VITAMIN STABILITY AND WATER-SOLID INTERACTIONS

by

Adrienne L. Voelker

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STATEMENT OF COMMITTEE APPROVAL

Dr. Lisa J. Mauer, Chair

Department of Food Science

Dr. Lynne S. Taylor

Department of Industrial and Physical Pharmacy

Dr. Cordelia A. Running

Department of Nutrition Science/Food Science

Dr. Lavanya Reddivari

Department of Food Science

Approved by:

Dr. Arun K. Bhunia

To my parents

For the sacrifices you have made that have led me to this point

I am forever indebted

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ABBREVIATIONS

a_w	water activity
Cl^-	chloride ion
DSC	differential scanning calorimetry
E_a	activation energy
FTIR	Fourier-transform infrared spectroscopy
HCl	hydrochloric acid
HNO_3	nitric acid
HPLC	high performance liquid chromatography
k_{obs}	observed reaction rate constant
NaOH	sodium hydroxide
NO_3^-	nitrate ion
PLM	polarized light microscope
PXRD	powder x-ray diffraction
RH	relative humidity
RH_0	deliquescence point
$RH_{0,mix}$	mutual deliquescence point
SEM	scanning electron microscopy
t_{90}	time when 90% of the initial thiamine concentration remains
TClHCl	thiamine chloride hydrochloride
T_{crys}	crystallization temperature
T_g	glass transition temperature
T_{gel}	gelatinization temperature
T_m	melting temperature
TMN	thiamine mononitrate

ABSTRACT

This dissertation investigates two major structure-function relationships important to food science: vitamin stability and water-solid interactions. Thiamine, vitamin B_1, is an essential micronutrient in the human diet. While thiamine is found naturally and as a fortification supplement in many foods, it is chemically unstable on exposure to heat and some co-formulated ingredients, with degradation exacerbated in prolonged shelf-life products. The instability of thiamine is a concern for the development of dietary deficiencies, which are prevalent even in developed countries; however, thiamine stability is not widely studied in the food or pharmaceutical industries. Thiamine is commercially available in two salt forms: thiamine mononitrate (TMN) and thiamine chloride hydrochloride (TClHCl). This study focused on documenting the storage stability of thiamine in solution, considering the effects of which commercially available salt form of the vitamin was used, vitamin concentration, pH, and ions present in solution by monitoring chemical stability and degradation kinetics over a 6-month to 1-year period following storage at 25-80ºC, and expanded these studies into food systems (bread doughs). The results from these studies, including the reaction kinetics of thiamine degradation, the degradation pathway, and the sensory impacts of the degradation products formed, especially as affected by pH and food matrix, can be used to improve thiamine stability and delivery in foods.

The studies of water-solid interactions in this dissertation covered two topics: 1) the effects of formulating a variety of food-relevant additives on the crystallization tendency of amorphous sucrose; and 2) the effects of formulation on the moisture sorption behaviors and physical stability of spices, herbs, and seasoning blends. Sucrose lyophiles were co-formulated with a variety of additives and stored at 11-40% relative humidity (RH). The structural compatibility of sucrose with the additive, and related intermolecular interactions, dictated the tendency of the additive to either delay, prevent, or accelerate sucrose crystallization. Spices, herbs, and seasoning blends were exposed to increasing RH (23-75%) and temperature (20-50ºC) to determine the effect of storage and formulation on a variety of physical properties. In general, as complexity of blends increased, physical stability decreased. While this dissertation covers a wide variety of food chemistry and food materials science topics, including vitamin chemical stability, amorphous sucrose physical stability, and moisture sorption behaviors of spices, herbs, and seasoning blends, the findings provide valuable information on the chemical and physical stability of ingredient

systems and how the structure-function relationships of the systems can be controlled for optimal ingredient functionality.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

The following sections will present relevant background literature introducing two major food chemistry concepts to be addressed in this research: vitamin stability and water-solid interactions. Vitamin stability includes a review of the fundamentals of thiamine (vitamin B_1), chemical stability of thiamine as a function of environment, the effect of food formulation on thiamine stability, and the nutritional and sensory implications of thiamine degradation. Water-solid interactions includes a review of how water interacts with solids, specifically in powder systems, the effect of water on the physical and chemical stability of dry food ingredients, and the impact of water-solid interactions on the quality of complex food ingredients.

1.2 Vitamin Stability

Vitamin stability has major implications for human health, specifically due to potential deficiencies resulting from degradation and lack of bioavailability. Thiamine (vitamin B_1) is sensitive to heat, light, alkali, oxygen, sulfites, and salts/electrolytes (Farrer, 1955; Gregory III, 2008; Pizzoferrato, 1992; Spitzer & Schweigert, 2007). In addition to nutritional implications, thiamine degradation also has implications for sensory properties due to sulfur-containing degradation products. Regardless of this, thiamine stability is not widely studied in the food or pharmaceutical industries, particularly with respect to thiamine stability during storage.

1.2.1 Thiamine

Thiamine was the first vitamin to be characterized (Funk, 1912). It is an essential micronutrient for humans, meaning it cannot be synthesized by the human body. It is found naturally in many foods and as a fortification ingredient in foods and dietary supplements. Plants, most often grains, are the main source of thiamine in the diet, but thiamine is also found naturally in legumes, nuts, pulses, meats, and yeast. In grains, thiamine is found mainly in the germ and the bran, which are the outer layers of the grain kernel. Thus, during the refining or polishing processes, thiamine content can be reduced by up to 89% (Fitzpatrick et al., 2012; Pourcel, Moulin, & Fitzpatrick, 2013). Since the refining of grains effectively eliminates thiamine, two different salt

forms of thiamine are used to enrich or fortify many grain-based products (O'Brien & Roberton, 1993). However, due to the relative instability of thiamine, specifically to heat and alkali, up to 84% of thiamine in foods can still be lost during cooking or processing (Gregory III, 2008; O'Brien & Roberton, 1993; Spitzer & Schweigert, 2007).

TMN vs. TClHCl

Thiamine is commercially available in two salt forms, thiamine mononitrate (TMN) and thiamine chloride hydrochloride (TClHCl), which are used to enrich or fortify many food products, including polished grains, breads, breakfast cereals, infant formula, and functional beverages like energy drinks or nutrition shakes (O'Brien & Roberton, 1993). The two salt forms differ in their solid-state properties (Table 1.1). Due to the aqueous solubility, hygroscopicity, and chemical stability, TMN is usually used for enrichment and fortification of dry food products, and TClHCl is generally found in liquid or beverage products or in liquid coating systems.

1.2.2 Nutritional implications

The Recommended Dietary Allowance (RDA) and Daily Value (DV) of thiamine are both 1.2 mg/day in the US (Institute of Medicine, 1998; U.S. Food & Drug Administration, 2018). Thiamine pyrophosphate (TPP) is the precursor to coenzymes for metabolism of carbohydrates, branched-chain amino acids, and lipids, including transketolase, pyruvate dehydrogenase, α-ketoacid dehydrogenase, and α-ketoglutarate dehydrogenase. Thiamine also plays major roles in the nervous, muscular, and cardiovascular systems, specifically affecting nerve conduction, cerebral degeneration, peripheral motor and sensory neuropathy, muscle contraction, blood flow, and sodium and water retention in the blood (Bémeur & Butterworth, 2014; Institute of Medicine, 1998). Thiamine deficiency can cause minor health problems, such as insomnia, fatigue, and irritability; however, prolonged deficiency can cause severe symptoms and diseases, specifically Beriberi and Wernicke-Korsakoff syndrome (Spitzer & Schweigert, 2007). Thiamine deficiency is a cause for concern in both developed and undeveloped countries. The major cause for thiamine deficiency in undeveloped countries is attributed to the lack of a nutritious diet, especially when the main dietary component is an unfortified and/or refined grain, such as white rice (Ball, 2006). In developed countries, proper fortification results in very low deficiency cases (~10%), especially

in healthy individuals with no underlying conditions (Shepherd & Gibson, 2013); however, some groups of people, specifically alcoholics, people with HIV/AIDS, strict weight loss dieters, and people avoiding fortified grains, such as those with Celiac disease, are much more susceptible to thiamine deficiency (Bémeur & Butterworth, 2014; Shepherd & Gibson, 2013).

1.2.3 Chemical stability

As in most compounds, thiamine stability is highly dependent on the time-temperature profile of storage. Thiamine is known to be more chemically stable in acidic conditions than in close to neutral or basic pHs (Farrer, 1955). Specifically, thiamine is more stable below a pH of 6 due to its food-relevant pK_a of 4.8 (Arnold, Libbey, & Lindsay, 1969; Dwivedi & Arnold, 1972). Using a speciation plot of thiamine (Figure 1.1), it can be shown that above a pH of 6, the predominant species present contains an unprotonated pyrimidine N1, which is the less stable thiamine species. The most commonly used method for thiamine quantification to measure chemical stability, in accordance with AOAC method 942.23, uses reverse-phase HPLC with a gradient method (Eitenmiller, Landen Jr, & Ye, 2008).

Physical state-dependent chemical stability

Thiamine is commonly found in dry products, and thus, in the solid-state. The activation energy (E_a) for degradation of thiamine in the solid state has been found to be dependent on salt form: 26.3 kcal/mol for TMN and 22.4 kcal/mol for TClHCl (Labuza & Kamman, 1982). Due to this difference in E_a, TMN in the solid state is consistently reported to be more stable than is TClHCl in the solid state (Hollenbeck & Obermeyer, 1952; Labuza & Kamman, 1982). The difference in activation energy between the two salts also suggests a difference in degradation pathway and therefore the resulting degradation products.

Solids may exist in either the amorphous or the crystalline state, which is known to affect stability. Amorphous solids usually exhibit lower chemical stability than their crystalline counterparts due to increased molecular mobility and hygroscopicity leading to increased chemical reactivity (Hancock & Zografi, 1997). Previous research in our lab has shown this to be true of thiamine as well, wherein type of polymer in the amorphous matrix greatly affects thiamine stability as a result of differences in intermolecular interactions with thiamine (Arioglu-Tuncil,

Voelker, Taylor, & Mauer, 2020); however, there is not adequate research comparing the stability between the two thiamine salt forms in the amorphous state.

Thiamine stability in the solution state has been previously studied; however, it has most commonly been studied using buffers to control pH or at higher temperatures than those reached during storage to emulate thermal processing temperatures (Beadle, Greenwood, & Kraybill, 1943; Farrer, 1945; K. C. Kwok, Shiu, Yeung, & Niranjan, 1998). The use of buffers is likely to affect the stability of thiamine in solution due to ionic constitution, irrespective of pH (Beadle et al., 1943; Farrer, 1945, 1947; Pachapurkar & Bell, 2005), and individual degradation mechanisms are partially temperature-dependent (Windheuser & Higuchi, 1962). It is clear, though, that in solution, the mechanism of thiamine degradation is highly dependent on additional components in the solution and on pH (Dwivedi & Arnold, 1973; Windheuser & Higuchi, 1962).

Food products/co-formulation

Many different components that may be present in food systems with thiamine have a significant effect on the chemical stability of thiamine. For example, both type and concentration of buffer salts in a system with thiamine greatly affect the stability due to ionic constitution (Beadle et al., 1943; Farrer, 1945, 1947; Pachapurkar & Bell, 2005). It has also been shown that α- and β-amino acids decrease the rate of thiamine degradation (McIntire & Frost, 1944). Other food constituents, such as gelatin, egg albumin, gums, dextrins, soluble starch, cereals, and some amines, have also been shown to have a stabilizing effect on thiamine (Atkin, Schultz, & Frey, 1943; Farrer, 1955; McIntire & Frost, 1944). Greater thiamine stability in natural foods, such as a variety of pureed meats and vegetables, compared to aqueous solutions, has been attributed to a protective action by proteins and starch, partially due to form (co-carboxylase, free form, etc.) of thiamine present in these products (Feliciotti & Esselen, 1957; Mulley, Stumbo, & Hunting, 1975a). On the other hand, sugars, including sucrose, lactose, and glucose, as well as salts and sulfites have been reported to decrease the chemical stability of thiamine (Farrer, 1955; Pizzoferrato, 1992; A. Watanabe & Sakaki, 1944). While a mechanism for differences in thiamine degradation due to food matrix components has not been well established, it is clear that many different food matrix components have significant effects on thiamine stability.

It has been reported that system properties, specifically pH and matrix components, affect the pathway of degradation that thiamine undergoes (Dwivedi & Arnold, 1972; Mulley, Stumbo,

& Hunting, 1975b; Pachapurkar & Bell, 2005; Windheuser & Higuchi, 1962). The degradation pathway and the resulting degradation products have also been shown to have a substantial effect on sensory properties due to the sulfur atom contained in a thiamine molecule, and therefore, the resulting sulfur-containing degradation products (Buttery, Haddon, Seifert, & Turnbaugh, 1984; Dwivedi, Arnold, & Libbey, 1973). Thus, the properties of a thiamine-containing product may be of great importance to the sensory properties of a food product due to the resulting degradation pathway.

1.2.4 Reaction kinetics

Thiamine degradation has been reported to follow pseudo first-order reaction kinetics, indicating the dependence of degradation rate on thiamine concentration (Arabshahi & Lund, 1988; Gregory III, 2008; Mauri, Alzamora, Chirife, & Tomio, 1989). Under first-order reaction kinetics, the kinetic rate constant of degradation (k) can be calculated using the following equation:

$$ln\frac{x}{x_0} = -kt$$

where x is the concentration of thiamine at time t, x_0 is the initial thiamine concentration, and k is the kinetic rate constant. In the case of thiamine degradation in conditions used in the following studies, time-related variables were calculated using the unit of days. In practice, the observed kinetic rate constant (k_{obs}) can be calculated as the negative slope of the line when the natural log of percent thiamine remaining is plotted against time (i.e., ln(% remaining) vs. time).

The Arrhenius equation is generally used to describe the temperature dependence of k, and thiamine degradation has been shown to obey this relationship as well (Arabshahi & Lund, 1988). The relationship is used to calculate the activation energy (E_a) of thiamine degradation when it is monitored over a range of temperatures using the following equation:

$$k = Ae^{\frac{-E_a}{RT}}$$

where k is the reaction rate constant, A is the frequency factor of collision, E_a is the activation energy (J/mol), R is the gas constant (8.3145 J/mol·K), and T is temperature (K). In practice, when the natural log of k_{obs} is plotted against 1/T (K^{-1}) (i.e., ln(k_{obs}) vs. 1/T), the resulting slope of the line is $-E_a/R$; thus, E_a can be calculated by multiplying the negative slope of the line by the gas constant R. Generally, the E_a of thiamine degradation is reported between 20-30 kcal/mol, dependent on system properties, including pH, other components in the system, and physical state

(Feliciotti & Esselen, 1957; Guzman-Tello & Cheftel, 1987; Kamman, Labuza, & Warthesen, 1981; Mauri, Alzamora, & Tomio, 1992; Mulley et al., 1975a; Ramaswamy, Ghazala, & Van de Voort, 1990; Windheuser & Higuchi, 1962).

1.3 Water-Solid Interactions

Water is ubiquitous in the environment. It is a very small and mobile molecule, and it has a strong tendency for hydrogen bond formation, forming up to four at one time (two hydrogen bond acceptors and two hydrogen bond donors). For these reasons, water commonly interacts with food solids during processing, storage, and use. Water is a near-universal solvent and plasticizer, so as water interacts with solids, chemical and physical changes are likely to occur, including degradation of ingredients, phase transformations, caking and decreased flowability of powders, and texture changes (Mauer & Allan, 2015; Slade & Levine, 1991). These changes can be both desirable and undesirable. The physical and chemical changes have a substantial effect on the product quality, including shelf-life of powdered ingredients; thus, a review of the fundamentals of water-solid interactions is essential to understanding quality and stability of solid foods and ingredients.

1.3.1 Amorphous and crystalline solids

Water-solid interactions are highly dependent on the physical state of the solid, which can be categorized into crystalline and amorphous solids. The major difference between crystalline and amorphous solids is in the molecular arrangement. A crystalline lattice has a periodic molecular arrangement with long-range three-dimensional order. Crystalline solids, which include ingredients like table salt or sugar, have a characteristic melting temperature at which point the bonds between molecules are weakened. Crystalline solids are more thermodynamically stable than their amorphous counterparts (Bhandari & Roos, 2017; Mauer & Allan, 2015). Amorphous solids lack the long-range order seen in crystalline solids and rather exhibit a disordered and random molecular arrangement (Bhandari & Roos, 2017). They behave like a liquid microscopically while maintaining their solid-state behavior macroscopically (Mauer & Allan, 2015). Amorphous solids have a characteristic temperature, the glass transition temperature (T_g), at which point a phase transition dictating macro and microscopic behavior occurs. Below the T_g,

an amorphous solid is in the 'glassy state' with limited mobility, such as in a Jolly Ranger, and above the T_g, an amorphous solid is in the 'rubbery, supercooled liquid' state with greater translational freedom, such as in honey or taffy (Bhandari & Roos, 2017). Ingredients can solidify into either the crystalline or amorphous state depending on conditions of solidification.

1.3.2 Five types of water-solid interactions

The five major mechanisms of water-solid interactions include adsorption, capillary condensation, deliquescence, crystal hydrate formation, and absorption (Figure 1.2). The five mechanisms can be categorized in three groups: surface interaction (adsorption), condensed water (capillary condensation and deliquescence), and internalized water (crystal hydrate formation and absorption), in which physical state affects which water-solid interactions are likely to occur (Mauer & Allan, 2015; Zografi, 1988). Crystalline solids are likely to experience moisture sorption in the form of adsorption, capillary condensation, deliquescence, and/or crystal hydrate formation; amorphous solids interact with water most significantly via absorption into the bulk of the matrix but may also experience adsorption and capillary condensation to a much smaller extent (Mauer & Allan, 2015; Zografi, 1988). Water-solid interactions may have a significant impact on physical and chemical properties of powder systems that may decrease shelf-life of powdered ingredients, including: agglomeration, powder flowability, caking, crystallization of amorphous materials, ingredient functionality, and degradation of components (Aguilera, del Valle, & Karel, 1995; Ahlneck & Zografi, 1990; Hartmann & Palzer, 2011; K. Kwok, Mauer, & Taylor, 2010). The five mechanisms are discussed in detail below.

Adsorption

Adsorption occurs when water molecules affix to the hydrophilic surface of a polar solid via hydrogen bonding. Water molecules can form monolayers on the surface of the solid but may also laterally diffuse across the surface, causing clustering and multilayer formation (Zografi, 1988). While the adsorbed molecules behave much differently than bulk water, only approximately 3 layers of water molecules can adsorb to the surface (Zografi, 1988). More water molecules will adsorb to the surface of smaller crystals or crystals with defects due to the higher surface area to mass ratio, and temperature and pressure also affect the amount of water adsorption (Mauer &

Allan, 2015; Zografi, 1988). While the physical and chemical characteristics of crystals affect the amount of water molecules that will adsorb onto the surface, the amount of adsorbed water is so small (estimated less than 0.002% weight gain for a 100μm cubic sucrose crystal) that no significant dissolution of the solid will occur (Mauer & Allan, 2015). Thus, adsorption has the least impact on moisture sorption behaviors of solids of the five major water-solid interactions.

Capillary condensation

Capillary condensation is a process in which water vapor condenses in a solid pore, at a contact point between two solid particles, or in surface irregularities of one solid particle, forming a liquid bridge. This occurs in the pores or contact points of solids before condensation on other parts of the surface due to the lowered vapor pressure of the liquid in the curved menisci compared to that of the surrounding atmosphere as a result of the constrained surface tension of the liquid in the small capillaries (Mauer & Allan, 2015; Zografi, 1988). Contact angle, particle size, particle arrangement, and RH all play a role in the extent of capillary condensation (Afrassiabian, Leturia, Benali, Guessasma, & Saleh, 2016; Billings, Bronlund, & Paterson, 2006). The bulk liquid formed by condensation may also result in dissolution of the surrounding area in which the initial condensation occurs. The liquid (and possibly solid) bridges formed by capillary condensation are likely to cause caking in powdered products (Afrassiabian et al., 2016; Billings et al., 2006). Capillary condensation may also precede deliquescence as RH approaches that required for deliquescence, often leading to deliquescence lowering when two types of crystals are present (K. Kwok et al., 2010; Stoklosa, Lipasek, Taylor, & Mauer, 2012).

Deliquescence

Deliquescence is a first-order phase transformation of a crystalline solid to a saturated solution that occurs when the environmental RH exceeds the critical RH characteristic of the crystal, known as the deliquescence point (RH_0) (Mauer & Taylor, 2010b; Zografi & Hancock, 1994). RH_0 is characteristic of a specific solid, dependent on temperature, and is equivalent to the water activity (a_w) of a saturated solution at the defined temperature (Zografi & Hancock, 1994). Some food-relevant deliquescent ingredients include organic and inorganic salts, sugars, vitamins,

preservatives, and sugar alcohols, and their wide range of RH_0s results from their aqueous solubilities, with more soluble crystals generally having lower RH_0s (Mauer & Taylor, 2010b).

The deliquescence process occurs as environmental RH increases. At RHs well below the RH_0, adsorption of moisture occurs on the surface of the crystal to create mono and multilayers of water. As RH is increased closer to the RH_0, capillary condensation may begin to take place, but dissolution does not occur until the environmental RH exceeds the RH_0 of the crystal (Mauer & Taylor, 2010b). When RH_0 is exceeded, a thin film of saturated solution forms around the crystal. As RH continues to increase above the RH_0, a vapor pressure gradient in which the vapor pressure of the film is less than that of pure water drives more vapor to continuously condense onto the film (Zografi, 1988). The crystal is subsequently further dissolved by the condensed vapor to maintain the saturated solution and bring the vapor pressure of the film back down to RH_0. The condensation-dissolution cycle alternates until complete dissolution of the solid occurs. Subsequent dilution of the solution will result if the environmental RH is maintained above the RH_0 (Salameh, Mauer, & Taylor, 2006; Van Campen, Amidon, & Zografi, 1983). Kinetics of dissolution can be very slow at RHs just above the RH_0, and deliquescence occurs more quickly as the RH-RH_0 increases (Van Campen et al., 1983). However, early deliquescence can be identified by caking and clumping of powders.

Deliquescence lowering

Deliquescence lowering occurs when two or more deliquescent ingredients are in physical contact with each other. The mutual deliquescence point ($RH_{0,mix}$) is lower than the RH_0 of any of the individual components (Mauer & Taylor, 2010b; Salameh et al., 2006). The Ross equation can be used to estimate the $RH_{0,mix}$, in which the $RH_{0,mix}$ of the blend is the product of the RH_0s of the individual ingredients: ($\frac{RH_{0,mix}}{100} = \frac{RH_{0,1}}{100} \times \frac{RH_{0,2}}{100} \ldots$) (Mauer & Taylor, 2010b; Ross, 1975). Deliquescence lowering does not depend on the ratio of deliquescent ingredients present in the blend; the phenomenon will occur as long as there is physical contact between the ingredients, indicating that even trace amounts of a deliquescent material can dramatically decrease the physical stability of the powder system (Mauer & Taylor, 2010b; Yamamoto & Takahashi, 1952). As shown by the Ross equation, as amount of deliquescent ingredients is increased, $RH_{0,mix}$ continues to decrease. Since many food products contain multiple deliquescent ingredients and

deliquescence has been shown to decrease both physical and chemical stability (Salameh & Taylor, 2006a, 2006b), deliquescence lowering plays a major role in stability and shelf-life of powdered ingredients.

Crystal hydrate formation

A crystal hydrate is a crystal in which water molecules are incorporated into the crystal lattice structure. Not all crystalline materials form hydrates; however, many food ingredients, like glucose, lactose, citric acid, sorbitol, and thiamine chloride hydrochloride are able to form hydrate structures (Allan & Mauer, 2017b). Water molecules in hydrates are stabilized by hydrogen bonds or coordinate covalent bonds within the solid (Khankari & Grant, 1995). Crystal hydrates are categorized into three classes: isolated site hydrates, ion-associated hydrates, and channel hydrates, which can be further classified as stoichiometric or nonstoichiometric hydrates (Vippagunta, Brittain, & Grant, 2001). In stoichiometric hydrates, a fixed molar quantity of water is in the crystal lattice in a stoichiometric ratio with the solid. In nonstoichiometric hydrates, the hydrate can take up or expel moisture continuously, usually due to changes in humidity, and the expanded or contracted lattice can cause changes in the dimensions of the unit cell (Vippagunta et al., 2001). Generally, hydrates are formed from anhydrates when the environmental RH reaches a critical RH, dependent on material, and the hydrated form has altered physical properties, like solubility, compared to the hydrated form (Vippagunta et al., 2001). The hydrated form of the crystal is usually more stable than the anhydrate (Mauer & Allan, 2015); however, dehydration of crystal hydrates, usually caused by increased temperature or decreased RH, can cause both physical and chemical stability concerns. Removal of water from the crystal lattice can be manifested in three ways: a complete lattice collapse, producing a metastable or amorphous phase; an altered lattice packing; or an unaltered lattice structure (Chakravarty et al., 2010; Vogt et al., 2006). Thus, mechanism of crystal hydrate formation or dehydration can substantially affect stability of ingredient formulations that contain crystalline hydrates.

Absorption

Absorption occurs only in amorphous solids, and it is the major mechanism of moisture uptake in those solids, in which water vapor is taken up into the bulk of the amorphous solid in

significant excess of what is seen in adsorption (Zografi, 1988). Amorphous solids generally have a type II sigmoidal moisture sorption isotherm, indicting their hygroscopicity and willingness to absorb moisture (Hancock & Zografi, 1993; Mauer & Allan, 2015). Water in amorphous solids may begin to have solvent-like properties due to the saturation of binding sites in the solid; however, the solvent-like properties may also result from the lowering of the glass transition temperature (T_g) (Zografi, 1988). When water is absorbed into the amorphous solid, it acts as a plasticizer, lowering the T_g due to the low T_g of water (-137ºC) (Slade & Levine, 1991). As the T_g is lowered to below the environmental temperature, the amorphous solid transitions from the glassy to the supercooled liquid state. The resulting decreased viscosity, increased molecular mobility, and presence of water for use in reactions increases the chemical reactivity of the material (Slade & Levine, 1991). The increased molecular mobility of the supercooled liquid state also promotes physical changes, such as collapse and/or crystallization (Roos & Karel, 1991b).

1.3.3 Physical stability of powders

The presence of water in low-moisture powders or in the storage environment can cause many undesirable physical and chemical changes, including caking, clumping, agglomeration, stickiness, crystallization of amorphous materials, and even degradation of powder ingredients (e.g. flavors, bioactives, vitamins, etc.) (Aguilera et al., 1995; Ahlneck & Zografi, 1990; Hartmann & Palzer, 2011; K. Kwok et al., 2010). Physical stability is a major area of concern for many powders due to water-solid interactions with both crystalline and amorphous powder components, and the induced changes can play major roles in both ingredient functionality and in manufacturing processes.

Caking

Physical stability of powders is often dictated by caking and clumping, which can cause serious problems not only for quality seen by consumers but also for processing of the powders (i.e. blockages in equipment). Caking can occur in both crystalline and amorphous materials, as well as blends of the two. Caking between crystalline particles occurs as a result of deliquescence. Caking begins when capillary condensation causes liquid bridges to form between particles, which then leads to agglomeration, compaction, and liquefaction as the particles deliquesce (Aguilera et

al., 1995; Lipasek, Li, Schmidt, Taylor, & Mauer, 2013). This can be further problematic if multiple different deliquescent ingredients are mixed together, as is the case in many powdered products (e.g. salts and sugars), due to deliquescence lowering, which lowers the $RH_{0,mix}$ to below the RH_0 of any individual ingredients and increases the moisture sensitivity of the blend (Hiatt, Ferruzzi, Taylor, & Mauer, 2008; Ross, 1975; Salameh & Taylor, 2006b).

Caking of amorphous materials occurs mainly due to plasticization by water that is absorbed into the bulk of the amorphous matrix, usually as a result of increasing environmental RH (Slade & Levine, 1988; Zografi, 1988). The plasticization by the absorbed water lowers the T_g of the amorphous powder, eventually leading to the transition from the glassy to the supercooled liquid state. This can cause a process called sintering, in which molecules move to and close the gap between two powder particles by creating a sinter bridge, leading to caking and clumping of the amorphous material (Feeney & Fitzpatrick, 2011; Palzer, 2005). Additionally, if the RH is lowered following the formation of liquid and/or sinter bridges in crystalline and/or amorphous solids, the water in these bridges can evaporate, forming solid bridges and hard cakes in powders (Feeney & Fitzpatrick, 2011; Salameh & Taylor, 2006a).

When amorphous and crystalline materials are mixed, additional caking mechanisms are likely, further increasing the sensitivity of the blends to moisture. In addition to a blend of amorphous and crystalline ingredients that commonly exists in food products, a mix of crystalline and amorphous forms of the same material may also be present as an amorphous solid slowly crystallizes. Regardless of the composition of the blend, when both amorphous and crystalline ingredients are present in a closed system, a lowering of the RH_0 of the crystalline ingredient(s) and/or the T_g of the amorphous ingredient(s) is likely to occur (Ghorab, Marrs, Taylor, & Mauer, 2014; Ghorab, Toth, Simpson, Mauer, & Taylor, 2014; Thorat, Marrs, et al., 2017). Generally, as temperature is increased in a closed system (no change in water), the a_w of an amorphous ingredient is increased and the RH_0 of a crystalline ingredient is decreased (Gorling, 1958; Greenspan, 1977). If amorphous and crystalline ingredients are both present in a closed system, there exists a temperature at which the increasing a_w and the decreasing RH_0 intersect (Figure 1.3). At temperatures above this point, referred to as the crossover point, the a_w of the amorphous ingredient causes the deliquescence of the crystalline ingredient (Thorat, Marrs, et al., 2017). This crossover point occurs at even lower temperatures if multiple crystalline ingredients are blended, due to

deliquescence lowering. Thus, as complexity of powered blends is increased, sensitivity to moisture and caking is also increased.

Crystallization

Crystallization from the amorphous state is another common consequence of water-solid interactions in foods. Since crystalline solids are more thermodynamically stable than their amorphous counterparts, amorphous materials easily crystallize as a function of their processing or storage conditions, specifically temperature and RH (Bhandari & Roos, 2017; Mathlouthi, 1995; Shamblin & Zografi, 1999). Amorphous solids are especially likely to crystallize when they are stored in conditions above their glass transition temperature (T_g), resulting in increased molecular mobility (Roe & Labuza, 2005; Roos & Karel, 1991b). T_g is lowered as water is absorbed into the amorphous matrix due to increasing RH; thus, crystallization of amorphous materials is likely to occur as processing or storage RH is increased. Specific formulation and processing conditions tend to produce amorphous solids, including freeze drying and spray drying (Písecký, 2012; Schuck & Dolivet, 2002; Vuataz, 2002). Some foods benefit from the presence of amorphous solids due to the desirable dissolution properties, ability to absorb volatiles, softer texture, and a number of other sensory attributes compared to crystalline solids. Thus, the crystallization of amorphous solids is likely to lead to undesirable changes in texture, flavor, solubility, and susceptibility to chemical reactions (Buera, Schebor, & Elizalde, 2005; Chirife & Karel, 1974; Slade & Levine, 1991).

When crystallization of amorphous ingredients occurs in powders (i.e. lactose in milk powder), the release of water from the amorphous matrix significantly increases the a_w of the system. The excess water can cause caking and clumping, as previously described, causing undesirable changes in the powder (Hartmann & Palzer, 2011). The excess water may also become available for the remaining amorphous ingredients (i.e. proteins in milk powder), potentially triggering unintended chemical reactions, such as Maillard browning, to occur, especially in the presence of increased heat often found in processing, transportation, and storage of powdered materials (Vuataz, 2002).

1.3.4 Glass transition

The glass transition is a reversible physicochemical event that occurs in amorphous systems which is temperature, time, composition (specifically moisture content), and material-dependent. The glass transition manifests itself as a change in the physical state from a glassy state, with limited molecular mobility, to the supercooled liquid state, with increased molecular mobility, as the storage temperature is increased above the glass transition temperature (T_g) (Bhandari & Roos, 2017; Slade & Levine, 1991). The increased molecular mobility in the supercooled liquid state enables both molecular rearrangement, which often promotes crystallization of amorphous materials, and/or increases the chemical reactivity, and therefore chemical instability of components in an amorphous matrix (Hancock & Zografi, 1997; Roos & Karel, 1991b). The rate of chemical or physical changes above the T_g is dependent on T-T_g, wherein as temperature continues to increase above the T_g, reaction rates are increased (Bhandari & Roos, 2017; Slade & Levine, 1991). For this reason, matrices with higher T_gs are generally considered to be more stable. However, it has also been reported that a higher T_g does not always result in increased chemical stability, with materials sometimes still degrading in the glassy state (Bell & Hageman, 1994; Sanchez, Ismail, Christina, & Mauer, 2018).

T_g is known to increase as molecular weight increases; thus, water has a very low T_g (-137ºC) (Damodaran, 2017; Slade & Levine, 1991). This low T_g causes a plasticizing effect by water, significantly lowering the T_g of a matrix as moisture content is increased. This relationship can be modeled by the Gordon-Taylor equation (Gordon & Taylor, 1953). Since T_g, and in effect, the glassy vs. supercooled liquid state of a material, has implications for both physical and chemical stability, storage of amorphous materials in an increased RH environment that may cause absorption is often a focus in food materials science.

1.3.5 Moisture sorption profiles

Moisture sorption profiles are a tool used to study the moisture sorption behavior, and thus, the moisture content, of solid materials when exposed to specific RHs (or a_ws) and temperatures. Solids with the same water content often times have different a_ws (Mauer & Allan, 2015). The amount of moisture sorbed by a solid at a specific RH is dependent on composition of a material, specifically the affinity of the solid for water, physical state of the components (e.g. amorphous

vs. crystalline), temperature, RH, and surface area (Damodaran, 2017). The term "moisture sorption isotherm" indicates that this measurement is completed at constant temperature and pressure with only changing RH. Thus, moisture sorption isotherms are defined as the equilibrium plot of water content of a solid as a function of RH (or a_w) (Bell & Labuza, 2000; Damodaran, 2017). Moisture sorption isotherms are measured by incubating a solid material in a controlled temperature and humidity environment, either in a desiccator or a gravimetric moisture sorption instrument, until equilibrium is reached. The weight change of the material indicates the increase or decrease in water content (Damodaran, 2017; Mauer & Allan, 2015). Moisture sorption profiles can also be measured by exposing the solid to increased RH for longer periods of time to monitor other moisture induced physical changes, such as crystallization of amorphous solids.

More often, however, a moisture sorption isotherm consists of equilibrium measurements at a number of increasing RHs. The shapes of these moisture sorption isotherms can be classified into six major types, generally dependent on the types of solids present (Sing et al., 1985). Type I isotherms are characterized by a concave curve to the a_w axis with an asymptote as a_w reaches 1; they are common for microporous solids, such as anticaking agents and certain salts (Damodaran, 2017; Sing et al., 1985). Type II isotherms are exhibited by most foods with amorphous content, such as proteins and gums, and are characterized by a sigmoidal shaped curve, in which the first inflection point indicates that monolayer sorption is complete and multilayer sorption begins (Sing et al., 1985). Type III isotherms are characterized by a J-type curve convex to the a_w axis with limited moisture sorption until a critical RH is reached, which is common for deliquescent crystalline materials (Damodaran, 2017; Mauer & Taylor, 2010b). Most foods exhibit either a type II or III isotherm (Mauer & Allan, 2015). Type IV isotherms are characterized by a hysteresis loop, often caused by capillary condensation. Type V isotherms occur for highly porous adsorbents with weak adsorbent-adsorbate interactions, and type VI isotherms are characterized by a stepwise multilayer adsorption, which may occur during crystal hydrate formation (Sing et al., 1985).

1.4 Summary and Overview of Research

The objectives to be addressed in this research focus on two major food chemistry concepts: vitamin stability and water-solid interactions. The major focus of this dissertation was on the chemical stability of thiamine in a variety of environments and the implications on nutritional value and sensory attributes. This topic was supplemented with studies to better understand the

water-solid interactions occurring in a wide variety of food products, specifically amorphous sugar systems and spices and seasoning blends, and the resulting implications on food characteristics.

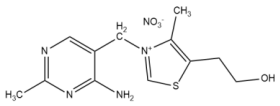
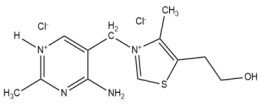
To better understand chemical stability of thiamine in a variety of environments, three studies will be discussed. The first study involves a fundamental overview of the chemical stability and reaction kinetics of degradation of the two salt forms of thiamine (TMN and TClHCl) in solution as a function of storage condition and thiamine concentration, including sensory analysis of degraded samples as a function of pH. The second study involves another shelf-life analysis of the chemical stability and reaction kinetics of thiamine degradation as a function of pH, thiamine concentration, and counterion in solution, without the use of buffers to adjust pH. The third and final thiamine study involves the chemical stability and reaction kinetics of thiamine degradation in the aqueous phase of bread dough to model thiamine stability in food formulations, specifically in bread dough.

The water-solid interactions topic of this work includes three studies focusing on the physical stability of amorphous sucrose. The three studies involve the effect of specific food ingredients on the crystallization tendency of amorphous sucrose co-lyophilized with the three classes of ingredients: emulsifiers, polyphenols, and polymers. The three classes of ingredients were chosen due to their applicability in the food industry as well as their structural features that may promote interaction with sucrose in the lyophiles. The water-solid interaction topic of this work also includes a study focusing on the physical stability of spices and seasoning blends, specifically focusing on how deliquescence and absorption lead to caking and clumping. The study also focuses on how increasing blend complexity affects physical stability.

Overall, the objectives of this work focus on understanding the chemical stability of thiamine and the physical stability of amorphous sucrose and spices and seasoning blends. Both major topics reviewed can be used to improve or maintain the stability and quality of food ingredients and formulations.

1.5 Tables and Figures

Table 1.1 Solid state properties of TMN and TClHCl.

	Thiamine Mononitrate	Thiamine Chloride Hydrochloride
Structure		
Molecular weight [1]	327.36 g/mol	337.26 g/mol
Melting point [1]	196-200°C	248°C
Deliquescence point (RH_0) [2]	98.5% RH	88% RH
Aqueous solubility	30 mg/mL	570 mg/mL

[1] ChemSpider (2015)
[2] Hiatt et al. (2008)

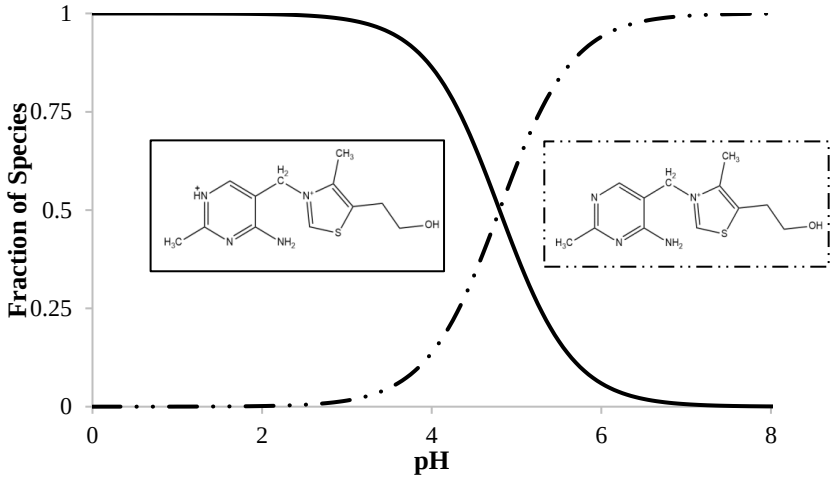


Figure 1.1 Speciation plot of thiamine based on the food-relevant pK_a of 4.8.

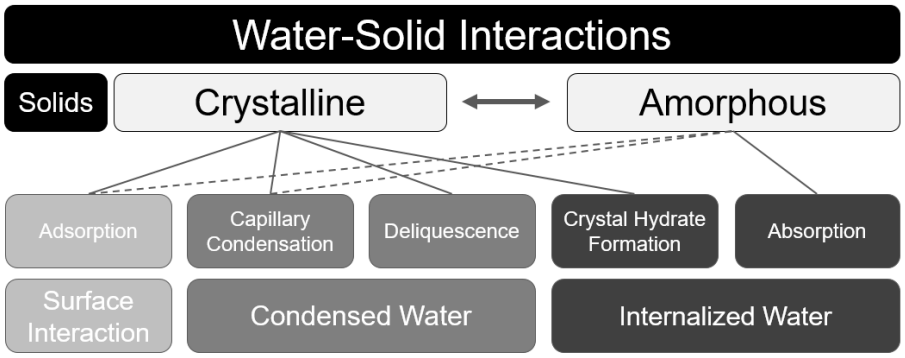


Figure 1.2 The major mechanisms of water-solid interactions adapted from Mauer and Allan (2015).

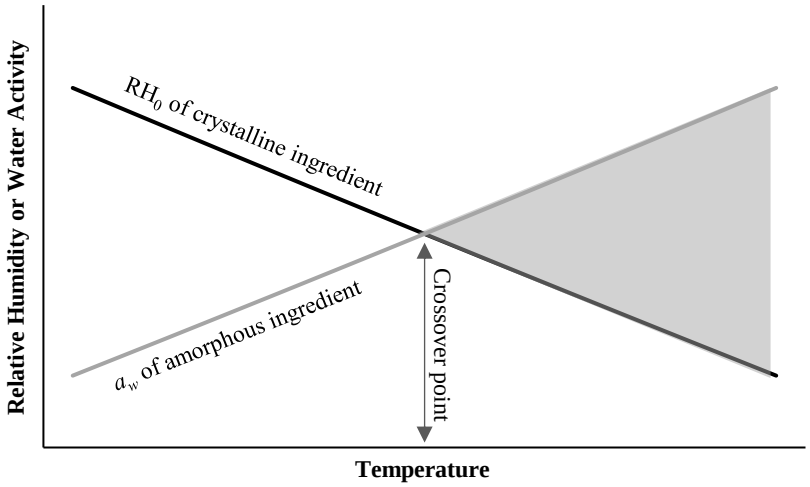


Figure 1.3 Determination of crossover point in amorphous-crystalline blends, adapted from Thorat, Marrs, et al. (2017).

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CHAPTER 2. CHEMICAL STABILITY AND REACTION KINETICS OF TWO THIAMINE SALTS (THIAMINE MONONITRATE AND THIAMINE CHLORIDE HYDROCHLORIDE) IN SOLUTION

Voelker, A. L., Miller, J., Running, C. A., Taylor, L. S., & Mauer, L. J. (2018). Chemical stability and reaction kinetics of two thiamine salts (thiamine mononitrate and thiamine chloride hydrochloride) in solution. *Food Research International, 112*, 443-456. doi:https://doi.org/10.1016/j.foodres.2018.06.056

2.1 Abstract

Two types of thiamine (vitamin B_1) salts, thiamine mononitrate (TMN) and thiamine chloride hydrochloride (TClHCl), are used to enrich and fortify food products. Both of these thiamine salt forms are sensitive to heat, alkali, oxygen, and radiation, but differences in stability between them have been noted. It was hypothesized that stability differences between the two thiamine salts could be explained by differences in solubility, solution pH, and activation energies for degradation. This study directly compared the stabilities of TMN and TClHCl in solution over time by documenting the impact of concentration and storage temperature on thiamine degradation and calculating reaction kinetics. Solutions were prepared containing five concentrations of each thiamine salt (1, 5, 10, 20, and 27 mg/mL), and three additional concentrations of TClHCl: 100, 300, and 500 mg/mL. Samples were stored at 25, 40, 60, 70, and 80°C for up to 6 months. Degradation was quantified over time by high-performance liquid chromatography, and percent thiamine remaining was used to calculate reaction kinetics. First-order reaction kinetics were found for both TMN and TClHCl. TMN degraded significantly faster than TClHCl at all concentrations and temperatures. For example, in 27mg/mL solutions after 5 days at 80°C, only 32% of TMN remained compared to 94% of TClHCl. Activation energies and solution pHs were 21-25 kcal/mol and pH 5.36-6.96 for TMN and 21-32 kcal/mol and pH 1.12-3.59 for TClHCl. TClHCl degradation products had much greater sensory contributions than TMN degradation products, including intense color change and potent aromas, even with considerably less measured vitamin loss. Different peak patterns were present in HPLC chromatograms between TMN and TClHCl, indicating different degradation pathways and products. The stability of essential vitamins in foods is important, even more so when degradation contributes to sensory changes, and this study

provides a direct comparison of the stability of the two thiamine salts used to fortify foods in environments relevant to the processing and shelf-life of many foods.

2.2 Introduction

Vitamin B_1, also known as thiamine (Figure 2.1), is an essential micronutrient in the human diet that is found both naturally and as a fortification supplement in many foods. Thiamine acts as a coenzyme for metabolism of carbohydrates and branched-chain amino acids and has roles in digestion, the nervous system, and muscle contraction (Institute of Medicine, 1998). Thiamine deficiency persists in both developing and developed countries. In developing countries, a lack of nutritious food or nutritional variety, which may occur when unfortified grains such as polished rice are the main dietary component, are the main contributors to thiamine deficiency, which is found in up to 25% of the population (Ball, 2006; Prinzo, 1999). In developed countries, where fortification efforts have reduced overall rates of thiamine deficiency to near 10%, deficiency is more likely found in alcoholics, people on strict weight loss diets, and people avoiding consumption of fortified grain products, including those with Celiac's disease (Ball, 2006; Shepherd & Gibson, 2013). Thiamine deficiency can cause both minor symptoms, such as fatigue, insomnia, irritability, and other neurological indicators, as well as severe diseases resulting from prolonged deficiency, e.g., Beriberi and Wernicke-Korsakoff syndrome (Spitzer & Schweigert, 2007). Thiamine stores in the body are very small and last only weeks, which contributes to the concern of deficiency (Baumgartner, Henderson, Fox, & Gondi, 1997). The Recommended Dietary Allowance (RDA) and Daily Value (DV) for thiamine in the U.S. are both 1.2 mg/day (Institute of Medicine, 1998; U.S. Food & Drug Administration, 2018). To combat the likelihood of deficiency, thiamine salts are often used to enrich and fortify many food and beverage products.

Thiamine is found naturally in foods, such as meats, yeast, whole grains, nuts, pulses, and legumes, in a phosphorylated form, most commonly thiamine triphosphate (Gregory III, 2008). Additionally, two salt forms are used as food additives: thiamine mononitrate (TMN) and thiamine chloride hydrochloride (TClHCl) (Figure 2.1). TMN is a mono-salt, with only one nitrate anion present, and TClHCl is a di-salt with two chlorides present. TClHCl is often interchangeably called 'thiamine hydrochloride' (Ash & Ash, 2008); however, it is important to note that the molecular formula contains two chlorides ($C_{12}H_{17}ClN_4OS \bullet HCl$), as shown in Figure 2.1. While thiamine has two pK_as ($pK_{a1} = 4.8$ for the pyrimidine N1 and $pK_a2 = 9.2$ for the thiazole quarternary nitrogen

(Edwards et al., 2017)), pK_{a1} is the only relevant pK_a for the majority of food products. Solid state properties of TMN and TClHCl differ widely from one another (Table 2.1). TMN is often used in dry food products due to its low hygroscopicity, and TClHCl is often used in liquid or beverage products due to its high solubility (Labuza & Kamman, 1982). The higher solubility of TClHCl compared to TMN is due to the higher free energy of the TClHCl crystalline salt form (Atkins & de Paula, 2006). The two salt forms also have substantial stability differences that have been explained by different activation energies, reported as 22.4 kcal/mol for TClHCl and 26.3 kcal/mol for TMN in solid state systems, with E_a decreasing as water activity (a_w) increased (Labuza & Kamman, 1982).

