies in the bio-preservation field [1]. The amorphous glass state can be produced either by the melting and rapid cooling of the sugar melt, or by removal of the dispersing medium (Ex. water) from the material. Figure (1.1) shows several different approaches used to produce the glassy state in materials, including heating, cooling, or lowering the pressure (vacuum). In the pharmaceutical and food industries the amorphous glass state of sugars is often produced intentionally as an inactive compound (excipient) to stabilize active materials [2]. Due to the very high viscosity and low molecular mobility in the amorphous glass state, preservation of bio-materials, such as protein or membrane components, is possible. Although, in the amorphous glass state the molecular mobility is extremely low, compared with crystals, the glassy state is relatively unstable and not in thermodynamic equilibrium. To preserve food products during storage for extended times a glassy amorphous form with an unchanging/stable state is desired. The glass transition temperature (T_g) is defined as thetemperature at which a material transitions from a fluid-like “rubbery” state to a solid state “glass”. The viscosity in the glassy state is around 10^{12} Pa. s and this value decreases to $10^6 – 10^8$ Pa. s in the rubbery state. Both of these states (e.g. high viscous glass and liquid-like rubbery) are categorized as the amorphous state.

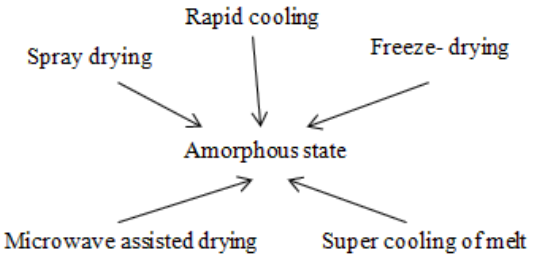

Figure 1.1: Schematic diagram of the most common ways in which amorphous character is induced

Researchers have recently shown that disaccharides such as trehalose, sucrose and lactose stabilize various bio-materials during lyophilization, microwave assisted drying, or spray-drying processes [3]. In nature species can survive drought or dehydration conditions by producing large amount of sugars like sucrose and trehalose. The glass-forming ability of these sugars has been used widely in the confectionary and pharmaceutical industries. Crowe *et al* [4] investigated the properties of trehalose and asserted that the ability of sugars to form a glassy state is not the only requirement for stabilization. They found that although poly-saccharides such as hydroxyethyl starch and dextran are considered as good glass formers, none were able to preserve liposomes during dehydration and they revealed that direct interaction between sugars and polar groups of bio-materials has a significant function in stabilization. To perform as a superior excipient both conditions (glass formation and direct interaction) should be satisfied.

Trehalose is a natural alpha-linked disaccharide formed by an α,α-1,1-glucoside bond between two α-glucose units. It is a naturally occurring non-reducing sugar which is widespread in nature with molecular formula $C_{12}H_{22}O_{11}$ and a molar mass of 342.31g/mol (anhydrous) [5]. It is usually prepared and sold as trehalose dihydrate. Compared to other

sugars with a similar structure trehalose is slightly more soluble in water at 80°C and higher temperatures [6]. Owing to its distinct molecular structure and physico-chemical properties, trehalose is a very stable disaccharide and in 1991 it was approved as a cryoprotectant for freeze-dried foods. Leslie *et al* [5] used trehalose and sucrose to lyophilize two different types of bacteria and after rehydration, samples containing trehalose had approximately 15% more viability compared with samples containing sucrose and this protective aspect of trehalose was attributed to preservation of the proteins and lipid membranes. Kawait *et al* [5] proposed that trehalose preserves lipids either by direct interaction with polar groups on bio-molecules in the absence of water or by the organization of water molecules in the membrane.

1.2 Retention of the Glassy State

In addition to forming a glassy state, in order to achieve long-term storage, retention of the glassy state is important. The glassy amorphous state is at a meta-stable state and tends to convert to a crystal eventually, with a rate dependent on the temperature and moisture content. Also, crystal formation in compositions with a small meta-stable range is initiated faster than one with a large meta-stable range [7].

Water is a major food component that has a significant effect on the T_g of sugars, acting as a strong plasticizer. The T_g value of binary mixture of sugars-water (i.e. trehalose, sucrose) decreases with an increase of the moisture content, following a non-linear function as described by the Gordon–Taylor equation [8],

$$T_g = \frac{c_s T_{g,s} + k c_w T_{g,w}}{c_s + k c_w} \quad (1.1)$$

where c_s and c_w are the mass fractions of the sugar and of water, $T_{g,s}$ and $T_{g,w}$ are their T_g values, respectively, and k is a constant.

The plasticizing effect of water, including the depression of T_g down to a temperature lower than room temperature, is the main cause of sugar crystallization in amorphous products stored at room temperature [2]. If the temperature or water content is further increased (the product being above its T_g), the viscosity is not high enough to support the structure of the solid material and collapse occurs. Physical changes such as crystallization and structure collapse can therefore be successfully explained and predicted with references to the glass transition temperature. The high glass transition temperature, compared with other disaccharide, and good glass forming ability makes trehalose more effective than other disaccharides for preserving bio-material during storage or transportation. For example, Crowe and coworkers [5] prepared sugar-liposome compositions and stored them at 40°C in a 58% RH environment. These studies showed that trehalose and sucrose absorbed similar amounts of water initially. In the case of sucrose the sorbed water decreased the glass transition temperature (≈56 °C), whereas the absorbed water in trehalose samples contributed to the formation of dihydrate in mixture and the glass transition temperature never decreased below 65 °C. Thus the T_g remained high in the non-transformed portion of the sample despite water uptake. This offers some protection against total loss of product, enabling a portion of the sample to remain in a glassy state. Crowe *et al* [5] demonstrated that adding a small amount of water to amorphous glass samples enabled the formation of trehalose dihydrate on the exterior boundary of the glass, which may isolate the remaining glass from the detrimental effect of crystallization. The moisture content of trehalose dihydrate stays

constant (9.54%) in an environment with RH up to 92%. Others have provided data that shows for pure trehalose at or above 44% RH, there exists a maximum in adsorption isotherm curve, which indicates the initial point of crystallization [9,10]. However, crystallization causes the cessation of the interactions between trehalose (protectant) and the bio-materials and the crystallization of trehalose from the amorphous state leads to loss of the protective effect of amorphous matrices and consequent deterioration of biomolecules and biological structures. Therefore, it is desired to retain the sugar amorphous state and suppress crystallization.

To understand the behavior of biomaterials in various environments, and thus preserve them in the glassy state and/or inhibit crystal forming during storage or transportation, knowledge of the conditions that induce crystallization is necessary. Nucleation is considered as the first step in crystallization, and the crystal characteristics (i.e. size and quantity) in the final products are highly dependent on the nucleation step. Although crystals form only under supersaturating condition, it is not a sufficient condition for crystallization because either molecular inhibitors or limited molecular mobility in the glassy state, can suppress this action. Temperature and other environmental factors can also induce nucleation in supersaturated solutions. For example, different kinds of energy inputs such as mechanical or ultrasonic energies have been shown to enhance the crystal formation process. As a result, in order to minimize crystallization, products should generally not be agitated. Generally high viscosity is associated with low molecular diffusivity and in most cases these two parameters are considered inversely proportional [7].

Following nucleation, crystal growth proceeds until the concentration of the solute in the liquid portion decreases below the saturated solution level, or the molecular motion (translational and rotational) drops below the molecular mobility level necessary for crystal formation. Mass and heat transfer both play major roles during the crystal growth stage. Additives can also impact crystal growth dramatically. They may modify the driving forces for crystallization or inhibit nucleation by restricting rearrangement of product molecular structure (Ex. Forming a hydrogen bond with the solute) in a way that a portion of the molecules cannot participate in further crystal growth. This latter case is the subject of this thesis, and will be discussed further in section 1.4.

1.3 Water Sorption Behavior

Although retention of the glassy state is preferred for preserved biologics, sometimes adverse environmental conditions can lead to short-term excursions in environmental humidity and thus water content. This uptake in water can lead to the irreversible formation of the crystal, or alternatively to a reversible rubber state. In the current work we are interested in understanding how the addition of additives can help to delay the onset of crystallization when samples are exposed to high humidities and thus enable the sample to return to the glassy state when ideal moisture conditions are restored. An understanding of the rate and extent of water uptake in high RH is an important element of understanding this behavior.

Equilibrium moisture content (EMC) is defined as the water content in the sample, when the vapor pressure in the system (i.e food, bio-material product) reaches equilibrium with that of surrounding environment, and the relation between the relative humidity (RH) and the equilibrium moisture content at constant temperature is called the

“moisture sorption isotherm”. Increasing the relative humidity results in an increase in the EMC. Also, the temperature and EMC are inversely proportional. Any elevation in temperature decreases the relative humidity and thus the equilibrium moisture content. Sometimes the equilibrium moisture content during adsorption has a different trend from that of desorption, which is called “hysteresis”, and this phenomenon has been depicted in figure (1.2) [11].

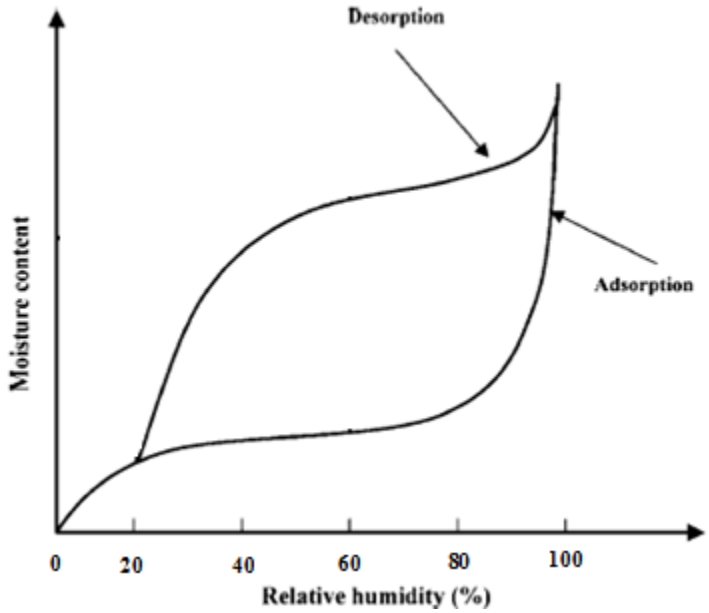

Figure 1.2: The hysteresis occurrence depicted in an isothermal sorption graph

Presenting water vapor sorption data in the form of an isotherm is a standard approach for characterizing the water sorption behavior of materials in different environmental humidities. A sorption isotherm gives the relation between water activity and water content at a constant temperature. Water activity is a thermophysical property that determines the boundness of water molecules in a solution and its ability to participate in chemical, physical reactions. It is defined as the ratio of water vapor

pressure in a mixture (P_{sys}) to the vapor pressure of pure water (P_{sat}) at the same temperature.

$$a_w = \frac{P_{sys}}{P_{sat}} = \frac{ERH}{100} \tag{1.2}$$

Water activity is also directly related to the equilibrium relative humidity (ERH) of air surrounding the system.

Brunauer, Emmett and Teller (BET) [12] determined water vapor sorption isotherm curves empirically based on multilayer adsorption assumptions, which is still considered the best known modelling in this field. Later Brunauer, Deming and Teller [11,12] proposed five different types of adsorption isotherms (1940). Type I is known as a Langmuir isotherm which represents monomolecular adsorption of gas in a vacuum finite volume. In figure (1.3), the sigmoid isotherm is considered as Type II which mathematically is described in the Oswin equation as series expansion:

$$M = K(\frac{a_w}{1-a_w})^N \tag{1.3}$$

where *K* is a linear function of temperature, a_w is the water activity and N is a constant. Type III is the well-known Flory-Huggins sorption isotherm which expresses adsorption of gases on non-porous solids. “Type II isotherms are commonly encountered with hydrophilic polymers such as wool, silk and cellulose acetate. For less hydrophilic ones (ethylcellulose, polydimethyl siloxane) Type III isotherms are observed” [13]. Type IV represents sorption by a swellable hydrophilic solid. The type II and type IV isotherms are the ones that are often used to predict the water sorption behavior of food products [14]. All five types of water vapor sorption isotherms are displayed in figure (1.3).

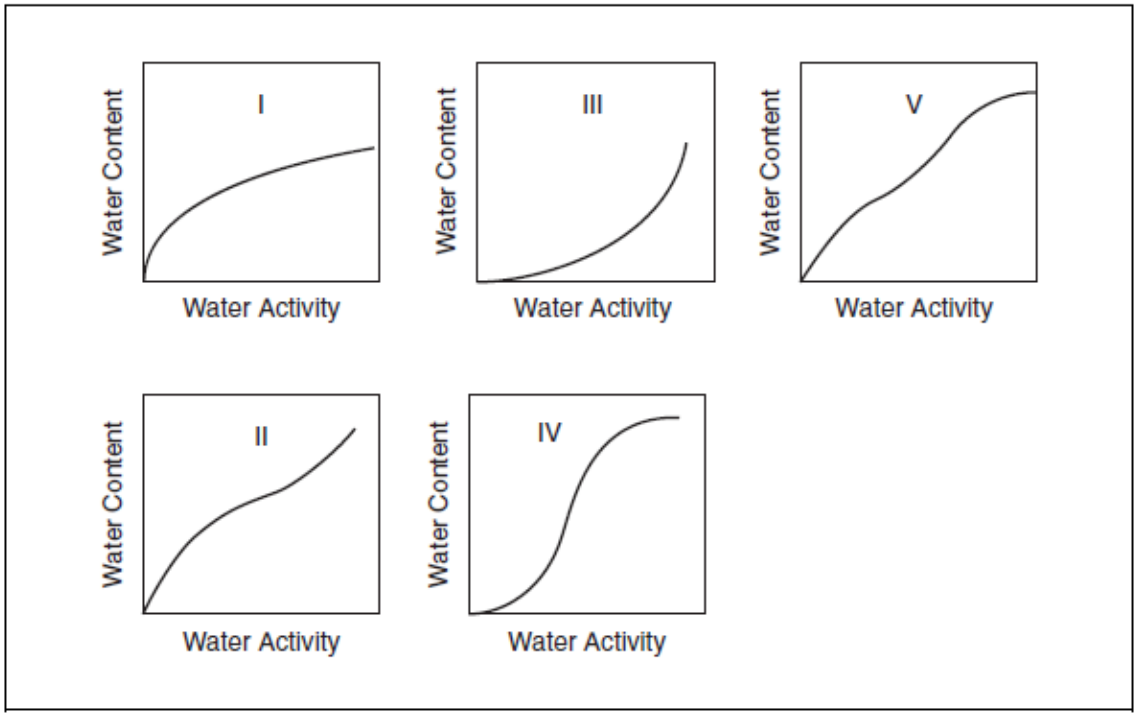

Figure 1.3: Sorption isotherm graphs presented by Brunauer *et al* [12]

There are three dominant methods for water sorption measurement [11]: "1.Manometric, 2.Gravimetric and 3.Hygrometric." Manometric methods involve measuring the water vapor pressure at the specific moisture content, which is in equilibrium with the product. In the gravimetric method mass changes are measured over time and this can be performed in static or dynamic systems. Hygrometric techniques involve the measurement of ERH of air over a system equilibrated at the particular moisture content. Amongst these methods the one which is used frequently is the use of thermostatic jars with constant relative humidity, prepared using supersaturated salt solutions [11]. Three distinctive stages of water uptake are expected in an amorphous sample: water vapor adsorption prior to crystallization, additional adsorption during or

after crystallization, and post-crystallization the final stage of water uptake by the crystalline material [9].

1.4 Effect of Additives on the Stability of Sugar Glasses

In general, increasing the amount of water in the amorphous state leads to a lower T_g. If, due to moisture uptake, the T_g decreases to a temperature below the storage temperature, nucleation and crystallization can commence. For many decades, various industries have utilized different material compositions to achieve product stability during transportation and storage of food and pharmaceutical compounds. Iglesias *et al* [15] investigated the effect of additives on the crystallization behavior of aqueous trehalose solutions and showed that maltodextrin (MD), 50:50 dry weight, can delay crystallization in a 44% and 52% RH environment. At lower RH (less than 44%) there is good agreement between predicted water uptake values and data from experiment but at higher RH the moisture content was higher than expected. At 75% RH the samples did not reach equilibrium. This effect may be ascribed to the fact that the high molecular mass of MD increases T_g at low RH but is less effective as a glass former at high RH (52%, 75% RH). Maio *et al* [16] also evaluated the crystallization kinetics of lactose, trehalose, and lactose/trehalose mixtures at RH $\geq$ 44% and showed the dependence of sugar crystallization on temperature, RH and water content. In this time-dependent study of crystallization water loss in trehalose and lactose/trehalose mixtures was considered as the initiation of crystal formation. It was concluded that existence of trehalose in lactose/water binary solution delays the crystallization of the component sugars which is attributed to interaction of component sugars and effects on lactose crystal forms (α-lactose monohydrate, anhydrous mixture of α- and β-lactose). Mazzobre *et al* [1]

investigated the crystallization kinetics of lactose in the presence of trehalose (up to 40% of total weight) in environments with 33% and 44% RH. Based on the shoulder on the crystallization peak in an isothermal DSC run it was concluded that lactose crystallized to different crystalline forms (monohydrate and anhydrous) at lower temperature. Trehalose delays lactose crystallization without any effect on T_g and it is attributed to lattice interference which modifies the molecular environment and impacts crystal growth.

Studies have shown that the addition of salts can also influence both the T_g of compositions as well as the ability to retain the glassy state during extended storage. The effect of salts on improvement of the thermophysical properties of sugars in solution and the enhancement of glass stability have been cited in various studies [17,18]. Considering the pervasiveness of electrolytes in biological systems and the known ability of salts to affect water structure, and understanding of the effect of sugar/salt mixtures is important for bio-formulations.

For example, Sitaula *et al* [19] explored mixtures of trehalose-PBS (phosphate-buffered saline) with various mass ratios from 20:1 to 1:1 (trehalose:PBS) and determined that increasing the PBS mass fraction caused a shift in the sorption isotherm from type II to type III. This phenomenon in the higher range of water activity (>0.54) would lead to a detrimental effect on the stabilization of cells (i.e. a higher water activity/molecular mobility at the same moisture content). Also, it was shown that adding PBS to trehalose decreased the T_g and increased the crystallization potential of trehalose in proportion to the increasing of PBS fraction in the mixture. Although NaCl is the predominant compound in PBS, and Miller *et al* [20] confirmed that sodium chloride

increases the T_g temperature of salt/sugar/water ternary solution, here the plasticizing effect of the other components in PBS were considered to contribute to the decline in T_g.

M.F. Mazzobre *et al* [17] evaluated the effect of inorganic salts ($MgCl_2$, NaCl, KCl and $CaCl_2$) in sugar/salt/water ternary mixtures on the crystallization and glass stability. The amorphous glassy state was produced by freeze-drying solutions (20% w/v) of trehalose, sucrose or sugar mixtures with salt added in a molar ratio of 5:1 sugar:salt. After freeze-drying, the samples were transferred into a vacuum desiccator and then stored over a constant relative humidity environment created using the saturated salts: LiCl, $KCOOCH_3$, $MgCl_2$, K_2CO_3, NaBr with corresponding water activities of 0.11, 0.225, 0.33, 0.428, 0.577, respectively. Samples were exposed to these environments for 33 days at 25°C. They determined that suppression of crystallization depended on the magnitude of charge/size ratio of the cation, i.e. Mg^{2+}>Ca^{2+}>Na^{+}>K^{+} in terms of effectiveness. Moreover, adding salt reduced the amount of ice (water crystallization) as measured with dynamic DSC. This property of the salt, increasing the water uptake while delaying or inhibition crystallization, could be inferred as the interaction of the cations with water molecules. Furthermore, results of the water sorption isotherm, plotted in sorbed water moles per solute mole, showed that for water activity $a_w \geq 0.44$ trehalose formed the crystalline dihydrate (each mole of sugar absorbed two water molecules).

Ohtake *et al* [18] evaluated the effect of pH and counter ion on the T_g of trehalose/sucrose-phosphate mixtures. Amorphous glasses were prepared by freeze-drying solutions containing 20% ($^{wt}/_{wt}$) sugar. Sugars were added at various ratios to completely dissolved salt in order to obtain a range of molar ratios of sugar-phosphate mixtures (0.1-1.0). Also, to reach the specified pH, different ratios of NaH_2PO_4,

Na_2HPO_4, KH_2PO_4 or K_2HPO_4 were mixed in solutions. It was shown that glass transition temperature in sucrose-phosphate compounds increased with increasing phosphate ratio. But in the case of trehalose, adding phosphate increased the T_g only at pH values greater than 6.6. This was ascribed to the fact that at lower pH the phosphate ion mostly appears as the H_2PO4^{1-} form, which does not interact effectively with sugar, while the majority of phosphates in the high pH regime are in the $HPO4^{2-}$ form, which creates a strong intermolecular network.

Miller and coworkers [21] examined the T_g and viscosity of trehalose with NaCl/$Na_2B_4O_7$ (borax) mixtures. Their results showed that the T_g increment, ΔT_g(the difference between glass temperature of the ternary solution and that of aqueous trehalose at the same mole fraction of trehalose) in the presence of borax is about 3 times greater than adding NaCl to mixture. Because borax decomposes to boric acid and sodium borate they further investigated the components and concluded that the borate ion, $B(OH)_4$, was the major contributor to the T_g effect, attributed to the affinity of sodium borate for water. For the same mole fraction of trehalose, the viscosities of ternary solutions of trehalose/borate/water are remarkably higher than pure trehalose solution.

1.5 Aim of the Experiment

The goal of the present work is to examine the effect of different salt additives on the water uptake and stability of amorphous trehalose compositions. From the standpoint of preserving bio-materials (i.e. cells and tissues) a review of the literature suggests that because PBS is commonly used as a solvent and it can increase the T_g of trehalose at certain pH levels, that this would be an appropriate anion for further study. Unlike borate, phosphate is not toxic in high concentrations [18]. In terms of the cation, besides the

effect of sodium mentioned previously, different studies have evaluated the effect of adding choline to cryopreservation formulations. Choline has been proven to be a bio-compatible ion which improves cryopreservation outcome [22]. Also, it has been shown that CDHP (choline dihydrogen phosphate) is an effective stabilizer for proteins and DNA [23]. These indicated properties of choline/sodium and phosphate are potentially useful additives for trehalose based cell preservation formulations and thus will be evaluated for their effects on the retention of the amorphous state in high RH conditions.