Thiamine is one of the most heat sensitive vitamins (Feliciotti & Esselen, 1957). It is often destroyed during thermal processing and, in addition to heat, is also sensitive to alkali, oxygen, radiation, sulfites, and the food matrix (Gregory III, 2008; Spitzer & Schweigert, 2007). Bis(2-methyl-3-furyl) disulfide, one possible degradation product of thiamine, delivers one of the lowest reported odor threshold values of any organic compound in water, at 0.02 parts per trillion (Buttery et al., 1984). The presence of water has been shown to negatively impact the stability of thiamine in the solid state, with degradation rates increasing as relative humidity or a_w increase, especially when the deliquescence point is exceeded (Dennison, Kirk, Bach, Kokoczka, & Heldman, 1977; Hiatt et al., 2008; Labuza & Tannenbaum, 1972). Many studies have also monitored the short-term stability of thiamine, primarily in its chloride hydrochloride form, in solution at very high temperatures, specifically as a function of pH (Dwivedi & Arnold, 1972; Farrer & Morrison, 1949; Feliciotti & Esselen, 1957; Williams & Ruehle, 1935). However, long term observations are lacking regarding the stability of thiamine in solution at temperatures to which foods are likely exposed, and few studies have directly compared the stability of TClHCl and TMN.

The objectives of this study were to: 1) investigate the impacts of concentration and storage temperature on the stability of thiamine in solutions prepared from TClHCl or TMN, 2) calculate activation energies of thiamine degradation using the temperature-dependent stability data collected from TClHCl and TMN solutions, 3) directly compare thiamine stability over time in solutions prepared from TMN and TClHCl, and 4) document if a difference in sensory impact exists in thiamine degraded in solutions prepared from TClHCl and TMN. The results of this study will provide a practical approach for understanding the delivery of thiamine salts in beverages and

products containing varying amounts of water in which higher concentrations of thiamine could be found.

2.3 Materials and Methods

2.3.1 Materials

Two thiamine salt forms were studied: thiamine mononitrate (TMN), $C_{12}H_{17}N_4OS \bullet NO_3$, obtained from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ), and thiamine chloride hydrochloride (TClHCl), $C_{12}H_{17}ClN_4OS \bullet HCl$, obtained from Fisher Scientific (Fair Lawn, NJ). For use in high performance liquid chromatography (HPLC), HPLC grade acetonitrile was obtained from Fisher Scientific and HPLC grade trifluoroacetic acid was obtained from Sigma-Aldrich Inc. (St. Louis, MO). Water used in all experiments was deionized and purified using a Barnstead E-Pure ultrapure water purification system with a resistivity at 25°C greater than 17.5 MΩ·cm (ThermoScientific, Waltham, MA).

2.3.2 Solubility measurement

The maximum solubility of each vitamin salt form in water at ambient temperature was determined, using a method adapted from Young (Young, 1957), to later use as a basis for preparing different solution concentrations of each sample. Beginning with 125 mg TMN or 50 mg TClHCl and 50 mL of water for each trial (based on reported solubility values), a mass balance was used to determine the saturation point by alternating additions of water (dropwise) and vitamin solid (1 mg). Saturation point was characterized by the inability of additional crystalline vitamin to be dissolved in solution. Volume was measured in a volumetric flask to quantify solubility in mg/mL of total solution.

2.3.3 Sample preparation

To understand the impact of thiamine concentration in solution on vitamin stability, series of TMN and TClHCl solutions were prepared containing 5 thiamine concentrations: 1, 5, 10, 20, and 27 mg/mL (the latter is just under the maximum solubility of TMN). Solutions containing higher concentrations of TClHCl were also prepared (100, 300, and 500 mg/mL) to investigate behaviors in solutions nearing the saturation point of TClHCl. The range and number of

concentrations chosen provided data for calculating reaction kinetics. The samples were prepared in terms of mass concentration rather than molar concentration, and although the two salt forms have slightly different molecular weights (Table 2.1), all calculations were done using percent remaining values, which account for this small discrepancy. Solutions (10 mL) containing each vitamin concentration were prepared in triplicate in 20 mL amber glass scintillation vials with PE cone-lined phenolic caps that were sealed with duct tape to prevent evaporation. Headspace in these vials was not modified prior to storage.

2.3.4 Sample storage

To monitor the effect of temperature on thiamine stability, solutions were stored at 5 temperatures: 25°C, 40°C, 60°C, 70°C, and 80°C. These temperatures were chosen to provide a large range of temperatures for calculating temperature-dependent reaction kinetics. The 25°C condition was used as an ambient temperature control and was maintained within ± 1°C using a temperature-controlled room (Commercial Fixture Company Inc., Indianapolis, IN). The 40°C, 60°C, and 70°C temperatures were maintained using Forma Scientific water-jacketed incubators (Thermo Fisher Scientific Inc., Marietta, OH). The 80°C temperature was maintained using a digital heatblock (VWR International, Radnor, PA). To monitor storage conditions, temperature was confirmed by liquid-in-glass partial immersion thermometers. Solutions were stored in controlled temperature environments for up to 6 months, depending on temperature and vitamin form, and were analyzed for percent vitamin remaining at a minimum of 5 selected timepoints.

2.3.5 Vitamin quantification

The chemical stability of thiamine in solution was monitored by measuring vitamin concentration over time using a high performance liquid chromatography (HPLC) method adapted from Xia et al. (Xia et al., 2006). A Waters 2690 Separations Module (Waters Corp. Milford, MA) equipped with a Waters 2996 Photodiode Array detector (Waters Corp.) was used with a 100 mm x 3.9 mm, 3.5 μm particle size XTerra RP-C_{18} column (Waters Corp.). The wavelength scan used was 235-400 nm. Mobile phase A: 0.1% trifluoroacetic acid (TFA) in water (*v/v*) and mobile phase B: acetonitrile (MeCN) were used with a flow rate of 1 mL/min and the following gradient method: 100/0 at 0 min, 97/3 at 4 min (linear), 90/10 at 6 min (linear), 100/0 at 10 min (linear), and 100/0

at 15 min. Prior to analysis, solutions were removed from controlled temperature storage, cooled in an ice bath, and diluted with mobile phase A to an estimated thiamine concentration of 500 ppm, or 0.5 mg/mL. Standard curves of TMN and TClHCl ($R^2 > 0.999$) at a concentration range of 10 ppm to 1000 ppm were prepared prior to each day of analysis and used to calculate the concentration of each sample. Integration was performed at 254 nm.

2.3.6 Reaction kinetics

To understand the kinetics of thiamine loss due to specific treatments, the data collected on the concentration of thiamine remaining in solution over time from the different initial solution concentrations and storage temperatures were applied to first-order reaction kinetic models, and the Arrhenius equation was used to model temperature-dependence of the reaction rate constants. Microsoft Excel 2016 (Redmond, WA) was used for the calculations.

Previous work has shown that thiamine degradation follows pseudo first-order reaction kinetics (Gregory III, 2008; Mauri et al., 1989) wherein thiamine concentration is described by:

$$ln\frac{x}{x_0} = -kt \quad (1)$$

where x is the concentration of thiamine at time t (days), x_0 is the initial thiamine concentration, and k is the reaction rate constant ($days^{-1}$). The Arrhenius equation can be used to describe temperature dependence of rate constant k:

$$k = Ae^{\frac{-E_a}{RT}} \quad (2)$$

where k is the reaction rate constant ($days^{-1}$), A is the frequency factor of collision, E_a is the activation energy (kJ/mol), R is the gas constant (8.3145 J/mol·K), and T is temperature (K). Since some foods have multiple degradation patterns that may have different temperature dependencies, it is possible to find non-linear Arrhenius plots (Gregory III, 2008), and therefore nonlinear Arrhenius plots were also considered.

2.3.7 pH measurement

The pH of solutions containing both vitamin forms, at all concentrations, and at all temperatures, was measured to document how these variables affected the pH. The pH of each solution was measured in duplicate at all temperatures studied using an Orion pH probe

(ThermoScientific) that had been calibrated from pH 5 to 7 for TMN and pH 1 to 4 for TClHCl using calibration standards obtained from Fisher Scientific.

2.3.8 Photography and color analysis

The color of the TMN and TClHCl solutions was documented in solutions removed from the different storage temperatures. Samples were photographed at their endpoints in a Deep Professional LED Photography light box using an iPhone 6s camera. The Hunter L, a, and b color scale values of the solutions were determined by using the Color Companion iPhone application as described in N. Li, Taylor, Ferruzzi, and Mauer (2013); N. Li, Taylor, and Mauer (2014) to analyze the photographs. In this color scale, L represents lightness (in percent), a represents red (positive) vs. green (negative), and b represents yellow (positive) vs. blue (negative) colors.

2.3.9 Sensory study of odor differences between degraded vitamin solutions

Thiamine degradation is known to produce aromas and flavors (Buttery et al., 1984; Dwivedi & Arnold, 1973). To determine if differences in the odors produced by degraded TMN and TClHCl could be detected by untrained panelists, 5 mg/mL solutions of each vitamin salt form were again prepared in the 20 mL amber vials with PE cone-lined caps, heated for 2 days at 80^{o}C, and frozen until the day of the sensory test. These conditions were chosen as a representation of the odor produced by each vitamin salt form, and the amount of thiamine degradation in these samples was determined by HPLC.

Eligibility requirements for participants in the sensory test included no food allergies or sensitivities, no known problems with sense of smell or taste, and no illness that may interfere with smelling capabilities. All procedures were approved by the Purdue University Human Subjects Research Protection Institutional Review Board as exempt under category 6 (taste and food quality evaluation and consumer acceptance studies). Samples (5 mL, in capped amber vials) were thawed at ambient temperature for 2 hours prior to the sensory analysis. The amber vials prevented color changes from affecting responses, and 3-digit codes were used for blinding purposes. A two-alternative forced choice test was used to evaluate which sample smelled stronger. Participants were presented with two vials (one containing each vitamin form) in counterbalanced order and instructed to: *"Start with the sample on the left. Open the bottle and* ***smell the cap****. Then put the*

*cap back on the bottle. Then open the bottle on the right and **smell the cap**. Then put the cap back on the bottle. Which sample smelled stronger? You may smell the samples again if you need to, but please smell just the cap."* Instructing participants to smell only the cap of the vials ensured that smelling techniques were more consistent across all participants.

After selecting the sample with the stronger smell, participants were given the option to describe the odor of the stronger smelling sample. This was done to surreptitiously determine if the participants found the samples to be unpleasant without biasing them for or against the "stronger" sample.

Data were analyzed by GraphPad Software using a two-tailed binomial distribution with $\alpha = 0.05$. Using a rearrangement of Abbott's formula to adjust for chance (Lawless & Heymann, 2010), 75% of the participants needed to select the same sample as "stronger" in order to conclude that participants found the aroma of one sample stronger than the other. This formula was also used to determine the percentage of participants who were true discriminators.

2.3.10 Statistical analysis

Samples were prepared and analyzed in triplicate for each time point of analysis. Single-variable ANOVA using SAS 9.4 (SAS Institute, Cary, NC) was used to determine significant differences in percent thiamine remaining between the initial solution and the degraded sample over time, between varying concentrations of solution at each time point, between both salt forms, and between temperatures. Single-variable ANOVA was also used to determine significant differences in pH and color change. Regression analysis was used to determine 95% confidence intervals for k_{obs} values. Differences were determined using Tukey's post hoc test for multiple comparisons at a significance level of $\alpha = 0.05$.

2.4 Results and Discussion

2.4.1 Effects of concentration and temperature on stability of thiamine in TMN solutions

Both temperature and concentration significantly ($p < 0.05$) affected thiamine stability in TMN solutions. Typical degradation profiles of thiamine across varying TMN solution concentrations are shown in Figure 2.2. Increasing temperature increased thiamine degradation rates at all TMN concentrations. Thiamine degraded in an exponential manner for all

concentrations of TMN solutions at all temperatures. Degradation patterns were related to the concentration of thiamine in solution, with more thiamine degradation occurring in solutions with higher TMN concentrations. As an example, in TMN solutions stored at 80°C, solutions containing the lowest TMN concentration, 1 mg/mL, had 48% thiamine remaining after 7 days (the least degradation), while solutions containing the most TMN (27 mg/mL) exhibited the greatest degradation (31% thiamine remaining) (Figure 2.2). A table containing all the thiamine percent remaining data from all TMN solution concentrations at all temperatures is included in the appendix (Table A.2.1).

A clear trend was found at all temperatures that indicated there was a relationship between increasing concentration and decreasing stability of thiamine in TMN solutions. This finding conflicts with older reports that increasing thiamine concentrations in solutions adjusted to pH 6 resulted in increasing thiamine stability (Farrer, 1947; McIntire & Frost, 1944). Differences between those studies and this one include: lower concentrations in the previous reports (the μg/mL scale rather than the mg/mL scale), and controlled pH versus unmodified pH. Controlling pH using a buffer system would be beneficial to better understand the dependency of TMN stability on pH independently from TMN concentration. However, this study did not explore buffer systems due to the possibility of thiamine interactions with the buffer affecting the degradation kinetics. The pH of TMN solutions in this study ranged from 5.36 to 6.96 due to the range of concentrations studied (Table 2.2). It is likely that pH, rather than concentration, was the main reason for differences in stability.

The thiamine degradation patterns found in all TMN solution concentrations and temperature treatments were consistent with those reported in previous TMN studies (Gregory III, 2008; Mauri et al., 1989), showing apparent first-order reaction kinetics (a typical example is shown in Figure 2.3). As expected, reactions proceeded faster as temperature increased. High correlations in linear regressions of the natural log of percent thiamine remaining over time for all TMN concentrations and temperature treatments were obtained (R^2 = 0.86-0.99). These results confirmed that the initial thiamine degradation in TMN solutions followed first-order reaction kinetics. Reaction rate constants, or k_{obs} values, were obtained using linear regressions and eq 1 (Arrhenius plots shown in Figure 2.4), and t_{90} values were calculated using each respective rate constant to describe the time it took for 10% of thiamine to degrade, or when 90% of the initial concentration of thiamine remained. The k_{obs} and t_{90} values are provided in Table 2.3. After the

initial degradation which ended when the samples had approximately 40% TMN remaining, the first order reaction rate was lost. This was likely due to interactions of thiamine with increasing amounts of degradation products along with change in concentration (Ahmad et al., 2018; Dhakal, Balasubramaniam, Ayvaz, & Rodriguez-Saona, 2018) . While kinetic parameters of thiamine degradation have been estimated using an endpoints method in food systems (Peleg, Normand, & Goulette, 2016), which would require a smaller number of experimental data points than used in this study and provide useful information on amount of thiamine remaining in the system, such an approach assumes first order reaction rate and thus could miss inflection points during the course of thiamine degradation when the first order reaction rate is lost.

HPLC chromatograms of TMN solutions before and after storage treatments (and degradation) are provided in the appendix (Figure A.2.1) to facilitate comparisons of the number and retention time of degradation peaks between TMN and TClHCl solutions. The main thiamine degradation peaks in the TMN solutions were found at retention times of approximately 3.26, 4.08, 5.79, 8.15, and 8.28 min. L, a, and b values that documented the color of TMN 27 mg/mL solutions over time are included in Table 2.4, and photographs are included in the appendix (Figure A.2.2). Little color change was found in TMN solutions wherein a large proportion of the thiamine had degraded. For example, when only 31% of thiamine remained in the TMN 27 mg/mL solution, after 7 days at 80°C, only a slightly yellow color in solution was present.

2.4.2 Effects of concentration and temperature on stability of thiamine in TClHCl solutions

Thiamine stability in TClHCl solutions was also significantly ($p < 0.05$) affected by temperature, with increasing temperature resulting in faster degradation. However, no trends were found between thiamine stability and the concentration of TClHCl in solution across all temperatures. The pH of TClHCl solutions in this study ranged from 1.12 to 3.59, due to the range of concentrations studied (Table 2.2). A typical degradation profile of TClHCl in varying concentrations of solution at 80°C is shown in Figure 2.5. Thiamine in solutions across all concentrations of TClHCl degraded in an exponential manner. A table containing all the thiamine percent remaining data from all TClHCl solution concentrations at all temperatures is provided in the appendix (Table A.2.2).

The thiamine degradation patterns found in all TClHCl solution concentrations and temperature treatments were consistent with those reported in the literature for TClHCl (Gregory

III, 2008; Mauri et al., 1989). Similar to the findings for thiamine stability in TMN solutions, apparent first-order reaction kinetics were found for thiamine in TClHCl solutions (Figure 2.6), and the first order reaction rate was lost after reactions had proceeded to approximately 40% thiamine remaining due to possible interactions with new solution components (thiamine degradation products) (Ahmad et al., 2018; Dhakal et al., 2018). The degradation of thiamine in TClHCl solutions was slower than in the TMN solutions, and thus only values from 60°C, 70°C, and 80°C were used for reaction kinetics calculations. High correlations in linear regressions of the natural log of percent thiamine remaining over time for all TClHCl concentrations and temperature treatments were obtained (R^2 = 0.79-0.99), which again confirmed the first-order reaction kinetics of the initial thiamine degradation. Reaction rate constants, or k_{obs} values, were obtained using linear regressions and eq 1 (Arrhenius plots are shown in Figure 2.7), and t_{90} values were calculated to describe the time it took for 10% of thiamine to degrade, as shown in Table 2.3.

HPLC chromatograms of TClHCl solutions before and after storage treatments (and degradation) are provided in the appendix (Figure A.2.1) to facilitate the comparison of the degradation peaks of thiamine in TClHCl and TMN solutions. The main thiamine degradation peaks found in TClHCl solutions were at retention times of approximately 2.13, 4.05. 5.72, and 6.95 min. The L, a, and b values that documented the color of selected TClHCl solutions after storage are included in Table 2.4, and photographs of the color change are included in the appendix (Figure A.2.2). Unlike what was found in the TMN solutions, much more color change was found in the TClHCl solutions, even when less thiamine had degraded. For example, when 56% of thiamine in TClHCl 27 mg/mL solutions remained after 31 days at 80°C, the solutions were nearly black, compared to minimal color change when more thiamine had degraded in a shorter timeframe in 27 mg/mL TMN solutions (31% thiamine remaining after 7 days at 80°C in solutions that were light yellow). After only 5 hours at 80°C, a 500 mg/mL solution of TClHCl in which no significant degradation of thiamine was found had a very similar color to that same 27 mg/mL TMN solution with only 31% thiamine remaining. The color changes found in solutions of TMN and TClHCl at various points during degradation were significantly different ($p < 0.05$). The difference in color change was attributed to the different degradation products that were formed by the different thiamine salts, exemplified by their differing HPLC chromatograms.

2.4.3 Sensory study of odor differences between degraded vitamin solutions

Throughout the course of the thiamine degradation studies, differences in both the color and aroma of TMN and TClHCl solutions were noted by the investigators, in addition to documenting the differences in thiamine degradation rates and degradation product patterns in the HPLC chromatograms. Investigators had noticed an intense odor and color change in TClHCl solutions that occurred before thiamine degradation in the TClHCl solutions was even statistically significant. In contrast, the investigators had also noticed that TMN solutions had not produced an intense smell or color change even when only ~30% of thiamine remained.

To further pursue these initial observations, a sensory study was completed to determine if a larger audience noted a difference in aromas produced by thiamine degradation in TMN and TClHCl solutions. Using the two-alternative forced-choice test, 51 of 68 panelists chose the TClHCl sample as having a stronger aroma than the TMN sample. Adjusting for chance, this was sufficient to conclude that the TClHCl sample had a stronger aroma than the TMN sample. From the adjusted Abbott's formula (Lawless & Heymann, 2010), 34 of the 68 panelists would be considered true discriminators, indicating that approximately 50% of people should truly find the TClHCl sample more potent. A two-tailed binomial test yielded $p < 0.0001$, again indicating that the TClHCl solution had a significantly stronger aroma than the TMN solution (see appendix Figure A.2.3). A cursory evaluation of the words used to describe the TClHCl solution odor indicated that subjects found the aroma unfavorable. Descriptive words used by panelists are provided in the appendix (Table A.2.3). The percent thiamine remaining in each of these solutions, as determined by HPLC, was 66% thiamine remaining in the TMN solution with no significant degradation found in the TClHCl solution. Thus, it was concluded that the thiamine degradation products in TClHCl solutions had a significantly more potent odor than the degradation products in TMN solutions.

2.4.4 Comparison of thiamine stability in TMN and TClHCl solutions

There was a significant difference ($p < 0.05$) in thiamine stability between TMN and TClHCl solutions, as shown by the comparison graphs in Figure 2.8 and by k_{obs} and t_{90} values reported in Table 2.3. Thiamine in TMN solutions degraded faster than thiamine in TClHCl solutions, with more substantial differences in stability manifesting as the temperature increased

(Figure 2.8, Table A.2.1, Table A.2.2). The differences between the two salt forms were also exemplified by sensory implications, including aroma and color change (Table 2.4, Figure A.2.2). Some possible degradation products that may contribute to differences in TMN and TClHCl solutions were identified by Dwivedi and Arnold (1973), including thiochrome, dihydrothiochrome, thioketones, pyrimidine and thiazole derivatives, and disulfides, among others.

TMN and TClHCl salts dissociate in solution to become the thiamine cation (with one or two positive charges, depending on pH (Figure 2.9)) and the respective anions. The main differences in solution traits between these thiamine salt forms are the type of anion present and the resulting solution pH. The pH values of TMN and TClHCl solutions at all concentrations and temperatures studied are shown in Table 2.2. It has been well-documented that pH affects thiamine stability; specifically, thiamine is much more stable in acidic conditions than in approximately neutral or alkaline conditions (Dwivedi & Arnold, 1973; Farrer, 1947; Gregory III, 2008; McIntire & Frost, 1944). Thus, it was not surprising to find that thiamine in TClHCl solutions was much more stable than thiamine in TMN solutions, since the TClHCl formed more acidic solutions than the TMN.

It has also been reported that pH affects the degradation pathway of thiamine (Dwivedi & Arnold, 1972). Thiamine has a pK_a of 4.8 (for the pyrimidine N1 nitrogen) (Edwards et al., 2017). In acidic conditions (pH < 6), degradation occurs by cleavage of the methylene bridge to release intact pyrimidine and thiazole moieties; while in conditions above pH 6, degradation involves the same cleavage, but also further fragmentation of the thiazole ring (Gregory III, 2008). These varying pathways support the observation of different degradation products formed in the close to neutral pH TMN solutions and the acidic TClHCl solutions, as noted in the HPLC chromatograms (Figure A.2.1). By comparing the retention times of the thiamine degradation products in the HPLC chromatograms, common degradation products found in both TMN and TClHCl solutions had retention times of approximately 4.05 and 5.75 min, while differences were found in degradation products appearing at 3.26, 8.15, and 8.28 min in TMN solutions, and at 2.13 and 6.95 min in TClHCl solutions. These different degradation products likely caused the differences in color and aroma between the TMN and TClHCl solutions.

Thiamine stability was significantly affected by TMN concentration, with thiamine degradation rates increasing as the concentration of TMN increased. This observation was likely more dependent on the changing solution pHs as TMN concentration increased rather than on the

solution concentration of the thiamine *per se*. It has been well-documented that there is a dramatic decrease in stability of thiamine as pH reaches and exceeds pH 6.0 (Feliciotti & Esselen, 1957; Mulley et al., 1975b; Williams & Ruehle, 1935). This change in stability is a result of the pK_a of thiamine (4.8). As illustrated in the speciation plot of thiamine in Figure 2.9, the more stable protonated species of thiamine is present as a notable fraction in acidic conditions up to approximately pH 6.0. As pH increases above 6.0, the less stable unprotonated species of thiamine dominates, and the stability of thiamine dramatically decreases. This noteworthy pH value (6.0) could be used to explain the dependence of thiamine stability on TMN concentration since the pH values found for TMN solutions were between pH 5.36 and 6.96. Small increases in pH due to increases in TMN concentration would have led to major changes in the fraction of protonated/unprotonated thiamine species present, which in turn would have caused the large decrease in thiamine stability that was found to be so dependent on TMN concentration. Conversely, in the pH range found in TClHCl solutions (from 1.12 to 3.59), the protonated species of thiamine would have been predominant, which was likely why thiamine was not only more stable in the TClHCl solutions but also exhibited no stability dependence on TClHCl concentration.

Over a large range of temperatures, pH is known to vary slightly (Clark, 2017): as temperature increases, pH decreases. As shown in Figure 2.10 and Table 2.2, this trend was found in the TMN and TClHCl samples. Although this is of interest to note, it is not likely that this temperature-dependent pH change significantly affected thiamine stability, especially since this stability trend is in opposition to the effect of temperature. However, K_w also changes with temperature (Clark, 2017), meaning that although pH changes, acidity/alkalinity does not change, which led to the conclusion that pH change with temperature was an inconsequential factor in this thiamine stability study.

2.4.5 Degradation kinetics of thiamine salt forms

The degradation kinetics of thiamine in various matrices (different from the solutions studied here) have been reported, including solid state with varying water activities, controlling for pH, and in the presence of various humectants (Kamman et al., 1981; Labuza & Kamman, 1982; Mauri et al., 1992). Thiamine was generally reported to have an activation energy of 20-30 kcal/mol (80-125 kJ/mol) (Kamman et al., 1981; Mauri et al., 1992). When controlling for pH, the activation energy was reported to be 27.4 kcal/mol at pH 5.5 and 29 kcal/mol at pH 4.0 (Mauri et

al., 1992). When specifically looking at the different salt forms, activation energy was reported as 22.4 kcal/mol for TClHCl and 26.3 kcal/mol for TMN, with the E_a decreasing as water activity increased (Labuza & Kamman, 1982). This difference in activation energies is the reason for the greater stability of TMN compared to TClHCl in the solid state, but these values do not agree with the stability trends of thiamine in solution found in this study. In the current study, pH and vitamin form were assumed to influence activation energy in solution, with the main factor being pH change due to variations in the ionization of each thiamine salt in solution.

It was reported previously that thiamine degradation in buffered solutions from 50°C to 110°C exhibited no deviation from Arrhenius behavior (Farrer & Morrison, 1949), but temperatures below 50°C were not included in the study. In the current study, non-linear Arrhenius plots were found to occur as the concentration of degradation products increased; however, in the early stages of thiamine degradation linear Arrhenius plots were found. These linear Arrhenius plots were used to calculate reaction kinetics. Using the k_{obs} values from temperatures 25, 40, 60, 70, and 80°C, the TMN activation energies were consistent with previous reports, ranging from 21-25 kcal/mol (88-105 kJ/mol), dependent on concentration. All values are included in Table 2.5. Using the k_{obs} values from temperatures 60, 70, and 80°C, TClHCl activation energies were found to range from 21-32 kcal/mol (90-135 kJ/mol). While these values are slightly higher than those previously reported, the extremely low pH found in the TClHCl solutions was not studied elsewhere. The low pH values (1.12-3.59) and consequently the predominance of the more stable protonated form of thiamine (Figure 2.9) led to the higher stability of thiamine in TClHCl solutions observed in this study (for example, 91% of TClHCl remained in the 10 mg/mL solution after 7 days at 80ºC compared to 38% TMN remaining in the same conditions). Additionally, the high thiamine stability in TClHCl solutions at 25°C and 40°C allowed the use of only 3 (higher) temperatures for the kinetics calculations, rather than the preferred 5 temperatures. However, the R^2 values for the Arrhenius calculations for TClHCl solutions were high correlations (0.87-0.99). All E_a values are reported in Table 2.5.

Overall, the reaction kinetics found in the current study agree reasonably well with previous reports. TClHCl was found to have a higher activation energy than TMN, presumably due to the difference in pH values between the two salt forms in solution. The low pH conditions in the TClHCl solutions studied caused the protonated thiamine species, the more stable of the two species, to be predominately present in solution. The low pH samples had a higher activation

energy of thiamine degradation and were significantly ($p < 0.05$) more stable than thiamine in the close to neutral pH TMN solutions.

2.4.6 Potential implications in food formulations

Although the concentrations of thiamine investigated in this study were higher than concentrations found in most food products, the implications for trends in thiamine stability at different pHs and temperatures are relevant for foods naturally containing or fortified with thiamine. Many food products act as acidic environments that will protect thiamine stability, including fruit products and energy drinks. In these acidic conditions, no significant thiamine degradation was found at ambient temperature over the 6 month period of this study. However, there are also many food sources of thiamine that are close to neutral pH or slightly alkaline, including milk, teas, beans, eggs, peas, and peanuts. The higher pHs in these foods may contribute to degradation of thiamine during storage. For example, in close to neutral pH or slightly alkaline samples at ambient temperatures, the t_{90} was 130-310 days, depending on pH, compared to t_{90} values that could not be calculated in acidic conditions due to lack of significant degradation. While some products (e.g., fruits, yeast, meats, eggs, and legumes) naturally contain thiamine, many other food products are enriched with the salt forms of thiamine investigated in this study. Some of the products enriched with TMN or TClHCl that have close to neutral pH or slightly alkaline pH include various dairy products, powdered or liquid infant formulas, dietary supplements, and enriched flour (Bettendorff, 2012). Enriched flours are commonly combined with leavening agents in baked goods formulations, and these leavening agents produce slightly alkaline conditions (Cauvain & Young, 2006) which, as shown in this study, provide an unstable environment for thiamine. Further heating these products, such as during baking, could contribute to more thiamine degradation. Additionally, common food products or dietary supplements with limited water but high thiamine content include nutritional yeast, dried milk, infant formula, dried seaweed, and vitamin B complex supplements (U.S. Department of Agriculture Agricultural Research Service, 2018). Since thiamine has the potential to begin to dissolve in small amounts of water and is known to degrade faster in solution than in the solid state (Hiatt et al., 2008), the thiamine found in these products may act more like the thiamine in this study at high concentrations in the water present.

Although thiamine is often found in the presence of excipients in supplements or other ingredients in food products that can improve (or worsen) chemical stability (Kandutsch & Baumann, 1953), the degradation kinetics found in this study for pure thiamine in solution provide valuable information on the fundamental behavior of thiamine. Analyzing thiamine stability in buffered solutions to control for pH or in the presence of co-formulated ingredients would extend the implications of this study to more representative food systems and provide useful information on additional factors that contribute to the stability and/or degradation of thiamine.

2.5 Conclusion

Degradation of thiamine in solution was dependent on the form of thiamine salt dissolved, the resulting solution pH, and the storage temperature. All thiamine degradation was found to follow first order reaction kinetics until degradation products were present in high concentrations (< 40% vitamin remaining), which were thought to alter the degradation pathway. Thiamine in TClHCl solutions was found to be much more stable in all conditions than thiamine in TMN solutions, which was attributed to the low pH of TClHCl solutions. Although acidic conditions delayed the degradation of thiamine in solution, the low pH also altered the degradation pathway and produced different degradation products than were found in close to neutral pH conditions. This was demonstrated by differing peak positions in HPLC chromatograms between solutions of TMN and TClHCl. Thiamine degradation products in TClHCl solutions also contributed a potent odor and intense color change even before degradation became significant ($p < 0.05$). However, even with very large amounts of thiamine degradation in TMN solutions, sensory impacts were minimal. This study developed shelf-life studies that directly compared the stabilities and reaction kinetics of the two most common salt forms of thiamine, used in dietary supplements and as food additives, as a function of concentration and temperature. The results can aid in improving the understanding of thiamine degradation in a variety of products that are enriched or fortified with thiamine.

2.6 Tables and Figures

Table 2.1 Solid state property comparison between TMN and TClHCl.

	Thiamine Mononitrate	Thiamine Chloride Hydrochloride
Molecular weight [1]	327.36 g/mol	337.26 g/mol
Melting point [1]	196-200°C	248°C
Deliquescence point (RH_0) [2]	98.5% RH	88% RH
Aqueous solubility	30 mg/mL	570 mg/mL

[1] ChemSpider (2015)
[2] Hiatt et al. (2008)

Table 2.2 The pH values of A) pure water and B) TMN and TClHCl solutions at each concentration and temperature studied. Uppercase superscript letters on values denote statistical significance within temperatures for each vitamin salt form (down columns). Lowercase superscript letters on values denote statistical significance within concentration for each vitamin salt form (across rows).

A)

	25°C	**30°C**	**40°C**	**50°C**	**100°C**	**Ref**
Pure Water	7.00	6.92	6.77	6.63	6.14	(Clark, 2017)

B)

Vitamin Salt Form	**Concentration (mg/mL)**	**25°C**	**40°C**	**60°C**	**70°C**	**80°C**
TMN	1	6.42 ± 0.04^{Aa}	6.23 ± 0.09^{Aa}	5.95 ± 0.07^{Ab}	5.76 ± 0.03^{Ab}	5.46 ± 0.07^{ABc}
	5	6.6 ± 0.3^{Aa}	6.2 ± 0.4^{Aab}	5.9 ± 0.3^{Aab}	5.6 ± 0.2^{Aab}	5.36 ± 0.05^{Bb}
	10	6.8 ± 0.2^{Aa}	6.5 ± 0.2^{Aab}	6.1 ± 0.1^{Abc}	5.8 ± 0.1^{Ac}	5.5 ± 0.2^{ABc}
	20	6.93 ± 0.03^{Aa}	6.57 ± 0.09^{Ab}	6.14 ± 0.09^{Ac}	5.9 ± 0.1^{Acd}	5.61 ± 0.05^{ABd}
	27	6.96 ± 0.03^{Aa}	6.67 ± 0.03^{Aa}	6.24 ± 0.08^{Ab}	5.86 ± 0.09^{Ac}	5.8 ± 0.1^{Ac}
TClHCl	1	3.59 ± 0.03^{Aa}	3.2 ± 0.1^{Aab}	2.9 ± 0.1^{Abc}	2.6 ± 0.1^{Abc}	2.6 ± 0.2^{Ac}
	5	3.30 ± 0.01^{Ba}	2.8 ± 0.1^{Aab}	2.5 ± 0.1^{ABbc}	2.2 ± 0.1^{Bc}	2.2 ± 0.2^{ABc}
	10	3.17 ± 0.00^{Ca}	2.67 ± 0.08^{Bb}	2.3 ± 0.1^{BCc}	1.95 ± 0.08^{BCcd}	1.89 ± 0.08^{BCd}
	20	3.05 ± 0.00^{Da}	2.51 ± 0.00^{BCb}	2.19 ± 0.04^{BCc}	1.97 ± 0.07^{BCcd}	1.9 ± 0.1^{BCd}
	27	2.99 ± 0.01^{Da}	2.46 ± 0.02^{BCb}	2.14 ± 0.01^{BCc}	2.0 ± 0.1^{BCcd}	1.8 ± 0.1^{BCDd}
	100	2.77 ± 0.01^{Ea}	2.36 ± 0.02^{Cb}	1.9 ± 0.1^{CDc}	1.7 ± 0.1^{CDcd}	1.51 ± 0.03^{CDEd}
	300	2.53 ± 0.01^{Fa}	2.05 ± 0.06^{Db}	1.6 ± 0.1^{Dc}	1.44 ± 0.03^{DEcd}	1.28 ± 0.06^{DEd}
	500	2.35 ± 0.03^{Ga}	1.93 ± 0.01^{Db}	1.5 ± 0.2^{Dc}	1.3 ± 0.1^{Ecd}	1.12 ± 0.09^{Ed}

Table 2.3 Rate constants and t_{90} values for thiamine in solutions. Uppercase and lowercase superscript letters denote statistical significance within concentration (down columns) and within temperature (across rows) for each vitamin salt form, respectively.

Vitamin Salt	Temperature (ºC)		1 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL	27 mg/mL	100 mg/mL	300 mg/mL
TMN	25	k_{obs}* (day^{-1})	$3.4x10^{-4} \pm 0.5x10^{-4}$ Aa	$6.3x10^{-4} \pm 0.6x10^{-4}$ Abc	$8.1x10^{-4} \pm 0.1x10^{-4}$ Ac	$4.8x10^{-4} \pm 0.5x10^{-4}$ Aab	$7.2x10^{-4} \pm 0.8x10^{-4}$ Ac	N/A	N/A
		R^2	0.8601	0.9560	0.8551	0.9259	0.9309		
		t_{90}** (days)	310	167	130	220	146		
	40	k_{obs} (day^{-1})	$1.74x10^{-3} \pm 0.7x10^{-4}$ Aa	$3.1x10^{-3} \pm 0.2x10^{-3}$ Aa	$5.1x10^{-3} \pm 0.4x10^{-3}$ Ab	$7.6x10^{-3} \pm 0.6x10^{-3}$ Ac	$9.7x10^{-3} \pm 0.1x10^{-3}$ Ad		
		R^2	0.9858	0.9680	0.9629	0.9554	0.9321		
		t_{90} (days)	60.6	34.0	20.7	13.9	10.9		
	60	k_{obs} (day^{-1})	$1.7x10^{-2} \pm 0.1x10^{-2}$ Ba	$2.7x10^{-2} \pm 0.1x10^{-2}$ Ba	$3.9x10^{-2} \pm 0.3x10^{-2}$ Bb	$6.3x10^{-2} \pm 0.6x10^{-2}$ Ac	$7.7x10^{-2} \pm 0.6x10^{-2}$ ABd		
		R^2	0.9840	0.9898	0.9832	0.9661	0.9762		
		t_{90} (days)	6.20	3.90	2.70	1.67	1.37		
	70	k_{obs} (day^{-1})	$4.6x10^{-2} \pm 0.5x10^{-2}$ Ca	$7.2x10^{-2} \pm 0.5x10^{-2}$ Cab	$9.7x10^{-2} \pm 0.6x10^{-2}$ Cb	$1.4x10^{-1} \pm 0.1x10^{-1}$ Bc	$1.9x10^{-1} \pm 0.2x10^{-1}$ Bd		
		R^2	0.9577	0.9803	0.9837	0.9686	0.9611		
		t_{90} (days)	2.29	1.46	1.09	0.753	0.555		
	80	k_{obs} (day^{-1})	$1.17x10^{-1} \pm 0.6x10^{-2}$ Da	$1.4x10^{-1} \pm 0.1x10^{-1}$ Da	$2.0x10^{-1} \pm 0.2x10^{-1}$ Da	$4.6x10^{-1} \pm 0.5x10^{-1}$ Cb	$6x10^{-1} \pm 1x10^{-1}$ Cb		
		R^2	0.9920	0.9764	0.9622	0.9819	0.9577		
		t_{90} (days)	0.901	0.753	0.527	0.229	0.176		
TClHCl	60	k_{obs} (day^{-1})	$2.1x10^{-3} \pm 0.2x10^{-3}$ Abc	$1.9x10^{-3} \pm 0.2x10^{-3}$ Aabc	$1.8x10^{-3} \pm 0.3x10^{-3}$ Aabc	$1.5x10^{-3} \pm 0.1x10^{-3}$ Aa	$1.6x10^{-3} \pm 0.2x10^{-3}$ Aab	$1.4x10^{-3} \pm 0.2x10^{-3}$ Aa	$1.5x10^{-3} \pm 0.1x10^{-3}$ Aab
		R^2	0.9304	0.9260	0.7922	0.9384	0.9283	0.9028	0.9382
		t_{90} (days)	50.2	55.5	58.5	70.2	65.9	75.3	70.2
	70	k_{obs} (day^{-1})	$1.7x10^{-2} \pm 0.3x10^{-2}$ Bc	$7.3x10^{-3} \pm 0.8x10^{-3}$ Bab	$7x10^{-3} \pm 1x10^{-3}$ Bab	$5.3x10^{-3} \pm 0.7x10^{-3}$ Ba	$4.4x10^{-3} \pm 0.4x10^{-3}$ Ba	$7x10^{-3} \pm 1x10^{-3}$ Bab	$7.8x10^{-3} \pm 0.9x10^{-3}$ Bab
		R^2	0.8215	0.9283	0.8745	0.8823	0.9498	0.8682	0.9110
		t_{90} (days)	6.20	14.4	15.1	19.9	23.9	15.1	13.5
	80	k_{obs} (day^{-1})	$3.1x10^{-2} \pm 0.1x10^{-2}$ Cd	$1.5x10^{-2} \pm 0.1x10^{-2}$ Ca	$2.3x10^{-2} \pm 0.3x10^{-2}$ Cbc	$2.4x10^{-2} \pm 0.1x10^{-2}$ Cbc	$1.89x10^{-2} \pm 0.09x10^{-2}$ Cab	$2.0x10^{-2} \pm 0.1x10^{-2}$ Cbc	$2.4x10^{-2} \pm 0.2x10^{-2}$ Cc
		R^2	0.9873	0.9500	0.8928	0.9736	0.9857	0.9753	0.9567
		t_{90} (days)	3.40	7.02	4.58	4.39	5.57	5.27	4.39

* Error values indicated for k_{obs} values represent a 95% confidence interval

Table 2.4 Color parameters L, a, and b values of selected TMN and TClHCl solutions at 80°C. Superscript letters denote statistical significance within their respective parameters.

Vitamin Form	Concentration	Time	L (0-100%, black-white)	a (negative=green, positive=red)	b (negative=blue, positive=yellow)
TMN	27 mg/mL	0 days	$80.0 \pm 0.7\%^{AB}$	-7.2 ± 0.4^{E}	6.2 ± 0.6^{D}
		7 days	$82 \pm 2\%^{A}$	-11.6 ± 0.4^{G}	15.8 ± 0.2^{B}
TClHCl	27 mg/mL	0 days	$77 \pm 1\%^{BC}$	-5.3 ± 0.6^{D}	2.8 ± 0.3^{F}
		31 days	$16 \pm 1\%^{E}$	2.9 ± 0.6^{B}	2.7 ± 0.4^{F}
	100 mg/mL	0 days	$77 \pm 2\%^{BC}$	-5.8 ± 0.3^{DE}	3.1 ± 0.1^{F}
		31 days	$15 \pm 3\%^{E}$	-2 ± 1^{C}	0 ± 1^{G}
	500 mg/mL	0 days	$76 \pm 1\%^{C}$	-6.8 ± 0.3^{DE}	4.8 ± 0.4^{E}
		5 hours	$80.7 \pm 0.6\%^{A}$	-9.3 ± 0.7^{F}	10.4 ± 0.9^{C}
		31 days	$40 \pm 2\%^{D}$	38 ± 2^{A}	35.7 ± 0.7^{A}

Table 2.5 Calculated activation energies of TMN and TClHCl as a function of temperature.

Vitamin Salt Form	Concentration (mg/mL)	E_A (kcal/mol)	E_A (kJ/mol)
TMN	1	22	94
	5	21	88
	10	21	88
	20	25	105
	27	25	103
TClHCl	1	32	133
	5	24	100
	10	30	124
	20	32	136
	27	29	120
	100	31	131
	300	32	135
	500	21	90

A) B) C)

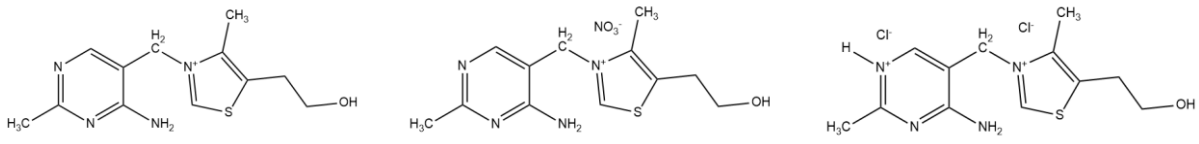

Figure 2.1 Chemical structures of A) thiamine, B) thiamine mononitrate, and C) thiamine chloride hydrochloride.

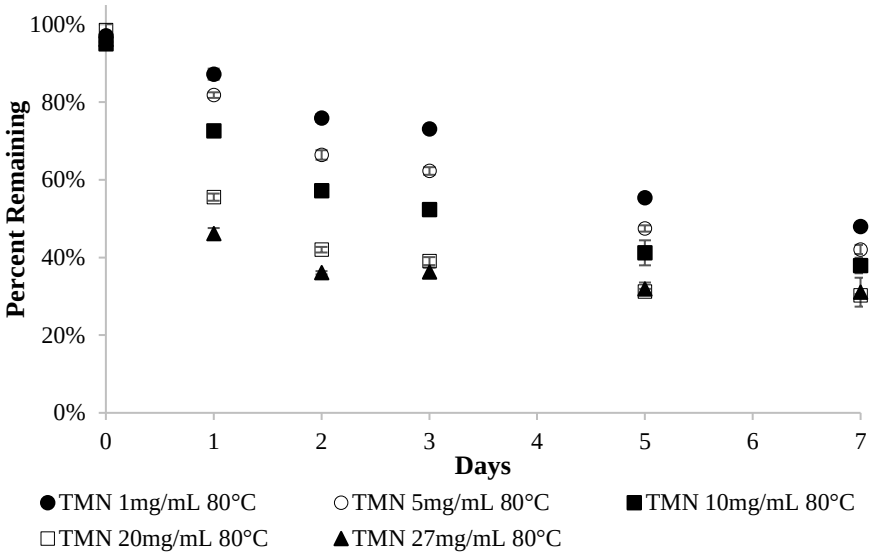

Figure 2.2 Degradation profiles of thiamine in TMN solutions in varying concentrations (1-27 mg/mL) at 80°C over time.

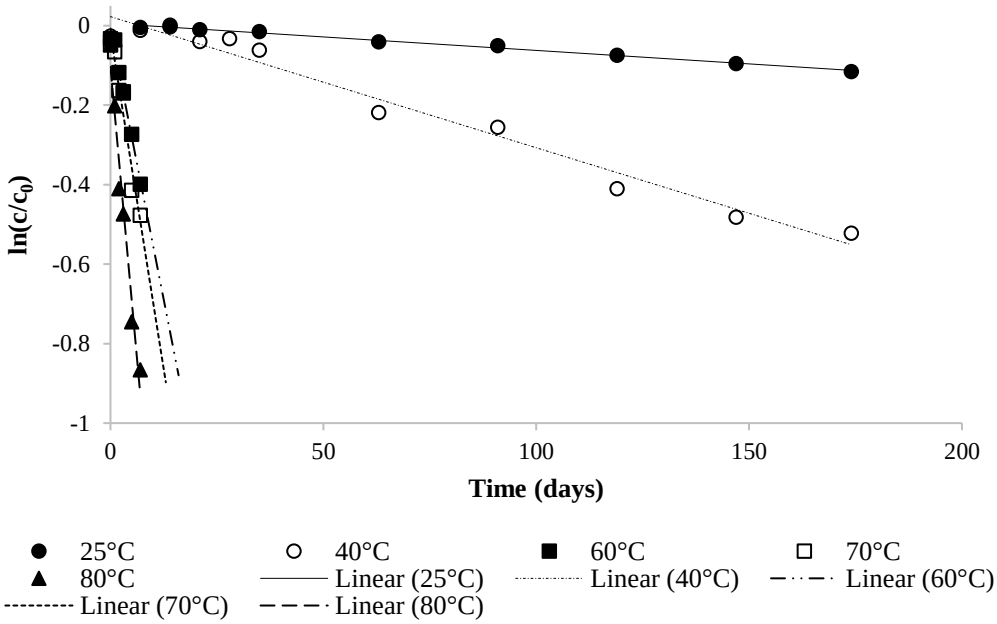

Figure 2.3 First-order degradation regression lines of thiamine in 5 mg/mL TMN solutions at temperatures from 25°C to 80°C.