CHAPTER 2: MATERIALS AND METHODS

2.1 Solution Preparation and Properties

In order to study the effects of additives on the crystallization tendency of trehalose, compositions that are typically used in preservation applications were chosen for study. A 30.8 wt% stock solution of trehalose, prepared in 1X TE buffer (1X Tris-EDTA buffer, pH 8.0, consisting of 10 mM Tris-HCL and 1 mM EDTA), was used to prepare solutions with varying amounts of phosphate salts. This concentration is close to the solubility limit of trehalose in water, thus gentle heat was used to facilitate dissolution. The osmolality of the stock solution was measured with a vapor pressure osmometer (Vapiro 5520, Logan).

As a next step compositions of salts with trehalose in buffer solution were made by adding aliquots of the trehalose stock solution together with the appropriate mass of salt in order to achieve the desired molar ratios of salt in a final volume of 1X TE buffer. Previous work from the Biostability Lab, in which the glass transition temperature at specific mass fractions of choline dihydrogen phosphate to trehalose were determined, indicated interesting deviations from ideal behavior in the high mass fraction of trehalose range (0.7-1.0), so molar ratios were chosen to also include this area of interest [24].

Because pH has significant effect on the speciation of ions in solution, the pH of all solutions was measured. For example, depending on the pH, the phosphate salts used in this study can be present in solution in the form dihydrogen phosphate ($H_2PO_4^-$) or monohydrogen phosphate (HPO_4^{-2}). The pH of 1M salt solutions were also recorded for

comparison. To enable a better understanding of the extent of dissociation, the conductivity of solutions was also measured using a Mettler Toledo (SevenMulti) pH/conductivity meter.

2.2 Dry Weight Determination

In order to allow water contents to be expressed in terms of mass of water per mass of solids, two different methods were used to determine the dry weight of samples with different compositions, a gravimetric method and a titration method. For the gravimetric method, small volumes of solution were pipetted onto a CEM company glass fiber absorbent pad and then heated in an oven for 48 hours at 95 ºC. The sample was then cooled in a desiccator containing phosphorus pentaoxide (P_2O_5), which maintained the humidity near 0% RH. By recording the initial wet mass and the post bake-out dry mass, the dry weight of each composition was acquired (See Appendix A for detailed protocol).

The second method was based on a determination of the water content of samples by employing volumetric Karl Fischer titration. Known masses of solution were titrated for water content, and the difference between initial mass and water mass was identified as the total solids content (dry weight). For both methods, dividing the dry weight by the total solution mass yielded a weight fraction of solids that could be used to estimate the dry weight in any given volume of this same solution.

2.3 Humidity Control

In order to investigate water sorption in different sugar-salt compositions at specific relative humidity, a closed environment with a stable RH over the test duration was required. Specific saturated salt solutions are capable of producing an environment

with constant relative humidity and this capacity was utilized in our experimental set-up [25]. Kilner glass jars with rubber seals and clamps or Mason glass jars with screw caps are used as controlled humidity vessels (1L, total height 159 mm, mouth Dia.89 mm) as shown in figure (2.1).

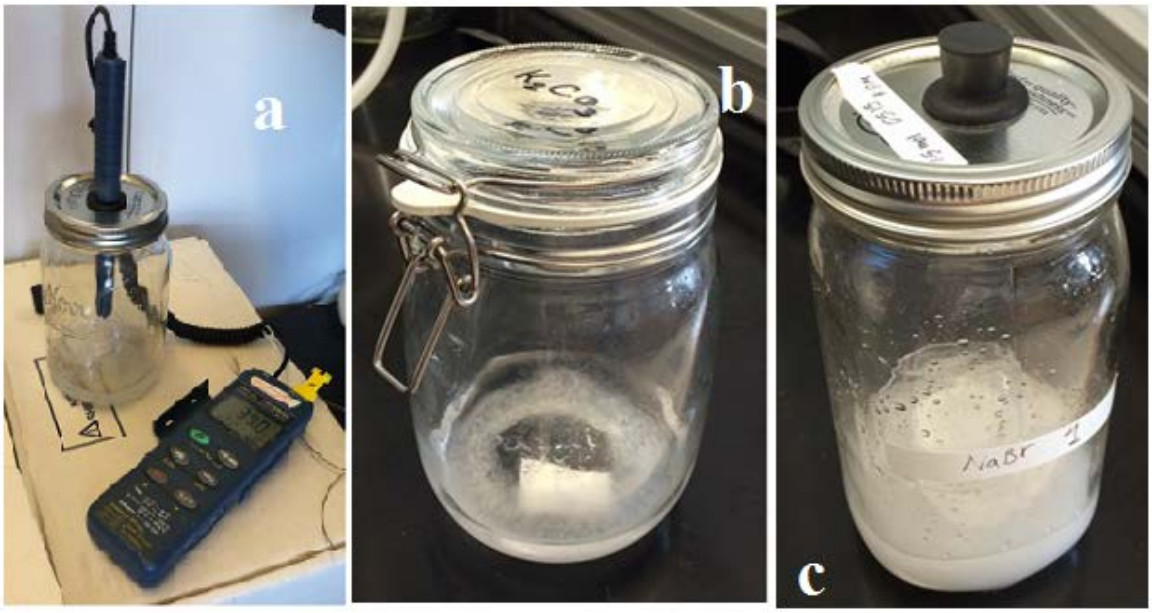

Figure 2.1: a. EOMEGA HH134A humidity meter with wired probe, b. Kilner jar, c. Mason jar

2.3.1 ASTM Protocol E 104-02: Preparation of Saturated Salt Solutions

ASTM Protocol E 104-02 was followed in order to achieve the desired relative humidity control, ranging from “dryness to near saturation at temperature spanning from 0 to 50 °C” [25]. Here, terms and conditions are defined according to Terminology D 1356 and purity of water, produced by ion exchange or distillation, is specified according to D 1193 (specification for reagent water). According to this protocol, the container (e.g. controlled humidity vessel) should be made of non-hygroscopic material, i.e. material

that neither absorbs nor retains water vapor, such as glass, and if the saturated salt solution is retained in a tray which made of appropriate material a plastic or metal container can be used. According to the ASTM standard the selected saturated salts in this standard can be utilized for one year or more to achieve an environment with constant relative humidity.

After preparing the slurry according to the ASTM standard protocol the slurry was added to the jar, the lid was clamped and the preservation jars were kept in a dark cabinet (sunlight may cause fluctuation in humidity inside the jars) for 1 week to reach equilibrium. During this period the solution was stirred daily using a spatula. Relative humidity values (% RH) were measured using an OMEGA HH134A humidity & temperature meter.

2.3.2 Stability of Controlled RH Environment

To evaluate the perturbation effect of temporarily exposing the established environment in the controlled humidity vessels to a different RH environment, a vessel equilibrated at 61% RH was opened in an 11% RH environment in a glove box for 12 seconds, and then closed. This was previously determined as the amount of time that it takes to open the jar, place samples inside, and close the lid again. To facilitate RH measurement inside the jar, lids were bored to the diameter of the humidity meter probe plus a clearance space and the hole was sealed by plastic O-ring to minimize leakage. As shown in figure (2.1) the RH probe was inserted in the jar and RH values were recorded for a minimum period of 10 hours (device measurement accuracy ±2.5%). The experiment was repeated three times.

2.4 Preparation of Sugar Glasses by Microwave Processing

In the pharmaceutical and food industries the glassy state of sugar is exploited as stabilizing excipient. The materials to be preserved are generally suspended in an aqueous solution containing various salts, sugars, and other stabilizers, and then different methods are used to remove water to reach a solid amorphous state (i.e. freeze-drying, freeze-thawing, spray drying). Here, we dehydrated samples into a solid amorphous state by microwaving samples at specific power rate to dehydrate samples within the shortest possible time period, without causing excessive heating ($T<100°C$). During microwave processing of samples electromagnetic radiation excites polarized molecule and the buildup of thermal energy enhances water evaporation from samples.

In order to decrease the water content to values that enable room temperature glasses to be created, all samples were processed into a dry state within an 11% RH environment. A chamber was designed to contain all of the necessary equipment within this environment, here after called the glove box. The chamber was built from aluminum frame (T-slotted type) with transparent plastic as walls. A bored hole in one of the lateral wall is used as the air inlet. Compressed air was used to fill the chamber, which was passed through desiccation chambers prior to entering the glove box, as shown in figure (2.2). A screw compressor housed in the facility room of the building provides air for service valves in the BioStability Lab. It was determined that 11% was the lowest consistent relative humidity that could be achieved in the glove box using a constant stream of air. Prior to experimentation, the air valve was opened and the RH was monitored using an OMEGA HH134A humidity & temperature meter. After establishing a 11% RH (±0.7) in the glove box, microwave processing could be started.

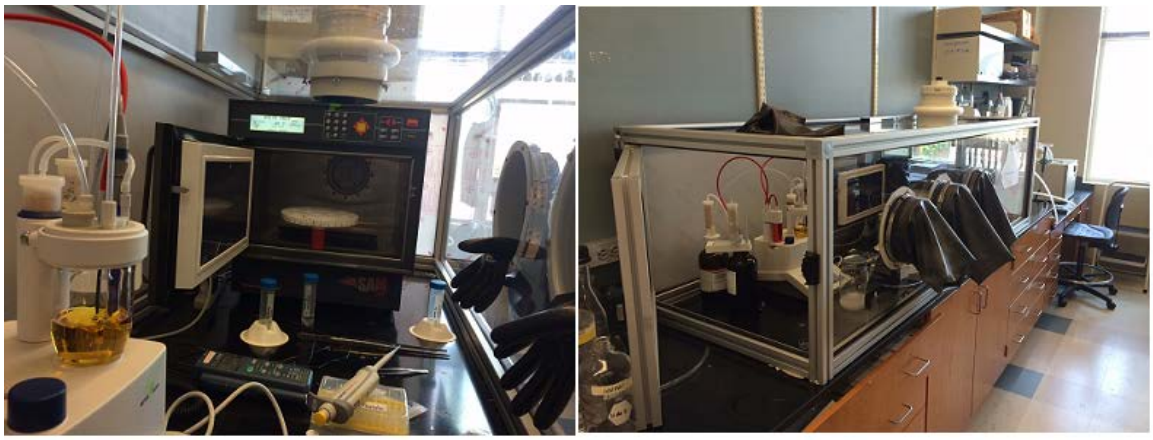

Figure 2.2: Arrangement of volumetric KF titration and microwave device inside the glove box

An Eppendorf Research Plus adjustable pipette was used to pipette 100μL droplets of the experimental compositions on the treated side of Thermanox coverslips with Dia. 22mm. This process was performed in the 11% RH stabilized glove box. In order to facilitate quick handling, samples were placed on top of a plastic petri dish as shown in figure (2.3), which was then placed on the outer ring of a custom turntable. The turntable with the Petri dish was then transferred into the microwave device for processing.

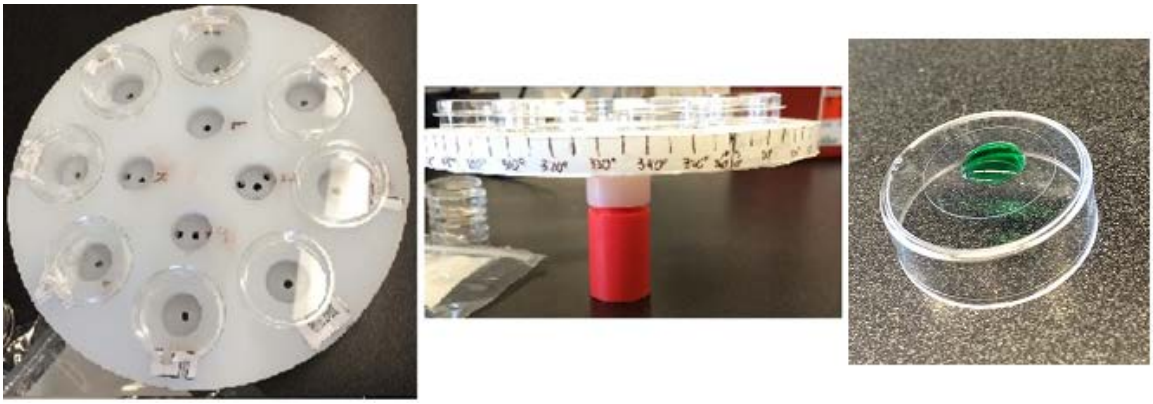

Figure 2.3: Microwave turn table with plastic petri dishes on outer ring, sample on a cover slip on top of a plastic petri dish

Iglesias *et al* [15] made samples using a freeze-drying method. For adsorption isotherm experiments the measured ratio of $\frac{gr(H_2o)}{gr(dw)}$ was reported as 0.038 at 11% RH (samples were in glassy state). In the freeze-drying method, the high vacuum rate leads to removing almost the entire water content from trehalose samples [4]. In our experiment the goal was to reach the lowest possible water content with only moderate temperature rises in the samples. To find the optimum time interval and input power, samples were processed for different combinations of time, varying from 30 to 60 minutes, and power level. Samples were titrated right after microwaving to determine end moisture content using V20 Mettler Toledo volumetric Karl Fischer titration. Sample dry weights were calculated according to the method explained in section 2.2. The goal was to process samples to moisture contents lower than 0.105 gH_2O/ gdw, which is known to produce a T_g above room temperature in aqueous trehalose solutions [15]. The microwave was allowed to cool down to room temperature (around 23.5±1.5 °C) prior to repeating experiments. The interior temperature of the microwave was measured using a General IRT 207 infrared handheld thermometer.

2.5 Experimental Design

Various studies have revealed a strong correlation between the glass transition temperature of a bio-material and its stability. Consequently, the kinetics of water uptake as a stimulant for crystallization (strong plasticizer and influential parameter on T_g) has received great consideration. Dynamic Vapor Sorption (DVS) has been established as one of the effective procedures to evaluate water adsorption. In this technique, the sample is exposed to a controlled RH environment (closed chamber) using saturated salt solutions and allowed to reach equilibrium with the surrounding atmosphere. Then water moisture sorption is monitored and recorded as a function of time. The DVS protocol used in this work is very similar to the isopiestic method which is used for measuring water activity. Here, the DVS method was used to monitor water sorption in trehalose samples (40-100 µL of solution) before and after crystallization (crystals were detected visually in samples) and results were compared with established trends. In subsequent studies water uptake in salt-trehalose samples were monitored to evaluate the effect of salt on end moisture content at time zero after microwave processing at 11% RH and water sorption trends in samples over the experiment duration while held in preservation jars (61% RH).

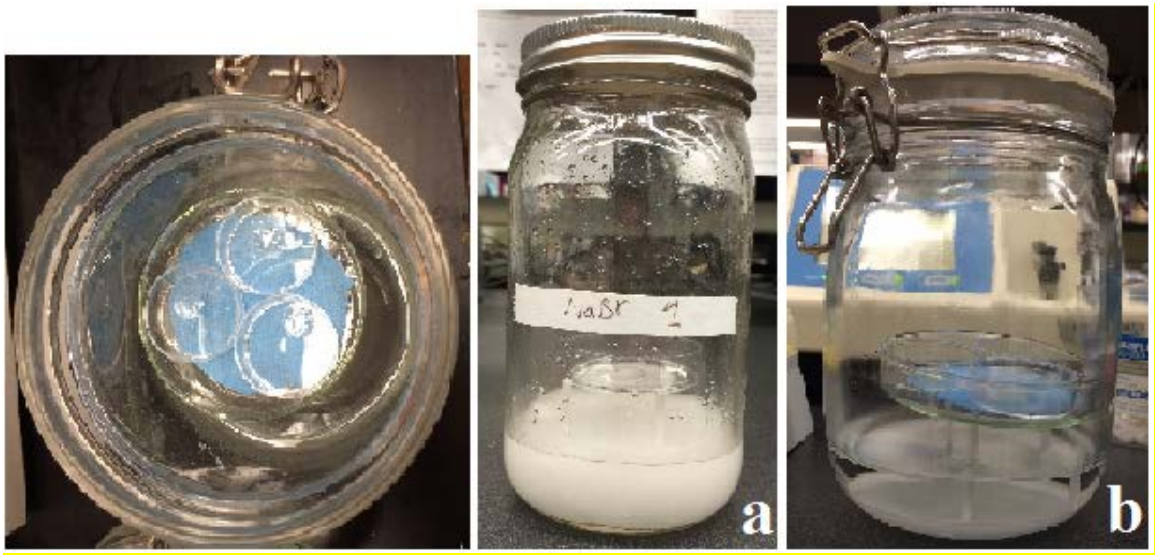

Figure 2.4: Position of samples within plastic petri dishes on pizza stand above saturated salt surface level in preserving jars, a. Mason jar, b. Kilner jar

In order to measure the moisture sorption only on cover slips and thus determine the error in subsequent determination of sorption in samples, blank cover slips were kept over P_2O_5 for 48 hours and the water content was determined on sacrificial cover slips then the rest were transferred to 61% RH. After 10 days these cover slips were titrated and the moisture content was recorded.

2.5.1 Experiment 1: Study the Effect of Choline Dihydrogen Phosphate on Water Sorption, Tendency to Crystallization and End Moisture Content in Salt-Trehalose in Mixture

Studies have cited the beneficial effect of choline in bio-preservation, including work from the BioStability Lab on proteins [22,23]. Satoshi Ohtake *et al* [18] studied the interaction of phosphate with trehalose and asserted that phosphate-trehalose mixtures could have T_g values much higher than those observed for the pure trehalose and several interesting features of phosphate-trehalose mixtures make them useful for preservation of

labile molecules. Here, in the first experiment choline as cation and phosphate in the form of dihydrogen phosphate as a counter ion were mixed with trehalose aqueous solution at various molar ratios. After drying via microwave processing at 11% RH, samples were transferred to thermostatic container with 61% RH and the onset of crystallization, water sorption were monitored over the time.

2.5.2 Experiment 2: Study the Effect of Sodium as Cation on Water Sorption, End Moisture Content and Tendency to Crystallization and in Sodium Dihydrogen Phosphate: Trehalose Mixture

Mazzobre and coworkers [17] evaluated the effect of cation (Na^+, Ca^{2+}, Mg^{2+}) on water sorption and crystallization of salt-sugar aqueous solution and contended that these criteria were modified by the presence of the inorganic salt, therefore as a next experiment, in order to study the effect of cation, choline was replaced by sodium, using the same form of phosphate as anion. The experiment was performed with same salt-sugar ratios used in previous experiment for choline dihydrogen phosphate-trehalose mixtures. The same data was acquired for these mixtures.

2.5.3 Experiments 3: Study the Effect of Anion with Sodium as Cation on Water Sorption, End Moisture Content and Tendency to Crystallization in Sodium Hydrogen Phosphate:Trehalose Mixture

Depending on the pH, phosphate as anion could be found in solution either in form of HPO_4 which interacts effectively with trehalose or H_2PO_4 which does not interact effectively with trehalose. In the previous two experiments cations were compared and the effects on water sorption and crystal suppression were evaluated. In this step a

dynamic vapor sorption was setup and the effect of anions were studied with sodium as a cation.

2.5.4 Experiment 4: Study the Effect of Anion with Choline as Cation on Water Sorption, End Moisture Content and Tendency to Crystallization in Choline Hydrogen Phosphate Mixture

Based on the results of previous experiments, in order to combine the crystal suppression effects observed from choline and monohydrogen phosphate, choline hydrogen phosphate was chosen as the additive to trehalose solution and the last DVS experiment was performed. Results of experiments are givenin Chapter three.

CHAPTER 3: RESULTS

3.1 Solution Properties: pH, Osmolality, and Conductivity

Because pH has a significant effect on the predominant form of phosphate ions, the pH of all solutions was measured. For example, phosphate salts can be present in solution in the form $H_2PO_4^-$ or HPO_4^{-2} depending on the pH [18]. Table (3.1) demonstrates pH values for reference salt solutions (1M of salt prepared in 1X TE buffer). Regardless of the cations, solutions with hydrogen phosphate as anions had a basic pH (≈ 9), whereas the pH of dihydrogen phosphate reference solutions were acidic, in the pH range of 4-5. The pH of trehalose in buffer solution (30.8 wt% stock solution of trehalose, prepared in 1X TE) was determined to be 7.69.

Table 3.1: pH of 1M of aqueous solutions of salts

Composition	pH
Choline hydrogen phosphate	8.73
Sodium hydrogen phosphate	8.99
Choline dihydrogen phosphate	4.85
Sodium dihydrogen phosphate	4.25

The pH of salt-trehalose aqueous mixtures is reported in table (3.2). It can be observed that for compositions in which the phosphates are predominantly in form of dihydrogen phosphate (H_2PO_4), increasing the trehalose ratio increases the final value of pH, whereas in mixtures where the majority of phosphates are in the monohydrogen

phosphate (HPO_4) form, increasing the trehalose ratio decreases the pH of the solution, which is in good agreement with expectations.

Table 3.2: pH values of different salt:trehalose compositions with different molar ratios

	Ratio		
	1:0.7	1:2	1:4.8
Composition	pH		
Sodium dihydrogen phosphate : tre	4.33	4.52	4.91
Choline dihydrogen phosphate : tre	5.24	5.19	5.27
Sodium hydrogen phosphate : tre	8.68	8.46	8.37
Choline hydrogen phosphate : tre	8.38	8.15	8.03

As a next step, the osmolality of solutions was measured and results are given in table (3.3). Osmolality is defined as the sum of active particles in solution and as salt dissociates into ions (i.e. Na^+, HPO_4^{-2}) it is expected that composition with the higher salt mole fraction has the highest value. It can be observed that the osmolality does not change considerably between compositions with the same ratio of salt to sugar, but as the sugar mole fraction in solution was increased, the osmolality decreased.

Table 3.3: Osmolality values of salt:trehalose compositions with different molar ratios

	Ratio		
	1:0.7	1:2	1:4.8
Composition	Osmolality		
Sodium dihydrogen phosphate : tre	2152.3±8.504	1964.3±4.618	1607.3±18.33
Sodium hydrogen phosphate : tre	2077±6.363	1950±5.656	1695±7.071
Choline dihydrogen phosphate : tre	2430±5.196	2004.66±11.59	1787±3.605
Choline hydrogen phosphate : tre	2479.5±7.77	2145±6.78	1916±5.853

Conductivity measurements were also acquired and are shown in figure (3.1) below. As the fraction of salt in solution increased, so did the conductivity, as expected. Both sodium based salts exhibited similar conductivity profiles, suggesting that a similar amount of ions were dissociated in solution, consistent with osmolality results. The conductivity of the choline dihydrogen phosphate series was lower than both of the sodium phosphate salts at the same salt to sugar ratio, consistent with a lower charge density on the choline cation.