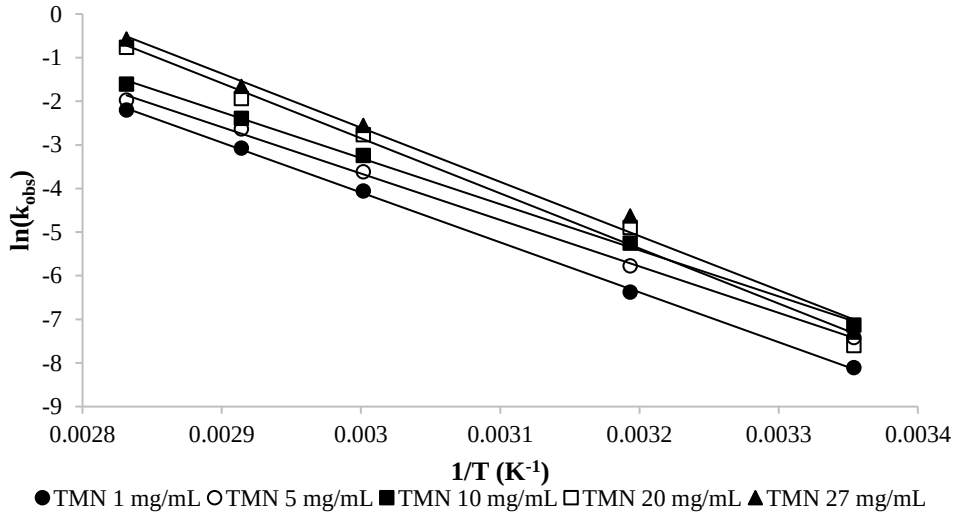

Figure 2.4 Arrhenius plots used to calculate temperature-dependent activation energy for TMN solutions (1-27 mg/mL) at temperatures from 25ºC to 80ºC.

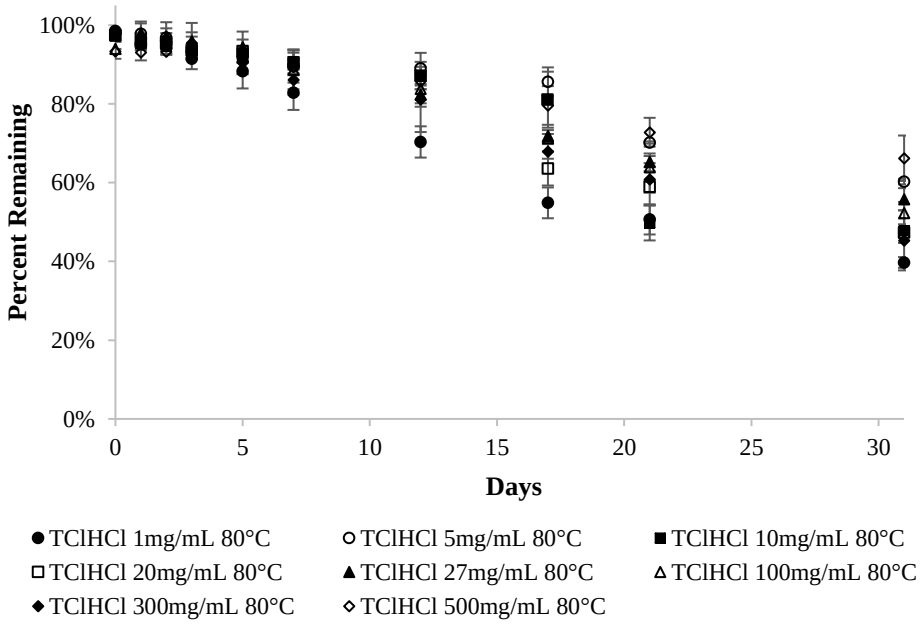

Figure 2.5 Degradation profiles of thiamine in TClHCl solutions at varying concentrations (1-500 mg/mL) at 80°C over time.

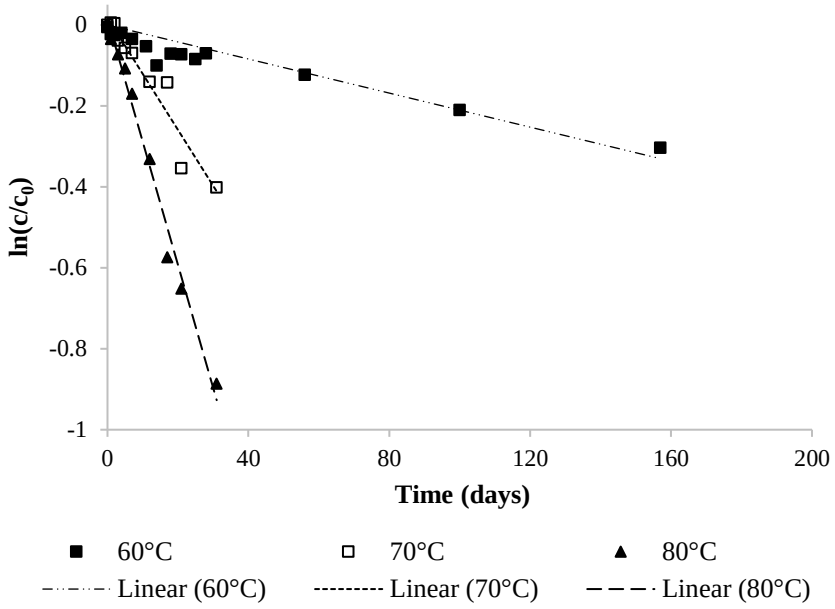

Figure 2.6 First-order degradation regression lines of thiamine in 1 mg/mL TClHCl solutions at temperatures from 60°C to 80°C.

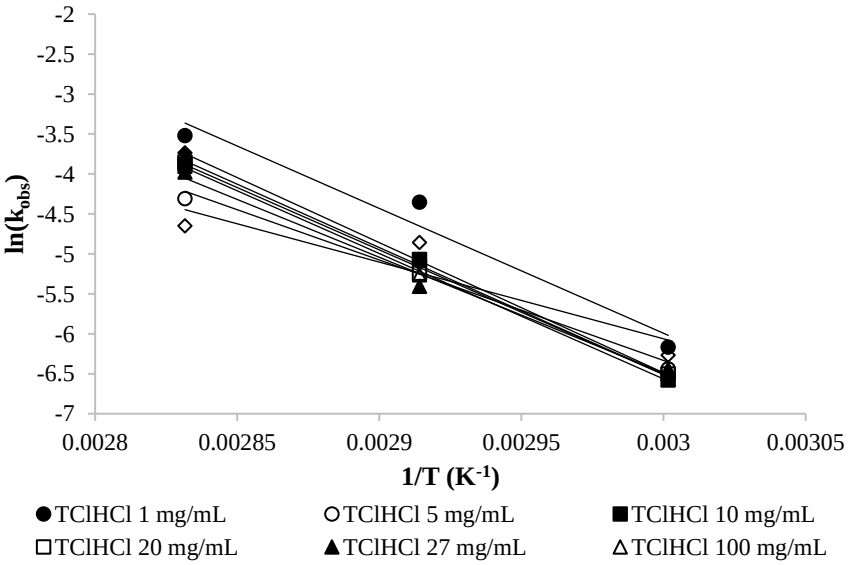

Figure 2.7 Arrhenius plots used to calculate temperature-dependent activation energy for TClHCl solutions (1-500 mg/mL) at temperatures from 25ºC to 80ºC.

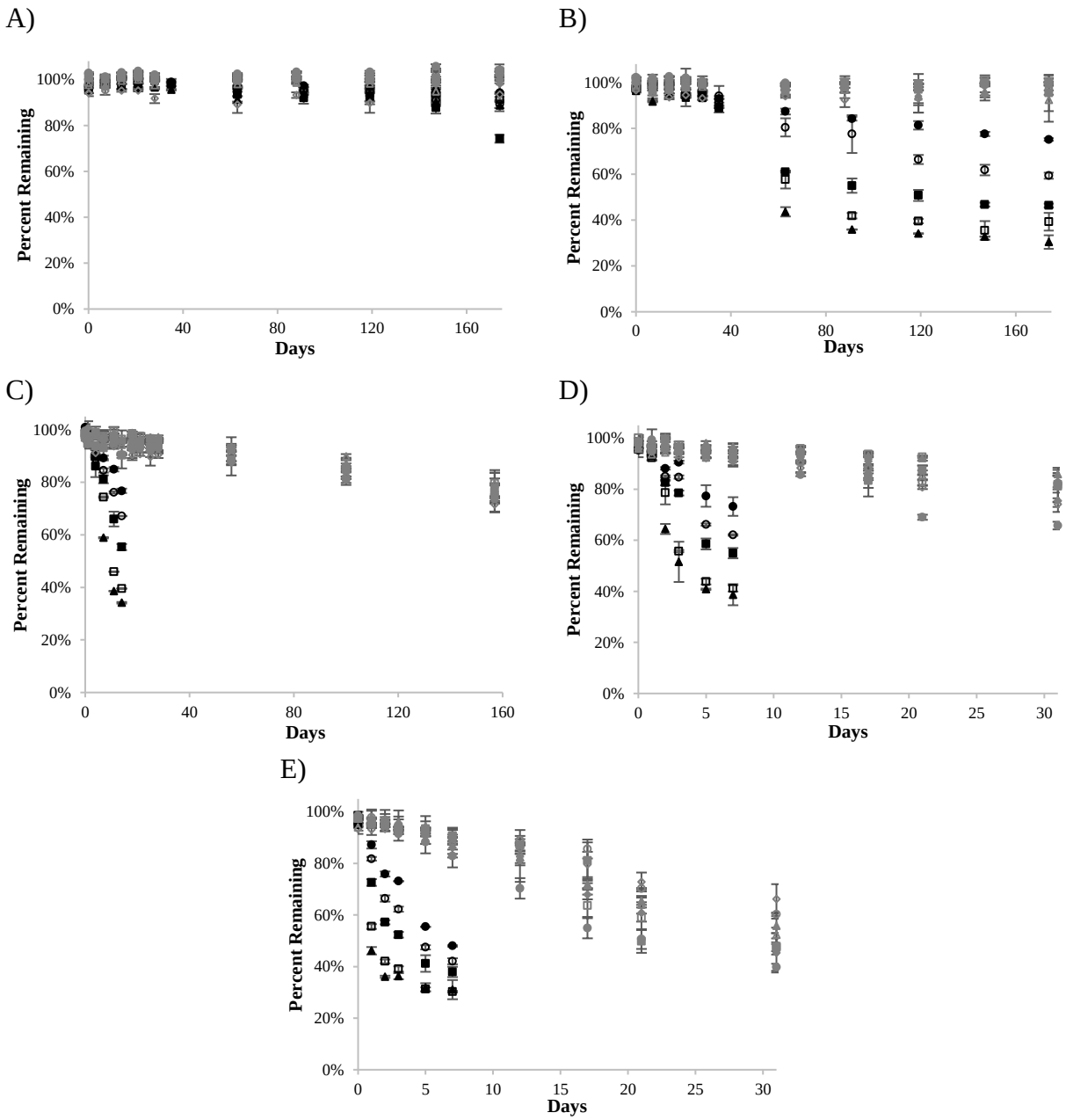

Figure 2.8 Comparison of chemical stability over time of TMN and TClHCl in multiple concentrations of solution at A) 25°C, B) 40°C, C) 60°C, D) 70°C, and E) 80°C:

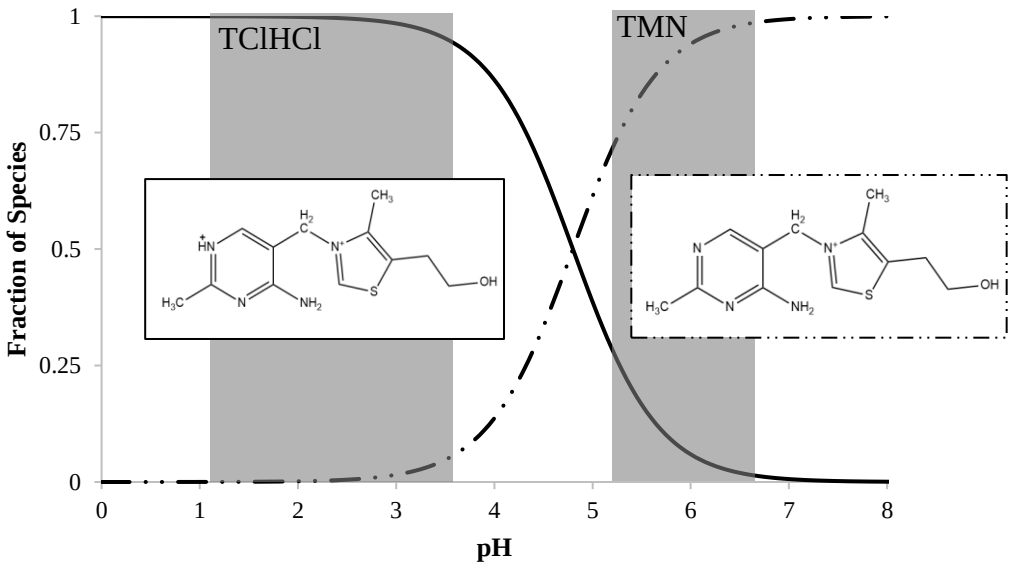

Figure 2.9 Speciation plot of thiamine as a function of pH prepared using only the pK_{a1} of thiamine (4.8) for the N1 nitrogen on the pyrimidine ring. Shaded areas indicate pH ranges of TClHCl and TMN samples, respectively.

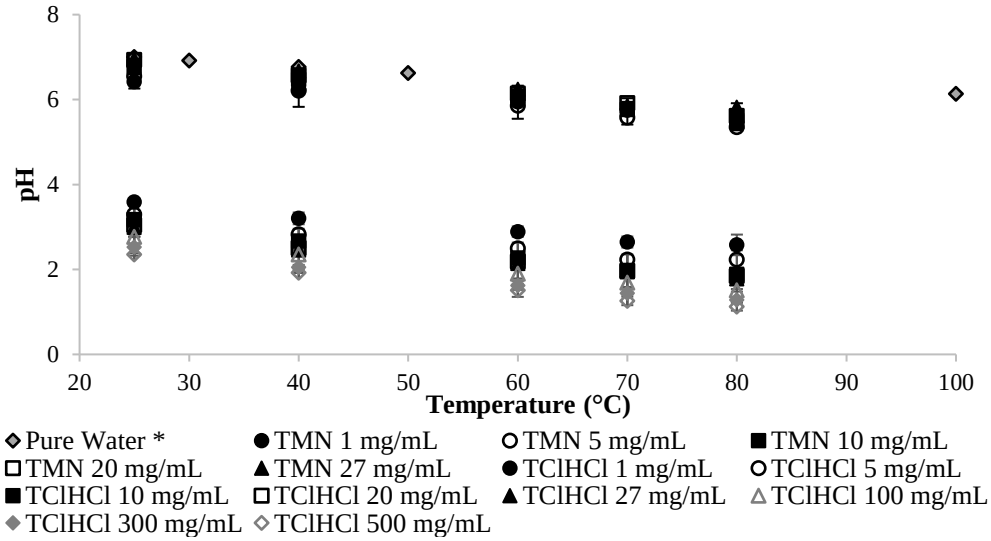

Figure 2.10 The pH change with temperature of pure water (Clark, 2017), TMN, and TClHCl for all concentrations studied.

2.7 References

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CHAPTER 3. EFFECT OF pH AND CONCENTRATION ON THE CHEMICAL STABILITY AND REACTION KINETICS OF THIAMINE MONONITRATE AND THIAMINE CHLORIDE HYDROCHLORIDE IN SOLUTION

3.1 Abstract

Thiamine (vitamin B_1) is an essential micronutrient in the human diet, found both naturally and as a fortification ingredient in many foods and supplements. However, it is susceptible to degradation due to heat, light, alkaline pH, and sulfites, among effects from other food matrix components, and its degradation has both nutritional and sensory implications as in foods. Thiamine storage stability in solution was monitored over time to determine the effect of solution pH and thiamine concentration on reaction kinetics of degradation without the use of buffers, which are known to affect thiamine stability independent of pH. The study directly compared thiamine stability in solutions prepared with different pHs (3 or 6), concentrations (1 or 20 mg/mL), and counterion in solution (NO_3^-, Cl^-, or both), including both commercially available salt forms of thiamine (thiamine mononitrate and thiamine chloride hydrochloride). Solutions were stored at 25, 40, 60, and 80ºC for up to one year, and degradation was quantified by high-performance liquid chromatography (HPLC) over time, which was then used to calculate degradation kinetics. Thiamine was significantly more stable in pH 3 than in pH 6 solutions. In pH 6 solutions, stability was dependent on initial thiamine concentration, with higher concentrations increasing reaction rate constant (k_{obs}). In pH 3 solutions, k_{obs} was not dependent on initial concentration, attributed to differences in degradation pathway dependent on pH. Activation energies of degradation (E_a) were higher in pH 3 solutions (21-27 kcal/mol) than in pH 6 solutions (18-21 kcal/mol), indicating a difference in stability and degradation pathway due to pH. The fundamental reaction kinetics of thiamine reported in this study provide a basis for understanding thiamine stability and therefore improving thiamine delivery in many foods containing both natural and fortified thiamine.

3.2 Introduction

Thiamine (vitamin B_1; Figure 3.1) was the first vitamin to be characterized (Funk, 1912). It is an essential micronutrient in the human diet, with a Recommended Dietary Allowance (RDA) and Daily Value (DV) of 1.2 mg/day in the United States (Institute of Medicine, 1998; U.S. Food

& Drug Administration, 2018). It is found naturally in foods, such as grains, legumes, nuts, and meats (Paucean et al., 2018). Thiamine acts as a precursor for a coenzyme in the metabolism of carbohydrates, branched-chain amino acids, and lipids, and plays major roles in muscle contraction and in the nervous system (Bémeur & Butterworth, 2014; Institute of Medicine, 1998). While grains are the main source of thiamine in the diet, the thiamine is mostly located in the germ and the bran, the outer layers of the kernel, so thiamine content is reduced by 89% during the refining process (Fitzpatrick et al., 2012; Pourcel et al., 2013). For this reason, thiamine deficiency is a concern in both developed and developing countries. A lack of a nutritious diet is the main cause of thiamine deficiency in developing countries, especially when the main dietary component is an unfortified grain, e.g., polished rice (Ball, 2006). In developed countries where malnutrition or lack of fortification is less of a concern, deficiency is still common in certain groups of people, including alcoholics, people with HIV/AIDS, and people on diets that avoid fortified grains, such as those with Celiac's disease (Bémeur & Butterworth, 2014; Shepherd & Gibson, 2013).

Two salt forms of thiamine (thiamine mononitrate (TMN) and thiamine chloride hydrochloride (TClHCl)) are commonly added to foods as enrichment or fortification supplements. Although this has substantially reduced thiamine deficiency in developed countries, up to 84% of thiamine in foods can still be lost during cooking or processing due to the instability of the vitamin (O'Brien & Roberton, 1993). Thiamine is sensitive to heat, alkali, salts, oxygen, and sulfites (Farrer, 1955; Gregory III, 2008; Pizzoferrato, 1992; Spitzer & Schweigert, 2007). Previous studies have shown that TMN and TClHCl have different activation energies (E_a) of degradation both in the solid state (26.3 and 22.4 kcal/mol, respectively) and in solution (21 and 32 kcal/mol, respectively in 10 mg/mL solutions) (Labuza & Kamman, 1982; Voelker, Miller, Running, Taylor, & Mauer, 2018). Differences in E_a suggest that the degradation pathway differs between the two salt forms, which also has sensory implications due to sulfur-containing degradation products (Buttery et al., 1984; Güntert et al., 1992; Voelker et al., 2018). However, the salt form of thiamine is dissociated when dissolved in solution, so it was proposed that the difference in E_a in solution and therefore difference in degradation pathway was due to the pH of the solution rather than the stability of the salt form itself (Dwivedi & Arnold, 1972; Feliciotti & Esselen, 1957; Voelker et al., 2018).

Thiamine degradation has been reported to be a pseudo-first order reaction and therefore dependent on concentration (Arabshahi & Lund, 1988; Gregory III, 2008; Mauri et al., 1989). It is

known to be more stable in acidic conditions, specifically below a pH of 6.0 due to thiamine's food-relevant pK_a of 4.8, wherein the less stable thiamine species (unprotonated pyrimidine N1) is the predominant species above pH 6.0 (Arnold et al., 1969; Dwivedi & Arnold, 1972; Farrer, 1955). However, pH-dependent thiamine stability is most often studied by employing the use of common buffer systems despite studies that have shown both type and concentration of buffer salts to affect thiamine degradation independent of pH (Beadle et al., 1943; Dwivedi & Arnold, 1972; Farrer, 1955; Pachapurkar & Bell, 2005). Although many kinetic studies on thiamine degradation have been published (Goulette et al., 2020; K. C. Kwok et al., 1998; Nisha, Singhal, & Pandit, 2004), more research is needed to understand the true effect of pH on the long-term stability of thiamine in solution at food-relevant temperatures without the unintended effect of common buffer salts or protective action of other food components.

It was hypothesized that the salt form of vitamin (TMN vs. TClHCl) and, in effect, counterion in solution (NO_3^- vs. Cl^-) would not affect the stability of thiamine, but rather pH of solution and thiamine concentration would play the most significant roles in dictating thiamine stability. Thus, the objectives of this study were to: 1) investigate the impacts of thiamine concentration and solution pH on thiamine stability in the absence of common buffer salts, 2) compare the effect of counterion of thiamine salts in solution on thiamine stability, and 3) calculate and compare reaction kinetics of pH-dependent thiamine degradation. The results of this study can be used to improve the nutritional quality of food products by better understanding the role of pH on thiamine stability.

3.3 Materials and Methods

3.3.1 Materials

Two salt forms of thiamine were used in this study: thiamine mononitrate, $C_{12}H_{17}N_4OS \bullet NO_3$ (TMN) (Spectrum Chemical Mfg. Corp., New Brunswick, NJ) and thiamine chloride hydrochloride, $C_{12}H_{17}ClN_4OS \bullet HCl$ (TClHCl) (Fisher Scientific, Fair Lawn, NJ). Nitric acid (HNO_3) (J.T. Baker, Center Valley, PA), hydrochloric acid (HCl) (Acros Organics, Fair Lawn, NJ), and sodium hydroxide (NaOH) (Sigma-Aldrich, St. Louis, MO) were used to adjust the pH of thiamine solutions. For use in high performance liquid chromatography (HPLC), HPLC grade acetonitrile and trifluoroacetic acid (TFA) were obtained from Fisher Scientific. All water used

throughout the study was deionized and purified using a Barnstead E-pure ultrapure water purification system with a resistivity greater than 17.5 MΩ·cm at 25°C (ThermoScientific, Waltham, MA).

3.3.2 Sample preparation

Previous studies have investigated the effect of thiamine concentration on stability in solution (Voelker et al., 2018). It was found that while TMN stability was influenced by concentration, TClHCl was less affected, which was attributed to pH. Therefore, to understand the impact of solution pH and concentration on thiamine stability a series of TMN and TClHCl solutions were prepared at two pHs and two concentrations: pHs 3 and 6 at thiamine concentrations 1 and 20 mg/mL. The samples were prepared on a weight basis rather than by molar concentration. Although TMN and TClHCl have slightly different molecular weights, degradation calculations were done using percent remaining, which accounts for the differences in molecular weights. Although the concentrations used in this study were higher than those found in foods, the higher concentration was used to enable more accurate thiamine analysis.

TMN solutions were adjusted to pH 3 and 6 using HNO_3 and NaOH. Nitric acid was used to adjust TMN solutions to limit counterions to only nitrate. TClHCl solutions were adjusted to the same pHs using HCl and NaOH. Hydrochloric acid was used to adjust TClHCl solutions to limit counterions to only chloride. Solutions were also prepared with the alternate acid (TMN with HCl and TClHCl with HNO_3) to determine if counterion influenced thiamine degradation patterns. A previous study by our group was completed in which solution pHs were not adjusted (Voelker et al., 2018). This data was used as a control point for comparison. All solutions (10 mL) were prepared in triplicate in 20 mL amber glass scintillation vials with PE cone-lined phenolic caps and sealed with duct tape to prevent evaporation.

3.3.3 Sample storage

Solutions were stored at 5 temperatures: 25, 40, 60, 70, and 80ºC using a method by Voelker et al. (2018) to investigate the effect of temperature on chemical stability. These temperatures were chosen based on conditions that may be experienced in the food industry, specifically during storage, processing, or accelerated shelf-life testing, and for temperature-

dependent reaction kinetics calculations. The 25°C condition was maintained using a temperature-controlled room. Samples were kept in 40°C, 60°C, and 70°C environments using Forma Scientific water-jacketed incubators (Thermo Fisher Scientific Inc., Marietta, OH). The 80°C temperature was maintained using a digital heatblock (VWR International, Radnor, PA). Temperature was confirmed over the duration of the study using thermometers. Depending on temperature and pH, solutions were stored in controlled temperature environments for up to 1 year. Samples were analyzed in triplicate for percent thiamine remaining at a minimum of 5 selected timepoints.

3.3.4 Vitamin quantification

The chemical stability of thiamine in solution was measured in accordance with an adaptation of AOAC method 942.23 for quantification of thiamine (Eitenmiller et al., 2008). Reverse-phase HPLC (Waters Corp. Milford, MA) using a gradient method with 0.1% TFA in water (*v/v*) and acetonitrile as the mobile phases, A and B, respectively, was used in accordance with our previous study (Voelker et al., 2018). Briefly, a Waters 2690 Separations Module and a Waters 2996 Photodiode Array (PDA) detector were used with a Waters XTerra RP-C_{18} column and a wavelength scan of 235-400 nm. The gradient method was as follows: 100/0 at 0 min, 97/3 at 4 min (linear), 90/10 at 6 min (linear), 100/0 at 10 min (linear), and 100/0 at 15 min. Prior to analysis, solutions were cooled in an ice bath, and diluted with the 0.1% TFA in water mobile phase to an estimated thiamine concentration of 500 ppm, or 0.5 mg/mL (assuming no degradation). Standard curves of TMN and TClHCl (R^2 > 0.999) were prepared to calculate thiamine concentration of samples on each day of analysis using a concentration range of 10 ppm to 1000 ppm. Integration was performed at 254 nm.

3.3.5 Reaction kinetics

Reaction kinetics were calculated to monitor the kinetics of thiamine degradation as affected by pH and counterion in solution using similar calculations to our preceding study (Voelker et al., 2018). Previous work has shown thiamine degradation to be a pseudo first-order reaction (Gregory III, 2008; Mauri et al., 1989; Voelker et al., 2018), and under this assumption, the kinetic rate constants (*k*) were calculated using the following equation:

$$ln\frac{x}{x_0} = -kt \qquad \textit{(eq. 1)}$$

where x is the concentration of thiamine at time t (days), x_0 is the initial thiamine concentration, and k is the reaction rate constant (days $^{-1}$).

The Arrhenius equation was used to describe temperature dependence of k:

$$k = Ae^{\frac{-E_a}{RT}} \qquad \textit{(eq. 2)}$$

where k is the reaction rate constant (days $^{-1}$), A is the frequency factor of collision, E_a is the activation energy (kJ/mol), R is the gas constant (8.3145 J/mol·K), and T is temperature (K). Our previous study (Voelker et al., 2018) found that linear degradation patterns were generally lost when 40% or less of thiamine remained due to side-reactions of the degradation products, so calculations only included data up to that point. The t_{90} values were also calculated to indicate the time at which 90% of the initial thiamine concentration remained (10% had degraded).

3.3.6 pH measurement

The pH of all samples was measured over time to monitor how pH changed from the original pH 3 or 6 value over the duration of the experiment. An Orion pH probe (ThermoScientific) that had been calibrated using pH 1.68, 4.01, and 7.00 calibration standards obtained from ThermoScientific was used in this study. Solution pHs were measured at least 3 times over the duration of the experiment, including a measurement at the first HPLC timepoint (following day 0), at least one midpoint, and the final timepoint of HPLC analysis. Solution pHs were measured in duplicate.

3.3.7 Statistical analysis

All samples were prepared and analyzed by HPLC in triplicate for each timepoint of analysis, and single variable ANOVA using SAS 9.4 (SAS Institute, Cary, NC) with Tukey's post-hoc test for multiple comparisons ($\alpha = 0.05$) was used to determine significant differences in: 1) percent thiamine remaining between the initial solution and the partially degraded sample over time, and 2) percent thiamine remaining between sample types at the same time point. Regression analysis was used to determine standard error of the slopes used to calculate k_{obs} and E_a values, and t_{90} values were calculated to indicate time when 90% of the initial thiamine remained. Single-variable ANOVA was also used to determine significant differences in pH.

3.4 Results and Discussion

3.4.1 Chemical stability of thiamine in pH 6 solutions

Thiamine is often reported to become less stable at a pH of 6 compared to more acidic conditions (Dwivedi & Arnold, 1973; Farrer, 1955; Mauri et al., 1989), so this study analyzed thiamine stability at that pH. Both temperature and concentration were found to significantly ($p < 0.05$) affect the stability of thiamine in pH 6 solutions, with higher temperatures and higher concentrations causing more degradation (Figures 3.2.A, 3.3.A). Generally, percents thiamine remaining in 1 mg/mL solutions at the same temperature and timepoint were not statistically different from one another ($p > 0.05$). Similarly, percents thiamine remaining in 20 mg/mL solutions were not statistically different from one another; however, 1 mg/mL solutions had significantly more thiamine remaining than 20 mg/mL solutions at the same timepoint ($p < 0.05$) (appendix Tables A.3.1, A.3.2). This can be exemplified by the percent thiamine remaining on day 2 following storage at 80ºC. The TMN with HNO_3, TMN with HCl, TClHCl with HNO_3, and TClHCl with HCl solutions (all 1 mg/mL) contained 74, 75, 81, and 74% thiamine, respectively, while the same sample types at the higher 20 mg/mL concentration contained 38, 42, 42, and 42% thiamine, respectively. Thus, all 1 mg/mL samples contained significantly ($p < 0.05$) more thiamine on day 2 than any of the 20 mg/mL samples. This in agreement with what was found in a previous study at similar pHs, but with the unmodified pH only dependent on concentration and thiamine salt form (Voelker et al., 2018).

An example of a typical degradation profile is shown in Figure 3.2.A, in which thiamine degraded in an exponential manner. All percent thiamine remaining data for all temperatures, concentrations, and counterions are provided in the appendix (Tables A.3.1, A.3.2). The pH was also monitored over the duration of the experiment, and an example of a typical pH change over time is shown in Figure 3.2.B. The pH of all samples that were initially at pH 6 remained above 4.5 following storage at all temperatures for the duration of the study, with many samples remaining above a pH of 5. The largest drop in pH came at the first timepoint of analysis, with smaller decreases coming at each subsequent timepoint. The 20 mg/mL solutions dropped to lower pHs than 1 mg/mL solutions. The change in pH was presumably caused by the degradation products present in solution following partial degradation. This same lowering of pH over time was also seen in our previous study (Voelker et al., 2018). Overall, the lowering of pH over the duration of the study was not considered to affect the thiamine stability due to the high correlations

of linear regressions used to calculate reaction kinetics even as pH decreased. Tables including all pH data over time for all temperatures, concentrations, and counterions are also provided in the appendix (Tables A.3.3, A.3.4).

3.4.2 Degradation kinetics of thiamine in pH 6 solutions

Due to the apparent first-order reaction behavior observed in the pH 6 solutions, eq. 1 was used to calculate the observed reaction rate constant (k_{obs}) for each sample preparation. High correlations were observed for all linear regressions of the natural log of percent thiamine remaining vs. time (R^2 = 0.82 – 0.99). These high correlations verified that initial degradation followed first-order reaction kinetics. All k_{obs}, R^2, and t_{90} values are reported in Table 3.1, and a typical example of linear regressions for the range of temperatures studied is shown in Figure 3.3.A. The general trend was that at a specified temperature, all 1 mg/mL solutions had k_{obs} values that were not statistically different from one another ($p > 0.05$), and 20 mg/mL solutions all had k_{obs} values that were not statistically different from one another; however, k_{obs} values for 20 mg/mL solutions were significantly ($p < 0.05$) higher than k_{obs} values for 1 mg/mL solutions. For example, at 70ºC, solutions of TMN with HNO_3, TMN with HCl, TClHCl with HNO_3, and TClHCl with HCl (all 1 mg/mL) had k_{obs} values of 0.043, 0.040, 0.046, and 0.047 day^{-1}, respectively; conversely, the same sample types at the higher 20 mg/mL concentration had k_{obs} values of 0.43, 0.40, 0.39, and 0.38 day^{-1}, respectively. Thus, all k_{obs} values of 20 mg/mL solutions were significantly higher ($p < 0.05$) than the k_{obs} values of 1 mg/mL samples. Differences between k_{obs} values for each thiamine concentration were found to be larger in this study than the previous study by Voelker et al. (2018); however, the previous study did not control the initial pH, thus the difference in pH due to difference in concentration could account for the discrepancy. The k_{obs} values found in this study were consistent with those found at ambient temperatures in buffered solutions at 0.2 mg/mL TClHCl and pH 5-6, though it was noted that buffer type and concentration greatly affected these values (Pachapurkar & Bell, 2005). Although there have been more reports of thiamine degradation kinetics in aqueous solutions, most are done at much higher temperatures so are therefore not compared here (Guzman-Tello & Cheftel, 1987; Mulley et al., 1975b; Ramaswamy et al., 1990).

Generally, a first-order reaction should have the same k value, regardless of starting concentration, and only rate should change, which is consistent with some thiamine degradation

kinetics studies (Ryan-Stoneham, Tong, & Clark, 1997; Windheuser & Higuchi, 1962); however, this was not the case in the current study. Previous studies have shown concentration in solution to affect k_{obs} values, for example, in some green tea catechins (N. Li, Taylor, Ferruzzi, & Mauer, 2012). This is generally attributed to the existence of multiple degradation pathways, which is known to be true of thiamine degradation. Another possible explanation for the change in k may be that the reaction order is not actually 1. Using the van't Hoff method, the order of this reaction was calculated to be approximately 1.3. Fractional order reactions are common when degradation products participate in subsequent chemical chain reactions, which is probable in the case of thiamine degradation (Dwivedi & Arnold, 1973; Laidler, 1987). In weakly acidic to neutral solutions (e.g., pH 6), thiamine is susceptible to hydrolysis in which the methylene bridge is broken, resulting in intact pyrimidine and thiazole moieties (Güntert et al., 1992; Hosny, Zaki, Mokbel, & Abdelhamid, 2019; Mabkhot et al., 2019; Windheuser & Higuchi, 1962). The resulting intact rings are then likely to undergo subsequent reactions. If consecutive reactions are occurring, i.e. the degradation products are further reacting, the degradation reaction becomes:

$$Thiamine \overset{k_1}{\rightarrow} degradation\ product\ 1 \overset{k_2}{\rightarrow} degradation\ product\ 2$$

in which concentration of thiamine and concentration of degradation product 1 both affect the reaction order, and k_1 and k_2 both contribute to k_{obs} (Yoshioka & Stella, 2000). It is also possible that thiamine and its degradation products react with one another, contributing an additional k value that also affects k_{obs}. This consequently results in a reaction order between 1 and 2, a range which encompasses the reaction order of 1.3 in the case of this study. Since the concentration of degradation product 1 is affected by the initial concentration of thiamine, it is therefore possible that the observed k value, which incorporates both k_1 and k_2, and was calculated with first-order reaction equations, was affected by the initial concentration of thiamine. Concerning thiamine, the model of the participation of degradation products in consecutive degradation reactions is simplified, in which probable degradation products and subsequent consecutive reactions are much greater (Dwivedi & Arnold, 1973; Güntert et al., 1992). In agreement with the proposed consecutive reaction mechanisms, it has also been suggested previously that the overall observed rate of thiamine degradation is actually a summation of a large number of separate reactions (Windheuser & Higuchi, 1962). Thus, since the k_{obs} values reported in this study were presumably a function of a substantial number of k values, the variation in k_{obs} was dependent on initial thiamine concentration.

Additionally, as ionic strength increases in thiamine solutions, k values for thiamine degradation are significantly increased, specifically at weakly acidic or neutral pHs (Windheuser & Higuchi, 1962). Since thiamine solutions in this study were prepared using salt forms of thiamine, it is possible that increased ionic strength in higher concentration solutions played a role in the increased k values. Although rate constant has been reported to be independent of initial thiamine concentration in some previous studies, these systems were pH adjusted using buffers (Windheuser & Higuchi, 1962) or unadjusted in food systems (Ryan-Stoneham et al., 1997), which provide additional considerations to thiamine stability.

E_a was calculated using the natural log of the temperature-dependent k_{obs} values for each sample type (R^2 = 0.9465 – 0.9718). The Arrhenius plots used to calculate E_a are provided in Figure 3.3.B, and calculated E_as are reported in Table 3.2. In pH 6 solutions, E_as ranged from 18-21 kcal/mol, with only TMN with HCl 1 and 20 mg/mL significantly differing from one another ($p < 0.05$); thus, it was concluded that all pH 6 samples underwent the same degradation pathway. These values were slightly lower than E_as found for dilute solutions in previous studies at similar pHs (Guzman-Tello & Cheftel, 1987; Mulley et al., 1975a; Ramaswamy et al., 1990; Voelker et al., 2018; Windheuser & Higuchi, 1962); however, the calculated values in this study are still in the general range reported for thiamine degradation overall (20-30 kcal/mol) (Kamman et al., 1981; Mauri et al., 1992).

3.4.3 Chemical stability of thiamine in pH 3 solutions

To analyze thiamine stability in an acidic environment, thiamine solutions were adjusted to pH 3 and monitored for stability over time. Both temperature and molar concentration were found to significantly ($p < 0.05$) affect the stability of thiamine in pH 3 solutions, with higher temperatures and higher molar concentrations causing faster degradation (Figures 3.4.A, 3.5.A). Generally, percent thiamine remaining in 1 mg/mL and 20 mg/mL solutions at the same temperature and timepoint were not statistically different from one another ($p > 0.05$). This can be exemplified by the percent thiamine remaining on day 91 following storage at 60ºC. Solutions of TMN with HNO_3, TMN with HCl, TClHCl with HNO_3, and TClHCl with HCl (all 1 mg/mL) contained 81, 80, 83, and 78% thiamine, respectively, and the same sample types at 20 mg/mL contained 82, 83, 82, and 84% thiamine, respectively. However, when comparing molar concentrations instead of percent thiamine remaining, the 20 mg/mL solutions tended to degrade

faster than 1 mg/mL solutions. This is typical of a first-order reaction and is in agreement with what was found in previous studies at a similar pH (Gregory III, 2008; Voelker et al., 2018). Thiamine was exceptionally stable over the one-year experiment period in pH 3 solutions when stored at 25ºC or 40ºC. After 392 days of storage at these temperatures, the thiamine content in all solutions remained above 91% of the initial concentration; in most cases, there was no significant ($p < 0.05$) degradation over the 392-day period. This suggests that in an acidic environment, thiamine will remain quite stable if kept below 40ºC.

An example of a typical degradation profile of thiamine at pH 3 is shown in Figure 3.4.A, with data for all temperatures, concentrations, and counterions provided in the appendix (Tables A.3.1, A.3.2). The pH was also monitored over the duration of the experiment, and an example of a typical pH change over time for solutions that were initially pH 3 is shown in Figure 3.4.B. The pH of all samples following storage remained above 2 for the duration of the study, with most samples remaining above a pH of 2.5. The pH gradually decreased over the duration of the experiment, with 20 mg/mL solutions dropping to lower pHs than 1 mg/mL solutions. Tables including all pH data over time for all temperatures, concentrations, and counterions can be found in the appendix (Tables A.3.3, A.3.4).

3.4.4 Degradation kinetics of thiamine in pH 3 solutions

Using the van't Hoff method, the reaction order of thiamine degradation in pH 3 solutions was calculated to be 1, consistent with reports of thiamine degradation as a first-order reaction (Gregory III, 2008; Voelker et al., 2018). Therefore, eq. 1 was used to calculate the observed reaction rate constant (k_{obs}) for each sample preparation of pH 3 solutions. High correlations were observed for all linear regressions of the natural log of percent thiamine remaining vs. time (R^2 = 0.92 – 0.995), which, in addition to the van't Hoff calculations, verified that initial thiamine degradation in pH 3 solutions followed first-order reaction kinetics. All k_{obs}, R^2, and t_{90} values are reported in Table 3.1, and a typical example of linear regressions for the range of temperatures studied is shown in Figure 3.5.A. Although Figure 3.5.A includes linear regressions for all temperatures studied, not enough thiamine degradation at 25ºC or 40ºC occurred over the duration of the one-year experiment to allow subsequent reaction kinetics calculations from these temperatures. Thus, reaction kinetics for pH 3 solutions were only calculated for the temperatures 60, 70, and 80ºC.

At a specified temperature, 1 mg/mL and 20 mg/mL thiamine solutions had no k_{obs} values that were statistically different from one another ($p > 0.05$). For example, at 60ºC, TMN with HNO_3, TMN with HCl, TClHCl with HNO_3, and TClHCl with HCl (all 1 mg/mL) had k_{obs} values of 0.00243, 0.00253, 0.00246, and 0.00275 day^{-1}, respectively, and the same sample types at 20 mg/mL had k_{obs} values of 0.00251, 0.00249, 0.00268, and 0.00259 day^{-1}, respectively. The k_{obs} values obtained in this study for thiamine solutions at pH 3 were similar to those reported in a previous study in solutions of the same concentrations and a similar pH range, although pH was unmodified in that study (Voelker et al., 2018).

Unlike what was found in pH 6 solutions in this study, thiamine degradation in the different pH 3 solutions all had the same k value, regardless of initial thiamine concentration, which follows what is expected of a first-order reaction and is in agreement with studies by Ryan-Stoneham et al. (1997) and Windheuser and Higuchi (1962). Based on previous studies in which sensory tests were completed to compare sensory properties of thiamine degraded in acidic vs. close to neutral solutions, we know that the degradation pathway differs between pH 3 and pH 6 solutions (Voelker et al., 2018). The difference in degradation pathway was presumably due to hydrolysis of the pyrimidine and thiazole moieties of thiamine not being the major degradation pathway in the pH 3 environment, as has been suggested previously (Windheuser & Higuchi, 1962). Thus, the first thiamine degradation step in pH 3 solutions was presumably the rate-determining step. Consequently, k values of the consecutive reactions of the degradation products did not significantly affect the k_{obs} values, resulting in k_{obs} values at pH 3 that were not statistically different from one another. Additionally, it has been reported that although k values of thiamine degradation are highly dependent on ionic strength in pH 6 solutions, k values in acidic pHs are independent of ionic strength (Windheuser & Higuchi, 1962); thus, the difference in ionic strength resulting from the different concentrations of thiamine salt forms did not play a role in k values of thiamine degradation in pH 3 solutions as was found in pH 6 solutions.

E_a was calculated using the natural log of the temperature-dependent k_{obs} values for each pH 3 sample type ($R^2 = 0.9861 – 0.9990$). The Arrhenius plots used to calculate E_a are shown in Figure 3.5.B, and calculated E_a values are reported in Table 3.2. In thiamine solutions at pH 3, E_a values ranged from 21-27 kcal/mol. There were some significant differences between E_a values ($p < 0.05$); however, the small range of E_a values indicates that all pH 3 sample preparations likely underwent the same degradation pathway. Although reports of reaction kinetics of thiamine

degradation at approximately pH 3 are limited, the E_a values found in this study are in ranges reported previously (20-30 kcal/mol), albeit at different pHs and complexity of the systems (Feliciotti & Esselen, 1957; Guzman-Tello & Cheftel, 1987; Kamman et al., 1981; Mauri et al., 1992; Mulley et al., 1975a; Ramaswamy et al., 1990). In similar pH systems, including without the use of buffer salts, the E_a values in this study are also in accordance with what has previously been reported (Voelker et al., 2018; Windheuser & Higuchi, 1962).

3.4.5 Comparison of pH- and counterion-dependent thiamine stability

The stability of thiamine in solution was significantly higher in pH 3 solutions than in pH 6 solutions (Figure 3.6), consistent with reports at many temperatures (from ambient to those found during processing) as well as in a variety of matrices (buffer systems and food products), commonly attributed to different thiamine degradation mechanisms at different pHs (Dwivedi & Arnold, 1972; Mulley et al., 1975b; Pachapurkar & Bell, 2005; Windheuser & Higuchi, 1962). Although all pH 3 and 1 mg/mL pH 6 solutions tended to have k_{obs} values that were not statistically different from one another ($p > 0.05$), the 20 mg/mL pH 6 solutions had k_{obs} values a factor of 10 greater than k_{obs} values for pH 3 solutions in all cases (Table 3.1), indicating increased stability in all pH 3 solutions. In addition, the E_as of thiamine degradation in pH 6 solutions tended to be significantly ($p < 0.05$) lower than the E_as in pH 3 solutions (Table 3.2). The k_{obs} values, E_as, and the percent remaining graphs over time at each temperature (Figure 3.6) verify previous reports that thiamine is more stable in acidic environments (Dwivedi & Arnold, 1973; Voelker et al., 2018). Additionally, since the E_a of thiamine degradation was higher in pH 3 solutions than in pH 6 solutions, it was concluded that the thiamine degradation pathway in the two pH environments differed, as was also suggested by sensory data in our previous study (Voelker et al., 2018). However, as expected, both concentrations had the same E_a at their respective pHs, indicating that thiamine concentration does not affect degradation pathway.

Since the presence of salts is known to affect thiamine stability, we prepared thiamine solutions adjusted to both pHs (3 and 6) using both salt forms of thiamine (TMN and TClHCl) adjusted with acids (HNO_3 or HCl) that would either isolate one counterion in solution or introduce both salt form counterions (NO_3^- or Cl^-) in solution to determine if counterion had an effect on stability. The k_{obs} and E_a values (Tables 3.1 and 3.2) as well as percent thiamine remaining over time (Figure 3.6) illustrated that regardless of counterion(s) present in solution, the thiamine

degradation reaction proceeded in the same manner. Additionally, all E_as calculated in this study were similar to those reported at the same concentrations and pHs without pH adjustment (Voelker et al., 2018); thus, it was determined that the presence of Cl^- or NO_3^- did not trigger a change in the thiamine degradation pathway in this study. Therefore, pH and concentration were considered as the sole factors contributing to thiamine degradation kinetics in this study.

The extent of the effect of pH on reaction kinetics of thiamine degradation can be quantified by graphing the log of k_{obs} as a function of pH, in which a resulting slope of 1 would indicate an ideal acid-base catalyzed reaction. In studies by Pachapurkar and Bell (2005) and Windheuser and Higuchi (1962), it was found that although there is a high correlation between log of the rate constant and pH (in the pH range 4-7), the slope of the plot indicates that the effects of pH are more complex than the ideal acid-base catalyzed reaction. They also found that the sensitivity of thiamine degradation to pH is dependent on the type of buffer, in which thiamine in a phosphate buffer system is more sensitive to pH than in a citrate buffer system, and correlation between reaction rate constant and pH is lower in citrate buffers than in phosphate buffers, presumably due to the stronger ability of phosphate to deprotonate thiamine than citrate (Pachapurkar & Bell, 2005). Although the current study used only two pHs (3 and 6), when the data were plotted as $\log(k_{obs})$ vs. pH, the slopes for 1 mg/mL and 20 mg/mL thiamine solutions were 0.23 – 0.30 and 0.45 – 0.68, respectively, dependent on temperature. As in the previous studies, these slopes indicated that the thiamine degradation reaction is less dependent on pH than an ideal acid-base catalyzed reaction. Additionally, these values suggest that the stability of thiamine in 20 mg/mL solutions is more influenced by a change in pH than in 1 mg/mL solutions. Overall, it was concluded that a change in solution pH caused a change in the rate of thiamine degradation (and rate constant), the E_a of degradation, the degradation pathway, and therefore, the resulting degradation products, which has been shown in previous studies to have a significant sensory impact due to sulfur containing degradation products (Buttery et al., 1984; Dwivedi et al., 1973; Voelker et al., 2018).

3.4.6 Potential impact on food formulation

Although this study investigated thiamine stability in simple aqueous solutions at higher concentrations than are often found in food products, the fundamental reaction mechanisms of thiamine degradation can be used to predict the responses in a variety of food formulations and vitamin supplements. Thiamine has been reported to be more stable in food systems than in buffer

systems, and thiamine degradation has been shown to both deviate from and to follow first-order reaction kinetics in food products (Bendix, Heberlein, Ptak, & Clifcorn, 1951; Mulley et al., 1975a, 1975b). Regardless of order of reaction, certain components in food are known to affect the stability of thiamine. For example, α- and β-amino acids and their derivatives, proteins, and starch have been shown to stabilize thiamine in foods, but salts and sulfites are known to destabilize thiamine (Farrer, 1955; McIntire & Frost, 1944; Mulley et al., 1975a; Pizzoferrato, 1992).