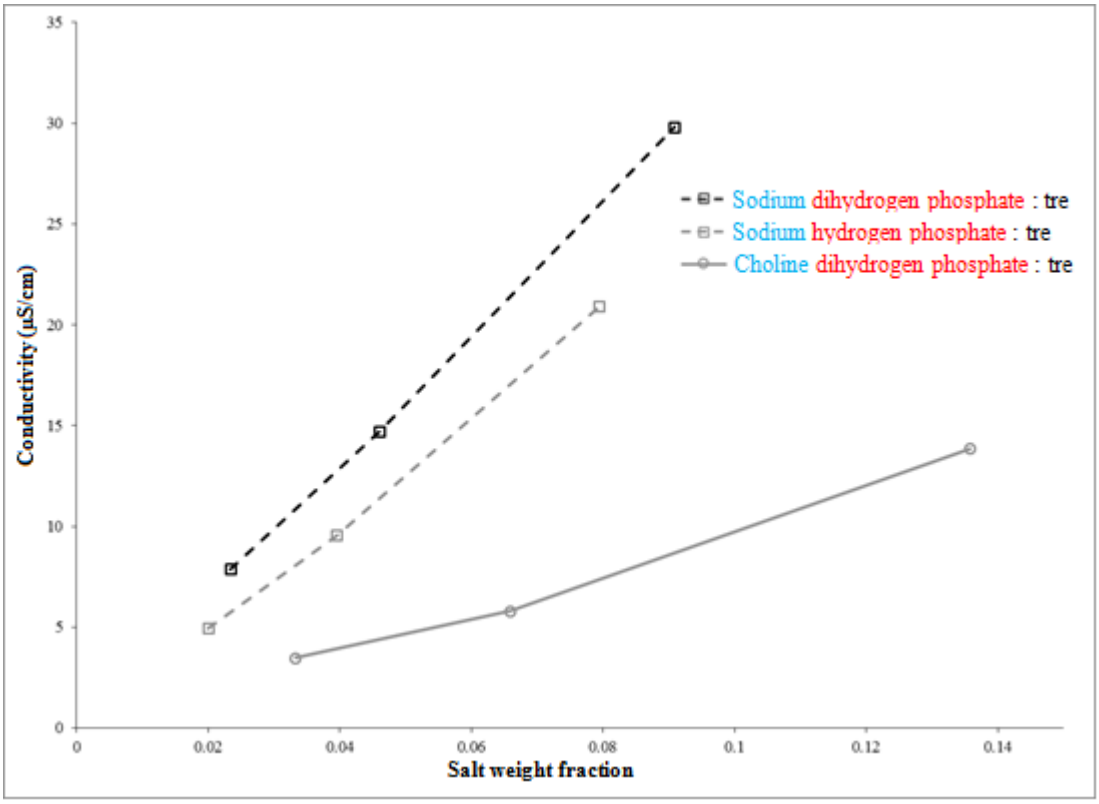

Figure 3.1: Measurements of conductivity in salt-sugar aqueous solution as a function of salt mass fraction (22.9±0.8 °C)

3.2 Fraction of Solids in Experimental Compositions

To determine the dry weight of the composition, the total weight of 100 µL of solution with different salt-trehalose ratios, as well as 30.8 wt% of trehalose in 1XTE buffer were measured. The dry weight of 30.8 wt% trehalose was determined using two different methods and results are shown in table (3.4). Also, the wet weight, water loss, and dry weight, are shown in table (3.5) for different ratios of salt-trehalose mixtures, which enabled the mass fraction of solids to be determined.

Table 3.4: Determination of mass fraction of solids in solution by the gravimetric and KF titration methods

	Average mass fraction	Standard deviation
Gravimetric method	0.314	0.01
KF titration method	0.347	0.001

Table 3.5: Dry weight and mass fraction of solids determination of salt-trehalose mixtures. Samples were baked out in a convective oven for 48 hours at 90°C

Composition	Ratio	Wet weight (g)	Water loss (g)	Dry weight (g)	Mass fraction	
					Grav.	Density
$C_5H_{14}NOH_2PO_4 - Tre$	1:0.7	0.848±0.17	0.586±0.02	0.261±0.06	0.308	0.293
	1:2	0.923±0.17	0.635±0.03	0.287±0.05	0.312	0.295
	1:4.8	0.925±0.15	0.624±0.03	0.301±0.04	0.32	0.302
$(C_5H_{14}NO)_2HPO_4 - Tre$	1:0.7	0.656±0.12	0.449±0.01	0.207±0.07	0.315	N/A
	1:4.8	0.659±0.09	0.44±0.02	0.219±0.06	0.332	N/A
$NaH_2PO_4 - Tre$	1:0.7	0.535±0.08	0.393±0.07	0.141±0.01	0.241	0.233
	1:2	0.480±0.04	0.346±0.06	0.133±0.02	0.278	0.27
	1:4.8	0.587±0.08	0.407±0.05	0.179±0.01	0.285	0.29
$Na_2HPO_4 - Tre$	1:0.7	0.573±0.09	0.434±0.03	0.139±0.08	0.242	0.24
	1:2	0.593±0.08	0.424±0.05	0.168±0.04	0.285	0.273
	1:4.8	0.587±0.07	0.507±0.03	0.079±0.07	0.294	0.291

3.3 Relative Humidity Control

The ASTM protocol described in Chapter 2 was utilized to produce various values of relative humidity in a closed environment using saturated salt solutions. The measured results are shown in table (3.6) along with ASTM equilibrium relative humidity values and RH values reported by Greenspan, in which a different saturated salt preparation method was used [25]:

Table 3.6: Established Relative humidity of each saturated salt in preservation jars

Salts	Relative Humidity (%) measured at 25 °C	ASTM reported values at 25 °C	Greenspan measured values
LiCl	14.1	11.3	11.3
CH_3COOK	25.3	22.5	23.11
$MgCl_2$	35.1	32.8	32.78
K_2CO_3	45.2	43.2	43.16
$Mg(NO_3)_2$	55.1	N/A	52.89
NaBr	61	57.6	57.57

Also, changes in the established environment in preservation jars caused by exposing the contents to an environment with a different relative humidity were recorded as a function of time. As shown in figure (3.2), in the case of the 61% RH environment, after a brief exposure (12s) to an 11% RH, within 1 hour the relative humidity in the jar reached equilibrium again.

To establish the potential error associated with moisture sorption on cover slips, the moisture uptake on blank coverslips was determined. At first empty coverslips were dried in a desiccator over P_2O_5 and the water content was determined on sacrificial cover slips. Pre-dried cover slips were then kept in preservation jars at 61% RH, for 10 days. After this period water content were measured and are displayed in second column of table (3.7):

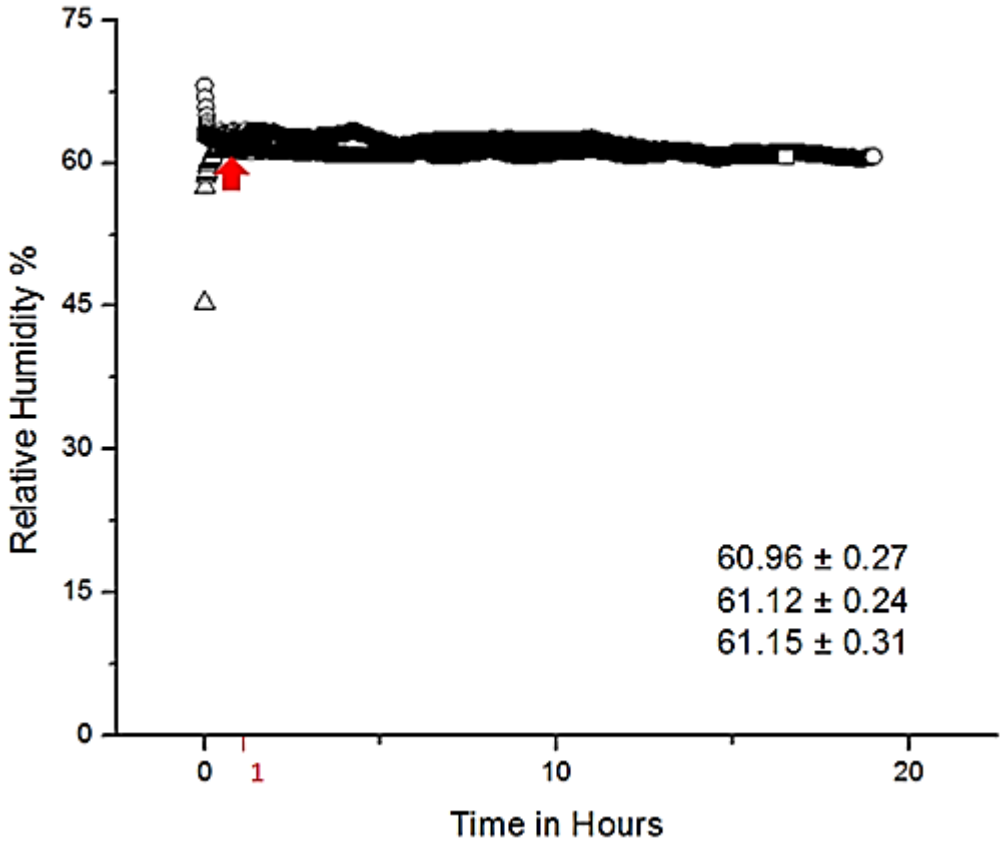

Figure 3.2: Recorded relative humidity in preservation jars over 20 hours. The 61% environment was achieved using a sodium bromide saturated salt solution prepared according to ASTM method E104-02 (61.12±0.24% RH)

Table 3.7: Residual moisture in Thermanox plastic coverslips after storage in 0% RH over P_2O_5 for 2 days (dry) and in 61% RH environment for 10 days (wet)

Dry cover slip (µg)	Wet cover slip (µg)
104.809±11.58	116.972±10.2

3.4 Experiment 1: Effect of Choline Dihydrogen Phosphate:Trehalose Ratio on Water Sorption and Tendency to Crystallize

It is well known that amorphous trehalose will crystallize in environments with 44% RH and higher [15]. Crystallization can have detrimental effects on cell membranes, proteins, and other biomolecules, and is generally avoided in preservation compositions. Here, the main aim of the experiment was to determine if the crystallization tendency of trehalose could be altered by the addition of choline dihydrogen phosphate to the mixtures. As described previously, samples were first dried into an amorphous state using microwave assisted drying in an 11% RH environment. It can be observed that right after microwaving at 11 % RH, samples with higher salt content have lower end moistures. In other words, increasing the mole fraction of choline dihydrogen phosphate in the samples leads to a decrease in the end moisture content after microwave processing.

Samples were then transferred to a 61% RH jar. The water uptake was measured as a function of time on sacrificial samples. Parallel samples were monitored for evidence of crystallization. As seen in figure (3.3) the amorphous trehalose samples took up water until enough was absorbed to enable the formation of trehalose dihydrate (≈ 0.105 gH_2O/gDryWeight). At day 1, crystal formation was observed in trehalose samples, and over the next 18 days, the sample moisture content decreased to that of the crystalline trehalose samples. The presence of choline dihydrogen phosphate in solution slowed the onset of crystallization, but also allowed the samples to equilibrate to higher moisture contents. The amount of water sorption in samples increases in proportion to the rising choline dihydrogen phosphate fraction in the mixture. In figure (3.3) the onset of crystallization (as observed visually) is indicated by stars. The more salt there is in

solution the longer the delay before crystallization. Pure choline dihydrogen phosphate was observed to be very hygroscopic.