Many foods, including fruit juices, sports drinks, and energy drinks, offer the protective effect of an acidic environment on thiamine. Many other thiamine-containing foods, such as eggs, milk, infant formulas, and other dairy-based nutritional beverages, have a close to neutral or even slightly alkaline pH, which was shown to significantly decrease thiamine stability. The pH- and concentration-based reaction kinetics in this study can be used to predict thiamine stability in a variety of food products. While thiamine will behave differently in most distinctive matrices, the degradation kinetics reported in this study provide a basis for this understanding using the fundamental stability of thiamine. Analyzing thiamine behavior in model food-formulations using guidance from the conclusions of this study may also extend the implications of this study to include an understanding of thiamine in specific food formulations.

3.5 Conclusion

Degradation kinetics of thiamine in solution were shown to be highly dependent on pH, concentration, and storage temperature, but the degradation was not affected by counterion present (NO_3^- vs. Cl^-) in the aqueous solutions. Thiamine was significantly ($p < 0.05$) more stable in pH 3 solutions than in pH 6 solutions. Additionally, differences in E_a values found for thiamine degradation at the two pHs indicated a difference in degradation reaction pathway between the two solution environments. All thiamine degradation was shown to follow first-order reaction kinetics; however, thiamine at pH 6 degraded via a pseudo first-order reaction (reaction order 1.3), whereas thiamine at pH 3 degraded via an ideal first-order reaction. The initial thiamine concentration was found to have a significant effect on thiamine stability in pH 6 solutions, with higher concentrations increasing k_{obs}, but k_{obs} values of thiamine in pH 3 solutions were not dependent on initial concentration. This difference was due to the difference in thiamine degradation pathway at different pHs as well as differences of response to ionic strength: ionic strength affects k values in pH 6 solutions but not in pH 3 solutions. This study developed long term thiamine stability studies

focusing on the effect of pH and thiamine concentration without the use of buffers. The fundamental understanding of the response of thiamine to a variety of matrices and temperatures can be used to improve thiamine delivery in food products.

3.6 Tables and Figures

Table 3.1 Rate constants and t_{90} values for thiamine in solutions of TMN and TClHCl.

pH	Vitamin Salt Form	Acid	Concentration (mg/mL)	Reaction Kinetics	25°C	40°C	60°C	70°C	80°C
3	TMN	HNO_3	1	k_{obs} (day^{-1})	-	-	0.00243 ± 5e-5 ^{C}	0.0082 ± 5e-4 ^{B}	0.0250 ± 6e-4 ^{C}
				R^2	-	-	0.9894	0.9340	0.9897
				t_{90}^{*} (days)	-	-	43.3 ± 0.9 ^{a}	12.9 ± 0.7 ^{c}	4.2 ± 0.1 ^{e}
			20	k_{obs} (day^{-1})	-	-	0.00251 ± 6e-5 ^{C}	0.0061 ± 4e-4 ^{B}	0.0207 ± 8e-4 ^{C}
				R^2	-	-	0.9847	0.9206	0.9710
				t_{90}^{*} (days)	-	-	42 ± 1 ^{ab}	17 ± 1 ^{a}	5.1 ± 0.2 ^{cd}
		HCl	1	k_{obs} (day^{-1})	-	-	0.00253 ± 9e-5 ^{C}	0.0084 ± 4e-4 ^{B}	0.0253 ± 5e-4 ^{C}
				R^2	-	-	0.9664	0.9598	0.9920
				t_{90}^{*} (days)	-	-	42 ± 2 ^{abc}	12.5 ± 0.5 ^{c}	4.17 ± 0.09 ^{e}
			20	k_{obs} (day^{-1})	-	-	0.00249 ± 7e-5 ^{C}	0.0069 ± 4e-4 ^{B}	0.0208 ± 3e-4 ^{C}
				R^2	-	-	0.9817	0.9349	0.9950
				t_{90}^{*} (days)	-	-	42 ± 1 ^{ab}	15.2 ± 0.9 ^{b}	5.06 ± 0.08 ^{d}
	TClHCl	HNO_3	1	k_{obs} (day^{-1})	-	-	0.00246 ± 5e-5 ^{C}	0.0068 ± 4e-4 ^{B}	0.0177 ± 6e-4 ^{C}
				R^2	-	-	0.9893	0.9283	0.9829
				t_{90}^{*} (days)	-	-	42.8 ± 0.9 ^{ab}	15.4 ± 0.9 ^{b}	6.0 ± 0.2 ^{b}
			20	k_{obs} (day^{-1})	-	-	0.00268 ± 8e-5 ^{C}	0.0068 ± 3e-4 ^{B}	0.0160 ± 4e-4 ^{C}
				R^2	-	-	0.9783	0.9657	0.9900
				t_{90}^{*} (days)	-	-	39 ± 1 ^{cd}	15.5 ± 0.6 ^{b}	6.6 ± 0.2 ^{a}
		HCl	1	k_{obs} (day^{-1})	-	-	0.00275 ± 6e-5 ^{C}	0.0088 ± 4e-4 ^{B}	0.0245 ± 6e-4 ^{C}
				R^2	-	-	0.9896	0.9584	0.9918
				t_{90}^{*} (days)	-	-	38.3 ± 0.8 ^{d}	12.0 ± 0.5 ^{c}	4.3 ± 0.1 ^{e}
			20	k_{obs} (day^{-1})	-	-	0.00259 ± 9e-5 ^{C}	0.0068 ± 3e-4 ^{B}	0.0195 ± 6e-4 ^{C}
				R^2	-	-	0.9674	0.9638	0.9837
				t_{90}^{*} (days)	-	-	41 ± 1 ^{bcd}	15.5 ± 0.6 ^{b}	5.4 ± 0.2 ^{c}

Table 3.1 continued

pH	Vitamin Salt Form	Acid	Concen-tration (mg/mL)	Reaction Kinetics	25°C	40°C	60°C	70°C	80°C
6	TMN	HNO_3	1	k_{obs} (day^{-1})	0.00111 ± 0.00004 ^{B}	0.00149 ± 0.00006 ^{C}	0.022 ± 0.001 ^{C}	0.043 ± 0.002 ^{B}	0.122 ± 0.004 ^{B}
				R^2	0.9712	0.9446	0.9330	0.9518	0.9883
				t_{90}^{*} (days)	95 ± 3 ^{c}	70. ± 3 ^{b}	4.7 ± 0.3 ^{e}	2.5 ± 0.1 ^{d}	0.86 ± 0.03 ^{f}
			20	k_{obs} (day^{-1})	0.0058 ± 0.0004 ^{A}	0.023 ± 0.002 ^{A}	0.31 ± 0.03 ^{A}	0.43 ± 0.06 ^{A}	0.48 ± 0.04 ^{A}
				R^2	0.9261	0.9431	0.9312	0.8945	0.9464
				t_{90}^{*} (days)	18 ± 1 ^{d}	4.6 ± 0.4 ^{c}	0.34 ± 0.03 ^{f}	0.24 ± 0.03 ^{e}	0.22 ± 0.02 ^{g}
		HCl	1	k_{obs} (day^{-1})	0.00067 ± 0.00005 ^{B}	0.00133 ± 0.00007 ^{C}	0.0196 ± 0.0007 ^{C}	0.040 ± 0.001 ^{B}	0.129 ± 0.005 ^{B}
				R^2	0.8943	0.9199	0.9771	0.9820	0.9803
				t_{90}^{*} (days)	160 ± 10 ^{a}	79 ± 4 ^{a}	5.4 ± 0.2 ^{e}	2.63 ± 0.09 ^{d}	0.82 ± 0.03 ^{f}
			20	k_{obs} (day^{-1})	0.0058 ± 0.0003 ^{A}	0.023 ± 0.002 ^{A}	0.29 ± 0.03 ^{AB}	0.40 ± 0.05 ^{A}	0.44 ± 0.06 ^{A}
				R^2	0.9259	0.9434	0.9238	0.9051	0.8809
				t_{90}^{*} (days)	18 ± 1 ^{d}	4.6 ± 0.4 ^{c}	0.37 ± 0.03 ^{f}	0.26 ± 0.03 ^{e}	0.24 ± 0.03 ^{g}
	TClHCl	HNO_3	1	k_{obs} (day^{-1})	0.00080 ± 0.00003 ^{B}	0.00129 ± 0.00005 ^{C}	0.019 ± 0.001 ^{C}	0.046 ± 0.001 ^{B}	0.098 ± 0.008 ^{BC}
				R^2	0.9616	0.9621	0.9422	0.9918	0.9244
				t_{90}^{*} (days)	131 ± 5 ^{b}	82 ± 3 ^{a}	5.5 ± 0.3 ^{e}	2.28 ± 0.05 ^{d}	1.08 ± 0.09 ^{f}
			20	k_{obs} (day^{-1})	0.0058 ± 0.0004 ^{A}	0.015 ± 0.002 ^{B}	0.26 ± 0.04 ^{B}	0.39 ± 0.05 ^{A}	0.43 ± 0.07 ^{A}
				R^2	0.8974	0.8154	0.8243	0.8890	0.8289
				t_{90}^{*} (days)	18 ± 1 ^{d}	6.8 ± 0.9 ^{c}	0.41 ± 0.06 ^{f}	0.27 ± 0.04 ^{e}	0.24 ± 0.04 ^{g}
		HCl	1	k_{obs} (day^{-1})	0.00102 ± 0.00006 ^{B}	0.00136 ± 0.00005 ^{C}	0.019 ± 0.001 ^{C}	0.047 ± 0.001 ^{B}	0.107 ± 0.003 ^{BC}
				R^2	0.9156	0.9576	0.9334	0.9896	0.9903
				t_{90}^{*} (days)	103 ± 6 ^{c}	77 ± 3 ^{ab}	5.4 ± 0.3 ^{e}	2.25 ± 0.06 ^{d}	0.99 ± 0.02 ^{f}
			20	k_{obs} (day^{-1})	0.0058 ± 0.0004 ^{A}	0.016 ± 0.002 ^{B}	0.25 ± 0.04 ^{B}	0.38 ± 0.05 ^{A}	0.42 ± 0.06 ^{A}
				R^2	0.8957	0.8263	0.8293	0.9076	0.8621
				t_{90}^{*} (days)	18 ± 1 ^{d}	6.7 ± 0.9 ^{c}	0.41 ± 0.06 ^{f}	0.27 ± 0.03 ^{e}	0.25 ± 0.04 ^{g}

* t_{90} indicates time when 90% of the initial concentration of thiamine remains

Uppercase superscript letters denote statistical significance of k_{obs} within a temperature (down columns)

Lowercase superscript letters denote statistical significance of t_{90} within a temperature (down columns)

Standard error of the slope was used for statistical calculations

Table 3.2 Calculated activation energies as a function of temperature.

pH	Vitamin Salt Form	Acid	Concentration (mg/mL)	E_a (kcal/mol)	E_a (kJ/mol)
3	TMN	HNO_3	1	27.2 ± 0.3 [a]	114 ± 1 [a]
			20	25 ± 1 [ab]	103 ± 5 [ab]
		HCl	1	26.9 ± 0.3 [a]	113 ± 1 [a]
			20	24.8 ± 0.5 [ab]	104 ± 2 [ab]
	TClHCl	HNO_3	1	23.1 ± 0.4 [bc]	97 ± 2 [bc]
			20	20.9 ± 0.3 [cd]	87 ± 1 [cd]
		HCl	1	25.6 ± 0.3 [ab]	107 ± 1 [ab]
			20	23.6 ± 0.5 [b]	99 ± 2 [b]
6	TMN	HNO_3	1	19 ± 1 [de]	79 ± 5 [de]
			20	18 ± 1 [de]	77 ± 5 [de]
		HCl	1	21 ± 1 [cd]	87 ± 4 [cd]
			20	18 ± 1 [e]	75 ± 5 [e]
	TClHCl	HNO_3	1	20. ± 1 [de]	82 ± 5 [de]
			20	18 ± 1 [de]	77 ± 5 [de]
		HCl	1	19 ± 1 [de]	80. ± 5 [de]
			20	18 ± 1 [de]	76 ± 5 [de]

Superscript letters denote statistical significance of E_a (down columns)
Standard error of the slope was used for statistical calculations

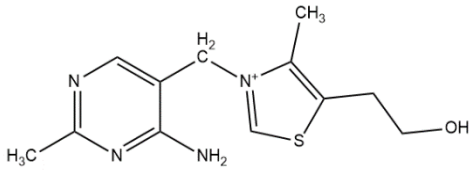


Figure 3.1 Chemical structure of thiamine.

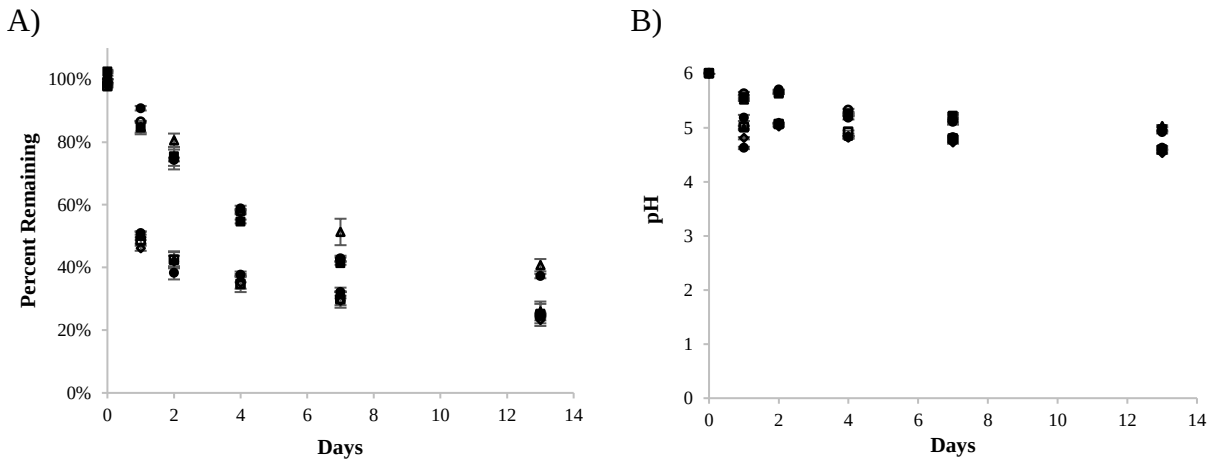
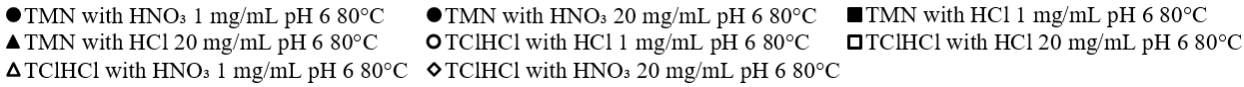


Figure 3.2 Chemical behavior of thiamine in pH 6 solutions with varying counterion (NO_3^- or Cl^-) and concentration (1 or 20 mg/mL) at 80ºC over time including: A) degradation profiles and B) pH profiles.



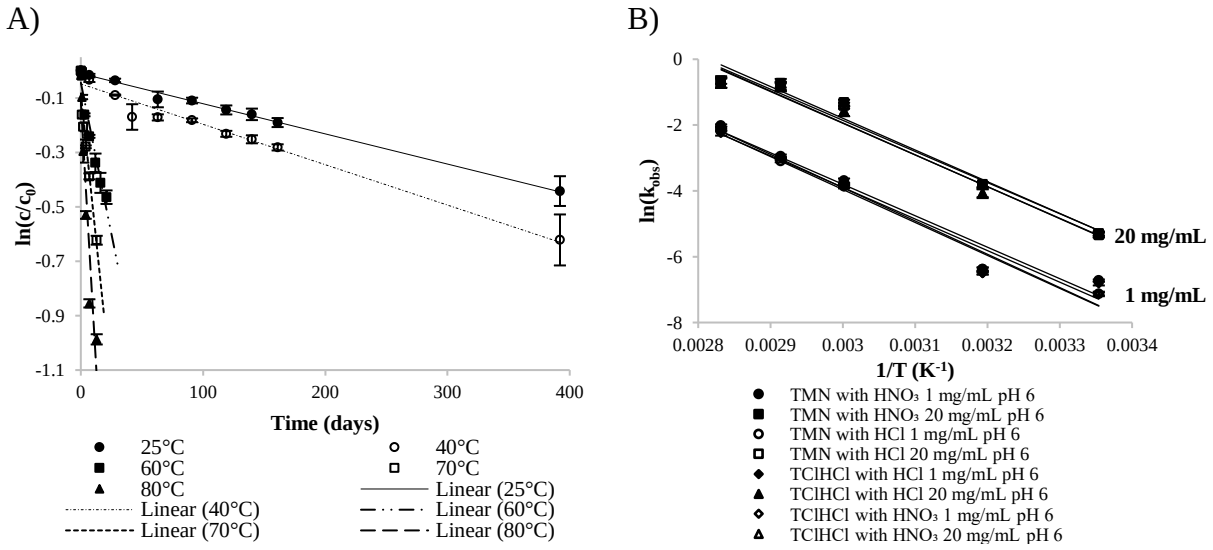


Figure 3.3 Reaction kinetics of thiamine degradation in pH 6 thiamine solutions: A) first-order degradation regression lines of 1 mg/mL TMN solutions adjusted to pH 6 with HNO_3 and NaOH at temperatures from 25-80ºC; and B) Arrhenius plots used to calculate temperature-dependent activation energy for thiamine degradation in 1 and 20 mg/mL thiamine solutions adjusted to pH 6 with HNO_3 or HCl and NaOH from 25-80ºC.

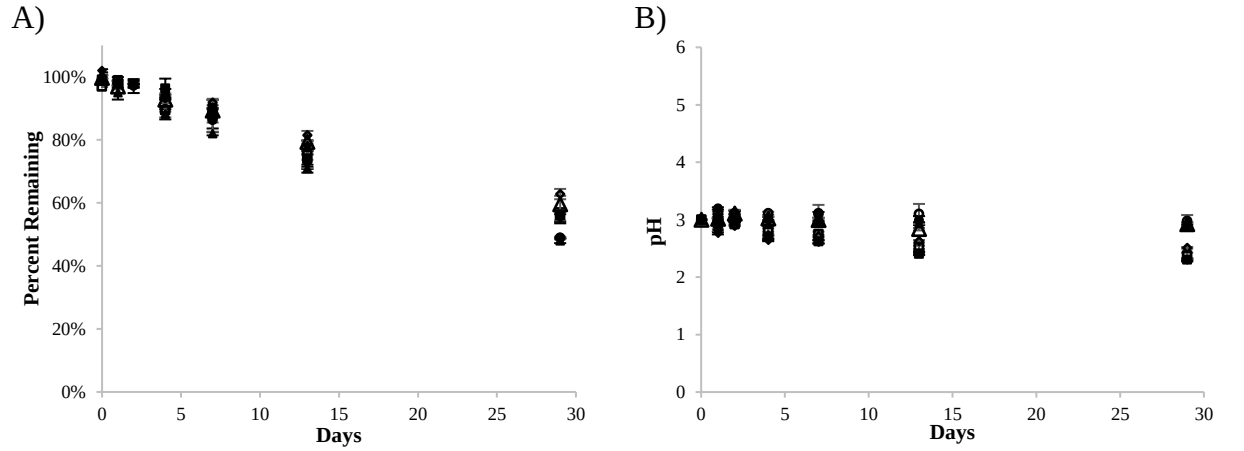


Figure 3.4 Chemical behavior of thiamine in pH 3 solutions with varying counterion (NO_3^- or Cl^-) and concentration (1 or 20 mg/mL) at 80ºC over time including: A) degradation profiles and B) pH profiles.

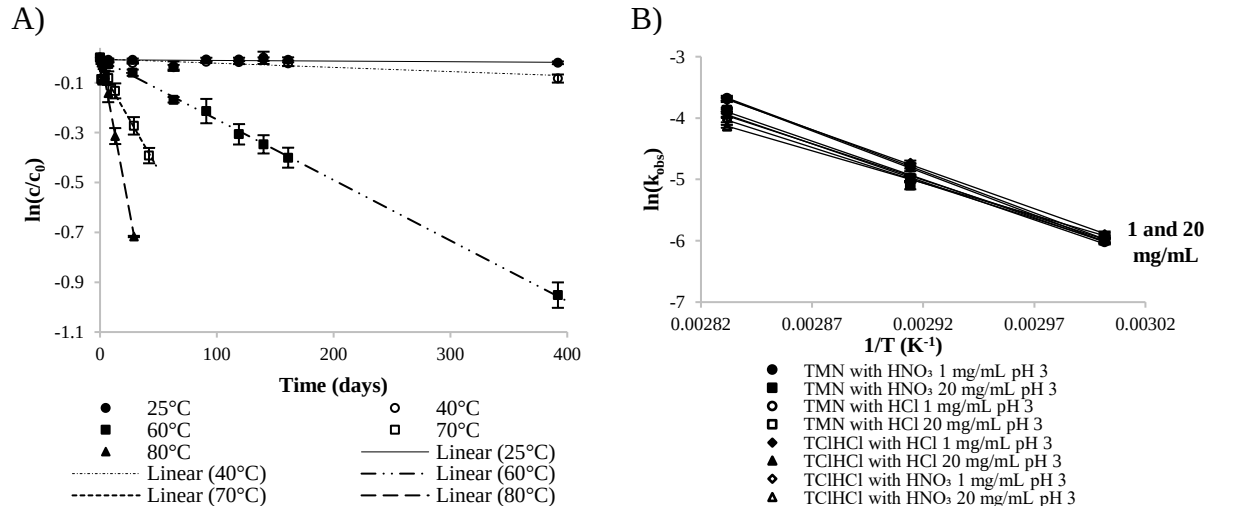


Figure 3.5 Reaction kinetics of thiamine degradation in pH 3 thiamine solutions: A) first-order degradation regression lines of 1 mg/mL TMN solutions adjusted to pH 3 with HNO_3 and NaOH at temperatures from 25-80ºC; and B) Arrhenius plots used to calculate temperature-dependent activation energy for thiamine degradation in 1 and 20 mg/mL thiamine solutions adjusted to pH 3 with HNO_3 or HCl and NaOH from 60-80ºC.

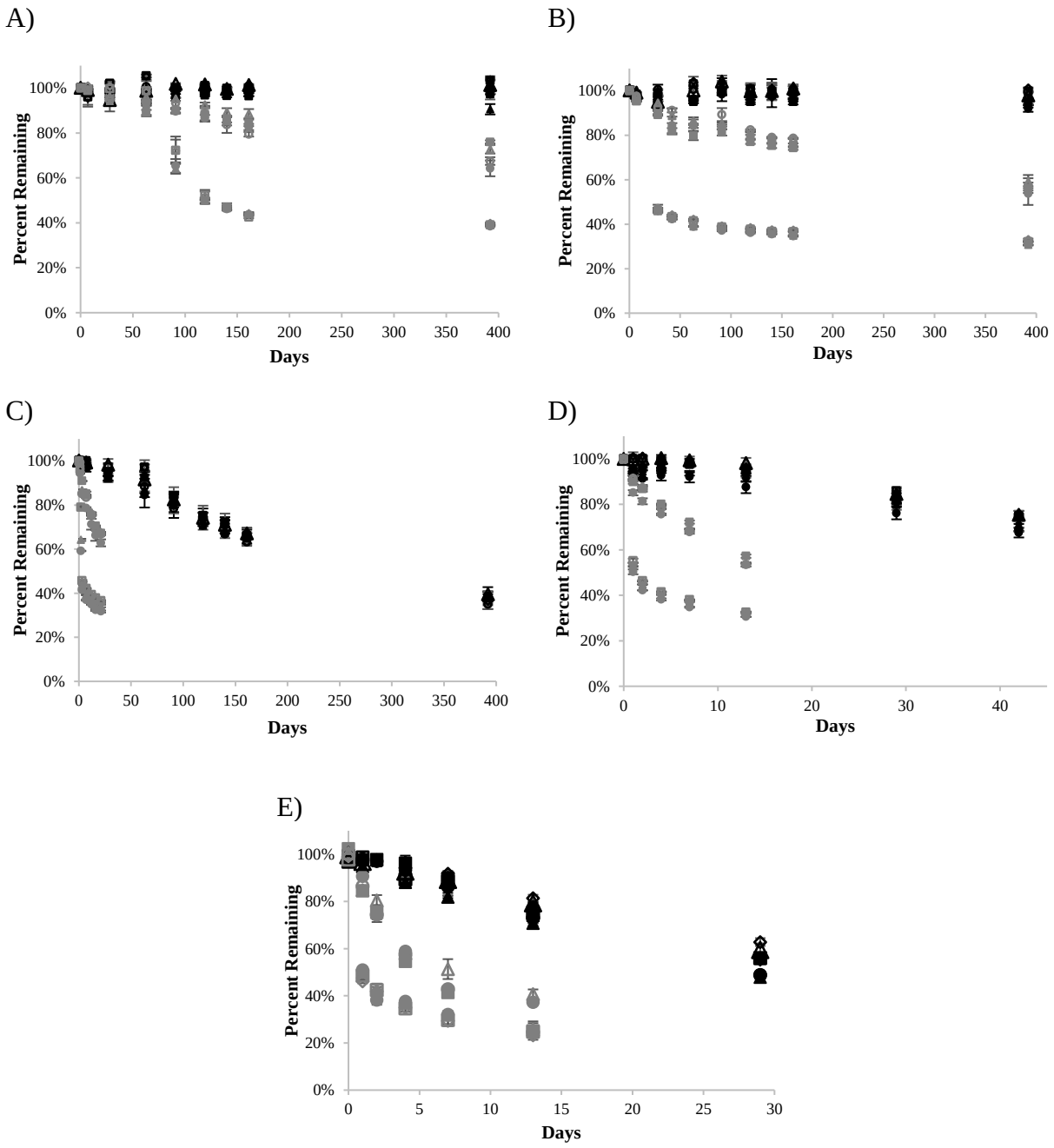


Figure 3.6 Comparison of chemical stability over time of thiamine in pH 3 vs. pH 6 solutions at 1 and 20 mg/mL concentrations stored at A) 25ºC, B) 40ºC, C) 60ºC, D) 70ºC, E) 80ºC.

3.7 References

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CHAPTER 4. CHEMICAL STABILITY AND REACTION KINETICS OF THIAMINE MONONITRATE IN THE AQUEOUS PHASE OF BREAD DOUGH

4.1 Abstract

Thiamine is a water-soluble essential micronutrient, and grains are the main source of thiamine in the human diet. Refining processes reduce thiamine content; therefore, many flours are enriched with thiamine. Further processes, such as heating (baking), destabilize thiamine. In doughs, thiamine partitions into the aqueous phase (dough liquor). The objective of this study was to document temperature effects on thiamine degradation reaction kinetics in dough liquor. Two concentrations of thiamine mononitrate (1 and 20 mg/mL) were added to dough liquor (the supernatant of centrifuged bread dough) and control solutions (water and pH 6-adjusted water). Samples were stored at controlled temperatures (25, 40, 60, 70, and 80ºC) for up to 6 months, and thiamine degradation was quantified over time using high-performance liquid chromatography. Thiamine degradation kinetics, including the observed reaction rate constant (k_{obs}) and activation energy (E_a) of degradation, were calculated. Dough liquor ingredients stabilized thiamine in most cases when compared to the pH 6 control solutions, especially in the samples containing more thiamine. Thiamine degradation in dough liquor generally followed similar trends to those in the controls: thiamine degraded more quickly in the 20 mg/mL solutions than in 1 mg/mL solutions (with one exception), and increasing temperature led to increased thiamine degradation. However, k_{obs} ranged from 0.0019-0.22 in dough liquor and 0.0003-0.46 in control solutions, with differences attributed to interactions with components in the dough liquor. The E_a of thiamine degradation was ~21 kcal/mol in the control samples regardless of vitamin concentration but differed between vitamin concentrations in the dough liquor (23 and 14 kcal/mol in 1 and 20 mg/mL solutions, respectively), indicating that a different degradation pathway may have occurred in dough liquor. The different thiamine stability trends in dough liquor compared to control solutions indicate that food formulation has a substantial impact on the chemical behaviors of thiamine.

4.2 Introduction

Thiamine (vitamin B_1; Figure 4.1) is an essential micronutrient in the human diet that has critical roles in both the cardiovascular and nervous systems and acts as a precursor for coenzymes involved in the metabolism of carbohydrates, lipids, and branched chain amino acids; thus, thiamine is essential for the growth, development, and function of cells (Bémeur & Butterworth, 2014; Institute of Medicine, 1998). The main cause of thiamine deficiency in developing countries is malnutrition, especially when the major component of the diet is an unfortified grain. Fortification and enrichment of foods keeps the rate of deficiency low in developed countries, although subsets of the population remain deficient (e.g., some medical conditions and restricted diets) (Bémeur & Butterworth, 2014). Grains are the main source of thiamine in the human diet; however, the majority of the thiamine in grains is located in the outer layers of the kernel (germ and bran), and the refining process reduces the thiamine content by approximately 89% (Batifoulier, Verny, Chanliaud, Rémésy, & Demigné, 2006; Fitzpatrick et al., 2012). Thus, refined flour is usually enriched with a synthesized salt form of thiamine, thiamine mononitrate (TMN), along with many other micronutrients, to replace what is lost during milling (Fitzpatrick et al., 2012).

In crystalline form, thiamine is quite stable; however, in amorphous form or solutions, which represent the majority of foods and dietary supplements, thiamine degradation occurs in the presence of alkali, salts, oxygen, sulfites, and especially when heated (Arioglu-Tuncil et al., 2020; Farrer, 1955; Gregory III, 2008; Pizzoferrato, 1992). In breads and other cereal products, ~60% of the added thiamine can still be lost due to the increase in pH by alkaline leavening agents and/or exposure to high temperatures during processing (e.g., baking, toasting, extrusion) (Guzman-Tello & Cheftel, 1987; O'Brien & Roberton, 1993). Overage policies allow for the addition of excess thiamine to foods to account for that lost by degradation (Yoo, Walfish, Atwater, Giancaspro, & Sarma, 2016). Since thiamine is not toxic even at high levels, thiamine overages in foods usually range from 15-20% but can reach as high as 150% (O'Brien & Roberton, 1993). Thiamine degradation is known to produce sulfur-containing compounds, many with potent flavors and aromas, which play a substantial role in the sensory properties following degradation (Güntert et al., 1992; Voelker et al., 2018). Thus, overage policies may successfully deliver thiamine in those foods (Yoo et al., 2016) but may also result in increased presence of thiamine degradation compounds that lead to altered and potentially undesirable sensory characteristics.

Thiamine degradation is reported to be a pseudo-first order reaction (Arabshahi & Lund, 1988; Gregory III, 2008; Mauri et al., 1989). However, food formulations also play a significant role in the stability of thiamine. Amino acids, proteins, starch, and salts have all been shown to affect thiamine degradation, either stabilizing or destabilizing the vitamin, and possibly altering the first order degradation pattern (Bendix et al., 1951; Farrer, 1955; Goulette et al., 2020; McIntire & Frost, 1944; Mulley et al., 1975b; Pizzoferrato, 1992). Studies have monitored the effects of vitamin type and concentration, buffer type and concentration, storage conditions, pH, water activity (a_w) and moisture content, etc., and it has been shown that thiamine is most stable in systems with a pH below 6 (Arabshahi & Lund, 1988; Arioglu-Tuncil et al., 2020; Dwivedi & Arnold, 1973; Labuza & Kamman, 1982; Pachapurkar & Bell, 2005; Ramaswamy et al., 1990; Voelker et al., 2018; Voelker, Taylor, & Mauer). Thiamine content has been occasionally studied in grain-based products, such as bread (pH 5.0-6.2) and pasta (pH 6.1-6.5) (Ayhan & Köksel, 2019; Kamman et al., 1981; U.S. Food & Drug Administration, 2007), but the complications of extracting thiamine from the matrix using a combination of enzymes and elevated temperatures may contribute to thiamine degradation prior to chromatographic analysis (Batifoulier, Verny, Chanliaud, Rémésy, & Demigné, 2005; Batifoulier et al., 2006; Martinez-Villaluenga et al., 2009; Mauritzen & Stewart, 1965). This may be why no publications were found that calculated the reaction kinetics of thiamine in breads or doughs. To avoid these complications, others have studied chemical relationships within doughs by centrifuging dough into discrete fractions (Mauritzen & Stewart, 1965). The term ‘dough liquor’ refers to the aqueous phase of bread dough, which is the resulting viscous supernatant following centrifugation of bread dough. Since this dough liquor presumably represents the water-soluble fraction of the dough, it may be used for subsequent chemical analysis of water-soluble components in bread dough (e.g., thiamine). A significant amount of thiamine in pasta noodles leaches into the cooking water (E. Watanabe & Ciacco, 1990), indicating that thiamine does in fact partition into the aqueous phase as expected. However, in the case of pasta, that aqueous phase is discarded, whereas in bread, the aqueous phase (including the thiamine) remains in the product. Thus, dough liquor presents an interesting opportunity to better understand thiamine stability in bread.

Thiamine is stable in the crystalline state, even at high temperatures (Arioglu-Tuncil et al., 2020), but is known to degrade in bread and dough (Batifoulier et al., 2005, 2006; Martinez-Villaluenga et al., 2009). Flour is enriched with a dry nutrient premix containing crystalline TMN.

Because it is water soluble, TMN will partition into the aqueous phase of bread dough, and this dissolution must contribute to the degradation of thiamine in bread. Therefore, it was of interest to investigate the stability of thiamine in dough liquor, which has not been reported before. The objectives of this study were to: 1) investigate the chemical stability of thiamine in dough liquor, 2) calculate reaction kinetics of thiamine degradation in dough liquor, and 3) compare the chemical behavior of thiamine in simple solutions to its behavior in dough liquor. The results of this study are the first to report reaction kinetics of thiamine in dough liquor and should contribute to the understanding of thiamine stability in breads and doughs.

4.3 Materials and Methods

4.3.1 Materials

Unbleached, unenriched bread flour (12.7% protein) (King Arthur Flour, White River Junction, VT) and deionized water were used to prepare bread dough. Thiamine mononitrate, $C_{12}H_{17}N_4OS \bullet NO_3$ (TMN) (Spectrum Chemical Mfg. Corp., New Brunswick, NJ) was the vitamin form used for stability analyses: it is the form of thiamine used to fortify/enrich flour and grain products (Eitenmiller et al., 2008). Potassium sorbate (J.T. Baker Inc., Philipsburg, NJ) was also used in some samples to control mold growth. High performance liquid chromatography (HPLC) grade acetonitrile and trifluoroacetic acid (TFA) were obtained from Fisher Scientific (Waltham, MA) for use in all HPLC experiments. All water used in this study was deionized and purified using a Barnstead E-pure ultrapure water purification system with a resistivity greater than 17.5 MΩ·cm at 25°C (ThermoScientific, Waltham, MA).

4.3.2 Preparation of the aqueous phase of bread dough

Dough liquor was prepared using an adaptation of methods reported by Mauritzen and Stewart (1965); Morimoto and Seguchi (2011); and Seguchi, Nikaidoo, and Morimoto (2003). First, bread dough was produced in batches by mixing 580 g of bread flour with 420 mL of water in a KitchenAid stand mixer equipped with the dough hook (KitchenAid, Benton Harbor, MI). The dough was mixed for 15 min at speed 4-6, allowed to rest for 5 min, and mixed again for 10 min at speed 4-6. No yeast was added to the bread dough due to problems arising from the expansion of dough during centrifugation in preliminary studies. Thus, no rising step was used in the

preparation. The bread dough was transferred to 400 mL centrifuge tubes and centrifuged at 17,696 x g for 2 h at 4ºC and then for 2 h at 30ºC (Beckman Coulter Avanti J-25i high performance centrifuge, Indianapolis, IN). The supernatant (dough liquor) was then decanted for further treatment. Approximately 31 mL of dough liquor was collected from the 1000 g of dough prepared using this approach (~1 mL/33 g of dough). An image of the centrifuged bread dough is included in the appendix Figure A.4.1. To reduce viscosity of the dough liquor so as not to contribute to chemical stability differences from thiamine in pure water solutions due to reduced a_w compared to control thiamine solutions in water (Labuza & Kamman, 1982), dough liquor was diluted 4x prior to addition of thiamine. The pH of the diluted dough liquor was 6.1.

4.3.3 Thiamine solution preparation

Thiamine dough liquor solutions

TMN was added to the diluted dough liquor at 2 concentrations: 1 and 20 mg/mL. Considering the molecular weights of TMN (327.36 g/mol) and thiamine (265.36 g/mol), these solutions contained 0.8 and 16.2 mg/mL concentrations of dissociated thiamine. For simplicity, the concentrations of TMN (1 and 20 mg/mL) will be used for reference. Following dosing, the pHs of the samples were initially 6.2 and 6.5 in the 1 and 20 mg/mL TMN dough liquor solutions, respectively.

Some mold growth was observed in dough liquor samples that had been stored at lower temperatures for longer periods of time, which caused a lowering of pH and changes in thiamine chemical stability. To combat this mold growth and potential fermentation, additional samples were prepared with 0.1% potassium sorbate. All solutions (10 mL) were prepared in triplicate in 20 mL amber glass scintillation vials with PE cone-lined phenolic caps and sealed with duct tape to prevent evaporation during storage. Although preliminary HPLC results indicated that no detectable thiamine was present in the initial dough liquor (which was then diluted 4x), all initial dough liquor samples were analyzed for thiamine content prior to further experimentation to account for any thiamine that may have been present prior to addition of TMN.

Thiamine control solutions

Two different groups of thiamine control solutions were prepared. The first set of controls were simple thiamine-water solutions in which TMN was added to water in 2 concentrations: 1 mg/mL and 20 mg/mL. The pH values of these solutions were 6.4 and 6.9, respectively. The second set of controls were thiamine-water solutions with adjusted pHs. TMN was added to water in 2 concentrations (1 and 20 mg/mL), and the pHs of the solutions were adjusted to exactly 6 following addition of TMN using nitric acid, limiting the counterion in solution to only nitrate, which was already present in solution from the TMN salt. All solutions (10 mL) were prepared in triplicate in 20 mL amber glass scintillation vials with PE cone-lined phenolic caps and sealed with duct tape to prevent evaporation during storage.

4.3.4 Sample storage

Following sample preparation, the thiamine solutions were stored at 5 temperatures (25, 40, 60, 70, and 80ºC) to enable temperature-dependent reaction kinetics calculations. Potassium sorbate-containing dough liquor samples were only stored at 25, 40, and 60ºC. These temperatures were chosen both as a comparison to previous studies (Voelker et al., 2018; Voelker et al.) as well as to emulate conditions that may be experienced in the food industry during storage, processing, or transportation. Samples were maintained in a 25ºC temperature-controlled room, a 40, 60, or 70ºC Forma Scientific water-jacketed incubator (Thermo Fisher Scientific Inc., Marietta, OH), or an 80°C digital heatblock (VWR International, Radnor, PA) for the duration of the study. Temperature was confirmed using thermometers, and solutions were stored in temperature-controlled environments for up to 6 months, depending on temperature and rate of thiamine degradation. Samples were analyzed in triplicate for percent thiamine remaining at a minimum of 5 selected time points, and sample pHs were also monitored over the duration of the study (in duplicate) using an Orion pH probe (ThermoScientific) that had been calibrated with pH 1.68, 4.01, and 7.00 calibration standards (ThermoScientific).

4.3.5 Vitamin quantification

Thiamine was quantified in this study using a high performance liquid chromatography (HPLC) method adapted from AOAC method 942.23 (Eitenmiller et al., 2008). The reverse-phase

HPLC (Waters Corp., Milford, MA) method used in our previous studies (Voelker et al., 2018; Voelker et al.) was also employed here using a Waters 2690 Separations Module, a Waters 2996 Photodiode Array (PDA) detector with a wavelength scan of 235-400 nm, and a Waters Xterra RP-C_{18} column. Briefly, a gradient method using mobile phases A (0.1% TFA in water (*v/v*)) and B (acetonitrile) was used as follows: 100/0 at 0 min, 97/3 at 4 min (linear), 90/10 at 6 min (linear), 100/0 at 10 min (linear), and 100/0 at 15 min. Prior to analysis, solutions were removed from storage conditions, cooled in an ice bath, and diluted with mobile phase A to an estimated thiamine concentration of 500 ppm (0.5 mg/mL), assuming no thiamine degradation. Standard curves of TMN were prepared on each day of analysis with a concentration range of 10-1000 ppm (R^2 > 0.999) to calculate thiamine concentration of samples. Integration was performed at 254 nm.

4.3.6 Reaction kinetics calculations

Reaction kinetics of thiamine degradation were calculated to monitor the degradation reaction in dough liquor compared to control solutions and previously published work (Voelker et al., 2018; Voelker et al.). For comparisons, these calculations were done similarly to our previous studies (Voelker et al., 2018; Voelker et al.). Thiamine degradation is commonly reported as a pseudo-first order reaction in a variety of liquid and solid systems, including meats, vegetables, buffer solutions, and other model systems (Gregory III, 2008; Mauri et al., 1989); however, our previous study proposed that thiamine degradation at pH 6 may be a fractional order reaction between first and second order, which is known to occur when degradation products participate in subsequent chemical chain reactions (Laidler, 1987), comparable to what occurs in thiamine degradation (Voelker et al.). Regardless, first-order reaction kinetics calculations were still used in this study due to the generally pseudo-first order behavior and for comparison purposes:

$$ln\frac{x}{x_0} = -kt \qquad (eq.1)$$

where x is the concentration of thiamine at time t (days), x_0 is the initial thiamine concentration, and k is the reaction rate constant ($days^{-1}$). The observed reaction rate constant in this study is further referred to as k_{obs}. The Arrhenius equation was also used to describe the temperature dependence of k using the following equation:

$$k = Ae^{\frac{-E_a}{RT}} \qquad (eq.2)$$

where k is the observed reaction rate constant (k_{obs}) (days^{-1}), A is the frequency factor of collision, E_a is the activation energy (kJ/mol), R is the gas constant (8.3145 J/mol·K), and T is temperature (K). In accordance with our previous studies (Voelker et al., 2018; Voelker et al.), calculations only included data up to the time at which logarithmic linear degradation patterns were lost due to side-reactions of degradation products, usually when 40% or less thiamine remained. The time at which 90% of the initial thiamine concentration remained (10% degraded) was also calculated and referred to as t_{90}.

4.3.7 Determination of gelatinization temperature and water activity

In order to explore the mechanisms by which thiamine stability in the aqueous phase of bread dough differed from that in simple solutions, a subset of samples were analyzed for the a_w of dough liquor samples and the gelatinization temperature (T_{gel}) of starch in the presence of thiamine. The a_ws of dough liquor, 4x diluted dough liquor, 1 mg/mL TMN in 4x diluted dough liquor, and 20 mg/mL TMN in 4x diluted dough liquor were measured at 25ºC using an Aqualab 4TE water activity meter (METER Group, Inc., Pullman, WA) that had been calibrated with a_w standards.

Starch T_{gel}s were measured for the dough liquor solutions with 1 and 20 mg/mL TMN using a differential scanning calorimetry (DSC) method adapted from Allan, Rajwa, and Mauer (2018) to enable comparisons between vitamin concentration and interpretation of vitamin stability trends at temperatures above and below the starch T_{gel}. Three samples were prepared with bread flour in a 1:2 (*w/w*) ratio with water, 1 mg/mL TMN in water solution, or 20 mg/mL TMN in water solution. Samples were vortexed to create homogenous slurries and stored overnight at 4ºC to allow thiamine migration into the starch granules. Samples were then vortexed again, and ~15 mg of each slurry was transferred to a 50 µL aluminum DSC pan (PerkinElmer, Waltham, MA) and hermetically sealed. Samples were analyzed using a PerkinElmer DSC 4000 that had been calibrated with indium and verified with the melting point of water. Dry nitrogen was used to purge the system at 20 mL/min. Samples were heated from 0ºC to 100ºC at a rate of 10ºC/min. The T_{gel} was defined as the endothermic event occurring at approximately 60ºC, and the onset and peak T_{gel}s were determined using Pyris software (PerkinElmer). Samples were measured in duplicate.

4.3.8 Statistical analysis

All samples for HPLC analysis were prepared and measured in triplicate. All samples for pH determination were measured in duplicate. Single variable ANOVA (SAS 9.4, SAS Institute, Cary, NC) with Tukey's post-hoc test for multiple comparisons ($\alpha = 0.05$) was used to determine significant differences between percent thiamine remaining, k_{obs}, and t_{90} values as a function of solution type, thiamine concentration, and storage temperature, E_a as a function of solution type and thiamine concentration, pH over time, and slurry T_{gel}s. Regression analysis using JMP Pro 14.0.0 (SAS Institute) was used to determine standard error of slopes used to calculate k_{obs} and E_a values, and t_{90} values were calculated from the reaction kinetics models.

4.4 Results and Discussion

4.4.1 Chemical stability of thiamine in dough liquor and control solutions

Degradation profiles of thiamine in dough liquor and control solutions are shown in Figure 4.2, and all data on percent thiamine remaining and change in pH over time are included in the appendix Tables A.4.1 and A.4.2, respectively. Both temperature and concentration of thiamine were found to significantly ($p < 0.05$) affect thiamine stability in all solution types, with increasing temperature causing increased degradation. In all temperatures studied in the control solutions and most temperatures studied in the dough liquor solutions, as thiamine concentration increased, chemical stability decreased, similar to concentration-related stability trends previously reported (Voelker et al., 2018; Voelker et al.). However, when the dough liquor was stored at 60ºC, the opposite trend was seen, especially after 7 days of storage, wherein thiamine degraded significantly ($p < 0.05$) more in dough liquor solutions containing 1 mg/mL TMN compared to those containing 20 mg/mL (Figure 4.2). The trend of thiamine stability in the control samples containing different amounts of thiamine did not change based on temperature.

4.4.2 Degradation kinetics of thiamine in dough liquor and control solutions

The k_{obs}, R^2, and t_{90} values are reported for all solution types in Table 4.1. Consistent with previous studies (Gregory III, 2008; Voelker et al.), apparent first-order reaction behavior was observed for thiamine degradation in all solutions. Eq. 1 was used to calculate the observed reaction rate constant (k_{obs}) for thiamine degradation in all samples. When natural log of percent

thiamine remaining is plotted against time, the negative slope of the line is the k_{obs}, as shown in Figure 4.3.A. Over the duration of the study, there was not significant ($p < 0.05$) thiamine degradation in dough liquor samples containing 1 mg/mL TMN that were stored at 25ºC, so reaction kinetics were not calculated for that sample type. High correlations (Table 4.1) were found for the linear regressions of natural log of percent thiamine remaining vs. time in dough liquor solutions ($R^2 \geq 0.75$), with increasing correlations at higher temperatures ($R^2 \geq 0.92$ from 60-80ºC), and high correlations were also found for the controls ($R^2 \geq 0.89$ from 40-80ºC). These high correlations were taken to indicate that thiamine degradation was proceeding as a pseudo-first order reaction in all cases.