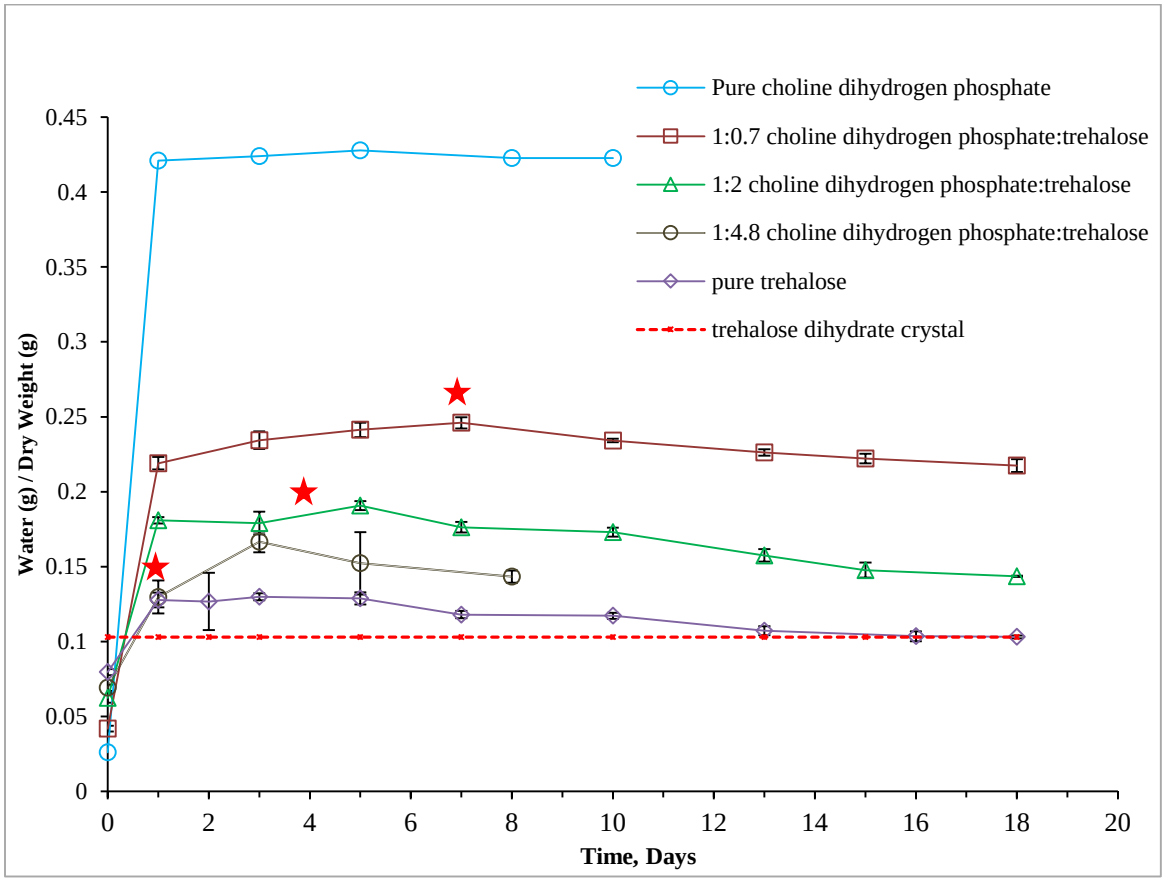

Figure 3.3: Water sorption of samples with different choline dihydrogen phosphate:trehalose ratios when held at 61% RH over the course of 18 days. The onset of crystallization as observed visually is indicated with a star. (n=3)

3.5 Experiment 2: Effect of Cation on Sorption Behavior and Tendency to Crystallize: Choline VS. Sodium with Dihydrogen Phosphate as the Anion

In the previous DVS experiment the amorphous trehalose composition crystallized within 24 hours, but adding choline dihydrogen phosphate delayed this event.

To determine how much each ion specifically contributed to this effect, in this experiment sodium was substituted for choline in the mixtures, and the DVS experiment was repeated. The results of this experiment for sodium dihydrogen phosphate-trehalose samples are displayed in figure (3.4). Because all samples crystallized by day 1, the experiment was not continued beyond 10 days. Samples with a higher salt mole fraction absorbed more water in the 61% RH environment and exhibited a distinct "rise and fall" pattern, first increasing in water content and then decreasing either directly after or within 2 days after observing crystallization. Sample water content right after microwave processing at 11% RH are shown in table (3.8). Differences between end moisture contents were not statistically significant (P-value>0.05). Samples with sodium as the cation held more water after microwave processing at 11% RH (e.g. samples are wetter) compared to equivalent compositions with choline as the cation.

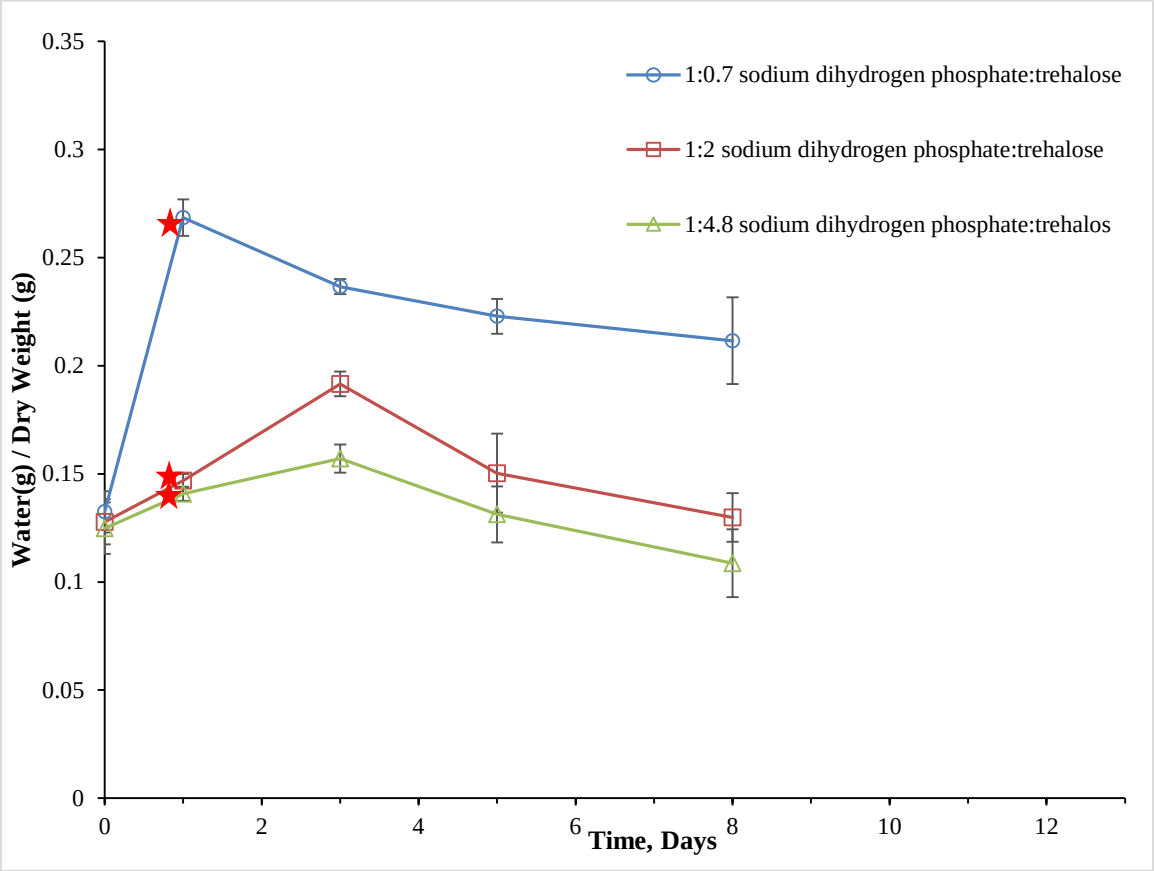

Figure 3.4: Water sorption of samples with different sodium dihydrogen phosphate:trehalose ratios when held at 61% RH over the course of 8 days. The onset of crystallization as observed visually is indicated with a star. (n=3)

Table 3.8: Result of statistical analysis using ANOVA single factor method

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
1:0.7	3	0.397276	0.132425	9.68E-05
1:2	3	0.363291	0.121097	0.000207
1:4.8	3	0.374481	0.124827	0.000346

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F critical*
Between Groups	0.0002	2	1E-04	0.461685	0.65088	5.143253
Within Groups	0.001299	6	0.000217			
Total	0.001499	8				

3.6 Experiment 3: Effect of Anion on Sorption Behavior and Tendency to Crystallize: Dihydrogen Phosphate VS. Hydrogen Phosphate with Sodium as the Cation

The effect of cation on crystal suppression was investigated in the previous section and it was revealed that choline dihydrogen phosphate delayed crystallization more effectively than sodium dihydrogen phosphate, an effect that was directly proportional to the mole fraction of salt in the solution. In the current experiment the effect of the anions on trehalose crystallization was explored, by determining the water sorption characteristics and crystallization tendency of compositions containing sodium hydrogen phosphate and comparing it to previous results for sodium dihydrogen phosphate. As observed in figure (3.5) for the lowest salt:sugar ratio in sodium hydrogen phosphate:trehalose samples crystallization occurs in same time interval as amorphous trehalose compositions. However, increasing the salt fraction led to an increase in the crystallization suppression time. Unlike the previous experiment with sodium dihydrogen phosphate, there were differences in the values for end moisture content at 11% RH but no trend with composition could be resolved.

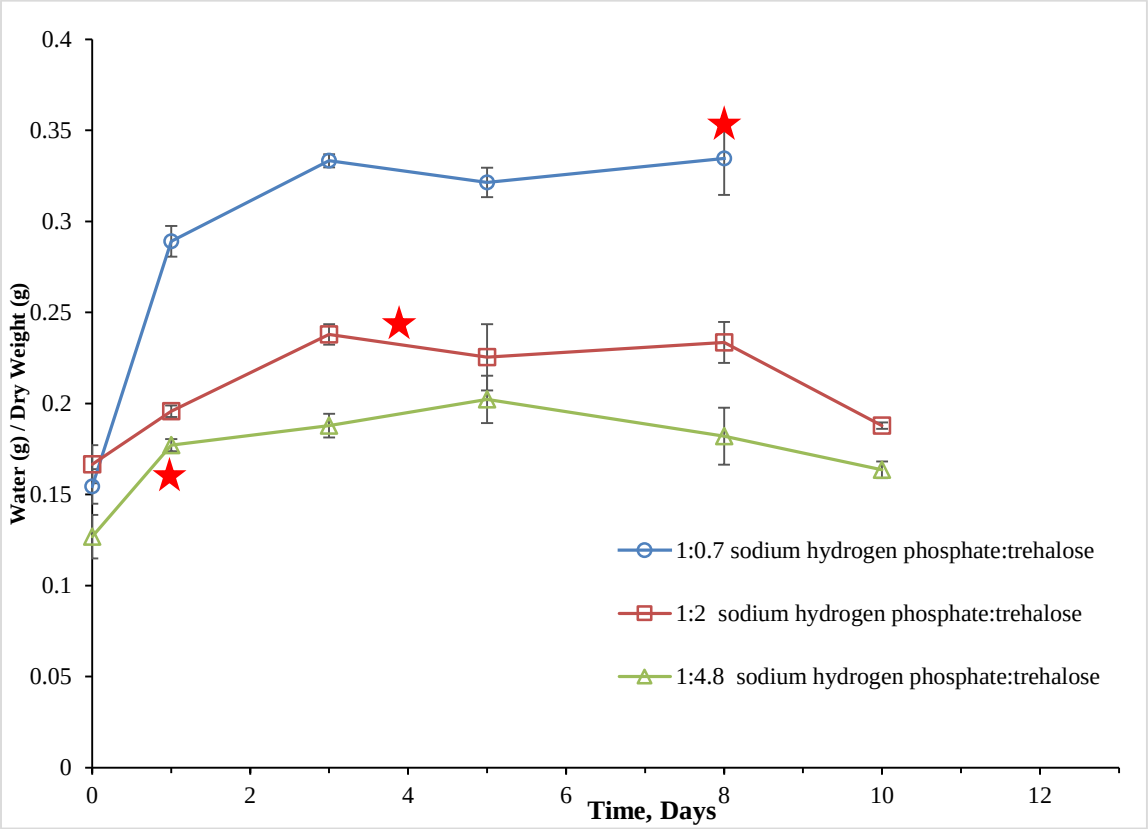

Figure 3.5: Water sorption of samples with different sodium hydrogen phosphate:trehalose when held at 61% RH over the course of 10 days. The onset of crystallization as observed visually is indicated with a star. (n=3)