The k_{obs} was significantly ($p < 0.05$) higher and t_{90} was significantly lower in the 20 mg/mL TMN samples than in the 1 mg/mL samples, in all samples except dough liquor solutions stored at 60ºC, indicating that degradation proceeded faster in samples with higher concentrations of thiamine (Table 4.1). Although a first-order reaction should generally have the same k value regardless of starting concentration, these results are consistent with the control samples at all temperatures (including 60ºC) (Table 4.1) and what has been previously shown to occur at a similar pH (Voelker et al., 2018; Voelker et al.). This was found to result from the reaction order being approximately 1.3 rather than exactly 1 (Voelker et al.), calculated using the van't Hoff equation. A fractional reaction order can arise when degradation products participate in subsequent chemical chain reactions or when multiple degradation pathways exist (Laidler, 1987), which is known to occur with thiamine degradation (Dwivedi & Arnold, 1973; Güntert et al., 1992) and is likely to be further complicated due to the presence of bread dough ingredients in the dough liquor system. The reaction order of thiamine degradation in the dough liquor solutions was also calculated using the van't Hoff equation as ~1.3, in agreement with the pH 6 control samples and a previous study (Voelker et al.). Interestingly, even in the 60ºC dough liquor samples, the reaction order was still ~1.3, since the increased stability of thiamine in the 20 mg/mL sample compared to the 1 mg/mL sample did not occur until day 7 (Figure 4.2), and the van't Hoff equation considers only the initial rate of degradation.

It is also worth noting that the k_{obs} values in the dough liquor solutions were significantly ($p < 0.05$) lower than the k_{obs} values in the pH 6 control samples in most cases, especially in the solutions containing more thiamine (e.g. k_{obs} = 0.22 and 0.48 following storage at 80ºC in dough liquor and in pH-adjusted solutions, respectively) (Table 4.1). At 80ºC the t_{90} values suggest that

90% thiamine would remain in dough liquor after 12 h, but it would only take 5-6 h in the control solutions to reach this thiamine level, indicating that the dough liquor may stabilize thiamine. Ingredients such as proteins and starch may have a protective effect on thiamine against degradation (Greenwood, Beadle, & Kraybill, 1943; Lawrence, Schultz, & Frey, 1943; McIntire & Frost, 1944). Additionally, the pHs of all dough liquor samples steadily decreased over the duration of the experiment, presumably due to the presence of degradation products in solution (Figure 4.4), and a decrease in pH suggests an increase in thiamine stability over time due to thiamine's relatively higher stability in an acidic environment (Dwivedi & Arnold, 1973). The magnitude of pH decrease in the dough liquor solutions was consistent with the control solutions during storage at 60, 70, and 80ºC. However, the decrease in solution pH during storage at 25 and 40ºC was much larger in dough liquor solutions than in the pH-adjusted control solutions at both concentrations (e.g. final pHs of 3.6 and 4.5 for 1 and 20 mg/mL dough liquor solutions, respectively, vs. 5.3 and 5.0 for 1 and 20 mg/mL pH-adjusted solutions, respectively, following storage at 25ºC). This difference in pH change may have also contributed to the significantly ($p < 0.05$) lower k_{obs} values calculated for thiamine degradation in dough liquor solutions compared to the pH-adjusted controls at 25 and 40ºC (Table 4.1). Alternatively, the k_{obs} values were often not statistically different in the dough liquor and simple water solutions, except in the 20 mg/mL solutions stored at 80ºC (k_{obs} = 0.22 and 0.43 in dough liquor and water solutions, respectively) (Table 4.1). This may indicate a better temperature stability of thiamine in dough liquor solutions compared to control solutions. Regardless, the trend seen in most storage conditions in which thiamine was more stable in 1 mg/mL solutions than in 20 mg/mL solutions was inverted when the dough liquor samples were stored at 60ºC. Namely, the k_{obs} value was significantly higher in the 1 mg/mL sample than the 20 mg/mL samples (k_{obs} = 0.105 and 0.050, respectively) (Table 4.1).

Eq. 2 was used to calculate the activation energy (E_a) of thiamine degradation by plotting the natural log of the k_{obs} values vs. the reciprocal of temperature wherein the negative slope multiplied by the gas constant R is the E_a. The calculated E_as of all solution types are reported in Table 4.2, and a typical example of the Arrhenius plots used to calculate E_a is shown in Figure 4.3.B. High correlations were found in the plots used for the calculations of degradation in dough liquor (R^2 = 0.98-0.99) and the controls (R^2 = 0.95-0.97). The E_as were significantly ($p < 0.05$) different between the two thiamine concentrations in dough liquor solutions studied (23 and 14 kcal/mol in 1 and 20 mg/mL solutions, respectively) (Table 4.2), which was not expected and

differed from the controls. In addition, the E_a of the 20 mg/mL dough liquor solution was lower than E_a ranges previously reported for thiamine degradation at similar pHs (20-30 kcal/mol) and of the control samples (18-26 kcal/mol) (Guzman-Tello & Cheftel, 1987; Mauri et al., 1989; Ramaswamy et al., 1990; Voelker et al., 2018; Voelker et al.; Windheuser & Higuchi, 1962). Taken with the stability data, this indicated a relatively better temperature stability of thiamine in dough liquor than in the simple control solutions, in agreement with the comparisons of the k_{obs} values in the 20 mg/mL 80ºC storage condition. This observation was consistent with what was reported by Ramaswamy et al. (1990) in which a thiamine mixture with sugars had an E_a of 17 kcal/mol vs. 28 kcal/mol in water. The difference in E_a between thiamine concentrations may result from specific interactions between thiamine and the matrix components.

In addition to differences between E_as being dependent on thiamine concentration in dough liquor, variations in the 1 mg/mL samples were found to cause the differing k_{obs} trend in the samples stored at 60ºC (in which thiamine in 20 mg/mL dough liquor solutions was more stable than in 1 mg/mL solutions). When considering the Arrhenius plots (Figure 4.3.B), the 1 mg/mL sample at 60ºC did not fit the trend line whereas the 20 mg/mL sample did. When the 60ºC sample was not considered in the linear regression of the 1 mg/mL sample, the R^2 value of the line was 0.98 vs. 0.77 when the 60ºC sample was included (Figure 4.3.B.i). Thus, variations of thiamine stability trends in the 1 mg/mL samples at different temperatures may have led to significant differences between the calculated E_as of 1 and 20 mg/mL dough liquor samples. It is also worth noting that differences in activation energy may indicate differences in degradation pathway, and variations in degradation peaks found in the HPLC chromatograms suggest that this is a possibility.

4.4.3 Behavior of thiamine in dough liquor compared to control solutions

Thiamine stability in dough liquor was found to differ in three major ways from thiamine stability in the simple control solutions at similar pHs, thiamine concentrations, and storage temperatures. First, thiamine was significantly more stable in dough liquor solutions than in the pH 6-adjusted control solutions, most notably at the 20 mg/mL TMN concentration (Table 4.1). This is presumably due to the protective effect of ingredients in the dough liquor, including proteins and starch (Greenwood et al., 1943; Lawrence et al., 1943; McIntire & Frost, 1944). Secondly, in dough liquor solutions stored at 60ºC, thiamine was significantly ($p < 0.05$) more stable in 20 mg/mL solutions than in 1 mg/mL solutions (Figure 4.2, Table 4.1), opposite of trends

at the other temperatures studied and opposite of the trends at all temperatures in the control solutions. Finally, the activation energy of thiamine degradation in dough liquor differed based on thiamine concentration (Table 4.2), which was not the case for thiamine degradation in the simple control solutions or what would be expected for most chemical reactions. The outlying behavior of the 1 mg/mL samples at 60ºC likely contributed to the concentration-dependent E_a trend in dough liquor that differed from the control solutions. All three of these variations in dough liquor compared to thiamine degradation in simple solutions presumably result from thiamine interactions with bread dough ingredients in the aqueous environment.

The physical properties of the ingredients contained in the aqueous phase of bread dough at specific temperatures (e.g., 60ºC) may be an underlying cause for why thiamine degradation behavior did not follow usual trends at 60ºC. Proteins and polysaccharides are known components of dough liquor (Mauritzen & Stewart, 1965), which may denature or gelatinize, respectively, as temperature is increased. Starch gelatinization is an irreversible process that occurs when starch is exposed to elevated temperatures in the presence of water, causing the melting of the crystalline amylopectin in starch and the loss of molecular order (BeMiller & Huber, 2008). The gelatinization temperature (T_{gel}) of starch in water is ~60ºC and is sometimes affected by additional ingredients in the system (Allan et al., 2018). The onset T_{gel} of starch in a 1:2 (*w/w*) slurry of bread flour and 1 mg/mL TMN solution was 60.58ºC, and the T_{gel} of starch in a 1:2 (*w/w*) slurry of bread flour and 20 mg/mL TMN solution was 61.7ºC; thus, the T_{gel} of starch in the dough liquor samples was significantly ($p < 0.05$) higher in the presence of 20 mg/mL TMN than 1 mg/mL TMN. This difference in T_{gel} indicates that the extent of starch gelatinization in dough liquor solutions containing 1 and 20 mg/mL TMN would have differed in samples stored at 60ºC, with the 1 mg/mL sample containing more gelatinized starch than the 20 mg/mL sample. Gelatinized starch presumably interacts differently with thiamine than ungelatinized starch, which may have led to differences seen in the expected trends of thiamine degradation between 1 and 20 mg/mL solutions at 60ºC (Figure 4.2, Table 4.1). This may have also contributed to the significantly ($p < 0.05$) higher k_{obs} value in the 1 mg/mL dough liquor solution than in either 1 mg/mL control solution when stored at 70ºC (Table 4.1).

Other potential interactions that could alter thiamine degradation in dough liquors include pH-related events in relation to the physical and chemical properties of proteins. The isoelectric point (pI) of gluten is ~6 (Bengoechea, Romero, Aguilar, Cordobés, & Guerrero, 2010), which is

the approximate pH of the dough liquor solutions prior to heat treatments. Although this seemingly indicates that a hydrophobic gluten protein would not be present in the aqueous phase of bread dough, Mauritzen and Stewart (1965) identified a gluten-containing sediment in their dough liquor, indicating some protein may be present in the dough liquors. As the pH of the dough liquor and thiamine systems decreased over the duration of the experiment (Figure 4.4), the changes in charge and solubility of gluten may have also affected thiamine stability. Regardless of the exact mechanism, it is clear that the dough liquor ingredients led to variations in thiamine stability trends, presumably resulting from changes in molecular conformation and intermolecular interactions between thiamine and bread ingredients.

In addition to the effect of pH on protein interactions, thiamine is significantly affected by changing ionic constitution (Farrer, 1955). For example, although no buffer systems were used in the pH 6-adjusted water solutions due to the effect of buffers on thiamine stability (Farrer, 1955), the presence of changing ionic constitution in the systems presumably contributed to the differences in thiamine stability compared to the water controls without adjusted pH even though a similar pH was achieved (Figure 4.2). This change in ionic constitution may have also occurred as pH changed over the duration of the dough liquor experiment, contributing to outlying trends of thiamine stability in the dough liquor solutions.

4.4.4 Effects of potassium sorbate on thiamine stability in dough liquor

As another example of ingredients in the aqueous phase of bread dough having an effect on thiamine stability, the presence of potassium sorbate significantly ($p < 0.05$) affected percent thiamine remaining over time compared to dough liquor samples without potassium sorbate in most cases, stabilizing or destabilizing thiamine depending on TMN concentration and storage condition (Figure 4.5, appendix Table A.4.4). Thiamine is known to be very sensitive to the presence of salts and electrolytes due to ionic constitution (Beadle et al., 1943; Farrer, 1955; Pachapurkar & Bell, 2005); thus, it was not surprising to find that potassium sorbate affected thiamine stability. Considering k_{obs} calculations in the presence of potassium sorbate in dough liquor solutions, correlations were high, especially at higher temperatures ($R^2 \geq 0.75$ from 40-60ºC), indicating pseudo-first order degradation similar to that shown in dough liquor without the potassium sorbate. The k_{obs} value in potassium sorbate-containing dough liquor solutions was significantly higher in the 1 mg/mL sample than the 20 mg/mL samples following storage at both

40 and 60ºC (Table 4.3), which is what was shown to occur in dough liquor without potassium sorbate at 60ºC but not at 40ºC. The effect of the changing solution pH with regards to the pI of gluten, described previously, could have played a role in why the stability trends differed between dough liquor with and without potassium sorbate, in which the change in pH differed over time. For example, in the 40ºC storage environment in the presence of potassium sorbate, the 20 mg/mL samples had a lower pH than the 1 mg/mL samples in the early time points, which is the opposite trend seen when potassium sorbate was not present (Figure 4.4.B). This may have contributed to the difference in thiamine stability trends in these same samples with and without potassium sorbate resulting from pI-related gluten behavior (Figure 4.5.B).

Thiamine stability is related to the electrolyte system, with some salts increasing stability and others decreasing stability (Beadle et al., 1943; Farrer, 1955). However, in general, as concentration of salt increases, thiamine degradation has been reported to increase, especially at weakly acidic or neutral pHs as in the current study (Pachapurkar & Bell, 2005; Windheuser & Higuchi, 1962). The largest effect of potassium sorbate on stability was found in 1 mg/mL TMN in dough liquor samples stored at 40ºC (Figure 4.5.B), wherein thiamine stability greatly decreased in the presence of potassium sorbate (e.g. 34% and 88% thiamine remaining on day 62 with and without 0.1% potassium sorbate, respectively). Since the ratio of potassium sorbate to thiamine decreased as TMN concentration increased from 1 to 20 mg/mL, it is logical that the greatest effect on thiamine stability was seen in the 1 mg/mL samples. This may also indicate why the trend of thiamine being more stable in 1 mg/mL samples than 20 mg/mL samples was not followed in the 40ºC storage conditions when potassium sorbate was present. Potassium sorbate did effectively decrease and/or prevent mold growth and fermentation in the dough liquor samples, which was the intended function. However, the effect of potassium sorbate on thiamine stability due to ionic constitution did not enable adequate comparisons of thiamine stability in dough liquor to the control samples in the current study. Still, potassium sorbate is commonly added to chemically leavened baked products (e.g. not containing yeast) and will partition into the aqueous phase of those doughs with thiamine. Thus, the dependence of the chemical stability of thiamine in dough liquor in the presence of potassium sorbate may be of interest in some baked goods.

4.4.5 Thiamine stability in bread

According to FDA regulations for thiamine content in enriched flour (0.64 mg/100 g flour) and assuming all thiamine partitions into the aqueous phase of bread dough, the thiamine concentration in dough liquor from bread dough made with enriched flour would be 0.12 mg/mL. Although this study explored thiamine stability in dough liquor with thiamine concentrations greater than what would be found in doughs made from enriched flour, the results and reaction kinetics of thiamine degradation reported in this study can be used as general guidelines to understand the behavior of thiamine in bread dough, including during baking. The classical breadmaking process with yeast has been shown to reduce the thiamine content by up to 56%, occurring both during processing as well as during baking, although fermentations sometimes increase thiamine content, resulting in a lower net loss after baking (Batifoulier et al., 2005, 2006; Martinez-Villaluenga et al., 2009). Other B vitamins have also commonly been shown to be affected by the breadmaking process, with yeast fermentations often leading to up to 350% increase in riboflavin content (due to yeast contribution) but up to 64% depletion of pyridoxine (Batifoulier et al., 2005, 2006). However, vitamin degradation in bread is generally studied such that an extraction process is required to analyze vitamin content, and these extraction processes may contribute to some of the degradation seen in these studies. Regardless, the degradation of thiamine during baking can lead to long term consequences, including depleted nutritional value as well as some altered, and perhaps undesirable, sensory properties resulting from thiamine degradation products (Voelker et al., 2018).

The temperature that thiamine reaches in the bread dough during baking is dependent on the location of the thiamine within the bread loaf. By definition, the aqueous phase of bread dough cannot exceed 100ºC. However, as water evaporates, thiamine contained in the crust and outer layers of the bread loaf may exceed that temperature. It has been reported that the internal temperature (crumb temperature) of bread dough can reach up to 99ºC before remaining constant for the duration of cooking (Therdthai, Zhou, & Adamczak, 2002). By extrapolating the degradation kinetics calculations for 1 and 20 mg/mL TMN in dough liquor in this study, it was shown that after 1 h of baking at 99ºC, ~97% thiamine would remain in the crumb. The t_{90} indicates that 90% of thiamine would remain at this baking temperature even after ~4.5 h. While this calculation indicates minimal thiamine degradation in the bread crumb, it is important to note that the higher concentration of TMN (20 mg/mL) in dough liquor had a better temperature stability

than the lower concentration of TMN (1 mg/mL) in dough liquor. This may indicate that the substantially lower concentration of thiamine found naturally in the aqueous phase of bread dough is less stable than that calculated in the current study.

High percentages of recovered thiamine have been reported in bread (up to 93% remaining), but extensive degradation is more commonly reported (O'Brien & Roberton, 1993). In addition, the temperature on the crust of bread can reach up to 150-205ºC during baking (Therdthai et al., 2002), and bread toasting can expose the crumb to temperatures up to 148-204ºC (O'Brien & Roberton, 1993). Although the reaction kinetics calculations in the current study cannot accurately predict thiamine stability at these high temperatures, and additional studies would be required to expose dough liquors to higher temperatures and pressures, thiamine degradation is likely much more extensive in the crust and outer layers of the bread loaf than in the crumb due to the different temperature conditions. The reaction kinetics of thiamine degradation could be used to better inform overage predictions for delivering an adequate amount of thiamine in bread products.

In addition to thiamine stability during the bread baking process, it was also of interest to explore thiamine stability in bread during storage. Although little to no thiamine loss has been reported during storage of dried noodles (Bui & Small, 2008; Kamman et al., 1981; E. Watanabe & Ciacco, 1990), the a_w of the dried noodles is very low. On the other hand, bread has a relatively high a_w of 0.94 (Mauer & Bradley, 2017). The a_w of the concentrated dough liquor in this study was 0.992 while the diluted dough liquor fortified with thiamine was 0.996-0.999 a_w, dependent on thiamine concentration. This comparatively high a_w of both bread and the dough liquor used in this study could create conditions wherein thiamine would degrade. Thiamine stability has been reported to decrease as a_w increased in an enriched pasta at much lower a_ws (0.44-0.65), even when riboflavin remained stable in the same conditions (Kamman et al., 1981). However, little thiamine degradation was found at 25ºC (Figure 4.2) and the t_{90} value in the 20 mg/mL TMN samples indicates the 90% thiamine will remain after 24 days of storage (Table 4.1). Lower concentrations of thiamine were even more stable, and the insignificant degradation did not enable t_{90} calculations at 25ºC. Bread proofing generally occurs at approximately 40ºC (Morimoto & Seguchi, 2011; Seguchi et al., 2003), and very little thiamine degradation was found in dough liquor at this temperature either. The t_{90} values indicated that 90% thiamine will remain after 56 and 7 days at 40ºC in 1 and 20 mg/mL TMN samples, respectively. Since bread proofing most commonly occurs for a maximum of several hours, thiamine degradation at bread proofing temperatures should not

be of concern. This high stability of thiamine at ambient and bread proofing temperatures has also been shown in amorphous solid dispersions, in which little to no degradation occurs at temperatures below 40ºC (Arioglu-Tuncil et al., 2020). This indicates that the majority of thiamine degradation in bread occurs during the baking process, not during storage at common storage temperatures or during bread proofing despite the high a_w conditions.

4.5 Conclusion

Reaction kinetics of thiamine degradation in the aqueous phase of bread dough indicated that bread dough ingredients provide a stabilizing effect on thiamine in most cases when compared to simple thiamine solutions at a similar pH, especially at the 20 mg/mL concentration. The initial thiamine concentration in the dough liquor solutions was found to have a significant effect on thiamine stability. Thiamine stability in dough liquor generally followed the commonly reported trend in which the higher thiamine concentration degraded more quickly than when present in lower concentrations; however, at 60ºC this trend was reversed. This was attributed in part to changes in molecular conformation of dough liquor ingredients, including starch and gluten, and thus changes in intermolecular interactions between the ingredients and thiamine. Additionally, it was shown that E_a of thiamine degradation differed based on the concentration of thiamine in the dough liquor. This may indicate that the degradation pathway differed between the two solutions, again due to changes in intermolecular interactions. The addition of potassium sorbate to dough liquors significantly ($p < 0.05$) affected thiamine stability, both stabilizing and destabilizing thiamine dependent on storage condition and thiamine concentration due to ionic constitution. Overall, this study presented a novel experimental approach for evaluating the stability of water-soluble ingredients in doughs by separating the aqueous phase and monitoring thiamine degradation therein. The results can be used to better understand how food matrix ingredients and thermal treatments affect the stability of thiamine in bread products.

4.6 Tables and Figures

Table 4.1 Effect of solution type, temperature, and thiamine concentration on the kinetic rate constants and t_{90} values for thiamine degradation. Uppercase superscript letters denote statistical significance of k_{obs} or t_{90} values between solution types at the same storage temperature and thiamine concentration (across rows). Lowercase superscript letters denote statistical significance of k_{obs} or t_{90} values between thiamine concentrations at the same storage temperature and solution type (down columns within each temperature). Shading indicates the 60ºC reaction kinetics outlier for the 1 mg/mL thiamine samples in dough liquor solutions.

	Thiamine Concentration (mg/mL)	Reaction Kinetics	Dough Liquor	Water	pH 6 Adjusted Water
25ºC	1	k_{obs} (day^{-1})	-	0.00029 ± 0.00004^{Ba}	0.00111 ± 0.00004^{Ab}
		R^2	-	0.6655	0.9712
		t_{90}^* (days)	-	360 ± 50^{Aa}	95 ± 3^{Ba}
	20	k_{obs} (day^{-1})	0.0044 ± 0.0004^{B}	0.00034 ± 0.00005^{Ca}	0.0058 ± 0.0004^{Aa}
		R^2	0.8400	0.5652	0.9261
		t_{90}^* (days)	24 ± 2^{B}	310 ± 50^{Aa}	18 ± 1^{Bb}
40ºC	1	k_{obs} (day^{-1})	0.0019 ± 0.0002^{Ab}	$0.00161 \pm 0.00004^{ABb}$	0.00149 ± 0.00006^{Bb}
		R^2	0.7737	0.9793	0.9446
		t_{90}^* (days)	56 ± 6^{Ba}	66 ± 2^{Aa}	$70. \pm 3^{Aa}$
	20	k_{obs} (day^{-1})	0.014 ± 0.003^{Ba}	0.0083 ± 0.0004^{Ca}	0.023 ± 0.002^{Aa}
		R^2	0.7499	0.9325	0.9431
		t_{90}^* (days)	7 ± 2^{ABb}	12.7 ± 0.6^{Ab}	4.6 ± 0.4^{Bb}
60ºC	1	k_{obs} (day^{-1})	0.105 ± 0.007^{Aa}	0.0183 ± 0.0008^{Bb}	0.022 ± 0.001^{Bb}
		R^2	0.9305	0.9704	0.9330
		t_{90}^* (days)	1.01 ± 0.07^{Cb}	5.8 ± 0.3^{Aa}	4.7 ± 0.3^{Ba}
	20	k_{obs} (day^{-1})	0.050 ± 0.002^{Bb}	0.070 ± 0.004^{Ba}	0.31 ± 0.03^{Aa}
		R^2	0.9632	0.9489	0.9312
		t_{90}^* (days)	2.10 ± 0.09^{Aa}	1.51 ± 0.09^{Bb}	0.34 ± 0.03^{Cb}
70ºC	1	k_{obs} (day^{-1})	0.066 ± 0.005^{Ab}	0.043 ± 0.004^{Bb}	0.043 ± 0.002^{Bb}
		R^2	0.9179	0.8905	0.9518
		t_{90}^* (days)	1.6 ± 0.1^{Ba}	2.5 ± 0.2^{Aa}	2.5 ± 0.1^{Aa}
	20	k_{obs} (day^{-1})	0.086 ± 0.006^{Ba}	0.14 ± 0.01^{Ba}	0.43 ± 0.06^{Aa}
		R^2	0.9281	0.9196	0.8945
		t_{90}^* (days)	1.22 ± 0.09^{Ab}	0.75 ± 0.06^{Bb}	0.24 ± 0.03^{Cb}
80ºC	1	k_{obs} (day^{-1})	0.096 ± 0.003^{Bb}	0.104 ± 0.003^{Bb}	0.122 ± 0.004^{Ab}
		R^2	0.9762	0.9873	0.9883
		t_{90}^* (days)	1.10 ± 0.04^{Aa}	1.02 ± 0.03^{Ba}	0.86 ± 0.03^{Ca}
	20	k_{obs} (day^{-1})	0.22 ± 0.01^{Ba}	0.43 ± 0.03^{Aa}	0.48 ± 0.04^{Aa}
		R^2	0.9708	0.9605	0.9464
		t_{90}^* (days)	0.48 ± 0.03^{Ab}	0.25 ± 0.02^{Bb}	0.22 ± 0.02^{Bb}

* t_{90} indicates time when 90% of the initial concentration of thiamine remains
Standard error of the slope was used for statistical calculations

Table 4.2 Effect of solution type and thiamine concentration on calculated activation energies as a function of temperature. Uppercase superscript letters denote statistical significance of E_a between solution types at the same thiamine concentration (across rows). Lowercase superscript letters denote statistical significance of E_a between thiamine concentrations in the same solution type (down columns).

Thiamine Concentration (mg/mL)	E_a (kcal/mol)		
	Dough Liquor	Water	pH 6 Adjusted Water
1	23 ± 1^{Aa}	22.7 ± 0.3^{Ab}	19 ± 1^{Ba}
20	14.3 ± 0.4^{Cb}	26 ± 1^{Aa}	18 ± 1^{Ba}

Standard error of the slope was used for statistical calculations

Table 4.3 Rate constants and t_{90} values for thiamine degradation in dough liquor solutions with 0.1% potassium sorbate under all concentrations and temperatures studied. Uppercase superscript letters denote statistical significance of k_{obs} within a specific temperature (down columns). Lowercase superscript letters denote statistical significance of t_{90} within a specific temperature (down columns).

Thiamine Concentration (mg/mL)	Reaction Kinetics	25ºC	40ºC	60ºC
1	k_{obs} (day^{-1})	0.0006 ± 0.0001^{B}	0.0094 ± 0.0008^{A}	0.14 ± 0.01^{A}
	R^2	0.6781	0.8517	0.9174
	t_{90}^{*} (days)	170 ± 30^{a}	11 ± 1^{b}	0.74 ± 0.06^{b}
20	k_{obs} (day^{-1})	0.0014 ± 0.0003^{A}	0.0020 ± 0.0002^{B}	0.037 ± 0.002^{B}
	R^2	0.5295	0.7539	0.9468
	t_{90}^{*} (days)	70 ± 20^{b}	52 ± 6^{a}	2.8 ± 0.2^{a}

* t_{90} indicates time when 90% of the initial concentration of thiamine remains
Standard error of the slope was used for statistical calculations

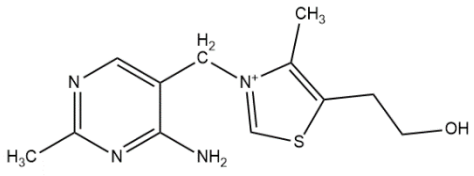


Figure 4.1 Chemical structure of thiamine.

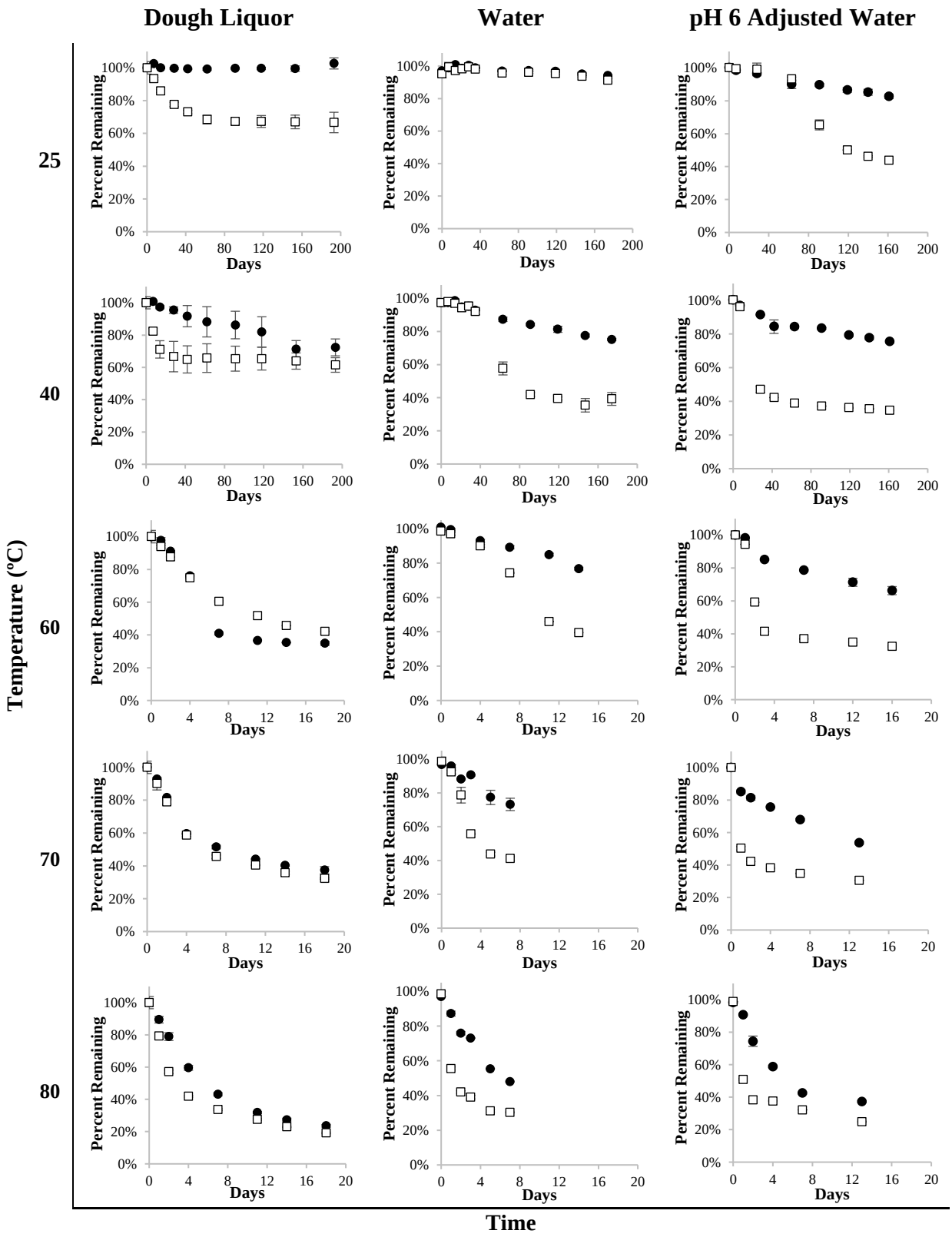
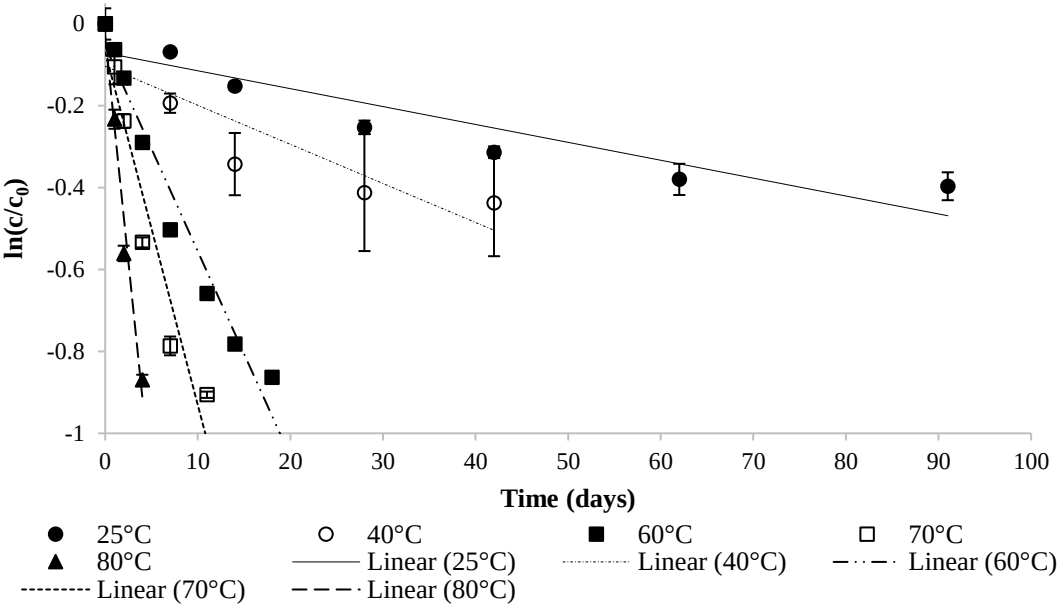


Figure 4.2 Effect of solution type, temperature, and TMN concentration (● 1 mg/mL and □ 20 mg/mL) on the chemical stability of thiamine over time in which all solution pHs were ~6.

A)



B)

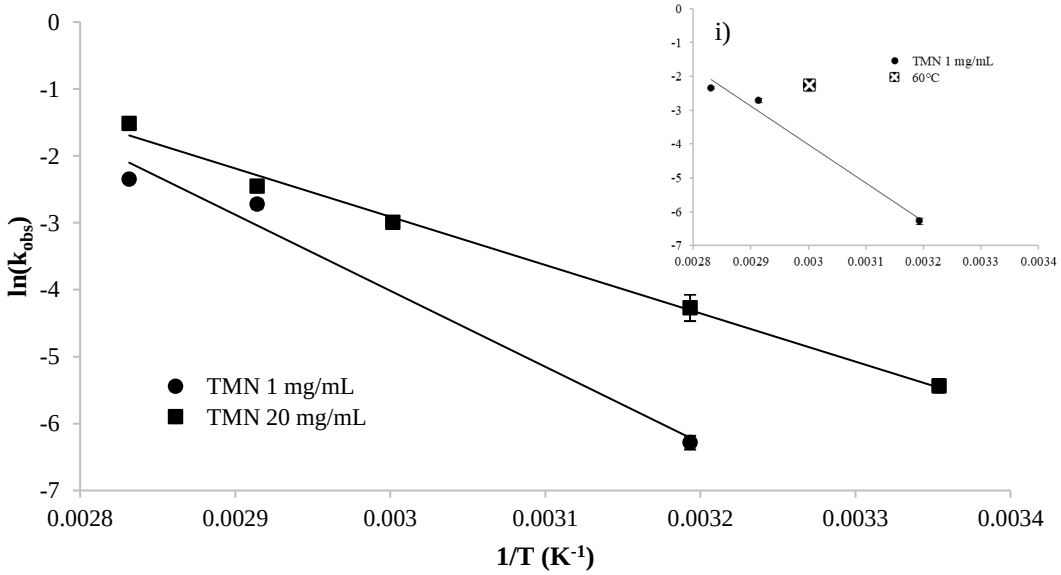


Figure 4.3 Reaction kinetics of thiamine degradation in dough liquor: A) first-order degradation reaction regression lines of 20 mg/mL TMN in dough liquor used to calculate k_{obs} at temperatures from 25-80ºC; and B) Arrhenius plots of temperature-dependent thiamine degradation used to calculate E_a of degradation in 1 and 20 mg/mL TMN in dough liquor from 25-80ºC, in which the insert graph i) indicates the 60ºC outlier in the Arrhenius calculations for the 1 mg/mL samples.

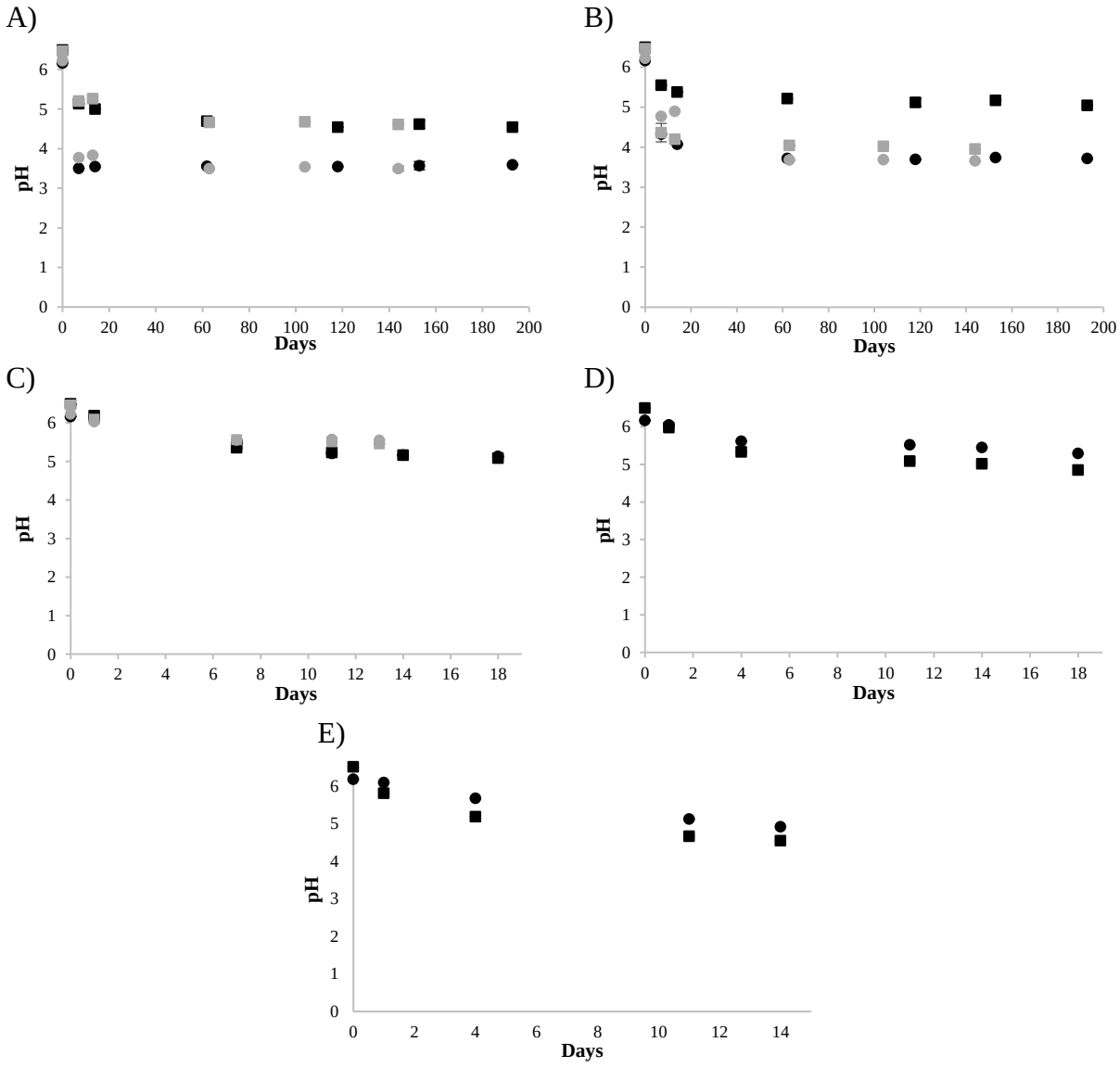


Figure 4.4 The pH profiles of thiamine-dough liquor solutions over the duration of chemical stability studies both with and without 0.1% potassium sorbate at: A) 25ºC, B) 40ºC, and C) 60ºC, and without potassium sorbate at D) 70ºC and E) 80ºC.

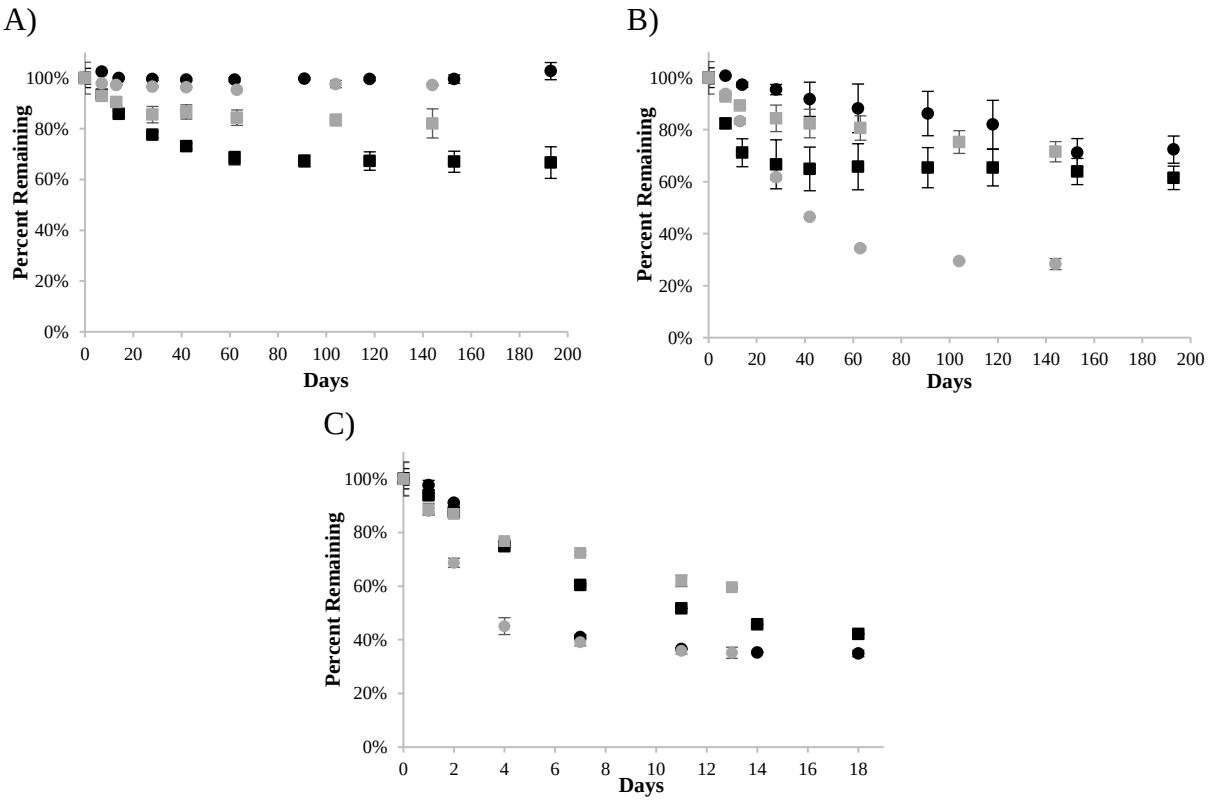


Figure 4.5 Comparison of the chemical stability of thiamine in dough liquor with and without 0.1% potassium sorbate at: A) 25ºC, B) 40ºC, and C) 60ºC.

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CHAPTER 5. EFFECTS OF EMULSIFIERS ON THE MOISTURE SORPTION AND CRYSTALLIZATION OF AMORPHOUS SUCROSE LYOPHILES

Voelker, A. L., Verbeek, G., Taylor, L. S., & Mauer, L. J. (2019). Effects of emulsifiers on the moisture sorption and crystallization of amorphous sucrose lyophiles. *Food Chemistry: X*, *3*, 100050. doi:https://doi.org/10.1016/j.fochx.2019.100050

5.1 Abstract

The crystallization of amorphous sucrose can be problematic in food products. This study explored how emulsifiers (a range of sucrose esters, polysorbates, and soy lecithin) impact the moisture sorption and crystallization of amorphous sucrose lyophiles. Solutions containing sucrose with and without emulsifiers were lyophilized, stored in desiccators, and analyzed by x-ray diffraction, infrared spectroscopy, and polarized light microscopy over time. Moisture sorption techniques, Karl Fischer titration, and differential scanning calorimetry were also used. Different emulsifiers had varying impacts on sucrose crystallization tendencies. Polysorbates enhanced sucrose crystallization, decreasing both the RH and time at which sucrose crystallized. These lyophiles did not collapse upon crystallization, unlike all other samples, indicating the likelihood of variations in nucleation sites and crystal growth. All other emulsifiers stabilized amorphous sucrose by up to a factor of 7x, even in the presence of increased water absorbed and independent of glass transition temperatures, indicating emulsifier structure governed sucrose crystallization tendencies.

5.2 Introduction

In addition to increasing the sweetness of foods, sucrose contributes to the structure, texture, dissolution, and/or taste perception of products ranging from various confectioneries and low moisture baked goods, to powder beverage and seasoning mixtures. The physical state of the sucrose solid affects many characteristics, including stability, dissolution, moisture sorption, and many sensory properties, such as texture and flavor perception (Chirife & Karel, 1974; Mathlouthi, 1995). Amorphous sucrose is often the preferred state for many confectionery products due to the

desirable dissolution properties and softer texture. However, amorphous sucrose has a tendency to crystallize to the more thermodynamically stable crystalline form during storage. Crystallization can lead to undesirable texture and flavor changes, impaired solubility, and acceleration of chemical changes such as oxidation and enzymatic activity in other materials in the food matrix (Buera et al., 2005; Slade & Levine, 1991). Therefore, sucrose crystallization is a major area of interest in the food industry, with emphasis placed on the effects of formulations and storage environments on crystallization kinetics (Buera et al., 2005; Kinugawa et al., 2015; Saleki-Gerhardt & Zografi, 1994; Thorat, Forny, Meunier, Taylor, & Mauer, 2017, 2018).

Numerous additives have been shown to disrupt and delay sucrose crystallization by a variety of mechanisms including: decreasing molecular mobility (Saleki-Gerhardt & Zografi, 1994), increasing the glass transition temperature (T_g) and/or viscosity of the co-lyophilized system (Roe & Labuza, 2005; Roos & Karel, 1991b), disrupting the crystal lattice due to molecular interactions between sucrose and the additive (Gabarra & Hartel, 1998; Shamblin & Zografi, 1999), and generally inhibiting nucleation and crystal growth (Carstensen & van Scoik, 1990). More recently, a study of the effects of chloride and sulfate salts on amorphous sucrose crystallization found that increasing the cation valency (and corresponding ion hydration shell) delayed or prevented sucrose crystallization even while decreasing T_g, presumably by altering the water dynamics in the matrix (Thorat, Forny, et al., 2017). A study on the effects of a series of mono-, di-, and tri-saccharides on amorphous sucrose stability found that saccharides containing regions of structural similarity as well as structural dissimilarity best inhibited sucrose crystallization, with these structural relationships seemingly having a greater influence on the delay of sucrose crystallization than that of a decrease in T_g due to moisture sorption (Thorat et al., 2018).

While many studies have explored the impact of additives on sucrose crystallization from the solid state, the role of emulsifiers in altering sucrose crystallization has primarily been studied in solutions and is not well-defined. Emulsifiers have been shown to alter the crystallization of compounds by different mechanisms. In solutions, emulsifiers have been shown to both reduce and increase the primary nucleation rate of different compounds (Canselier, 1993; van Hook, 1961). Emulsifiers have also been shown to have conflicting effects on crystal growth rates: the reduction of interfacial tension by the emulsifier can increase the crystal growth rate, but the slowing of mass transfer at the crystal-solution interface due to the presence of the emulsifier can slow the crystal growth rate (Canselier, 1993; van Hook, 1988; Vasanth Kumar & Rocha, 2009). Some emulsifiers

have even been shown to both increase and decrease the rate of crystallization, depending on the amount added (Michaels & van Kreveld, 1966). For example, sodium doecyl (tetrapropylene) benzene sulfonate increased the rate of lactose crystallization at low levels of addition but decreased the rate when added in larger amounts (Michaels & van Kreveld, 1966). Regardless, it is agreed upon that the changes emulsifiers cause on the adsorbed crystal surface layer are likely to affect secondary nucleation, and changes in surface energy due to emulsifiers are likely to affect crystal growth (Canselier, 1993; Hartel & Shastry, 1991; Vasanth Kumar & Rocha, 2009); however, these concepts have not been shown to correlate to crystallization from the amorphous state. While understanding formulation effects on crystallization from solutions is certainly important, foods and food ingredients tend to be solids. Therefore, understanding how emulsifiers alter sucrose crystallization from the amorphous state is relevant.

The objective of this study was to determine the effects of different types and concentrations of food-relevant emulsifiers on the crystallization of amorphous sucrose. It was hypothesized that the structure of the emulsifiers would play a significant role in stabilizing amorphous sucrose. Emulsifiers containing a region that is structurally similar to sucrose as well as a structurally dissimilar region were anticipated to provide the greatest inhibition to sucrose crystallization, consistent with the concept shown for the efficacy of how different saccharide structures altered sucrose crystallization (Leinen & Labuza, 2006; Thorat et al., 2018). The structures and properties of the emulsifiers used in this study are shown in Table 5.1. To test the hypothesis, these emulsifiers were selected to encompass a range of hydrophilic lipophilic balances (HLB, ~2-17), number of monosaccharide units (0-2), number of hydroxyl groups (~0-7), molecular weights, thermal and hygroscopic traits, and structural components.

5.3 Materials and Methods

5.3.1 Materials

The sucrose used in this study was obtained from Mallinckrodt Chemicals (Philipsburg, NJ), and the emulsifiers were a series of sucrose esters (stearic ester 30% (SP30), stearic ester 50% (SP50), stearic ester 70% (SP70), and palmitic ester 75% (PS750)) varying in the type of fatty acid as well as the percentage of mono-esters (30-75% as shown) relative to di- and tri-esters from Sisterna (Roosendaal, Netherlands); soy lecithin from Modernist Pantry (Eliot, ME); and

polysorbate 20 and polysorbate 80 from Florida Laboratories, Inc. (Fort Lauderdale, FL). The emulsifiers were chosen based on common usage in the food industry as well as variable structures of the compounds (as shown in Table 5.1).

Desiccators were prepared using phosphorus pentoxide (P_2O_5) (Fisher Scientific, Fair Lawn, NJ) to maintain a relative humidity (RH) of ~0% or by using the following saturated salt solutions to control the RH at higher levels: lithium chloride (~11% RH) obtained from Avantor Performance Materials (Center Valley, PA), potassium acetate (~23% RH) obtained from Fisher Scientific, and magnesium chloride (~33% RH) obtained from Fisher Scientific. For use in volumetric one-component Karl Fischer titrations, Karl Fischer reagents including HYDRANAL-Composite 2 (titrant), HYDRANAL-Methanol Rapid (working medium), and HYDRANAL-Water Standard 10 were purchased from Sigma-Aldrich (St. Louis, MO). Water used throughout the study was deionized and purified using a Barnstead E-Pure ultrapure water purification system (ThermoScientific, Waltham, MA) with a resistivity at 25ºC greater than 17.5 MΩ·cm.

5.3.2 Preparation of amorphous samples

Samples were prepared by freeze drying 10% *w/v* sucrose solutions with and without 1% and 5% (*w/w*) of the co-formulated emulsifier in which both the sucrose and the emulsifier were completely dissolved. There were 7 co-formulated emulsifier additives (Table 5.1), each added at two concentrations (1% and 5% *w/w* emulsifier/sucrose), giving a total of 14 dispersion preparations in addition to the control sucrose. The solutions were frozen at -20ºC for at least 12 h prior to lyophilization. Lyophilization was completed in a VirTis Genesis 25ES freeze dryer (SP Scientific, Warminster, PA). Samples were initially frozen in the freeze dryer at -40ºC and 300 mTorr (40 Pa) for 6 h. The freeze dryer was then held at -40ºC and 150 mTorr (20 Pa) for 24 h to allow for primary drying to occur. This was followed by an increase in temperature from -40ºC to 20ºC in increments of 10ºC, holding for 9 h at each step to allow for secondary drying. Finally, a heating step was completed at 25ºC and 300 mTorr (40 Pa) for 6 h, after which samples were immediately transferred to desiccators containing P_2O_5 (~0% RH). These samples were stored in the desiccators containing P_2O_5 at ambient temperature (22 ± 2ºC) until further analysis, and all subsequent sample handling was done in a glove box purged with nitrogen (to drop the ambient RH to ~5%).

5.3.3 Storage treatments

To initiate the RH storage treatments, the lyophiles were transferred from the desiccators containing P_2O_5 into desiccators containing saturated salt solutions of lithium chloride (~11% RH), potassium acetate (~23% RH), or magnesium chloride (~33% RH), which were then stored at 25ºC in a temperature-controlled room. Samples were removed from these desiccators and analyzed periodically over 4 weeks. A single desiccator was used for each timepoint of analysis to avoid exposing the samples to ambient RH until the day of their analysis. Samples were discarded after analysis.

5.3.4 Determination of crystallinity

A combination of powder x-ray diffraction (PXRD), polarized light microscopy (PLM), and Fourier-transform infrared spectroscopy (FTIR) was used to monitor the physical state of samples over time and to identify the onset of crystallization of the amorphous lyophiles (Figure 5.1). Lyophiles were analyzed on days 0, 7, 14, 21, and 28. Samples that were found to be crystalline on day 7 were further analyzed on days 2 and 4 by preparing fresh samples to narrow down the time at which the onset of crystallization occurred.

Powder x-ray diffraction

PXRD diffractograms were collected using a Rigaku Smartlab diffractometer (Rigaku Corporation, Tokyo, Japan) equipped with a Cu-Kα radiation source set in Bragg-Brentano geometry and operating at 40 kV and 40 mA. Samples were analyzed using a scan range of 10-35º 2θ at a scan speed of 15º/min and a step size of 0.02º. Samples with diffraction patterns consisting of peaks above a signal-to-noise ratio of 3 were considered PXRD crystalline. Samples with small peaks above the baseline were labeled partially crystalline, with increasing peak areas/intensities related to increasing crystallinity (Figure 5.1.A). Samples with no peaks and only an amorphous halo were considered to be PXRD amorphous.

Fourier-transform infrared spectroscopy

FTIR (TravelIR HCI, SensIR Technologies, LLC, Danbury, CT) with a fixed attenuated total reflectance (ATR) accessory was used to monitor crystallinity of sucrose in the lyophiles

using a method described in Lescure (1995) and Mathlouthi (1995). Briefly, crystalline sucrose can be identified by characteristic absorption peaks due to hydrogen bonding in the 2800-3800 cm^{-1} region. The FTIR was equipped with a TGA detector, resolution was set to 4 cm^{-1}, and samples were scanned 64 times from 650-4000 cm^{-1}. Spectra of control crystalline and amorphous sucrose samples were collected and used as comparisons to verify the physical state of the lyophiles. OMNIC Series Software (ThermoScientific) was used to analyze the spectra.

Polarized light microscope

Samples were observed with an Omano polarized light microscope (Omano, China), and crystal identification was done as described by Carlton (2011). Briefly, the appearance of birefringence in the lyophilized samples indicated crystallinity. Photographs to document sample appearance were taken using an iPhone 6s camera attached to the microscope eyepiece by an iDu LabCam adapter (Detroit, MI). The microscope was also paired with a RH-controlled stage (GenRH, Allentown, PA), and crystallization of a subset of samples was observed over time at 40% RH and ambient temperature (22 ± 2ºC). Timelapse videos of crystallization were taken using the iPhone 6s camera.

5.3.5 Dynamic vapor sorption

Three different moisture sorption profiles of all lyophiles were collected at 25ºC using a SPSx-1µ Dynamic Vapor Sorption Analyzer (Projekt Messtechnik, Ulm, Germany). For the first moisture sorption profile, samples (100-200 mg) were placed in a 23-ring sample holder and held at 0% RH for 48 h in the instrument. Samples were then analyzed from 0-80% RH in 5% RH increments, with a maximum residence time of 12 h per step and an equilibration end-point criterion of < 0.001% weight change within 30 min. The moisture sorption profile of each lyophile was plotted using the percent change in mass at the end of each RH step as the equilibration moisture gain at that RH. For the second moisture sorption profile, lyophiles (100-200 mg) were placed in a 23-ring sample holder, again held at 0% RH for 48 h, and then the RH was increased to 40% RH, at which samples were held for 96 h or until mass loss indicative of sucrose crystallization had occurred in all samples. The percent change in mass was plotted versus time to generate a moisture sorption/desorption profile with time, and the onset of mass loss was used to

identify the onset time of crystallization. For the third moisture sorption profile, lyophiles were prepared and handled the same as was done for the second profiling, but then the samples were held at 33% RH (instead of 40% RH) until mass loss (indicating crystallization) of most samples had occurred. The percent change in mass was plotted versus time, and onset of crystallization data were compared with those from the 40% RH moisture sorption profile as well as crystallization that occurred in the 33% RH desiccators.

5.3.6 Moisture content

The moisture contents of all initial lyophiles after exposure to 0% RH for 2-4 days, as well as all lyophiles that remained amorphous for the entire 4 weeks of exposure to 11%, 23%, or 33% RH, were determined using a one-component volumetric Karl Fischer titration method (V20S Volumetric KF Titrator, Mettler-Toledo, LLC, Columbus, OH). Approximately 50 mg of each lyophile was added directly to the HYDRANAL-Methanol Rapid working medium to extract water from the sample. The sample was then titrated using the HYDRANAL-Composite 2 titrant, which allowed moisture content to be measured in % moisture (*wb*). Calibration of the Karl Fischer titration system was completed prior to sample analyses using the HYDRANAL-Water Standard 10.0 (10 mg/g = 1% water content).

5.3.7 Differential scanning calorimetry

All lyophiles and starting materials were analyzed by differential scanning calorimetry (DSC) using a DSC 4000 (PerkinElmer, Waltham, MA). The instrument was calibrated with indium and verified with the melting point of water. Dry nitrogen was used to purge the system at 20 mL/min. Initial lyophiles that had been exposed to 0% RH for 2-4 days (5-10 mg) were weighed into 50 µL aluminum DSC pans (PerkinElmer), hermetically sealed, and punctured with a pinhole to allow water vapor to escape when determining 'dry' T_gs. The onset T_g was determined in a heat-cool-heat protocol from the second scan. Samples were first scanned by heating the samples in the DSC from 20ºC to 100ºC at a rate of 20ºC/minute. Samples were cooled to 20ºC at a rate of 50ºC/minute and held at 20ºC for 3 min to allow the temperature to equilibrate. A second scan then heated the samples from 20ºC to 100ºC at a rate of 20ºC/minute. All starting ingredients were also analyzed for T_g or melting point (T_m) using the heat-cool-heat protocol described above, only

varying the temperature range of the scans based on material. Pyris software (PerkinElmer) was used to calculate the onset T_g or onset T_m, which was defined as the temperature in which the endothermic event characterized by a baseline shift began in the second scan or the temperature in which a sharp endothermic peak began in the second scan, respectively.

5.3.8 Sample photography

Select samples were analyzed for appearance following crystallization in the second moisture sorption experiment, in which samples were held at 40% RH in the SPS moisture sorption instrument. These samples were photographed in a Deep Professional LED Photography light box and with the polarized light microscope using an iPhone 6s camera.

5.3.9 Scanning electron microscopy

Scanning electron microscopy (SEM) was completed using a NOVA nanoSEM Field Emission SEM (FEI Company, Hillsboro, OR) to identify differences in crystal morphology. Lyophiles in which sucrose had crystallized during storage at 33% RH were applied to double-sided carbon tape and coated using a platinum target coating system before analysis.

5.3.10 Statistical analysis

Samples were analyzed in duplicate for moisture sorption (time of crystallization), moisture content, T_g, and T_m. Single-variable ANOVA using SAS 9.4 (SAS Institute, Cary, NC) was used to determine significant differences in time of crystallization, moisture content, T_g, and T_m. Differences were determined using Tukey's post hoc test for multiple comparisons at a significance level of $\alpha = 0.05$. The HLB value, moisture content (initial, after 4 weeks at 11% RH, and after 4 weeks at 23% RH), T_g, molecular weight, and number of -OH groups were also plotted vs. time to crystallization to determine Pearson's correlation coefficients.

5.4 Results and Discussion

5.4.1 Stability of amorphous sucrose in RH-controlled desiccators

All of the sucrose lyophiles with and without emulsifiers were initially amorphous, as indicated by PXRD, FTIR, and PLM, except for the lyophiles containing the higher concentration (5% *w/w*) of polysorbate 80. The effects of storage RH on the time to sucrose crystallization in all lyophiles are summarized in Table 5.2, wherein it can be seen that different emulsifiers had different effects on the stability of amorphous sucrose. Most of the lyophiles that were initially amorphous remained so for the 4 week duration of storage in desiccators at 11% and 23% RH; however, all lyophiles containing polysorbates at both 1% and 5% (*w/w*) concentrations crystallized at these RHs. Increasing the concentration of either polysorbate and increasing the storage RH both resulted in shorter time to sucrose crystallization.

More varied times to sucrose crystallization (ranging from 2 days to 2 weeks) were found when the RH in the desiccators was increased to 33%. The lyophiles in which sucrose was fastest to crystallize (by day 2) at 33% RH included the control and those containing polysorbates, SP50 1%, and PS750 1%. The lyophiles that were slowest to crystallize at 33% RH (by day 14) were those containing SP70 5% and PS750 5%. Unlike the inverse stability trends seen with increasing polysorbate concentration resulting in decreased amorphous sucrose stability, it appeared that increasing the concentration of the sucrose esters tended to increase amorphous sucrose stability (delay time to crystallization). Sucrose esters containing higher percentages of mono-esters (instead of di- and tri-esters) generally resulted in longer amorphous sucrose stabilization. Based on the desiccator studies, the stabilizing trend of the emulsifiers, as documented by time to crystallization, followed the general trend: polysorbate 80 5% < polysorbate 20 5% < polysorbate 80 1% < polysorbate 20 1% < **sucrose control** ≈ SP50 1% ≈ PS750 1% < SP30 1% ≈ SP30 5% ≈ SP50 5% ≈ SP70 1% < soy lecithin 1% ≈ soy lecithin 5% < SP70 5% ≈ PS750 5%. No evidence of crystallization of the emulsifiers was found in PXRD diffractograms over time. Additional analyses were conducted to better understand the differing effects of the emulsifiers on sucrose crystallization.

5.4.2 Moisture content of amorphous sucrose lyophiles

The storage RH and sample moisture content are known to affect sucrose crystallization (Mathlouthi, 1995). When exposed to environments with RHs higher than the water activity of the sample, amorphous sucrose will absorb moisture, which results in a decrease in the T_g of the matrix and increase in molecular mobility. If conditions are favorable, molecular rearrangement and crystallization occur, at which point moisture is expelled (Makower & Dye, 1956). To enable comparisons between moisture contents and amorphous sucrose stability, the initial moisture contents of all lyophiles and the moisture contents of lyophiles that remained amorphous after 4 weeks of storage in 11% and 23% RH desiccators were measured (Table 5.3). All initial moisture contents of the lyophiles except for sucrose:SP30 1% were significantly lower ($p < 0.05$) than the sucrose control. The low initial moisture content found in sucrose:polysorbate 80 5% was likely due to the partially crystalline sucrose in the lyophile even immediately after lyophilization. Increasing the storage RH to 11% or 23% RH significantly increased all sample moisture contents but also resulted in no significant differences in moisture content between any of the lyophiles, including the control, at each RH. Taken together, these findings indicate that the addition of an emulsifier altered the moisture diffusion rates during lyophilization, generally resulting in lower initial moisture contents than the control, and that the addition of 1 and 5% (*w/w*) of the sucrose esters and soy lecithin did not alter the hygroscopicity of the samples at low storage RHs in desiccators compared to the control. Although these samples did not crystallize in these conditions, these data may indicate that matrix effects other than differences in hygroscopicity may contribute to the variations in sucrose crystallization onset times between the formulations.

5.4.3 Moisture sorption profile and sucrose crystallization

To enable direct comparisons between the samples of moisture sorption leading up to crystallization as well as the RH at which crystallization occurred (indicated by mass loss (Makower & Dye, 1956)), moisture sorption profiles were collected from 0-80% RH in a gravimetric moisture sorption instrument (Figure 5.2.A). A 48 h drying step at 0% RH was done in the instrument prior to this data collection to remove significant differences in the initial moisture contents. While most lyophiles (including the control) crystallized at 40% RH, two lyophiles exhibited delayed sucrose crystallization (sucrose:SP50 1% crystallized between 40 and

45% RH, and sucrose:soy lecithin 5% did not crystallize until 45% RH), and the lyophiles containing polysorbates crystallized at lower RHs (sucrose lyophiles containing polysorbate 20 and 80 at both 1% and 5% crystallized at 30% and 10-15% RH, respectively; however, sucrose:polysorbate 80 5% was partially crystalline initially, so crystallization shown by moisture sorption was affected).

The percent moisture gained before crystallization varied between samples, with some formulations crystallizing at lower moisture contents than the control and others not crystallizing until moisture contents were higher. The sucrose control gained 6% weight before crystallizing. The formulations that did not crystallize until moisture contents surpassed 6% were: sucrose:SP70 5% and sucrose:PS750 5%, which gained 8% weight, sucrose:SP50 5% and sucrose:soy lecithin 5%, which gained 7.5% weight, and sucrose:SP70 1%, sucrose:PS750 1%, sucrose:SP30 5%, and sucrose:soy lecithin 1%, which gained 7% weight. Lyophiles that sorbed less water than the control prior to sucrose crystallization were sucrose:polysorbate 20 and 80 1% and 5% lyophiles, gaining less than 5% and 1% weight before extensively crystallizing, respectively (though sucrose:polysorbate 80 5% was partially crystalline initially). Aside from the polysorbate-containing lyophiles, moisture sorption trends at ~11 and 23% RH shown in Figure 5.2.A indicated no differences between lyophiles, in agreement with the moisture content data (Table 5.3).

A second set of moisture sorption profiles was collected for samples exposed to a constant 40% RH after drying (Figure 5.2.B), and the times at which mass loss indicative of crystallization occurred in this treatment are recorded in Table 5.2. Crystallization differences between lyophiles have been clearly exhibited in a reasonable timeframe between 32% and 43% RH (Saleki-Gerhardt & Zografi, 1994; Shamblin & Zografi, 1999), as was the case in previous studies investigating additive effects on amorphous sucrose stability (Thorat, Forny, et al., 2017). Here again, differences in the amount of water sorbed prior to sucrose crystallization and the onset time for crystallization were found between the different emulsifier formulations. As in the desiccator studies, the presence of polysorbates resulted in faster sucrose crystallization onset times (1.5-3.95 h) compared to the control, which did not crystallize until hour 15 (Figure 5.2.B, Table 5.2). The emulsifier formulations that most delayed sucrose crystallization were: sucrose:SP70 5%, which crystallized at hour 27, and sucrose:soy lecithin 5%, which crystallized at hour 24. All other lyophiles crystallized between hours 14 and 19, times which were not significantly different than the control sucrose. Unlike trends in the desiccator studies, increasing the degree of mono-esters

(as opposed to di- and tri-esters) in the sucrose esters did not correlate to increased time before sucrose crystallization occurred ($R^2 = 0.047$), and increasing the amount of emulsifier (from 1 to 5% *w/w*) did not result in significantly delayed crystallization except for SP70 and soy lecithin.

Based on the 40% RH SPS experiment, the stabilizing trend of the emulsifiers for delaying sucrose crystallization was: polysorbate 80 5% < polysorbate 20 5% < polysorbate 80 1% < polysorbate 20 1% < soy lecithin 1% < SP30 1% ≈ SP70 1% < **sucrose control** ≈ PS750 5% < SP50 5% < SP50 1% < PS750 1% < SP30 5% < soy lecithin 5% < SP70 5% (Figure 5.2.B, Table 5.2). This trend differed in several places from that found in the 33% RH desiccator experiments (Table 5.2), with some formulations providing better stability and others no longer delaying sucrose crystallization compared to the control. Most notably, sucrose:PS750 5% was one of the most stable lyophiles in the desiccator experiment but did not significantly delay sucrose crystallization compared to the control in the 40% RH SPS experiment. Similarly, the sucrose:soy lecithin 1% formulation was the most stable of those containing 1% emulsifier in the desiccators but was not significantly different from the control in the 40% RH experiment. Conversely, sucrose:soy lecithin 5% had similar effects to other emulsifiers in the desiccator experiments but was one of the most stable lyophiles in the 40% RH SPS experiment, remaining amorphous until 24 h into the experiment. Sucrose:PS750 1% was also much more successful at stabilizing sucrose in the 40% RH SPS experiment than in the 33% RH desiccators. Aside from these differences, the polysorbates followed the same trends in both the desiccator and SPS experiments, resulting in more rapid sucrose crystallization than the control, and both experiments found sucrose:SP70 5% to be the most stable lyophile.

Differences in crystallization trends between the SPS experiment at a constant 40% RH and the desiccator studies could have been caused by the drying step done in the SPS experiment, the passive vs. active headspace differences between the treatments, and/or the higher RH of the SPS experiment (40% RH) compared to the 33% RH desiccator. To better determine whether the differences in the experiments were due to the method of storage or specifically as a result of the difference in storage RHs (33% vs. 40%), a third set of sorption profiles was collected in the SPS in which the lyophiles were held at a constant 33% RH (Figure 5.2.C, Table 5.2). The stabilizing trend of the emulsifiers in this experiment was: polysorbate 80 5% ≈ polysorbate 20 5% < polysorbate 80 1% ≈ polysorbate 20 1% < PS750 1% < **sucrose control** < soy lecithin 1% < SP50 1% < SP30 1% < SP70 1% < SP30 5% < SP50 5% < soy lecithin 5% < SP70 5% < PS750 5%.

Other than the low stability of sucrose:soy lecithin 1% in both the 33% and 40% RH SPS experiments (compared to high stability in the 33% RH desiccator), the overall trend of this 33% RH SPS experiment was more similar to the 33% RH desiccator experiment than the higher 40% RH moisture sorption results, indicating that the difference in RH (33 vs. 40% RH) was likely the main reason for the differing stability trends noted previously. The formulation that exhibited the most RH-dependent properties was sucrose:PS750 5%, which was the most stable amorphous lyophile (along with sucrose:SP70 5%) in both the 33% RH desiccator and SPS experiments, only partially crystallizing in the 3 week SPS experiment, but was not significantly different than the control in the 40% RH SPS experiment. While it is known that RH plays a key role in sucrose crystallization (Mathlouthi, 1995; Shamblin & Zografi, 1999), PS750 was the only emulsifier to show such a dramatic difference in stabilization of amorphous sucrose between 33% and 40% RH.

5.4.4 Effect of glass transition temperature on amorphous sucrose stability

The 'dry' T_gs of lyophiles in this experiment are shown in Table 5.3 and in the appendix Figure A.5.1. Although previous studies report an increase in T_g as the underlying reason additives delay crystallization in a variety of food systems, including sucrose matrices (Roos & Karel, 1991b; van Hook, 1961), no significant trends were found between T_g and sucrose crystallization time in the current study (when excluding polysorbates, $R^2 = 0.006$). The small amount of emulsifier added relative to sucrose (1% and 5% *w/w*) did not significantly alter the 'dry' T_g compared to the sucrose control (56.5°C), except for sucrose:SP70 1%, which had a T_g of 64°C. The other difference noted was that the sucrose:SP30 5% sample had 2 T_gs, presumably due to heterogeneity. Previous studies on sucrose crystallization in the presence of salts and saccharides have also shown that there is not a direct relationship between crystallization onset times and T_g (Thorat, Forny, et al., 2017; Thorat et al., 2018). Exposing lyophiles to increasing environmental RHs would be expected to drop the T_gs of all samples in a predictable manner based on moisture content (according to models such as the Gordon-Taylor equation), and therefore crystallization would be expected to correlate with moisture content since no significant differences were found between the majority of the 'dry' T_gs of the lyophiles. However, this was not the case, as shown in Figure 5.2. Discrepancies found in Gordon-Taylor modeling of sucrose:saccharide lyophiles with varying moisture contents suggested that factors beyond T_g, specifically structural compatibility of the saccharides with sucrose, contributed to the stabilization of amorphous sucrose (Thorat et al., 2018), and the lack

of correlation between T_g and crystallization onset times in sucrose:salt lyophiles was due to ion-water interactions and possible hydration pockets around the ions in the lyophiles affecting T_g and plasticization of amorphous sucrose (Thorat, Forny, et al., 2017).

Despite the lack of evidence that lyophile T_g correlated to delay in sucrose crystallization, the thermal behaviors of the individual emulsifiers were investigated. Experimental values for T_gs of sucrose and soy lecithin and T_ms of sucrose, sucrose esters, soy lecithin, and polysorbates are provided in Table 5.1. The T_gs of polysorbates were too low to be measured by this DSC (< -50ºC). Two T_ms were found for the sucrose esters (appendix Figure A.5.2), which agrees with the report by Szűts, Pallagi, Regdon, Aigner, and Szabó-Révész (2007), although the range of T_ms found differed. It is important to note that the sample storage temperature (25ºC) was above the melting temperature of some of the emulsifiers (Table 5.1), and T_g is always lower than T_m, often by a factor of T_g/T_m = 2/3 (Sakka & Mackenzie, 1971). Polysorbates are known to be plasticizers, having T_gs around -61ºC and T_ms from -15 to 20ºC (Amim, Blachechen, & Petri, 2012; Amim, Kawano, & Petri, 2009). Polysorbates have much lower T_gs and T_ms than sucrose or the other emulsifiers in this study (Table 5.1). These properties may have led to more localized plasticization of the sucrose matrix when polysorbates were added than in matrices with the other emulsifiers, which may have contributed to the more rapid sucrose crystallization onset times found in these samples. While the T_gs of other emulsifiers were also slightly lower than that of sucrose, indicating that if stability is related to T_g, the samples containing the other emulsifiers should theoretically have been less stable as well, the T_gs of the emulsifiers in this study (except polysorbates) were at most 15ºC less than that of sucrose. This magnitude of difference was small enough that the lowering effect on the T_g of the lyophiles by these emulsifiers was not significant (Table 5.3). Additionally, the T_gs of these lyophiles remained above room temperature.

5.4.5 Effect of emulsifier structural properties on amorphous sucrose stability

Role of emulsifier structural similarity to sucrose

It has previously been shown that when T_g is not significantly affected by additives, the structure of the additive plays the major role in influencing comparative stabilization of amorphous sucrose against crystallization (Leinen & Labuza, 2006; Saleki-Gerhardt & Zografi, 1994; Thorat et al., 2018). The stabilizing effect for delaying sucrose crystallization seems to be best when the

additive has a region that is structurally similar to sucrose, usually a glucose or fructose unit, that is able to interact with sucrose at the crystal interface and also has a dissimilar structural region that prevents further incorporation of sucrose into the crystal lattice (Thorat et al., 2018). When considering the structures of the emulsifiers used, the sucrose esters had a region that was most structurally similar to sucrose. Theoretically, the glucose and fructose units in the sucrose esters could have interacted with sucrose, and the fatty acid region could have disrupted sucrose crystal growth. This concept is similar to a report on how raffinose disrupts sucrose crystallization: the glucose and fructose units on raffinose attach to the sucrose crystal interface, and the galactose unit disrupts further incorporation into the sucrose crystal lattice, slowing crystal growth (Leinen & Labuza, 2006). However, the success of the sucrose esters at delaying crystallization was minimal in the 40% RH SPS experiment, with only the sucrose:SP70 5% and sucrose:PS750 5% formulations significantly delaying sucrose crystallization time compared to the control (Figure 5.2.B, Table 5.2). These emulsifiers (SP70 and PS750) had a higher fraction of mono-esters, and thus less di- and tri-esters, than the other sucrose esters studied. The lower molecular weight of these two sucrose esters led to a greater contribution of molecules since the samples were prepared on a weight basis. Assuming the species adsorb with the head group to sucrose, the presence of the fatty acid tails was what disrupted crystallization. It does not appear that length of the tail, and in effect hydrodynamic radius, played a role in efficacy of delaying crystallization, but rather the prevalence of sucrose head groups determined how effective the sucrose ester was at disrupting crystallization as long as any tail was present.

Role of emulsifier HLB values

While the efficacy of sucrose esters increased with increasing HLB value, when the HLB values of the other emulsifiers studied were considered, there was no correlation between the HLB value of emulsifiers and crystallization time ($R^2 = 0.197$). The ability of the sucrose esters to inhibit sucrose crystallization was more likely due to degree of ester substitution than HLB value since soy lecithin, which has a lower HLB value than the sucrose esters, was more effective at delaying crystallization than many of the sucrose esters, and polysorbates, which have higher HLB values, induced crystallization.

Role of emulsifier structural dissimilarity to sucrose

Although phosphatidylcholine (in soy lecithin) lacks a structurally similar region to sucrose, which may indicate that it would not be successful at delaying sucrose crystallization, soy lecithin contains other phospholipids as well, including phosphatidylethanolamine, phosphatidylinositol (which contains a monosaccharide unit), phosphatidylserine, and phosphatidic acid (Poirier, 2011). The heterogeneity of phospholipids found in soy lecithin may have contributed to the delay of sucrose crystallization seen in this study due to a wider variety of impurities present in the sample despite the absence of many structurally similar regions to sucrose (Gabarra & Hartel, 1998; Smythe, 1967).

The polysorbates also lack a structurally similar region to sucrose and have multiple long hydrophobic chains. However, unlike soy lecithin, both polysorbates at both concentrations induced a much faster rate of crystallization than occurred in the sucrose control (0.1x-0.3x in the 40% RH SPS experiment) (Figure 5.2.B, Table 5.2). The lyophile containing 5% polysorbate 80 was never fully amorphous and crystallized faster than lyophiles containing polysorbate 20 in all experiments conducted. The structural differences between these polysorbates (polysorbate 80 contains an oleic acid chain (18:1 *n*-9) and polysorbate 20 contains a lauric acid chain (12:0)) suggested that the longer fatty acid side chain may have played a role in inducing crystallization. Increasing the amount of either polysorbate from 1% to 5% resulted in faster sucrose crystallization.

Interestingly, it was observed that while most lyophiles (including the control) collapsed before crystallizing, the sucrose:polysorbate lyophiles did not collapse and did not change much in physical appearance upon crystallization. PLM and light box images of the physical polysorbate lyophiles compared to the control that better illustrate this anomaly are shown in Figure 5.3.A. The crystallization of these lyophiles at 40% RH was documented using PLM and a RH-controlled microscope stage, with videos of these events provided in the appendix (Figures A.5.3, A.5.4, and A.5.5). The videos show that while the sucrose control has a changed morphology when exposed to 40% RH as a response to moisture sorption, collapses and becomes rubbery (due to sorbed moisture lowering the T_g), and then crystallizes, the sucrose:polysorbate lyophiles did not undergo the same extent of physical collapse or plasticization before crystallizing. Collapse precedes crystallization and is caused by decreased viscosity as a response to moisture sorption (Roe & Labuza, 2005). The lack of collapse in the polysorbate-containing lyophiles was presumably

because the rate of crystallization was faster than collapse. The videos also suggest that there was a difference in nucleation between the control and the polysorbate-containing lyophiles. The control sucrose had few nucleation sites, which grew larger to eventually completely crystallize the sucrose. Conversely, the sucrose:polysorbate lyophiles had a large number of nucleation sites from which not much crystal growth was seen under the microscope.

The sucrose and sucrose:polysorbate lyophiles were also viewed by SEM after they had crystallized (Figure 5.3.B). Visual observation of these samples supports the supposition that increased nucleation occurred in the polysorbate-containing lyophiles. While the surface of the control was smooth, the crystals from the polysorbate samples had a bumpy and jagged surface. This rough surface indicated that nucleation was rampant and crystal growth was limited in the presence of the polysorbates. A smaller crystal size and higher surface area also demonstrated that crystal growth was less extensive (Canselier, 1993). The sucrose crystals formed in the polysorbate lyophiles were porous, as seen in SEM and PLM images, consistent with the lack of collapse and the increased nucleation rate causing formation of many small crystallites. The formation of this porous crystalline structure made of small crystallites generated a large surface area that facilitated rapid release of moisture from the crystallizing amorphous sucrose. Without such fast moisture release from the matrix, the water would have plasticized the remaining amorphous fraction, leading to the collapse that was observed in the other lyophiles in this study. The high surface area of the porous crystals has many additional implications, including altered texture and dissolution. The irregular shape of the crystals may also indicate heterogeneous nucleation and growth of the sucrose on the surface of the polysorbates (Verma, Zeglinski, Hudson, Davern, & Hodnett, 2018).

Role of emulsifier critical micelle concentration

Another emulsifier property of potential interest is the critical micelle concentration (CMC) since the presence of micelles could contribute to the regions of interaction between the emulsifiers and sucrose in solution prior to lyophilization. While CMCs of the emulsifiers were not measured in this study, the concentrations of polysorbates used (1% and 5%) are greater than reported aqueous CMCs for both polysorbates 20 and 80 (Mahmood & Al-Koofee, 2013; Wan & Lee, 1974), and the concentrations of sucrose esters used in this study were also above the CMC since the CMCs of sucrose esters are generally lower than for polysorbates (Becerra, Toro, Zanocco, Lemp, & Günther, 2008). Because lecithin has such a low HLB value, it does not have a well-reported

CMC in water. Since micelles were likely formed in all formulations (micelles were present in solution prior to lyophilization and presumably surfactant was trapped in this form following water removal), except perhaps the sucrose:soy lecithin lyophiles, and polysorbates induced sucrose crystallization while sucrose esters and soy lecithin delayed or had no effect on sucrose crystallization, it was concluded that CMC was not a significant factor in how the emulsifiers altered sucrose crystallization.

Role of emulsifier templating and intermolecular hydrogen bond lifetime with sucrose

Previous studies on the effect of surfactants on crystallization in the pharmaceutical industry have shown that, due to their inherent properties, surfactants with unbranched hydrophobic chains, including polysorbate 80, are more flexible than surfactants with bulky hydrophobic groups and are therefore able to act as a template and align molecules in the optimal configuration to promote nucleation (Berman, June Ahn, & Lio, 1995; Chen, Ormes, Higgins, & Taylor, 2015; Weissbuch, Addadi, Leiserowitz, & Lahav, 1988). Although the studies cited here describe a templating effect by a hydrophobic chain for a hydrophobic crystal, the steric properties indicate that the same effect is worth considering in the case of the numerous nucleation sites and more rapid sucrose crystallization observed in the sucrose:polysorbate lyophiles (Figures 5.2, A.5.4, and A.5.5 and Table 5.2). Generally, a surfactant that is less flexible is unable to have this templating effect and instead inhibits nucleation by mass transfer effects. Assuming the templating theory plays a role in this study, the structural differences between polysorbates and other emulsifiers may have contributed to the absence of this effect in the sucrose ester and soy lecithin lyophiles even though they do not contain exceptionally bulky hydrophobic groups. For example, although sucrose esters also have an unbranched hydrophobic chain, the presence of a sucrose head group caused the sucrose esters to act more like a raffinose additive in which the sucrose group adsorbs to the crystal interface and the hydrophobic chain prevents mass transfer of sucrose into the crystal lattice (Leinen & Labuza, 2006). However, it is interesting to note that when more di- or tri-esters (unbranched hydrophobic chains) were present in the sucrose esters (SP30 and SP50), the sucrose esters were not as successful at stabilizing the amorphous sucrose (Figure 5.2, Table 5.2). This may indicate that there is a contradictory effect between the presence of a sucrose group and the presence of unbranched hydrophobic chains which prevented the sucrose:SP30 and sucrose:SP50 lyophiles from being significantly more stable than the sucrose control. Additionally,

soy lecithin also has unbranched hydrophobic chains; however, the presence of multiple types of phospholipids introduces some branched chains and some monosaccharide units, and the higher prevalence of pi bonds decreases the flexibility of the hydrophobic groups. The presence of these bulkier groups in soy lecithin may have prevented the templating effect seen in the sucrose:polysorbate lyophiles.

While the templating effect is a plausible explanation for the increased sucrose nucleation seen in the sucrose:polysorbate lyophiles, an alternative, and possibly more likely, theory is the propensity for hydrogen bonding between sucrose and polysorbates (Cui, Zhang, Yin, & Gong, 2012; Galek, Fábián, Motherwell, Allen, & Feeder, 2007; Verma et al., 2018). It has been reported that when hydrogen bonding is favorable between a compound of interest and a heterosurface (polysorbates in the current study), nucleation is promoted in solution due to the lengthened lifetime of the favorable hydrogen bond (Cui et al., 2012; Verma et al., 2018). Since the polysorbates have only three hydrogen bond donors but have 26 hydrogen bond acceptors, there is a high propensity for hydrogen bonding between the polysorbates and the hydrogen bond donor-rich sucrose molecules. The lengthened lifetimes of these hydrogen bonds promote more sucrose-sucrose interactions and increase the chance that the crystal nucleus survives (Verma et al., 2018). Essentially, the polysorbates create a surface which allows for the clustering and therefore crystallization of the sucrose, which also accounts for the irregular crystal shape shown in Figure 5.3.B. While phosphatidylcholine found in soy lecithin contains no hydrogen bond donor groups, which could cause it to act like the polysorbates, other phospholipids contained in soy lecithin contain some hydrogen bond donor groups and also some monosaccharides units (Poirier, 2011). The hydrogen bond donor groups on phospholipids may hydrogen bond with other phospholipids rather than with sucrose, lowering the propensity for hydrogen bonding with sucrose. Monosaccharides found in the phospholipids may promote interactions with sucrose as was discussed in crystallization inhibition by raffinose (Leinen & Labuza, 2006), which is why they effectively delay crystallization despite their high density of hydrogen bond acceptor groups. Sucrose esters also contain some hydrogen bond acceptors; however, as previously noted, sucrose esters contain a structurally similar region to sucrose that allowed them to interact with sucrose and prevent further incorporation into the crystal lattice. The amorphous sucrose stabilization induced by the presence of soy lecithin and sucrose esters due to the presence of monosaccharide units despite the presence of hydrogen bond acceptors suggested that when a structurally similar

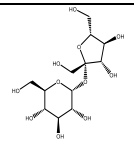
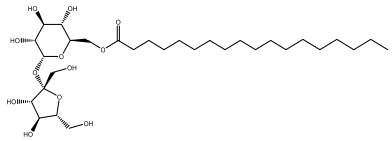
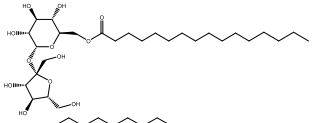
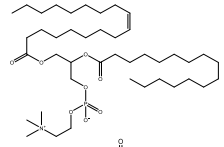
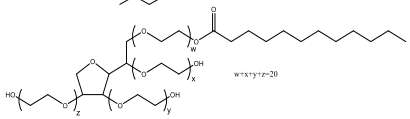
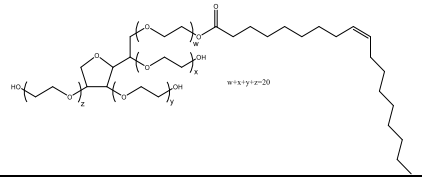
region to sucrose is present in the additive, that emulsifier property outweighed all others when considering the delay of sucrose crystallization.

5.5 Conclusion

Different emulsifiers had varying effects on the crystallization rates of amorphous sucrose, ranging from accelerating to delaying the onset time of crystallization. Most lyophiles remained amorphous in desiccators at low RHs (11 and 23% RH), except for lyophiles containing polysorbates, and increasing storage RH led to variations in moisture sorption and crystallization tendencies. The structure of the emulsifier was considered to be the major factor contributing to crystallization trends in the sucrose:emulsifier lyophiles, and no correlation was found between moisture sorption, critical micelle concentration, or T_g and crystallization onset time. Sucrose esters contained a structurally similar region to sucrose which was able to interact at the crystal interface, and the ester side chains prevented further incorporation into the crystal lattice, thereby delaying the crystallization of sucrose (by up to 1.8x that of the control at 40% RH and longer at lower RHs). When such a region of structural similarity was not present, intermolecular hydrogen bonding and structural heterogeneity seemed to influence the sucrose crystallization, contributing to the efficacy of soy lecithin (up to 1.6x at 40% RH). Polysorbates destabilized sucrose crystallization, with crystallization times as low as 0.1x that of the sucrose control at 40% RH, attributed to the long fatty acid and polyoxyethylene side chains that seemed to have a templating effect that increased sucrose nucleation and inhibited structural collapse during crystallization. These findings provide insight into mechanisms by which emulsifiers alter sucrose crystallization and could be useful for designing formulations to alter or control the crystallization of amorphous sucrose in low moisture products.

5.6 Tables and Figures

Table 5.1 Properties of sucrose and emulsifiers used in lyophiles. Uppercase superscript letters denote statistical significance between experimental T_gs, and lowercase superscript letters denote statistical significance between experimental T_ms (T_{m1}s and T_{m2}s).

Component	Average MW (g/mol)	HLB	HBD/HBA	Number of –OH units	Starting Physical State at RT	Structure	Amorphous	Crystalline	
							Onset T_g	Onset T_{m1}	Onset T_{m2}
Sucrose [1]	342.3	-	8/11	8	Crystalline solid		56.5 ± 0.5°C ^{A} (freeze-dried)	187.9 ± 0.7°C ^{a}	-
SP30 [2]	888.55	6	7/12	5.6 – 6.3	Crystalline solid		-	22 ± 2°C ^{e}	57 ± 1°C ^{b}
SP50 [2]	808.61	11	7/12	6 – 6.5	Crystalline solid		-	19 ± 2°C ^{e}	46 ± 1°C ^{d}
SP70 [2]	728.67	15	7/12	6.4 – 6.7	Crystalline solid		-	18.9 ± 0.9°C ^{ef}	52 ± 2°C ^{bc}
PS750 [2]	683.63	16	7/12	6.5 – 6.75	Crystalline solid		-	14 ± 1°C ^{f}	51 ± 1°C ^{cd}
Soy Lecithin (Phospha-tidylcholine) [3]	643.9	2-7	0/8	0	Amorphous solid		41 ± 2°C ^{B}	190.7 ± 0.5°C ^{a}	-
Polysorbate 20 [4,5]	1228	16.7	3/26	3	Liquid		< -50°C ^{C}	-23 ± 2°C ^{g}	-
Polysorbate 80 [4,5]	1310	15	3/26	3	Liquid		< -50°C ^{C}	-19.6 ± 0.8°C ^{g}	-

[1] Slade and Levine (1991)
[2] Szűts et al. (2007)
[3] Bueschelberger, Tirok, Stoffels, and Schoeppe (2015)
[4] Cottrell and van Peij (2015)
[5] Amim et al. (2012)

Table 5.2 Physical stability of sucrose lyophiles in controlled RH desiccators measured by a combination of PXRD, FTIR, and PLM as well as time of crystallization of amorphous sucrose lyophiles on exposure to 33% and 40% RH in the SPS instrument and the enhancement compared to the control based on the SPS data. Superscript letters denote statistical significance between times of crystallization.

	Co-formulated Additive	Percent Additive	Crystallization in Desiccators at			Crystallization in SPS at 40% RH		Crystallization in SPS at 33% RH	
			11% RH*	23% RH*	33% RH*	Crystallization time (hr)	Enhancement Compared to Control	Crystallization time (hr)	Enhancement Compared to Control
Sucrose	-	-	A	A	Day 2 (PC)	15 ± 2^{BC}	1x	85 ± 3^{E}	1x
Sucrose	SP30	1%	A	A	Day 4	14.6 ± 0.2^{BC}	1x	111 ± 1^{DE}	1.3x
Sucrose	SP30	5%	A	A	Day 4	18.5 ± 0.6^{B}	1.2x	120 ± 20^{CD}	1.4x
Sucrose	SP50	1%	A	A	Day 2 (PC)	17 ± 2^{BC}	1.1x	99 ± 8^{DE}	1.2x
Sucrose	SP50	5%	A	A	Day 4 (PC)	15.55 ± 0.07^{BC}	1x	143 ± 4^{CD}	1.7x
Sucrose	SP70	1%	A	A	Day 4	14.6 ± 0.4^{BC}	1x	120 ± 20^{DE}	1.4x
Sucrose	SP70	5%	A	A	Day 14 (PC)	27.0 ± 0.4^{A}	1.8x	200 ± 10^{B}	2.3x
Sucrose	PS750	1%	A	A	Day 2	18.2 ± 0.6^{BC}	1.2x	80 ± 10^{E}	0.9x
Sucrose	PS750	5%	A	A	Day 14	15 ± 2^{BC}	1x	260 ± 30^{A}	3x
Sucrose	Soy Lecithin	1%	A	A	Day 7	14 ± 1^{C}	1x	90 ± 20^{E}	1x
Sucrose	Soy Lecithin	5%	A	A	Day 7	24.1 ± 0.2^{A}	1.6x	169 ± 7^{BC}	2x
Sucrose	Polysorbate 20	1%	Day 14 (PC)	Day 4	Day 2	3.95 ± 0.07^{D}	0.3x	5.1 ± 0.6^{F}	0.06x
Sucrose	Polysorbate 20	5%	Day 2	Day 2	Day 2	1.8 ± 0.1^{D}	0.1x	0.8 ± 0.4^{F}	0.009x
Sucrose	Polysorbate 80	1%	Day 14 (PC)	Day 2 (PC)	Day 2	3.45 ± 0.07^{D}	0.2x	5.1 ± 0.1^{F}	0.06x
Sucrose	Polysorbate 80	5%	Never fully amorphous			1.5 ± 0.1^{D}	0.1x	0.9 ± 0.2^{F}	0.01x

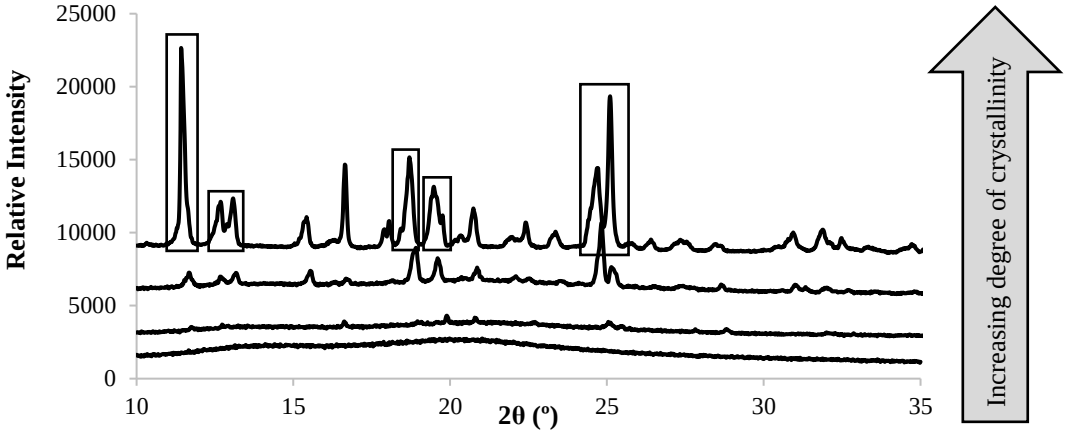
* Samples that remained amorphous for the entire 4 week desiccator study are marked "A"; length of time prior to evidence of crystallization is indicated otherwise. PC indicates the onset of crystallization before sample was largely crystalline.

Table 5.3 Percent moisture content (*wb*) of amorphous sucrose lyophiles prior to desiccator storage (Day 0) and samples that remained amorphous after 4 weeks of storage at 11% and 23% RH and onset T_gs of initial (dry) amorphous lyophiles. Uppercase superscript letters on moisture content data denote statistical significance between percent moisture of each lyophile at the specified timepoint, and lowercase superscript letters on moisture content data denote statistical significance between percent moisture of the specified lyophile at each timepoint. Superscript letters on T_g data denote statistical significance between T_gs only. Statistical analysis was run separately for each trial.