3.7 Experiment 4: Combining the Beneficial Effects of the Choline Cation and the Hydrogen Phosphate Anion

Given the results of previous experiments it is clear that both the cation and the anion can play a role in suppressing crystallization. It was logical to next test a salt with choline as the cation and hydrogen phosphate as the anion. This composition was prepared by increasing the pH of choline dihydrogen phosphate solution with choline hydroxide and then lyophilizing the composition to recover choline hydrogen phosphate. The salt was then added to an appropriate aliquot of 30.8 wt% trehalose solution to make the desired choline hydrogen phosphate:trehalose mixtures. At all ratios of salt:sugar,

there was a distinct increase in water content by day one, and then the samples largely equilibrated at a specific water content. As observed previously, samples with a higher salt mass fraction absorbed more water. Results were similar to the previous experiment with sodium hydrogen phosphate, with the exception of the 1:0.7 compositions, which had no observed crystals within the observation period. All salt:sugar ratios were observed to have similar end moisture contents at 11% RH.

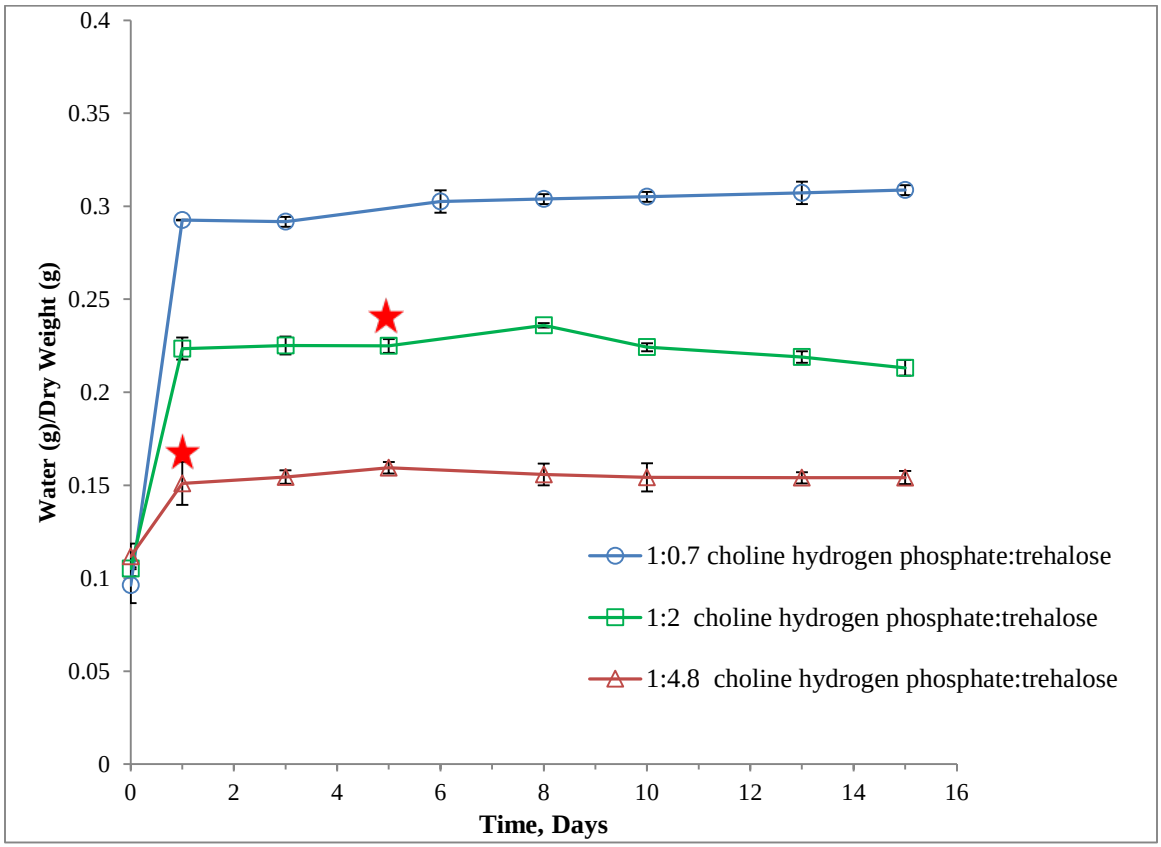

Figure 3.6: Water sorption of samples with different choline hydrogen phosphate:trehalose when held at 61% RH over the course of 15 days. The onset of crystallization as observed visually is indicated with a star. (n=3)

Figure (3.7) shows the time before crystallization for the different compositions from all 4 experiments. As observed in this figure, the replicates at each composition either crystallized on the same day or within 24 hours. Samples with a higher salt mole fraction suppressed crystallization for a longer time, except for the sodium dihydrogen phosphate compositions. None of the 1:0.7 choline hydrogen phosphate:trehalose samples were crystallized within the period of observation (15 days) and after 24 days one of the samples exhibited what appeared to be tiny cracks. Over the span of 24 days, compared with 100 µL volume of sample at day 1 of the experiment there was a significant volume increase in the other two samples which was observed visually. In summary, choline as the cation and hydrogen phosphate as the anion (individually in composition or a combination of both) resulted in a longer time span before observation of the onset crystallization.

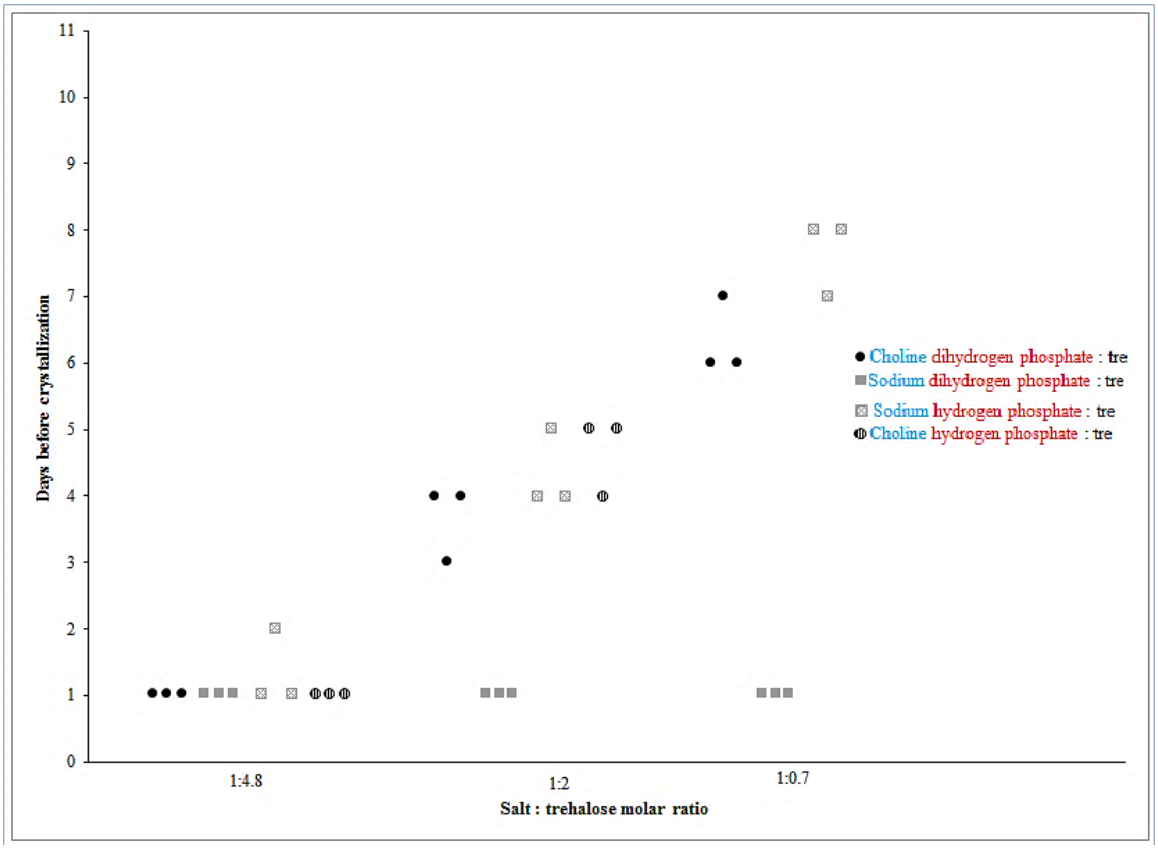

Figure 3.7: Days before the onset of crystallization in choline/sodium phosphate:trehalose samples (parallel replicates were monitored)

CHAPTER 4: DISCUSSION

4.1 Effect of Solution Properties on the Results of Experiments

The results of pH measurements of mixtures were shown in table (3.2), for each salt:trehalose ratio and type of anion. In order to make mixtures with particular salt:trehalose molar ratios and to remain consistent with these ratios in the experiments, differences in pH values of compositions with same anion is inevitable.

The Osmolality of solutions was given in table (3.3). The values given in table (3.3) deviate from the results one would expect based on chemical structure and if ions completely dissociated in solution. The osmolality of sodium salts are less than the choline ones with same salt ratio. As seen in figure (3.3) samples of choline hydrogen phosphate are very hygroscopic and this property of choline salt could lead to a higher level of ion dissociation compare with sodium salt in solution.

4.2 Investigation of Composition Dry Weight: Comparison of Methods

As seen in table (3.4) where the mass fraction of trehalose values are demonstrated, compared with the average values in each column, standard deviations were small. As observed in table (3.4) the mass fraction determined for pure trehalose via the gravimetric method was in good agreement with the theoretical calculation ($0.308\,\frac{dry\ weight(g)}{wet\ weight(g)}$), whereas the KF titration method deviated notably from theoretical one. Thus the gravimetric method was chosen to determine the dry weight for the balance of mixtures. In table (3.5) the solids mass fraction of different compositions are shown,

along with experimental measurements based on solution densities. In general there was good agreement between each of these measured values.

4.3 Consistency of Relative Humidity in Storage Containers

Measured values of relative humidity in preservation jars are shown in table (3.6). The saturated salts with the two lower relative humidities deviated significantly from values reported in the ASTM standard. Salt purity, jar sealing or water properties may have resulted in the observed errors. Although the Greenspan [25] published values were used as reference, their saturated salt preparation method may have varied from the ASTM protocol. For the DVS experiment it was desired that the RH in the preservation jar was higher than 44% with little fluctuation. Trehalose will crystallize in environments with RH >44% and a stable high RH environment was thus vital for evaluating the effect of salt on suppressing crystallization. Figure (3.2) shows that environment in 61% RH jars was relatively stable. Even after exposure to an environment with a significantly different relative humidity (11% RH), the content of the jar equilibrated in a relatively short time period. These results demonstrated that the environment in the container was suitable, i.e. stable with RH > 44%.