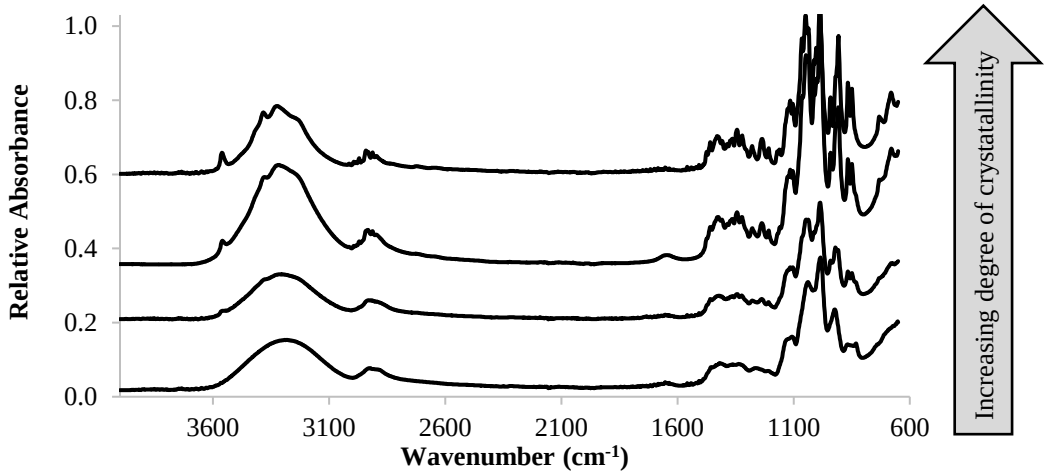
	Co-formulated Additive	Percent Additive	Week 0	Week 4 11% RH	Week 4 23% RH	Week 0	
						T_{g1}	T_{g2} *
Sucrose	-	-	2.2 ± 0.1% ^{Aa}	3.12 ± 0.07% ^{Ab}	5.07 ± 0.08% ^{Ac}	56.5 ± 0.5°C ^{BCD}	
Sucrose	SP30	1%	1.9 ± 0.2% ^{ABa}	3.08 ± 0.03% ^{Ab}	4.97 ± 0.03% ^{Ac}	61 ± 2°C ^{ABCD}	
Sucrose	SP30	5%	1.05 ± 0.04% ^{Ea}	2.99 ± 0.00% ^{Ab}	4.7 ± 0.3% ^{Ac}	57 ± 2°C ^{BCD}	66 ± 1°C
Sucrose	SP50	1%	1.29 ± 0.03% ^{CDEa}	3.10 ± 0.06% ^{Ab}	5.005 ± 0.007% ^{Ac}	58.4 ± 0.4°C ^{ABCD}	
Sucrose	SP50	5%	1.14 ± 0.02% ^{DEa}	3.0 ± 0.1% ^{Ab}	4.9 ± 0.1% ^{Ac}	57 ± 3°C ^{BCD}	
Sucrose	SP70	1%	1.29 ± 0.09% ^{CDEa}	2.99 ± 0.02% ^{Ab}	4.98 ± 0.05% ^{Ac}	64 ± 3°C ^{A}	
Sucrose	SP70	5%	1.4 ± 0.1% ^{CDEa}	3.3 ± 0.1% ^{Ab}	4.8 ± 0.1% ^{Ac}	58 ± 2°C ^{ABCD}	
Sucrose	PS750	1%	1.6 ± 0.2% ^{BCa}	3.2 ± 0.1% ^{Ab}	4.9 ± 0.2% ^{Ac}	58 ± 2°C ^{ABCD}	
Sucrose	PS750	5%	1.08 ± 0.06% ^{DEa}	3.0 ± 0.3% ^{Ab}	5.1 ± 0.1% ^{Ac}	55.1 ± 0.5°C ^{D}	
Sucrose	Soy Lecithin	1%	1.17 ± 0.04% ^{DEa}	3.24 ± 0.08% ^{Ab}	5.01 ± 0.01% ^{Ac}	62.3 ± 0.9°C ^{AB}	
Sucrose	Soy Lecithin	5%	1.285 ± 0.007% ^{CDEa}	3.1 ± 0.3% ^{Ab}	4.9 ± 0.1% ^{Ac}	62.5 ± 0.6°C ^{AB}	
Sucrose	Polysorbate 20	1%	1.51 ± 0.09% ^{BCD}	-	-	61.3 ± 0.8°C ^{ABC}	
Sucrose	Polysorbate 20	5%	1.06 ± 0.08% ^{DE}	-	-	-	
Sucrose	Polysorbate 80	1%	1.39 ± 0.03% ^{CDE}	-	-	56.1 ± 0.8°C ^{CD}	
Sucrose	Polysorbate 80	5%	0.9 ± 0.2% ^{E}	-	-	-	

* T_{g2} was found for one sample due to heterogenous nature of the sample at 5% additive.
No T_g was found for sucrose:polysorbate lyophiles at 5% additive

A)



B)



C)

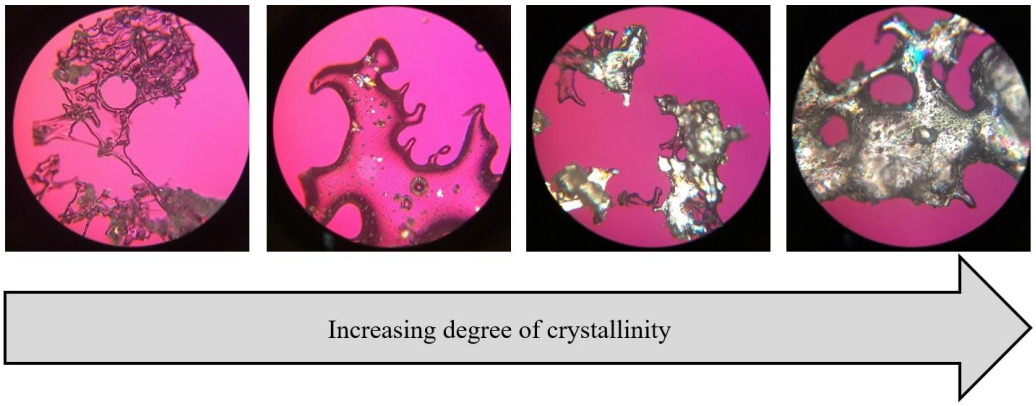
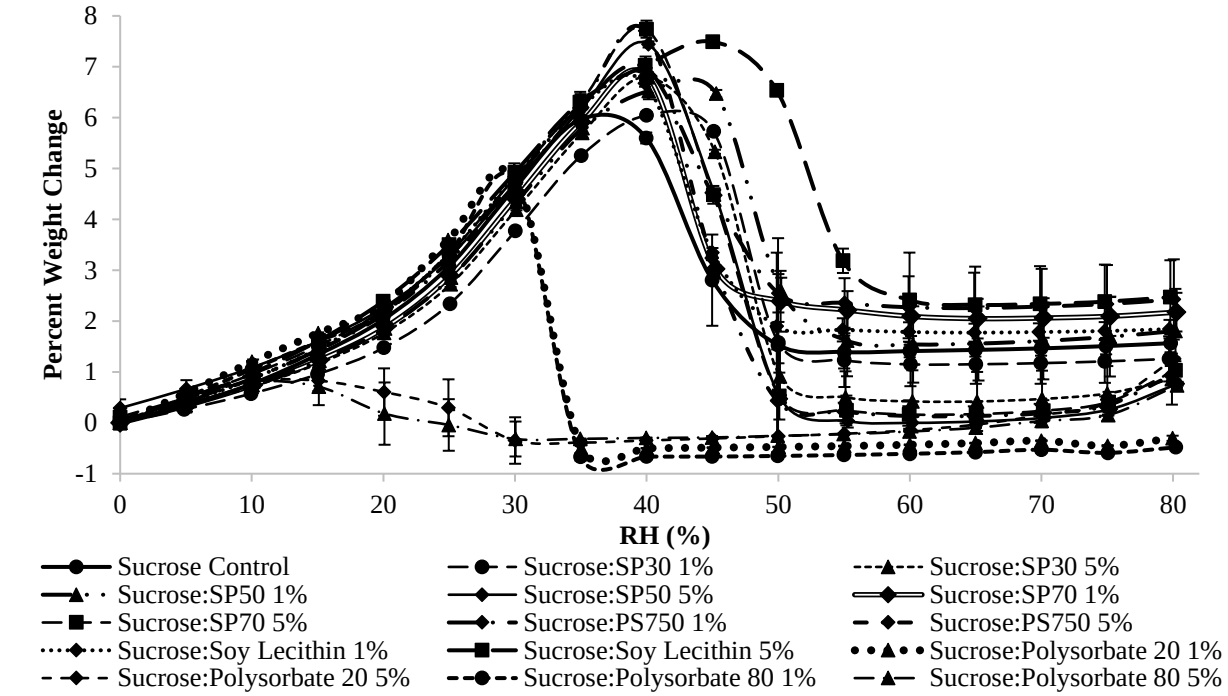


Figure 5.1 Analysis of select sucrose lyophiles over time indicating increasing degree of crystallinity, from completely amorphous to completely crystalline, interceded with increasing degrees of crystallinity of A) powder x-ray diffractograms, where boxed in peaks are the well-defined crystalline sucrose peaks (Leinen & Labuza, 2006), B) FTIR spectra, where crystallinity was evaluated by the characteristic absorption peaks of crystalline sucrose in the region of 2800-3800 cm^{-1} wavenumbers (Lescure, 1995; Mathlouthi, 1995), and C) PLM images, where birefringence indicates crystallinity.

A)



B)

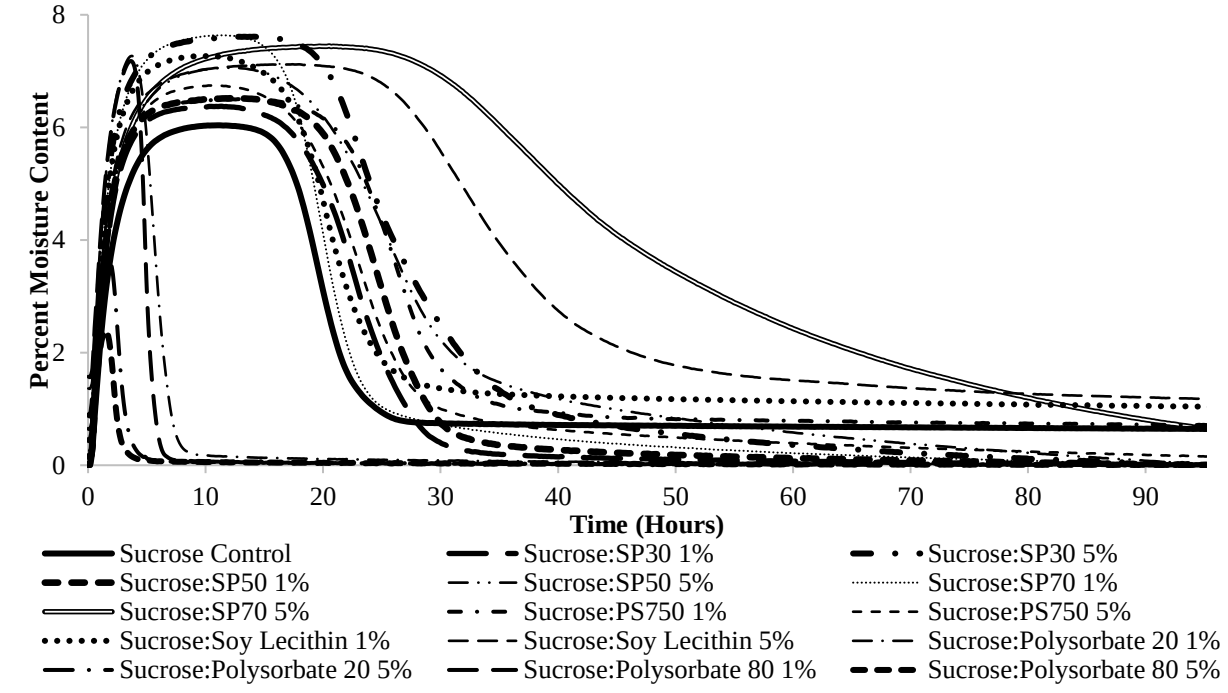
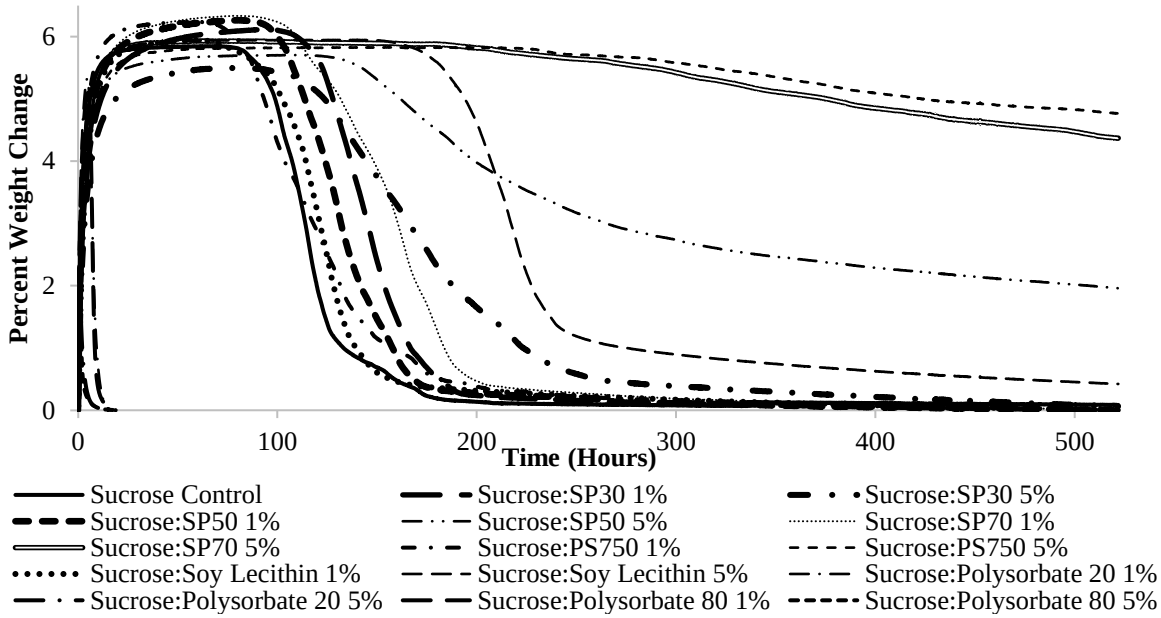


Figure 5.2 Moisture sorption profiles of sucrose lyophiles A) from 0-80% RH, B) held at 40% RH, and C) held at 33% RH.

Figure 5.2 continued

C)



A)

B)

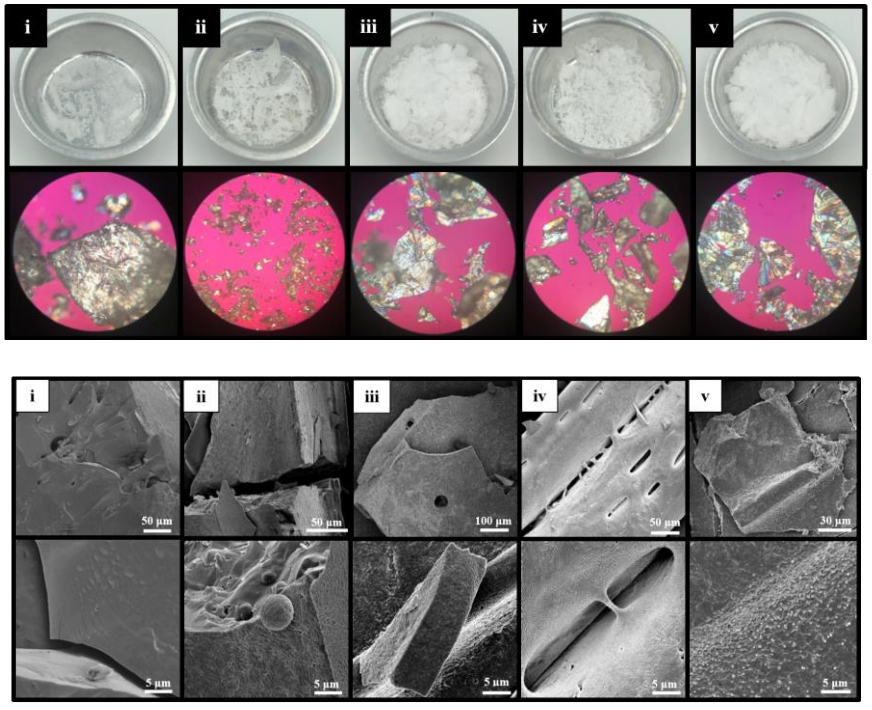


Figure 5.3 Images comparing the A) physical and microscopic (PLM) appearance and B) crystal morphology by SEM micrographs after crystallization of the following lyophiles: i) sucrose control, ii) sucrose:polysorbate 20 1%, iii) sucrose:polysorbate 20 5%, iv) sucrose:polysorbate 80 1%, and v) sucrose:polysorbate 80 5%.

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CHAPTER 6. EFFECTS OF POLYPHENOLS ON CRYSTALLIZATION OF AMORPHOUS SUCROSE LYOPHILES

Voelker, A. L., Felten, C., Taylor, L. S., & Mauer, L. J. (2021). Effects of polyphenols on crystallization of amorphous sucrose lyophiles. *Food Chemistry*, *338*, 128061. doi:https://doi.org/10.1016/j.foodchem.2020.128061

6.1 Abstract

The crystallization of amorphous sucrose in food products can greatly affect the quality of foods. This study investigated the effects of polyphenols on the crystallization of amorphous sucrose lyophiles. Monoglycosylated, polyglycosylated, and aglycones with differing polyphenol backbones were studied, in addition to bulk food ingredients containing a high concentration of polyphenols. Solutions containing sucrose with and without polyphenols (1 and 5%) were lyophilized, stored in RH-controlled desiccators, and analyzed by x-ray diffraction. Moisture sorption studies, Karl Fischer titration, and differential scanning calorimetry were also completed. Polyphenol addition delayed sucrose crystallization by up to 6.4x compared to the control. Structure played the most significant role in efficacy of polyphenols in delaying sucrose crystallization, more than T_g or hygroscopicity. Glycosylated polyphenols were more effective than aglycones, polyphenols with (2,1) glycosidic linkages were more effective than those with (6,1) linkages, and bulk food ingredients were the most effective at delaying sucrose crystallization.

6.2 Introduction

Sucrose is a common food ingredient used to increase the sweetness of foods. However, not all sucrose is the same: the physical state of sucrose contributes to food texture, taste perception, moisture sorption, dissolution properties, and rate of chemical reactions in food products (Buera et al., 2005; Chirife & Karel, 1974; Mathlouthi, 1995; Slade & Levine, 1991). Amorphous sucrose is often desired for its soft texture and more rapid dissolution, but the amorphous material quickly crystallizes into crystalline sucrose in many food storage conditions. In some food applications though, the properties of crystalline materials may be desirable, including the harder and more brittle texture, slower dissolution, low water holding capacity, and higher chemical stability.

Regardless, crystalline solids are more thermodynamically stable than amorphous solids, so crystallization of amorphous sucrose is likely to occur, especially in the presence of elevated temperature and/or relative humidity (RH) (Shamblin & Zografi, 1999; Slade & Levine, 1991). Physical changes in an amorphous material can decrease the quality of a food product, for example, caking caused by sintering of the amorphous materials or the release of excess moisture from the amorphous matrix during recrystallization (Hartmann & Palzer, 2011). Thus, the physical behaviors of amorphous sucrose, specifically concerning increased stability induced by formulation, have been widely studied in the food industry (Gabarra & Hartel, 1998; Saleki-Gerhardt & Zografi, 1994; Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker, Verbeek, Taylor, & Mauer, 2019).

Crystallization occurs in two steps: first nucleation, the rate-limiting step, followed by crystal growth (Makower & Dye, 1956). The overall rate of crystallization is determined by the combination of the nucleation and growth kinetics. The crystallization onset time describes the time required until evidence of crystallinity can be detected, and is typically dominated by the nucleation kinetics, with some contribution from the time required for the nuclei to grow to a detectable size. A variety of additives has been shown to delay sucrose crystallization by decreasing the rate of nucleation, specifically as a result of the increase in glass transition temperature (T_g) and/or viscosity of the system, decreasing molecular mobility in the system, and increasing intermolecular interactions between sucrose and the additives that disrupt ordering of the sucrose molecules into a crystal lattice (Carstensen & van Scoik, 1990; Gabarra & Hartel, 1998; Roe & Labuza, 2005; Roos & Karel, 1991b; Saleki-Gerhardt & Zografi, 1994; Shamblin & Zografi, 1999). While a large number of studies focus on the change in T_g as having the major effect on amorphous sucrose stabilization, recent studies have shown that additives such as inorganic salts, a variety of sugars, and some emulsifiers stabilize amorphous sucrose when T_g is unchanged or even decreased (Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker et al., 2019). Altering the water dynamics of an amorphous sucrose matrix by increasing cation valency and ion hydration shell of salts and increasing intermolecular interactions between sugar and structurally similar saccharides or emulsifiers were instead cited as the reasons for the stabilization of amorphous sucrose in these studies (Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker et al., 2019).

While many studies have found additives that stabilize amorphous sucrose, the ingredients are not always viable for use in food products in the amounts necessary for substantial stabilization.

Polyphenols, on the other hand, are food ingredients that could be an ideal natural food additive due to their wide presence in natural food sources and increasing consumer association with health benefits (Bresciani et al., 2017; Guo et al., 2017; Steluti, Fisberg, & Marchioni, 2017). Polyphenols are also naturally found in some of the same products in which amorphous sucrose is desirable. For example, tea, coffee, and chocolate are often used as the primary ingredients in many powdered beverages and all contain relatively high quantities of polyphenols (Gonzalez-Sarrias et al., 2017; Graham, 1992; Madhava Naidu, Sulochanamma, Sampathu, & Srinivas, 2008; Rimbach, Melchin, Moehring, & Wagner, 2009).

While the polyphenol category contains a wide range of ingredients, the chemical structures of many polyphenols align with the structural features which have been shown to cause an increase in amorphous sucrose stabilization (Thorat et al., 2018; Voelker et al., 2019). Generally, the backbone of a polyphenol consists of an aromatic ring with two or more hydroxyl groups. Glycosylated polyphenols contain both a region that is structurally similar to sucrose and a hydrophobic polyphenol backbone that could incorporate at the surface of a crystal lattice and disrupt further sucrose crystallization. Additionally, a patent publication has shown increased stabilization of amorphous sucrose with the addition of two glycosides, naringin and glycyrrhizic acid, attributed to the presence of both a structurally similar and a structurally dissimilar region in the glycoside to sucrose (Taylor, Mauer, & Thorat, 2019). Therefore, polyphenols with these structural features seem to have a promising potential for naturally stabilizing amorphous sucrose in a variety of food products, especially those that may already contain the ingredients. Enhancing amorphous sucrose stability using ingredients such as polyphenols that are already present in foods may lead to major improvement in product quality, shelf-life, and even nutritional benefits with potential for minimal or no negative impact on flavor or function.

Therefore, the objectives of this study were to determine the effects of a variety of polyphenols, glycosylated phenols, and other common food ingredients with structural similarities to polyphenols on the crystallization behavior of amorphous sucrose and to draw conclusions on the potential mechanisms behind the stabilizing effects. It was hypothesized that polyphenol structure would be the determining factor in efficacy of amorphous sucrose stabilization. Specifically, polyphenols that contain a structurally similar region to sucrose as well as a structurally dissimilar region were expected to most significantly inhibit sucrose crystallization, similar in principle to what was found for effects of other compounds on stabilizing amorphous

sucrose by Taylor et al. (2019), Thorat et al. (2018), and Voelker et al. (2019). This hypothesis was tested by incorporating a variety of polyphenol-like additives into an amorphous sucrose lyophile, including nonglycosylated, monoglycosylated, and polyglycosylated structures as well as polyphenol-containing bulk food ingredients. The structures and properties of the additives in this study are reviewed in Table 6.1.

6.3 Materials and Methods

6.3.1 Materials

The sucrose used in this study was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). The polyphenols used (shown in Table 6.1) included: nonglycosylated polyphenols (apigenin (Sigma-Aldrich, St. Louis, MO), quercetin dihydrate (Bulk Supplements, Henderson, NV), daidzein (Sigma-Aldrich), and resveratrol (Bulk Supplements)); a monoglycosylated polyphenol (puerarin (Sigma-Aldrich)); polyglycosylated polyphenols (hesperidin (Santa Cruz Biotechnology Inc., Dallas, TX), rutin (Bulk Supplements), naringin (Sigma-Aldrich), and glycyrrhizic acid (Sigma-Aldrich)); and raw food ingredients high in polyphenol content (cocoa powder (Nuts.com, Cranford, NJ), green tea extract (Nutrients Scientific, Diamond Bar, CA), ceremonial-grade matcha (Encha, San Jose, CA), green coffee (Bulk Supplements), and monkfruit (Julian Bakery, Oceanside, CA)). Additional materials used in the study included: phenyl β-D-glucopyranoside (Sigma-Aldrich) and α-arbutin (L'eternel World, LLC).

The relative humidity (RH) in desiccators used for sample storage was maintained using the following saturated salt solutions: lithium chloride 11% RH (Acros Organics, Fair Lawn, NJ), potassium acetate 23% RH (Fisher Scientific, Fair Lawn, NJ), and magnesium chloride 33% RH (Fisher Scientific) at 25ºC (Greenspan, 1977). Phosphorous pentoxide (P_2O_5) (Acros Organics) was also used in desiccators to maintain a RH of ~0%. All water used in this work was ultrapure, obtained using a Barnstead E-Pure Ultrapure water purification system (ThermoScientific, Waltham, MA) with resistivity greater than 17.5 MΩ-cm, total organic carbon concentrations less than 10 ppb, and a 0.2 µm filter that eliminated possible bacterial and particulate contamination. Karl Fischer reagents for volumetric one-component titrations included: HYDRANAL-Composite 2 (titrant), HYDRANAL-Methanol Rapid (solvent), and HYDRANAL-Water Standard 10.0 (Sigma-Aldrich).

6.3.2 Preparation of amorphous lyophiles

To prepare samples for lyophilization, 10% (*w/v*) sucrose solutions were made. Polyphenols and other additives (Table 6.1) were added to the sucrose solution at 1 and 5% (*w/w*), and a control sample was prepared containing no additive.

Prior to lyophilization, samples were frozen at -20ºC for at least 12 h. Freeze drying conditions adapted from Thorat, Forny, et al. (2017); Thorat et al. (2018); and Voelker et al. (2019) were used. Lyophilization was done in a HarvestRight Scientific freeze dryer (North Salt Lake, UT). Following lyophilization, the lyophiles were immediately transferred to desiccators containing P_2O_5 (~0% RH) and stored at 25ºC until analysis. Sample handling after lyophilization was done in a glove box purged with nitrogen (~5% RH). X-ray diffractograms of the initial lyophilized samples verified that the sucrose was fully amorphous.

6.3.3 Moisture content analysis

All lyophiles were initially stored for 2-4 days at 0% RH (P_2O_5) and analyzed for moisture content by a one-component Karl Fischer titration using a V20S Volumetric KF Titrator (Mettler-Toledo, LLC, Columbus, OH). Approximately 50 mg of each lyophile was added to the HYDRANAL-Methanol Rapid solvent, which extracted water from the sample, and was then titrated with HYDRANAL-Composite 2 titrant to calculate % moisture (*wb*). Calibration of the titration system was done using the HYDRANAL-Water Standard 10.0 (10 mg/g =1% water content).

6.3.4 Differential scanning calorimetry

Following moisture content determination, the same lyophiles were analyzed by differential scanning calorimetry (DSC) using a DSC Q2000 (TA Instruments, New Castle, DE) with a method adapted from Thorat, Forny, et al. (2017); Thorat et al. (2018). Initial lyophiles (5-10 mg) were weighed into aluminum DSC pans and hermetically sealed. The system was purged with dry nitrogen at a rate of 50 mL/min. The instrument was calibrated with indium and tin and verified with the melting point of water. The onset T_g and onset crystallization temperature (T_{crys}) were measured using the second scan of a modulated heat-cool-heat protocol. The first scan heated the samples from 0ºC to 80ºC (20-30ºC above the expected T_g) with a 5ºC/min heating rate to erase

thermal history. The samples were then cooled to 0ºC at 10ºC/min and heated in the second scan to 160ºC at 5ºC/min. The onset T_g was defined as the temperature at which an endothermic baseline shift characteristic of a glass transition began in the second scan. The onset T_{crys} was defined as the onset temperature of the exothermic peak occurring after the T_g.

6.3.5 Desiccator storage and physical state analysis

The procedures used for desiccator studies and related analyses were adapted from Voelker et al. (2019) to monitor crystallization of lyophiles over time in increasing storage RH conditions. Lyophiles were placed in desiccators containing one of three saturated salt solutions to control the RH of the headspace: lithium chloride (11% RH), potassium acetate (23% RH), or magnesium chloride (33% RH). The desiccators were stored in a 25ºC temperature-controlled room. The lyophiles were analyzed over a 4-week period, and a single desiccator was used for each day of analysis to prevent exposure of the sample to ambient RH prior to analysis.

Powder x-ray diffraction (PXRD) was used to determine the presence or absence of crystallinity in samples following storage using a method adapted from Thorat, Forny, et al. (2017); Thorat et al. (2018). Although crystallization involves nucleation and crystal growth, PXRD does not identify either individual step, but rather indicates the presence of crystallinity. Diffractograms were collected using a Shimadzu LabX XRD-6000 (Shimadzu Corporation, Kyoto, Japan), equipped with a Cu-Kα radiation source in Bragg-Brentano geometry and operating at 40 kV and 30 mA. Daily calibration was performed prior to analysis using a silicon standard peak. Samples were analyzed with a scan range of 10-35° 2θ, a scan speed of 4°/min, and a step size of 0.04°. Samples with diffractograms containing peaks above a signal-to-noise ratio of 3 were considered PXRD crystalline, and samples producing small peaks above the baseline were considered to be partially crystalline, with increasing intensity indicating increasing crystallinity (Figure 6.1). Samples with diffractograms containing an amorphous halo with no peaks were considered to be PXRD amorphous. Samples were discarded following analysis.

6.3.6 Dynamic vapor sorption analysis

Two moisture sorption experiments at 25ºC were conducted for this study using a SPSx-1μ Dynamic Vapor Sorption Analyzer (Projekt Messtechnik, Ulm, Germany) with methods used

by Voelker et al. (2019). For the first experiment, 100-200 mg of the lyophiles were weighed into sample pans that were then placed in the 23-ring sample holder and held at 0% RH for 48 h in the SPS. RH was then increased stepwise from 0-80% RH in 5% increments with a minimum step time of 50 min, a maximum step time of 12 h, and an equilibration end-point criterion of < 0.001% weight change within 30 min. The moisture sorption profiles were obtained by plotting percent mass change vs. RH in which the mass change at the end of each step indicated the equilibration moisture gain at that RH.

In the second moisture sorption experiment, 100-200 mg of each lyophile were weighed into pans and placed in the 23-ring sample holder. The samples were again held at 0% RH for 48 h, at which point the RH was increased to 40% for a 96 h iso-RH hold. The moisture sorption profiles were obtained by plotting percent mass change vs. time. Although crystallization involves both nucleation and crystal growth, the sensitivity of the moisture sorption instrument was only used to detect the onset of cooperative crystallization, in which the onset of mass loss was taken to indicate crystallization (Makower & Dye, 1956).

6.3.7 Statistical analysis

All samples were analyzed in duplicate. Single-variable ANOVA using SAS 9.4 (SAS Institute, Cary, NC) was used to determine significant differences in time or RH of crystallization, moisture content, T_g, and T_{crys} between lyophiles. Tukey's post hoc test was used to determine differences using a significance level of $\alpha = 0.05$.

6.4 Results and Discussion

6.4.1 Stability of amorphous sucrose lyophiles during desiccator storage

All sucrose lyophiles were initially amorphous, as determined by PXRD analyses. The physical stability of the lyophiles was significantly affected by storage conditions as well as the type and amount of added ingredient (Table 6.2). All lyophiles with and without additives remained amorphous for the 30-day duration of the experiment when stored in desiccators at 11% or 23% RH and 25ºC except for those containing glycyrrhizic acid, which showed evidence of sucrose crystallinity on day 30 at 23% RH. Major differences in the stability of the different lyophiles (delay to crystallization onset) emerged when the storage RH was increased to 33% RH.

The sucrose control crystallized after 7 days in these conditions. The addition of other ingredients to sucrose resulted in lyophiles that either maintained or increased amorphous sucrose stability in desiccators compared to the control with the exception of sucrose:apigenin 1% and sucrose:rutin 1%, which both exhibited sucrose crystallinity by day 4. Increasing the percentage of additive from 1 to 5% in the lyophile generally increased the amorphous sucrose stability.

Based on the controlled-RH desiccator studies, the stabilizing success of the additives from greatest to least in terms of delaying amorphous sucrose crystallization was as follows, in which each bullet point indicates a different day of crystallization:

- Naringin 5%, glycyrrhizic acid 5%, green tea extract 5%, green coffee 5%
- Puerarin 1%, puerarin 5%, green tea extract 1%, matcha 5%
- Glycyrrhizic acid 1%, cocoa powder 5%
- Apigenin 5%, quercetin 5%, α-arbutin 5%, phenyl β-D-glucopyranoside 5%, naringin 1%, green coffee 1%, monkfruit 1%
- **Sucrose control,** quercetin 1%, daidzein 1%, daidzein 5%, resveratrol 1%, resveratrol 5%, α-arbutin 1%, phenyl β-D-glucopyranoside 1%, hesperidin 1%, hesperidin 5%, rutin 5%, cocoa powder 1%, matcha 1%
- Apigenin 1%, rutin 1%

The lyophile formulations that prevented sucrose crystallization within the experimental space were the formulations containing 5% of naringin, glycyrrhizic acid, green tea extract, and green coffee.

Generally, of the single additives, the mono- and polyglycosylated additives had a greater sucrose stabilizing effect than the nonglycosylated additives, with puerarin, naringin, and glycyrrhizic acid having the greatest stabilizing effects on inhibiting sucrose crystallization. The bulk food ingredients containing complex mixtures of compounds, including polyphenols, also significantly enhanced amorphous sucrose stability. Lyophiles containing 5% of green tea extract or green coffee did not crystallize within the experimental space of the desiccator experiments. Crystallinity observed by PXRD indicated only sucrose crystallization and no additive crystallization occurred. Amorphous solids tend to exhibit faster dissolution rates than their crystalline counterparts (Mathlouthi, 1995), and thus co-formulating sucrose and polyphenols in lyophiles may be an approach for enhancing dissolution of all components.

Increasing environmental RH to above 30% RH has been shown to increase the rate of sucrose crystallization due to increased moisture sorption, increased molecular mobility, and decreased T_g (Makower & Dye, 1956; Palmer, Dye, & Black, 1956). While additives are generally known to increase amorphous sucrose stability above this RH, some ingredients, specifically emulsifiers, have been shown to have both stabilizing and destabilizing effects depending on emulsifier type (Voelker et al., 2019). For example, sucrose esters, which contain a structurally similar region to sucrose, were shown to interact at the surface of the crystal lattice and delay sucrose crystallization. However, polysorbate structures were shown to cause a templating effect, which increased the rate of sucrose nucleation and subsequent crystal growth (Voelker et al., 2019). Very few ingredients containing phenolic structures in this study were shown to increase the rate of sucrose crystallization in desiccators, with varying effects at delaying sucrose crystallization found between different additives, and thus further studies were completed to better understand the different effects of the additives on stability of the amorphous lyophiles.

6.4.2 Moisture content of amorphous sucrose lyophiles

Crystallization of amorphous lyophiles often occurs as a result of storage RH and moisture content (Makower & Dye, 1956; Mathlouthi, 1995). Amorphous sucrose can absorb moisture from an increased RH environment, thereby lowering the T_g, increasing molecular mobility, and promoting crystallization. The initial moisture contents of the lyophiles (after 2-4 days of storage at 0% RH) was measured by Karl Fischer to investigate any role moisture may have played in the stability of the lyophiles (Table 6.3). Moisture contents of the lyophiles were between 2.0-2.9%, and there was no significant difference ($p < 0.05$) between any of the measurements. Thus, initial moisture content was not considered to be a factor in the observed differences in amorphous sucrose stability.

6.4.3 Moisture sorption profiles

Moisture sorption profiles were measured to monitor differences in moisture sorption and crystallization occurring as a result of different additives in the lyophile matrices, including the amount of moisture sorption leading up to crystallization, RH at which crystallization occurred in an experiment wherein RH was increased stepwise from 0 to 80% RH, and time of crystallization

in an iso-RH experiment. Crystallization was considered to be the point at which mass loss occurred (Makower & Dye, 1956). Although there were no significant differences between moisture contents of the initial lyophiles, a 48 h drying step at 0% RH was completed prior to both moisture sorption experiments to remove any variation.

In the first moisture sorption experiment, moisture sorption profiles of all formulations were collected from 0-80% RH at 25ºC (Figure 6.2.A). Differences were found in both the amount of moisture sorbed prior to crystallization and the RH at which the onset of crystallization (mass loss) occurred between the different formulations. The RHs at which crystallization occurred in these conditions are listed in Table 6.2. While the sucrose control crystallized at 35% RH, most other lyophiles crystallized at higher RHs (up to 55% RH), with only sucrose:apigenin 1% and sucrose:hesperidin 1% also crystallizing at 35% RH. In contrast, a similar study by Voelker et al. (2019) found that very few of the emulsifiers studied increased the RH of crystallization in an amorphous sucrose lyophile compared to the control. The stabilizing trend of the additives from greatest to least as shown by RH of crystallization in the 0-80% RH step-wise experiment was as follows, in which each bullet point indicates statistical significance ($p < 0.05$):

- Green tea extract 5%, green coffee 5%
- Puerarin 5%, matcha 5%
- α-arbutin 5%, phenyl β-D-glucopyranoside 5%, cocoa powder 5%
- Quercetin 1%, quercetin 5%, daidzein 5%, α-arbutin 1%, puerarin 1%, phenyl β-D-glucopyranoside 1%, hesperidin 5%, rutin 1%, rutin 5%, cocoa powder 1%, green tea extract 1%, matcha 1%, green coffee 1%, monkfruit 1%
- **Sucrose control,** apigenin 1%, hesperidin 1%

Similar to the trend found in the desiccator experiments, the mono- and polyglycosylated additives generally had a greater sucrose stabilizing effect than the nonglycosylated additives. The bulk food ingredients again demonstrated the greatest enhancement to amorphous sucrose stability, with green tea extract and green coffee having the most significant effects, increasing the RH of crystallization from 35 to 55% RH. Increasing the percentage of additive from 1 to 5% also generally increased the RH at which crystallization occurred.

The percent moisture gained prior to crystallization varied between samples, with most lyophiles sorbing more moisture than the control, which gained 5.7% weight before crystallizing. The lyophiles that did not crystallize until reaching higher RHs gained the most moisture prior to

crystallizing, with sucrose:green coffee 5%, sucrose:matcha 5%, and sucrose:green tea extract 5%, gaining 7.5%, 7.6%, and 8.3% weight, respectively, prior to exhibiting crystallization (mass loss) within the experimental space.

In the second moisture sorption experiment, lyophiles were exposed to constant 40% RH and 25ºC conditions following the drying step and were monitored gravimetrically over time (Figure 6.2.B). Differences in the amount of moisture sorbed and time of onset of moisture loss (crystallization onset) were found between formulations. The times at which crystallization occurred in these conditions are listed in Table 6.2. As was found in the desiccator experiments and the 0-80% RH moisture sorption experiment, the mono- and polyglycosylated additives generally demonstrated a greater enhancement to amorphous sucrose stability than did the nonglycosylated additives. The monoglycosylated additives had a more significant ($p < 0.05$) effect on delaying crystallization than did the polyglycosylated additives. The bulk food ingredients most delayed the onset of crystallization of amorphous sucrose, again with green tea extract and green coffee having the most substantial effects. No evidence of crystallization was found in sucrose:green tea extract 5% lyophiles over the 96 h duration of the experiment, and the addition of 5% green coffee to the sucrose lyophiles delayed crystallization 4.6x compared to the control. The addition of 5% naringin also caused a substantial delay in crystallization, of 3.5x compared to the control (Taylor et al., 2019). Increasing the percentage of additive from 1 to 5% also generally increased the time prior to evidence of crystallization at 40% RH, with the exception of quercetin, phenyl β-D-glucopyranoside, hesperidin, and rutin. The gravimetric moisture sorption profiles also indicated that there were possibly differences in the crystal growth rates between the different lyophile formulations, evidenced by the time between onset of crystallization (onset of moisture loss) and equilibration of moisture content (flattening of the curve) (Figure 6.2.B). However, using the slope of moisture loss as a quantitative measure of the rate of crystallization was not done due to potential complicating factors such as differences in moisture diffusion between the different samples.

The stabilizing trend of the additives from greatest to least with respect to time of onset of crystallization at 40% RH and 25ºC, in the iso-RH experiment, was as follows, in which each bullet point indicates statistical significance ($p < 0.05$):

- Green tea extract 5%
- Green coffee 5%

- Naringin 5%
- α-arbutin 5%, puerarin 1%, puerarin 5%, phenyl β-D-glucopyranoside 1%, green tea extract 1%, matcha 5%, green coffee 1%
- α-arbutin 1%, phenyl β-D-glucopyranoside 5%, cocoa powder 5%, matcha 1%
- Quercetin 1%, rutin 1%, rutin 5%, cocoa powder 1%, monkfruit 1%
- Apigenin 1%, hesperidin 1%, naringin 1%, glycyrrhizic acid 5%
- **Sucrose control,** quercetin 5%, daidzein 5%, hesperidin 5%, glycyrrhizic acid 1%

In all desiccator studies and both gravimetric moisture sorption studies (increasing RH steps and iso-RH), bulk food ingredients had the greatest stabilizing effect compared to the control, followed by monoglycosylated additives and polyglycosylated additives, with nonglycosylated additives generally providing the least enhancement to amorphous sucrose stability.

While the trends between the iso-RH desiccator studies at 33% RH and the SPS study at 40% RH were generally the same, there were a few exceptions. For example, sucrose:α-arbutin 1%, sucrose:phenyl β-D-glucopyranoside 1%, sucrose:rutin 1%, sucrose:cocoa powder 1%, and sucrose:matcha 1% better delayed sucrose crystallization in the 40% RH SPS experiment than in the 33% RH desiccator experiment (Figure 6.2.B, Table 6.2). Interestingly, while most lyophiles gained 5.6-7% moisture weight prior to crystallizing, sucrose:glycyrrhizic acid 1 and 5% gained more than 8% weight prior to crystallization (Figure 6.2.B). Additionally, although the addition of glycyrrhizic acid and naringin to sucrose resulted in favorable delays of crystallization in the desiccator studies, these additives did not uniformly delay sucrose crystallization in the SPS study at 40% RH, wherein only sucrose:naringin 5% resulted in a significant increase in time to crystallization (3.5x compared to the control) (Taylor et al., 2019). Differences in moisture sorption and crystallization behavior between the iso-40% RH SPS experiments and 33% RH desiccator studies of lyophiles may have been caused by the drying step done in the SPS experiment, the difference in RH (33% vs. 40%), or the passive desiccator headspace vs. the active SPS headspace. It is known that RH plays a key role in sucrose crystallization (Makower & Dye, 1956), so it is not surprising to see slightly different trends in crystallization tendencies in different RH conditions.

6.4.4 Glass transition and crystallization temperatures

One mechanism by which additives are reported to stabilize amorphous food systems is by increasing the glass transition temperature (T_g) of the matrix (Roos & Karel, 1991b; van Hook, 1988). Briefly, when an amorphous system is in the supercooled liquid state (e.g. the T_g is below the environmental temperature), an increased molecular mobility enables molecular rearrangement, often promoting crystallization of amorphous solids to the lower energy crystalline state. Increasing the T_g of the matrix so that the amorphous system is in the glassy state (e.g. the T_g is above the environmental temperature) conversely slows molecular movement and inhibits crystallization. In many cases, the addition of a miscible higher molecular weight or higher T_g additive to an amorphous matrix results in an increase in the matrix T_g (Levine & Slade, 1986; Tant & Wilkes, 1981). In order to test this theory in the current study, the T_gs of the amorphous sucrose lyophiles with 1% additive were measured by DSC (Table 6.3). The T_g of dry sucrose has been reported to be in the range of 52 to 74°C (Saleki-Gerhardt & Zografi, 1994; Slade & Levine, 1991). In this study, the sucrose control lyophile had a T_g of 47°C at a moisture content of 2.4%. No significant differences were found in the initial moisture contents of the lyophiles that were analyzed by DSC (2.0-2.9% by Karl Fischer titration). The T_gs of the lyophiles were found to range from 47 to 56°C. Some of the initial lyophile T_gs were significantly ($p < 0.05$) higher than the sucrose control, including lyophiles in all groups of additives (non-, mono-, polyglycosylated, and bulk food ingredients). These increased matrix T_gs could have contributed to the stability of the amorphous sucrose lyophiles; however, it is important to note that crystallization was not found until the moisture contents of the samples had increased up to at least 5.5% in the SPS moisture sorption experiments. In general, crystallization from an amorphous matrix within the time scale of this experiment is often attributed to an increase in temperature above the T_g of the matrix, or depression of T_g below the experimental temperature due to moisture sorption, but rate of crystallization is affected by the extent of the difference between environmental temperature and T_g (Roos & Karel, 1991b). Water has a very low T_g (-135ºC), and thus an increase in moisture content of 5.5% could theoretically depress the T_g of the matrix by more than 40ºC (Gordon & Taylor, 1953; Roos & Karel, 1991a; Slade & Levine, 1991), to well below the experimental temperature. Although variations in the amount and rate of moisture sorption were found between samples in this study (Figure 6.2), the T_g immediately prior to crystallization was not studied in this experimental space. Previous studies have reported the effects of increasing storage RH (thus,

increasing moisture content) on the T_gs of amorphous sucrose lyophiles both with and without saccharide and salt additives (Thorat, Forny, et al., 2017; Thorat et al., 2018). It was found that T_g generally decreased to below ambient temperature following storage at 33% RH, the condition at which most samples crystallized; however, some lyophiles in these studies did not crystallize when stored above their respective T_gs, some crystallized when stored below their respective T_gs, and the overall stability of amorphous sucrose did not correlate with sample T_gs (Thorat, Forny, et al., 2017; Thorat et al., 2018). While more experimentation would be needed in the current study to identify if and when differences in T_g were present at the time crystallization began, in agreement with the previous studies, the measured T_gs in this study suggest that factors beyond T_g contributed to the stabilizing effect of polyphenols on amorphous sucrose.

T_{crys} as determined by DSC has also been used as a technique for predicting the stability of amorphous matrices (Kinugawa et al., 2015; Roos & Karel, 1991b). However, T_{crys} has also been reported in some cases to not correlate with RH-induced crystallization in lower temperature environments (Thorat, Forny, et al., 2017; Thorat et al., 2018). In some cases, the T_{crys} of freeze-dried and further dehydrated sucrose has been predictive of stability in increasing RH environments over time, in which amorphous matrices with higher 'dry' T_{crys} were found to be more stable when exposed to increasing RH than those with lower 'dry' T_{crys} (Kinugawa et al., 2015). When amorphous sucrose was exposed to increasing RH environments prior to measuring T_{crys}, increasing storage RH, and thus higher moisture contents, generally decreased T_{crys} (Thorat, Forny, et al., 2017; Thorat et al., 2018). Crystallization of lyophilized amorphous sucrose was reported to occur at 130°C (Saleki-Gerhardt & Zografi, 1994). In this study, the onset T_{crys} of amorphous sucrose containing 2.4% water was found to be 98°C, and the onset T_{crys} of the different lyophile formulations ranged between 91 and 117°C (Table 6.3). Lyophiles with a higher T_{crys} compared to the control also tended to have higher T_gs (R^2 = 0.7233). Consistent with time of crystallization in desiccator and SPS increasing RH environments, T_{crys} was highest in the lyophiles with bulk food ingredients, followed by lyophiles with monoglycosylated additives. However, T_{crys} did not correlate well with isothermal time to crystallization at 40% RH (R^2 = 0.2869). Thus, as was the case in similar studies done by Thorat, Forny, et al. (2017); Thorat et al. (2018) and Voelker et al. (2019), differences in T_g and T_{crys} between samples were not considered to be major factors in isothermal amorphous sucrose stabilization.