4.4 Effect of Choline Dihydrogen Phosphate as an Additive to Trehalose Aqueous Solutions

As was demonstrated in table (3.7), the amount of moisture absorbed by 22 mm Thermanox plastic coverslips over the period of 10 days in 61% RH is about 12μg which compared with the water sorption in samples is negligible (for Ex. moisture content of sodium dihydrogen phosphate:trehalose samples with 1:0.7 molar ration at day 1 in 61%

RH is equal to 3500 µg) and does not introduce any significant error to the results of the DVS experiments.

As displayed in figure (3.3) the amount of water in samples of trehalose after crystallization levels off is ~ 0.105 ($\frac{water\ (g)}{dry\ weight\ (g)}$), which is equivalent to the dry weight of trehalose dihydrate crystals. The samples of pure choline dihydrogen phosphate are very hygroscopic and as expected, the equilibrium water content in 61% RH in mixtures containing choline dihydrogen phosphate increases with increasing salt mole fraction. For the 1:0.7 ratio there is a sharp increase in water content by day 1 after transferring samples to the container with 61% RH, and then water sorption rises gradually until day 7, when crystallization was detected visually. Crystallization was followed by a slight decline in water content which plateaued around day 15. As seen for salt-trehalose composition of 1:2 molar ratio, considerable water sorption occurred within 24 hours in 61% RH. Interestingly, even after crystallization the water content increased within the next day and then decreased slightly and leveled off by day 15. In the case of the 1:4.8 ratio crystallization occurred at day 1, within the same span of time and at the same water content as samples of pure trehalose. A decrease in water content started two days after the observed crystallization.

Maximum suppression of crystallization was observed in choline dihydrogen phosphate-trehalose samples with the highest salt ratio. Compared with pure trehalose, choline dihydrogen phosphate-trehalose samples also retained more moisture in 61% RH while suppressing crystallization which may imply that despite the higher volume of moisture and plasticizer effect of water, formation of crystals has been suppressed. Before the onset observation of crystallization, samples absorbed moisture in 61% RH

and the ratio of $\frac{water(gr)}{DW(gr)}$ increased over time. Depending on the salt-trehalose ratio, water uptake can continue following observation of crystallization prior to re-equilibrating at a lower moisture content. The time at which the sample begins to lose moisture, which is cited in the results of other researchers [16], could be inferred as the point of complete crystallization in samples. Water uptake past the visualized start of crystallization would thus be associated with the portion of sample which has not crystallized.The end moisture content of samples right after microwave processing in an 11% RH environment, decreased in proportion to the rising choline dihydrogen phosphate fraction in mixture, as seen in figure (3.3). The water content in 11% and 61% RH revealed inverse trends in moisture uptake as a function of the salt mass fraction.

Sitaula *et al* [19] asserted that adding PBS to the trehalose mixture leads to a shift from a type II isotherm to a type III isotherm (characterized by the BET equation). Based on the moisture content observed at both 11% RH and 61% RH, the addition of choline dihydrogen phosphate could also be shifting the isotherm from a type II to a type III. In general, type III isotherms have higher moisture contents at high RH compared to type II isotherms. Due to the plasticizing effect of water in trehalose glasses, a higher moisture content in a given RH could decrease the T_g of the mixture. If this T_g value drops below the storage temperature, crystallization may occur in amorphous samples, which would have a detrimental effect on biomaterials. Based on the type of isotherm the water sorption kinetics (ad-sorption or ab-sorption) can be resolved. For hydrophobic compounds, it is proposed that water molecule does not permeate through the sample volume and instead accumulates in area near to the surface of sample (i.e. adsorption).

More data would be required to full evaluate these sorption characteristics in the compositions described here.

4.5 Role of Cation in Mixtures with Dihydrogen Phosphate as Anion: Choline VS Sodium

In the 1:0.7 molar ratio mixtures, crystallization was observed by day 1 and there was a major increase in the water content within the first 24 hours after storage in 61% RH ($\approx 0.27 \frac{water\ (g)}{Dry\ weight\ (g)}$). Then water content decreased continuously during the next 7 days after crystallization. In the two lower ratios, the onset of crystallization was also observed in the same time period (day 1) but there was no evidence of a large pick in water sorption at day 1. In compositions with a 1:2 molar ratio the water content increased up to 0.18 ($\frac{water(g)}{Dry\ Weight(g)}$) after visual onset of crystallization on day 1 and decreased to 0.13 ($\frac{water(g)}{Dry\ Weight(g)}$) during the next 5 days. As seen in figure (3.4) by substituting sodium for choline, all samples crystallized within 24 hours thus regardless of composition ratio, sodium dihydrogen phosphate as an additive did not delay crystallization.

Differences between water content values for samples equilibrated in 11% RH for the different ratios were also not statistically significant. As the cation, sodium has a higher charge/size in comparison with choline, even though choline was found to be a better candidate cation for crystal suppression. This result is in contradiction with other results for inorganic salts [17] and could be related to a different capacity of choline for interacting with other composition or its capability for making hydrogen bonds. Also it

has been cited in other research that molecular weight can play a vital role in crystal suppression [13].

4.6 Role of Anion in Mixtures: Dihydrogen Phosphate VS. Hydrogen Phosphate

In this experiment sodium hydrogen phosphate was used as an additive to trehalose aqueous solutions. Studies in the literature indicate that pH affects the glass transition temperature in phosphate-sugar mixtures. By increasing the pH of solutions, we studied the pH effect on crystallization tendency in the mixture. For the 1:4.8 molar ratio compositions the water content increased to 0.18 ($\frac{water(g)}{Dry\ Weight(g)}$) at day 5 with no significant water uptake at day 1 and then decreased over time. Crystallization was observed at day 1 at ≈ 0.17 ($\frac{water(g)}{Dry\ Weight\ (g)}$), which is higher than the water content of pure trehalose at the time of crystallization. In the case of the 1:2 molar ratio of salt:trehalose composition the water content increased until day 3 and after crystallization on day 4, the moisture content fluctuatedand then started to decrease as a function of time. In the highest salt:sugar ratio, the water content almost doubled to 0.3 ($\frac{water(g)}{Dry\ Weight\ (g)}$) within the first day and then rose gradually to 0.35 ($\frac{water(g)}{Dry\ Weight\ (g)}$) by day 3 and then fluctuated around this value before crystallization at day 8.

Here, unlike the sodium dihydrogen phosphate:trehalose samples, the suppression of crystallization was directly correlated with the salt proportion in the mixture, or in other words, the more salt there was in solution the longer the delay before crystallization. The crystal suppression behavior as a function of salt ratio in the sodium hydrogen phosphate:trehalose mixtures is comparable to that of the choline dihydrogen phosphate:trehalose samples.

4.7 Combination of Superior Effect of Choline as Cation and Hydrogen Phosphate as Anion

From previous experiments with other salts it was realized that the combination of choline and hydrogen phosphate could potentially be an effective crystal inhibitor mixture because each of these ions was individually observed to have a crystal suppression effect. As observed in figure (3.6) no crystallization was observed visually in the 1:0.7 molar ratio mixtures over the course of 15 days. Possible crystallization was observed for the first time in one of the samples after 24 days, the longest period before crystallization amongst all compositions. Ohtake *et al* [18] observed that phosphate ions in the form of hydrogen phosphate (HPO_4^{-2}) interact effectively with sugar (i.e. trehalose) thus providing some insights as to the crystallization suppression effect contributed by this ion. It was found that, compare with sodium, choline is a better candidate cation for crystal suppression. Even though as the cation, sodium has a higher charge/size in comparison with choline. It has been shown that molecular weight can play a crucial role in crystal suppression [13]. Moreover, the crystal suppression property of choline could be related to the capacity of this ion for interacting with other composition or its capability for making hydrogen bonds. During the period of storage at 61% RH, the majority of moisture is absorbed into the samples within the first day and within this span of time the 1:0.7 ratio compositions have the largest increase in water sorption compared with the other two ratios. It was noticed that in the case of 1:0.7 ratio composition, water content in the samples increased gradually after day one with no decrease, which is expected before the onset observation of crystallization.

CHAPTER 5: CONCLUSIONS AND FUTURE STUDIES

The results of these experiments show that choline hydrogen phosphate is a promising agent for crystal suppression. In relatively high salt mass fraction of choline hydrogen phosphate the crystallization of trehalose was delayed for a considerable period of time (>2 weeks). However, the samples absorbed a considerable amount of water compared to pure trehalose. Depending on the mass fraction, the water content increase ranged from 25% up to 145%. Increasing the water content can have a detrimental effect on the T_g and/or molecular mobility within the mixture, as water is a known plasticizer. Due to complexity of composition interactions in the mixture with three or more compounds, the glassy behavior of these mixtures is complicated and difficult to predict, therefore further studies would be needed to determine the effect of salts and the associated effect of additional water uptake on the mechanical properties of the storage matrix. It is possible that while potentially adverse crystallization effects are suppressed, the ability to immobilize biomolecules in this matrix may be compromised.

A high glass transition temperature is not the only influential parameter for choosing components as a protectant in bio-preservation. A good excipient acts through a combination of good glass forming abilities, effective interaction with biomolecules, and overall stability, and sometimes it is necessary to compromise between various desirable characteristics in order to achieve an effective composition for a given biomaterial. As described previously the combination of choline and hydrogen phosphate has interesting effect on crystal suppression. Thus in next steps, the state of choline hydrogen phosphate:

trehalose mixtures with 1:0.7 molar ratio at different water contents should be determined. In order to study the effect of each composition on T_g the simplified ternary mixture of choline hydrogen phosphate and trehalose in water could be utilized in future studies. Functional studies to understand the effect on biomaterial stabilization are also advised.

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APPENDIX A: DRY WEIGHT DETERMINATION

Protocol of Karl Fischer titration method

- Prior to experiment, run the Karl Fischer titration at least for one hour and make sure drift value is below 25 µg/min
- Use 3mL syringe and fill it with unknown amount of solution (trehalose in 1XTE buffer, CDHP:trehalose, sodium phosphate:trehalose)
- Zero the analytical balance and place syringe filled with solution on it and record the mass and tare off the balance
- Open the reaction well cap of Karl Fischer titration and put 1 droplet (max. 2 droplet) of solution from syringe into chamber and push Start sample button
- Quick and carefully place the syringe on analytical balance and the recorded value would be total mass of droplet(s) in the reaction chamber
- Subtracting total mass from water mass (given by Karl Fischer titration) result in dry mass of solution

Protocol of gravimetric method

- Put small 60 mm glass culture dishes on analytical balance and record the mass of them (zero analytical balance prior to measurement).
- Having culture dish on balance tare off the balance and place CEM absorbent pad (cut in 3x3 square) on the culture dish and record the value.
- Again zero the balance containing culture dish and CEM absorbent and pipette unknown amount of solution (trehalose, CDHP:trehalose, …) on the filter and record it as wet weight.

- move the culture dishes into convective oven (VWR), place silica gel desiccant around culture dishes or at bottom rack (color of silica gels must be dark blue indicating that they are recharged) and bake-out for 48 hour at temperature below crystallization temperature (e.g. 1.5 moles Trehalose at 95 °C).
- After 48 hours remove culture dishes and place them into desiccator over phosphorus pentaoxide (P_2O_5) and let them to cool down to room temperature in this near 0% relative humidity environment.
- Later transfer culture dishes to analytical balance and the measured values are considered as dry weight.