6.4.5 Effect of polyphenol structure on stability of amorphous sucrose lyophiles

The structure of the additives is sometimes considered to influence the efficacy of an additive at delaying sucrose crystallization, particularly in cases wherein T_g is not considered as the major factor for stabilization (Leinen & Labuza, 2006; Saleki-Gerhardt & Zografi, 1994; Thorat et al., 2018; Voelker et al., 2019). One hypothesis for how additive structure influences amorphous matrix resistance to crystallization is that when an additive contains both a structurally similar and structurally dissimilar region to sucrose, the molecular region that is similar to sucrose (e.g. a sugar unit) can interact with sucrose at the sucrose crystal interface and the structurally dissimilar region is able to prevent further incorporation of sucrose into the crystal lattice, therefore slowing crystal growth (Leinen & Labuza, 2006; Thorat et al., 2018). Consequently, this study chose to analyze the effect of polyphenols and other additives that are both glycosylated and nonglycosylated as well as some bulk food ingredients high in polyphenol content. The key trend in this study was that bulk food ingredients had the greatest stabilizing effect compared to the control, followed by monoglycosylated additives, polyglycosylated additives, and nonglycosylated additives showed the least effect, with no significant ($p < 0.05$) delay in onset of crystallization in the 40% RH SPS experiment compared to the control (Table 6.2).

Glycosylated additives delayed amorphous sucrose crystallization more than the nonglycosylated additives presumably due to the sugar units in the glycosylated compounds which were structurally similar to sucrose. For example, daidzein is the aglycone of puerarin, apigenin is the aglycone of naringin, and quercetin is the aglycone of rutin. While daidzein did not significantly ($p < 0.05$) affect the rate of sucrose crystallization in this study, puerarin consistently delayed sucrose crystallization, up to 2.6x compared to the control in the 40% RH SPS study. Similarly, apigenin did not significantly delay sucrose crystallization while naringin did, even preventing sucrose crystallization in the desiccator study at 33% RH. However, neither rutin nor its aglycone quercetin delayed sucrose crystallization. Regardless, it can be seen that when a sugar unit is a structural component of an additive, amorphous sucrose stability is generally increased compared to when no sugar unit is present.

It was also shown that monoglycosylated additives delayed sucrose crystallization to a greater extent than did polyglycosylated additives. This was presumably a similar response to that found by Voelker et al. (2019) in which emulsifiers with a higher fraction of mono-esters (compared to di- and tri-esters) led to a greater amorphous sucrose stabilization. Since the additives

were added on a weight basis, the lower molecular weight of the monoglycosylated additives compared to the polyglycosylated additives led to a greater contribution of molecules that could interact with sucrose and disrupt crystallization. Of the three monoglycosylated additives studied, puerarin was the most effective at delaying sucrose crystallization even though the sugar unit associated with puerarin was the least structurally similar to sucrose. Phenyl β-D-glucopyranoside and α-arbutin both contain phenol backbones while puerarin has a polyphenol backbone. It was therefore supposed that, within the monoglycosylated structures, a larger predominantly non-polar backbone (polyphenol) was more important to the efficacy of amorphous sucrose stabilization than was the similarity of the sugar unit to sucrose.

Considering the polyglycosylated additives, naringin and glycyrrhizic acid were more effective at delaying sucrose crystallization than were hesperidin and rutin (Taylor et al., 2019), which did not significantly ($p < 0.05$) delay crystallization compared to the control. The primary difference between hesperidin and rutin versus naringin and glycryrrhizic acid is the glycosidic linkage between sugar units, in which hesperidin and rutin have a (6,1) linkage and naringin and glycyrrhizic acid have a (2,1) linkage. It is proposed that the difference in linkage sites between the sugar units caused the observed differences in amorphous sucrose stabilization, specifically due to the fact that (6,1) glycosidic linkages are known to be more flexible than (2,1) linkages (Hardy, 1997). The less flexible glycosidic linkage may have provided a more rigid structure that was better able to disrupt incorporation of more sucrose into the crystal lattice following interaction of the sugar units and sucrose. While sugar groups as structural components were found to be necessary for polyphenols to delay amorphous sucrose crystallization, the specific unit and linkages between units were also of importance.

The bulk food ingredients were the most effective additives at delaying amorphous sucrose crystallization. Of these, the addition of green tea extract, a blend of primarily nonglycosylated polyphenols, resulted in the most significant delay in sucrose crystallization of all additives tested. Epigallocatechin gallate (ECG) comprised 51% of the green tea extract product by weight. Although ECG does not contain any sugar units, there are some known glycosylated ingredients that are commonly present in green tea extract, e.g. flavonol glycosides (Graham, 1992). ECG does however contain a large number of hydroxyl groups which may allow for interaction with sucrose even despite the lack of sugar units. Green tea extract delayed crystallization more significantly than matcha, which indicates that the high polyphenol content in green tea extract

compared to matcha may be responsible for this delay in sucrose crystallization rather than other components in tea.

Cocoa powder contains glycosylated forms of quercetin, specifically quercetin-3-O-α-D-arabinoside and quercetin-3-O-β-glucopuranoside (Rimbach et al., 2009), which are also structurally similar to rutin. These glycosylated structures likely contributed to the efficacy of cocoa powder in delaying sucrose crystallization. Green coffee's primary polyphenol constituent, chlorogenic acid, does not contain a sugar side group but does contain a quinic acid group, which is somewhat structurally similar to a sugar (Clifford, 1999; Madhava Naidu et al., 2008). Monkfruit, which was the least effective bulk food ingredient at delaying sucrose crystallization, is a mogroside, which contains many glucose units that may structurally overlap with sucrose (X. Li, Lopetcharat, & Drake, 2015). It is also probable that the pure heterogeneity of constituents within these bulk additives played a significant role in their stabilization of amorphous sucrose.

Since polyphenols are generally viewed positively by the public and are associated with numerous health benefits, the compounds studied may be useful for increasing the shelf life of foods wherein amorphous sucrose is desired. The efficacy of the polyphenolic additives from this study were compared to a variety of other ingredients previously reported to affect amorphous sucrose stability, including salts, sugars, and polysorbates (Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker et al., 2019). As shown in Figure 6.3, several of the polyphenolic ingredients were shown to be among the most effective additives at delaying sucrose crystallization.

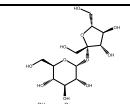
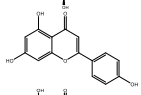
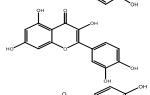
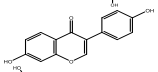
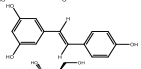
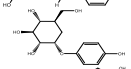
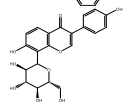
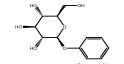
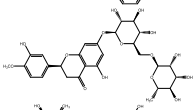
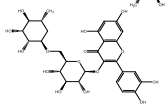
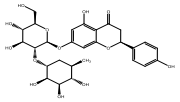
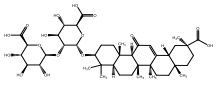
6.5 Conclusion

Polyphenols represent potentially viable additives to food products since they are already naturally present in foods, often in the same products in which amorphous sucrose is desirable, including tea, coffee, and chocolate-containing products. They are also strongly associated with many health benefits. The observation that different polyphenol structures had varying effects on the stabilization of amorphous sucrose allows for considerable potential flexibility in the product design space in terms of attaining and maintaining the desired sucrose physical form. Notably, for pure additives, glycosylated structures delayed sucrose crystallization more effectively than their nonglycosylated counterparts, presumably due to the presence of a region that is structurally similar to sucrose in addition to the dissimilar region. Of all additives studied, bulk food ingredients were the most successful at delaying crystallization, which can be attributed to the

heterogeneity of the material. Given the high feasibility of adding bulk food ingredients such as green tea to sucrose-containing products, future studies focusing on sucrose dissolution rate and sweetness perception of these systems seem warranted. Thus, this study increased understanding of amorphous sucrose stabilization by polyphenols, which may lead to improvement in product quality, shelf-life, and nutrition using natural ingredients with positive consumer perception.

6.6 Tables and Figures

Table 6.1 Properties of sucrose and polyphenols used in lyophiles. Grayscale indicates increasing degree of glycosylation.

Component	Average MW (g/mol)	Sample Type	Sugar Units	Glycosidic Linkage Between Sugar Units	Structure
Sucrose [1]	342.3	-	β-D-fructofuranosyl, α-D-glucopyranoside	(2,1)	
Apigenin [1]	270.24	Nonglycosylated	-	-	
Quercetin [1]	302.23	Nonglycosylated	-	-	
Daidzein [1]	254.24	Nonglycosylated	-	-	
Resveratrol [1]	228.24	Nonglycosylated	-	-	
α-Arbutin [1]	272.25	Monoglycosylated	α-D-glucopyranose	-	
Puerarin [1]	416.4	Monoglycosylated	1,5-anhydroglucitol	-	
Phenyl β-D-glucopyranoside [1]	256.25	Monoglycosylated	β-D-glucopyranose	-	
Hesperidin [1]	610.6	Polyglycosylated	β-D-glucopyranose, α-L-rhamnopyranose (terminal)	(6,1)	
Rutin [1]	610.5	Polyglycosylated	β-D-glucopyranose, α-L-rhamnopyranose (terminal)	(6,1)	
Naringin [1]	580.5	Polyglycosylated	β-D-glucopyranose, α-L-rhamnopyranose (terminal)	(2,1)	
Glycyrrhizic Acid [1]	822.9	Polyglycosylated	α-D-glucuronic acid, β-D-glucuronic acid (terminal)	(2,1)	
Cocoa Powder	-	Bulk Food Ingredient	-	-	-
Green Tea Extract	-	Bulk Food Ingredient	-	-	-
Matcha	-	Bulk Food Ingredient	-	-	-
Green Coffee	-	Bulk Food Ingredient	-	-	-
Monkfruit	-	Bulk Food Ingredient	-	-	-

[1] PubChem

Table 6.2 Physical stability of sucrose lyophiles in controlled RH desiccators measured by PXRD, time of crystallization of amorphous sucrose lyophiles exposed to iso-40% RH or increasing RH in SPS instrument, and enhancement of additives compared to the control based on SPS data. Superscript letters denote statistical significance between time or RH of crystallization. Grayscale indicates increasing degree of glycosylation.

	Co-formulated Additive	Percent Additive (*w/w*)	Crystallization in Desiccators at			Crystallization in SPS at 40% RH hold		Crystallization in SPS at increasing RH
			11% RH	23% RH	33% RH	Crystallization time (hr)	Enhancement Compared to Control	Crystallization RH
Sucrose	-	-	A	A	Day 7	18 ± 4^{JKL}	1x	35 ± 0^{E}
Sucrose	Apigenin	1%	A	A	Day 4	23 ± 1^{IJKL}	1.3x	35 ± 0^{E}
Sucrose	Apigenin	5%	A	A	Day 14	-	-	-
Sucrose	Quercetin	1%	A	A	Day 7	26.0 ± 0.9^{GHIJ}	1.4x	38 ± 4^{DE}
Sucrose	Quercetin	5%	A	A	Day 14	17.1 ± 0.1^{JKL}	1x	40 ± 0^{D}
Sucrose	Daidzein	1%	A	A	Day 7	-	-	-
Sucrose	Daidzein	5%	A	A	Day 7	13.8 ± 0.6^{L}	0.8x	40 ± 0^{D}
Sucrose	Resveratrol	1%	A	A	Day 7	-	-	-
Sucrose	Resveratrol	5%	A	A	Day 7	-	-	-
Sucrose	α-Arbutin	1%	A	A	Day 7	33 ± 1^{EFG}	1.8x	40 ± 0^{D}
Sucrose	α-Arbutin	5%	A	A	Day 14	42 ± 5^{DE}	2.3x	45 ± 0^{C}
Sucrose	Puerarin	1%	A	A	Day 30	43 ± 2^{D}	2.4x	40 ± 0^{D}
Sucrose	Puerarin	5%	A	A	Day 30	46 ± 5^{D}	2.6x	50 ± 0^{B}
Sucrose	Phenyl β-D-glucopyranoside	1%	A	A	Day 7	42.15 ± 0.07^{D}	2.3x	40 ± 0^{D}
Sucrose	Phenyl β-D-glucopyranoside	5%	A	A	Day 14	31 ± 1^{FGHI}	1.7x	45 ± 0^{C}
Sucrose	Hesperidin	1%	A	A	Day 7	23.5 ± 0.1^{HIJK}	1.3x	35 ± 0^{E}
Sucrose	Hesperidin	5%	A	A	Day 7	15.1 ± 0.2^{KL}	0.8x	40 ± 0^{D}
Sucrose	Rutin	1%	A	A	Day 4	24.45 ± 0.07^{GHIJ}	1.4x	40 ± 0^{D}
Sucrose	Rutin	5%	A	A	Day 7	24 ± 1^{GHIJ}	1.3x	40 ± 0^{D}
Sucrose	Naringin *	1%	A	A	Day 14	23.2 ± 0.7^{HIJK}	1.3x	-
Sucrose	Naringin *	5%	A	A	A	63 ± 6^{C}	3.5x	-
Sucrose	Glycyrrhizic Acid *	1%	A	Day 30	Day 21	17.3 ± 0.5^{JKL}	1x	-
Sucrose	Glycyrrhizic Acid *	5%	A	A	A	23 ± 1^{HIJK}	1.3x	-
Sucrose	Cocoa Powder	1%	A	A	Day 7	27.6 ± 0.1^{GHI}	1.5x	40 ± 0^{D}
Sucrose	Cocoa Powder	5%	A	A	Day 21	33 ± 1^{FG}	1.8x	45 ± 0^{C}
Sucrose	Green Tea Extract	1%	A	A	Day 30	38 ± 2^{DEF}	2.1x	40 ± 0^{D}
Sucrose	Green Tea Extract	5%	A	A	A	$> 96^{A}$	> 5.3x	55 ± 0^{A}
Sucrose	Matcha	1%	A	A	Day 7	32 ± 2^{FGH}	1.8x	40 ± 0^{D}
Sucrose	Matcha	5%	A	A	Day 30	40 ± 1^{DEF}	2.2x	50 ± 0^{B}
Sucrose	Green Coffee	1%	A	A	Day 14	43 ± 1^{D}	2.4x	40 ± 0^{D}
Sucrose	Green Coffee	5%	A	A	A	83.3 ± 0.8^{B}	4.6x	55 ± 0^{A}
Sucrose	Monkfruit	1%	A	A	Day 14	24 ± 1^{GHIJK}	1.3x	40 ± 0^{D}

* Data has been included in a patent publication (Taylor et al., 2019)

Samples that remained amorphous for the entire 30-day desiccator study are marked "A"; length of time prior to evidence of crystallization is indicated otherwise.

Table 6.3 Moisture content, onset glass transition temperature, and onset crystallization temperature of initial amorphous sucrose lyophiles prior to any treatment. Superscript letters denote statistical significance between moisture content, onset T_g, or onset T_{crys}, respectively. Grayscale indicates increasing degree of glycosylation.

	Co-formulated Additive	Percent Additive (*w/w*)	Moisture Content (*wb*)	Onset T_g	Onset T_{crys}
Sucrose	-	1%	2.4 ± 0.2% ^{A}	47 ± 3°C ^{D}	98 ± 6°C ^{EFG}
Sucrose	Apigenin	1%	2.5 ± 0.4% ^{A}	48.9 ± 0.4°C ^{BCD}	94.5 ± 0.5°C ^{FG}
Sucrose	Quercetin	1%	2.5 ± 0.2% ^{A}	47 ± 2°C ^{D}	95 ± 2°C ^{FG}
Sucrose	Daidzein	1%	2.6 ± 0.1% ^{A}	54 ± 1°C ^{ABC}	107.6 ± 0.6°C ^{ABCDE}
Sucrose	Resveratrol	1%	2.5 ± 0.2% ^{A}	47.8 ± 0.5°C ^{CD}	91.1 ± 0.3°C ^{G}
Sucrose	α-Arbutin	1%	2.7 ± 0.4% ^{A}	54 ± 3°C ^{AB}	104 ± 4°C ^{CDEF}
Sucrose	Puerarin	1%	2.7 ± 0.3% ^{A}	54 ± 1°C ^{ABC}	114.1 ± 0.2°C ^{ABC}
Sucrose	Phenyl β-D-glucopyranoside	1%	2.0 ± 0.1% ^{A}	52.3 ± 0.7°C ^{ABCD}	107.7 ± 0.9°C ^{ABCDE}
Sucrose	Hesperidin	1%	2.7 ± 0.3% ^{A}	53.3 ± 0.9°C ^{ABC}	104 ± 2°C ^{CDEF}
Sucrose	Rutin	1%	2.5 ± 0.3% ^{A}	52.1 ± 0.1°C ^{ABCD}	103.1 ± 0.6°C ^{DEF}
Sucrose	Naringin	1%	2.7 ± 0.6% ^{A}	53 ± 1°C ^{ABC}	109.63 ± 0.08°C ^{ABCD}
Sucrose	Cocoa Powder	1%	2.6 ± 0.1% ^{A}	52 ± 1°C ^{ABCD}	107 ± 3°C ^{BCDE}
Sucrose	Green Tea Extract	1%	2.9 ± 0.2% ^{A}	54.3 ± 0.2°C ^{AB}	114 ± 1°C ^{ABC}
Sucrose	Matcha	1%	2.8 ± 0.1% ^{A}	56 ± 1°C ^{A}	115 ± 2°C ^{AB}
Sucrose	Green Coffee	1%	2.6 ± 0.5% ^{A}	52 ± 3°C ^{ABCD}	112 ± 6°C ^{ABCD}
Sucrose	Monkfruit	1%	2.5 ± 0.3% ^{A}	53.5 ± 0.3°C ^{ABC}	117 ± 1°C ^{A}

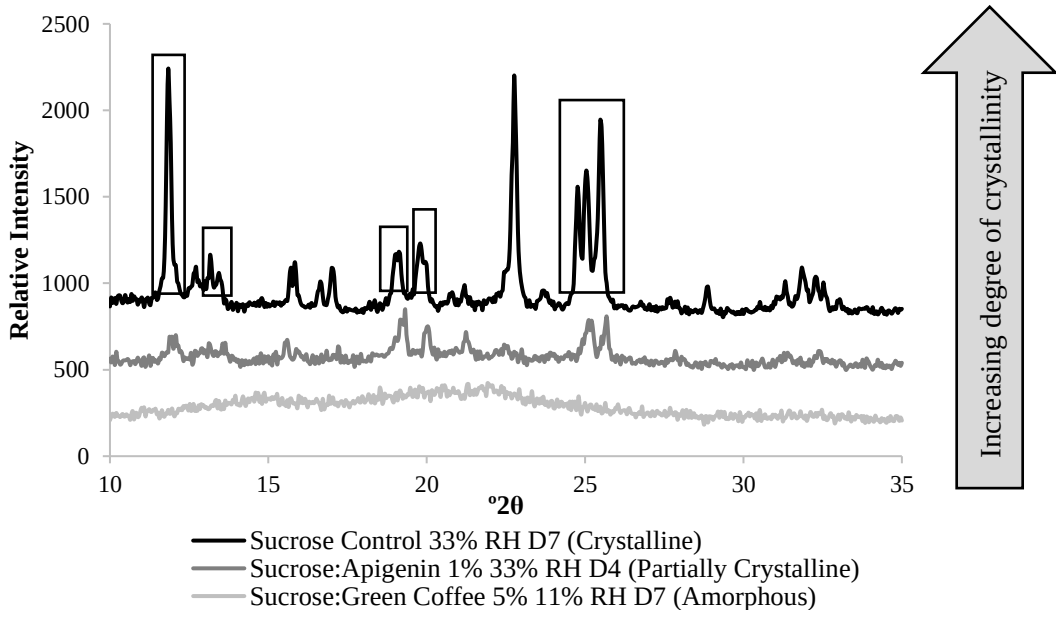


Figure 6.1 Increasing degree of crystallinity using powder x-ray diffraction, where well-defined crystalline sucrose peaks are indicated (Leinen & Labuza, 2006).

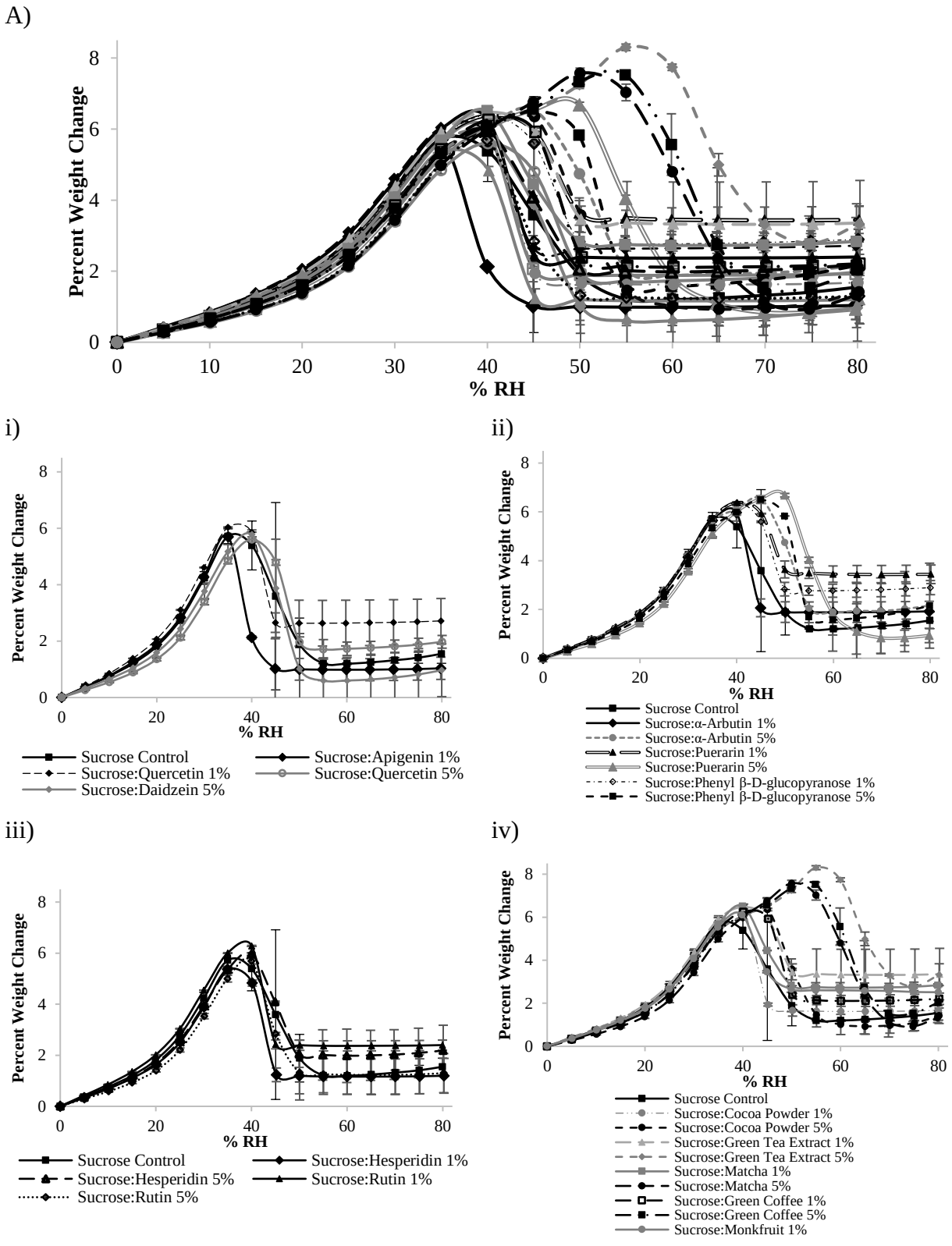
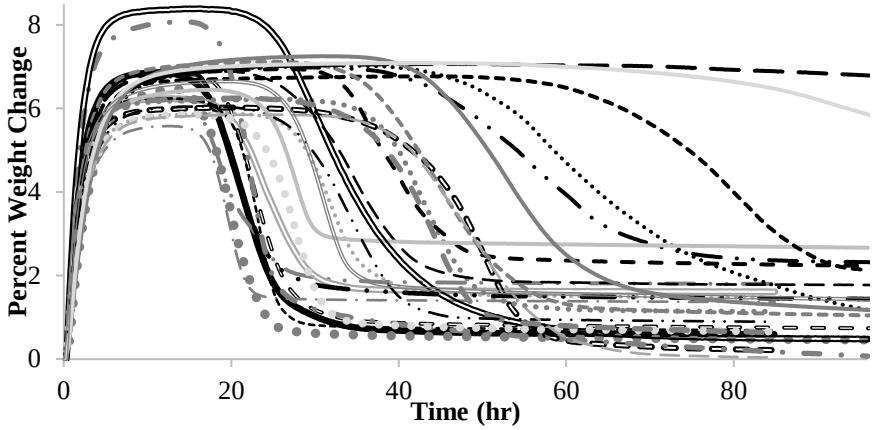


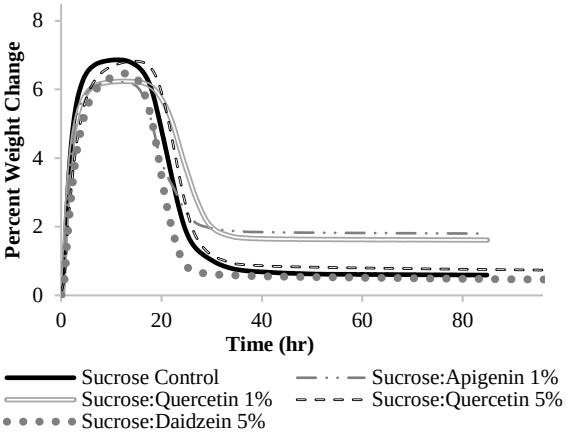
Figure 6.2 Moisture sorption profiles of sucrose:polyphenol lyophiles A) from 0-80% RH and B) iso-RH hold at 40% RH, where structural properties of additives are as follows: i) nonglycosylated, ii) monoglycosylated, iii) polyglycosylated, and iv) bulk food ingredients.

Figure 6.2 continued

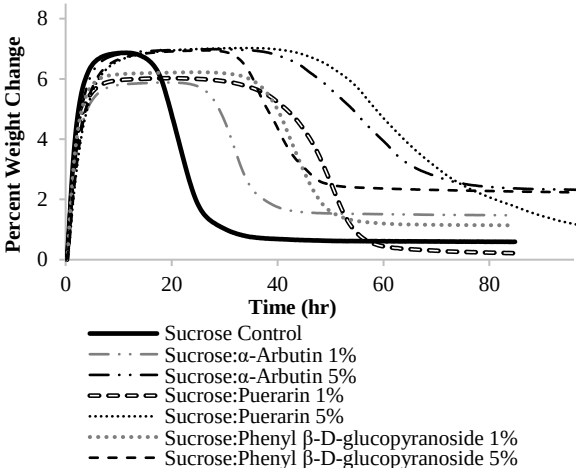
B)



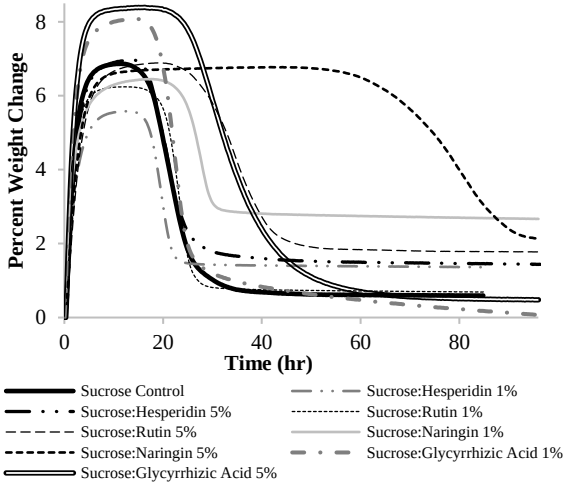
i)



ii)



iii)



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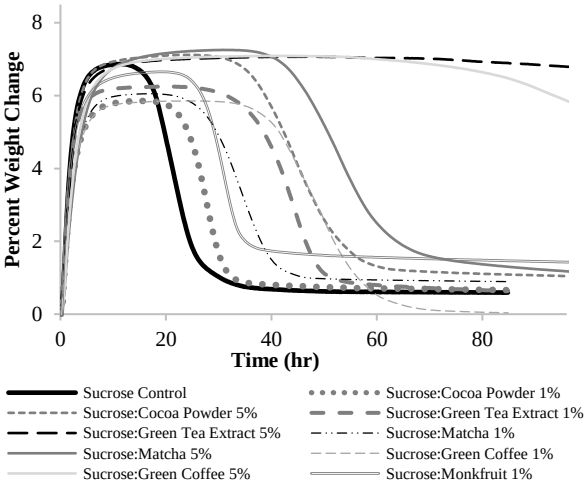

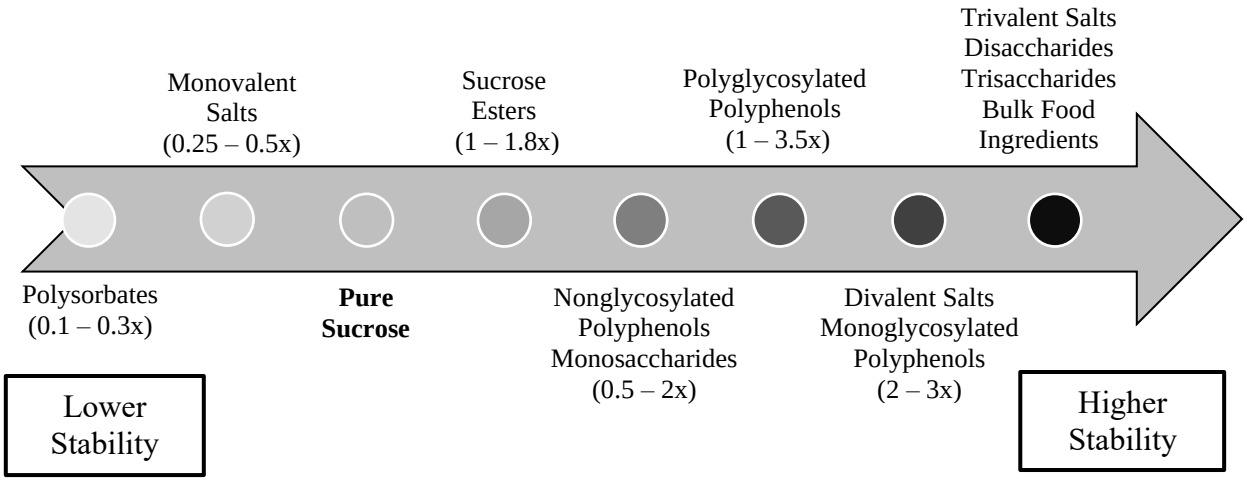

Figure 6.3 Stability trend of sucrose lyophiles prepared with various additives, including salts, sugars, polysorbates, and polyphenols (Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker et al., 2019).

6.7 References

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CHAPTER 7. ALTERING THE CRYSTALLIZATION TENDENCY OF AMORPHOUS SUCROSE UTILIZING A WIDE VARIETY OF POLYMERS AND OTHER ADDITIVES

7.1 Abstract

Amorphous sucrose tends to crystallize readily in the presence of increasing amounts of water, and the ability to stabilize amorphous sucrose in such environments may be advantageous for many foods. This study directly compared the sucrose crystallization altering properties of a variety of polymers and other additives that encompass a range of physicochemical traits and are commonly used in foods: inulin, guar gum, locust bean gum, xanthan gum, high- and low-methoxy (HM, LM) pectins, gum Arabic, polydextrose, β-lactoglobulin, gelatin, corn syrup solids, DE 10 and 18 maltodextrins, DE 47 glucose syrup, honey, and blue agave syrup. Solutions containing 10% *w/v* sucrose with and without 1% additive (*w/w* sucrose) were lyophilized, stored in desiccators (11, 23, 33% RH) at 25°C, and analyzed by powder x-ray diffraction, infrared spectroscopy, and polarized light microscopy over time to document physical states. Samples were also analyzed by moisture sorption techniques, Karl Fischer titration, and differential scanning calorimetry. All lyophiles were initially amorphous, and most additives delayed sucrose crystallization between 1.2x and 1.8x that of the control at 40% RH and up to 2.5x at 33% RH. Delays in crystallization related more to molecular structures (those able to overlap with the sucrose crystal lattice structure due to structural similarity or ability to hydrogen bond with sucrose) than glass transition temperature. Corn syrup solids, inulin, and pectins were the most effective at delaying sucrose crystallization. These findings indicate the mechanism by which common food additives may affect crystallization tendencies of amorphous sucrose.

7.2 Introduction

Sucrose is commonly used in many dry food products where it can exist in the crystalline and/or amorphous state. Amorphous solids lack the long-range three-dimensional order found in crystalline solids and behave more like liquids than solids due to their random arrangement of molecules (Hancock & Zografi, 1997). Physical state is dependent on food matrix, processing operations, and formulation, and the physical form of the solid will affect stability, dissolution rate,

moisture uptake, texture, and various sensory attributes, including mouthfeel (Mathlouthi, 1995). Amorphous carbohydrates may also release volatiles upon crystallization, which leads to changes in aroma and flavor perception (Chirife & Karel, 1974). Although crystalline sucrose is sometimes desired, the amorphous state is often preferable for many dry food products, especially many confectionary products and dry beverage mixes, due to its softer texture and faster dissolution. However, the amorphous state is less thermodynamically stable, easily crystallizing into the more stable crystalline form and releasing absorbed moisture in the process, which can lead to undesirable textural changes in the product, including caking and clumping in powders or graining in confections (Hartel & Hartel, 2014; Slade & Levine, 1991). Consequently, strategies to stabilize amorphous sucrose have been widely investigated (Cuè, Salvador, Morales, Rodriguez, & Gonzàlez, 2001; Leinen & Labuza, 2006; Liang, Hartel, & Berglund, 1989; Makower & Dye, 1956; Roe & Labuza, 2005; Saleki-Gerhardt & Zografi, 1994; Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker, Felten, Taylor, & Mauer, 2021; Voelker et al., 2019).

Sucrose crystallization is dependent on both temperature and relative humidity (RH) (Mathlouthi, 1995; Shamblin & Zografi, 1999). Exposure to RHs above 32% is known to increase the moisture content of amorphous sucrose enough to significantly increase the rate of crystallization, due to the plasticization properties of water, which lowers the glass transition temperature (T_g) of the material (Palmer et al., 1956). When the storage temperature is higher than the T_g, the material exists in the rubbery, supercooled liquid state, increasing its molecular mobility and susceptibility to chemical and physical changes, including crystallization (Buera et al., 2005; Labuza & Labuza, 2004; Roos & Karel, 1991b). For this reason, polymeric additives with high T_gs, including corn syrup solids, carboxymethylcellulose, guar gum, garrofin gum, sodium alginate, starch, microcrystalline cellulose, polyvinylpyrrolidone, and vinyl acetate, have often been combined with amorphous sucrose to study how the resulting increase in overall matrix T_g causes a disruption of sucrose crystallization (Gabarra & Hartel, 1998; Islesias & Chirife, 1978; Leinen & Labuza, 2006; Shamblin, Huang, & Zografi, 1996). This is also commonly done in commercial confections, for example, Jolly Ranchers®, wherein corn syrup is added as a doctoring agent to maintain the glassy amorphous state of sucrose.

However, it has also been shown that even when T_g is not significantly affected by a co-formulated additive, such as with small additions of salts, sugars, polymers, emulsifiers, and polyphenols, crystallization rate can still be dramatically slowed by two major mechanisms:

preventing the mass transfer of sucrose to the site of the crystal lattice, and by adsorbing to the surface of the crystal itself, specifically if the additive is structurally similar to sucrose, and thus preventing incorporation of additional sucrose molecules into the crystal lattice (Islesias & Chirife, 1978; Mazzobre, Soto, Aguilera, & Buera, 2001; Roe & Labuza, 2005; Smythe, 1967; Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker et al., 2021; Voelker et al., 2019). It has also been shown that polysorbates increase the rate of sucrose crystallization by acting as a template for sucrose to aggregate, due to structural properties, promoting nucleation and leading to rapid crystallization (Voelker et al., 2019).

Therefore, the objective of this study was to determine the effects of a wide variety of food-relevant polymers and other common food additives with varying structural characteristics (Table 7.1) on the physical stability and crystallization tendency of amorphous sucrose, specifically at low levels of addition in which T_g would not play a role. It was hypothesized that even when T_g was not significantly affected by the small addition of various polymeric additives, polymers with regions that were structurally similar to sucrose would most successfully delay crystallization. Understanding the structural interactions of sucrose with co-formulated ingredients common in dry, sugary food products will improve the stability of amorphous sucrose and reduce the undesirable effects of sucrose crystallization in foods.

7.3 Materials and Methods

7.3.1 Materials

Crystalline sucrose was obtained from Mallinckrodt Chemicals (Philipsburg, NJ). The polymers and other additives that were used as co-formulated ingredients in the sucrose lyophiles included: guar gum, xanthan gum, locust bean gum, and high- and low-methoxy (HM, LM) pectins from Sigma-Aldrich (St. Louis, MO); gum Arabic from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ); β-lactoglobulin from Davisco Foods International, Inc. (Le Sueur, MN); polydextrose from Tova Industries LLC (Louisville, KY); gelatin from Ward's Science (Rochester, NY); corn syrup solids from The SausageMaker, Inc. (Buffalo, NY); maltodextrin DE 10 and DE 18 from A.E. Staley Manufacturing Company (Decatur, IL); inulin from Bulk Supplements (Henderson, NV); Glucidex glucose syrup DE 47 (Roquette, Lestrem, France) donated by Nestlé Research (Lausanne, Switzerland); honey from Billy Bee Honey Products Company (London,

Ontario); and blue agave syrup from Bluava (Minneapolis, MN). The additives were chosen based on use in the food industry and to encompass a variety of structures and physicochemical properties (Table 7.1).

Controlled-RH desiccators were prepared using phosphorus pentoxide (P_2O_5) (~0% RH) (Fisher Scientific, Fair Lawn, NJ) and the following saturated salt solutions: lithium chloride (11% RH) (Avantor Performance Materials, Center Valley, PA), potassium acetate (23% RH) (Fisher Scientific), and magnesium chloride (33% RH) (Fisher Scientific) (Greenspan, 1977). Karl Fischer reagents for use in volumetric one-component Karl Fischer titrations included HYDRANAL-Composite 2 (titrant), HYDRANAL-Methanol Rapid (working medium), and HYDRANAL-Water Standard 10.0 (Sigma-Aldrich). All water used in this study was deionized and purified using a Barnstead E-Pure ultrapure water purification system (ThermoScientific, Waltham, MA) with a resistivity > 17.5 MΩ·cm at 25ºC.

7.3.2 Preparation of amorphous samples

Sucrose was amorphized by freeze drying a 10% *w/v* sucrose solution with and without 1% (*w/w* sucrose) of the co-formulated additive. There were 16 dispersion preparations in addition to the control sucrose: sucrose:inulin, sucrose:guar gum, sucrose:xanthan gum, sucrose:locust bean gum, sucrose:HM pectin, sucrose:LM pectin, sucrose:gum Arabic, sucrose:β-lactoglobulin, sucrose:polydextrose, sucrose:gelatin, sucrose:corn syrup solids, sucrose:maltodextrin DE 10, sucrose:maltodextrin DE 18, sucrose:glucose syrup DE 47, sucrose:honey, and sucrose:blue agave syrup. Additives were added at 1% (*w/w*) to enable a comparison of results to previous studies (Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker et al., 2021; Voelker et al., 2019) as well as due to the low solubility of some polymers. The solutions were frozen at -20ºC for at least 12 h prior to lyophilization. Using a lyophilization method described previously (Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker et al., 2019), lyophilization was completed in a VirTis Genesis 25ES freeze dryer (SP Scientific, Warminster, PA). Samples were initially frozen in the freeze dryer at -40ºC and 300 mTorr for 6 h. The samples were then held at -40ºC and 150 mTorr for 24 h to allow for primary drying to occur. Secondary drying was achieved by increasing the temperature from -40ºC to 20ºC in increments of 10ºC, holding for 9 h at each step. A heating step was then completed at 25ºC and 300 mTorr for 6 h before being immediately transferred to desiccators containing P_2O_5 (~0% RH). The samples were stored with P_2O_5 at ambient temperature

(22 ± 2ºC) until further analysis, and all subsequent sample handling was done in a glove box purged with nitrogen (~5% RH).

7.3.3 Storage treatments

Storage treatments were adapted from Voelker et al. (2019) and Voelker et al. (2021). Lyophiles were stored at different RHs in desiccators containing saturated salt solutions of lithium chloride (11% RH), potassium acetate (23% RH), or magnesium chloride (33% RH) at 25ºC (Greenspan, 1977) in a temperature-controlled room for up to 4 weeks. RH was verified by measuring the water activity of the saturated salt solutions. Samples were analyzed over a period of 4 weeks, and a single desiccator was used for each timepoint of analysis to avoid exposing the samples to ambient RH until the day of their analysis. Samples were discarded after analysis.

7.3.4 Determination of crystallinity

The physical state of samples was monitored over time by a combination of powder x-ray diffraction (PXRD), polarized light microscopy (PLM), and Fourier-transform infrared spectroscopy (FT-IR), as described in Voelker et al. (2019). Lyophiles were analyzed on days 0, 1, 2, 4, 7, 14, 21, and 28 to identify the onset of crystallization of the amorphous lyophiles.

Powder x-ray diffraction

PXRD diffractograms were collected using two different machines. A Shimadzu LabX XRD-6000 (Shimadzu Corporation, Kyoto, Japan) equipped with a Cu-Kα radiation source set in Bragg-Brentano geometry and operating at 40 kV and 30 mA was used to monitor crystallization. Calibration was done on each day of analysis using a silicon standard to confirm the accuracy of the º2θ angle. Samples were analyzed using a scan range of 10-35º 2θ at a scan speed of 4º/min and a step size of 0.04º. Due to instrument downtime, a Rigaku Smartlab diffractometer (Rigaku Corporation, Tokyo, Japan) equipped with a Cu-Kα radiation source set in Bragg-Brentano geometry and operating at 40 kV and 40 mA was also used. Samples were analyzed using a scan range of 10-35º 2θ at a scan speed of 15º/min and a step size of 0.02º. Samples with diffraction patterns consisting of peaks above a signal-to-noise ratio of 3 were considered PXRD crystalline. The characteristic peaks associated with crystalline sucrose occur at 11.6, 13.1, 18.8, 19.6, and

24.6º 2θ (Leinen & Labuza, 2006). Samples with only small peaks were considered partially crystalline, and samples with no peaks and only an amorphous halo were considered PXRD amorphous (Figure 7.1.A).

Fourier-transform infrared spectroscopy

FT-IR (TravelIR HCI, SensIR Technologies, LLC, Danbury, CT) with a fixed attenuated total reflectance (ATR) accessory was used to monitor crystallinity of sucrose in the lyophiles using a method described by Lescure (1995), Mathlouthi (1995), and Voelker et al. (2019). Crystalline sucrose has characteristic FT-IR absorption peaks due to hydrogen bonding in the region of 2800-3800 cm^{-1} that can be used to identify crystallinity (Figure 7.1.B). The FT-IR was equipped with a TGA detector, resolution was set to 4 cm^{-1}, and samples were scanned 64 times from 650-4000 cm^{-1}. Control crystalline and amorphous sucrose spectra were used to verify the physical state of the lyophiles. Analysis of the spectra was completed using OMNIC Series Software (ThermoScientific).

Polarized light microscope

Samples were examined with an Omano polarized light microscope (Omano, China) using crystal identification as described by Carlton (2011) and Voelker et al. (2019). Birefringence indicated crystallinity in the sucrose lyophiles. Photographs to document sample appearance were taken using an iPhone 6s camera attached to the microscope eyepiece by an iDu LabCam adapter (Detroit, MI). Multiple areas of the lyophiles were observed to accurately identify the degree of crystallinity (Figure 7.1.C).

7.3.5 Moisture content

The moisture contents of all initial lyophiles were analyzed following exposure to ~0% RH (P_2O_5) for 2-4 days. Moisture contents of lyophiles that remained amorphous for the entire 4 weeks of exposure to 11% or 23% RH in desiccators were also determined. Moisture contents were measured using a one-component volumetric Karl Fischer titration method (V20S Volumetric KF Titrator, Mettler-Toledo, LLC, Columbus, OH) with a method adapted from Voelker et al. (2019) and Voelker et al. (2021). Approximately 50 mg of each lyophile were added to the HYDRANAL-

Methanol Rapid working medium to extract water from the sample, which was then titrated using the HYDRANAL-Composite 2 titrant. Moisture content was determined in % moisture (*wb*). Calibration of the Karl Fischer titration system was completed each day prior to sample analyses using the HYDRANAL-Water Standard 10.0 (10 mg/g = 1% water content).

7.3.6 Dynamic vapor sorption

Three sets of moisture sorption profiles of all lyophiles were collected at 25ºC using a SPSx-1µ Dynamic Vapor Sorption Analyzer (Projekt Messtechnik, Ulm, Germany) with methods adapted from Thorat, Forny, et al. (2017); (Voelker et al., 2021; Voelker et al., 2019). The first moisture sorption profile was completed by weighing 100-200 mg of samples in a 23-ring sample holder and equilibrating at 0% RH for 48 h in the instrument to standardize initial moisture content. Samples were analyzed from 5-80% RH in 5% RH increments with a maximum residence time of 12 h per step and an equilibration endpoint of < 0.001% weight change in 30 min. The percent change in mass at the end of each RH step taken to indicate moisture gain at that RH was plotted to create a moisture sorption profile of each lyophile.

The second moisture sorption profile involved weighing 100-200 mg of lyophiles into a 23-ring sample holder and again equilibrating at 0% RH for 48 h in the instrument to standardize initial moisture content. Samples were then held at 40% RH for 96 h or until mass loss indicative of crystallization of all samples had occurred. A moisture sorption/desorption profile was plotted as the percent change in mass versus time. This profile was also used to calculate exact time of crystallization under the specified conditions, in which the onset of mass loss indicated onset time of crystallization (Makower & Dye, 1956).

The third moisture sorption profile was created by again weighing 100-200 mg of lyophiles into a 23-ring sample holder and equilibrating at 0% RH for 48 h in the instrument to standardize initial moisture content. Samples were then held at 33% RH until crystallization of all samples had occurred. A moisture sorption/desorption profile was plotted as the percent change in mass versus time, which was again used to calculate onset time of crystallization indicated by mass loss, which was used to directly compare crystallization at 33% RH in the SPS and in the desiccators.

7.3.7 Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to measure the glass transition temperature (T_g) with a DSC 4000 (PerkinElmer, Waltham, MA) following a method adapted from (Voelker et al., 2019). The DSC was calibrated with indium and verified with the melting point of water. The system was purged with dry nitrogen at 20 mL/min. Initial lyophiles that had been exposed to 0% RH for 2-4 days (5-10 mg) were weighed into 50 µL aluminum DSC pans (PerkinElmer), hermetically sealed, and pierced with a pinhole to measure the 'dry' T_g by allowing water vapor to escape through the pinhole on heating. The onset T_g was measured by heating the samples in the DSC from an initial temperature of 20ºC to 100-120ºC at a rate of 20ºC/min, cooling to 20ºC at a rate of 50ºC/min, and holding at 20ºC for 3 min to allow the temperature to equilibrate. Samples were then heated to 100-120ºC at a rate of 20ºC/min, and the onset T_g was defined as the onset of the endothermic baseline shift in the second scan and determined using Pyris software (PerkinElmer). All starting ingredients were also analyzed using the heat-cool-heat method described above, varying the temperature range of the scans based on the predicted T_g of the material.

7.3.8 Statistical analysis

Samples were analyzed in duplicate for moisture sorption (time of crystallization), moisture content, and T_g. Single-variable ANOVA using SAS 9.4 (SAS Institute, Cary, NC) was used to determine significant differences between lyophiles in time or RH of crystallization, moisture content, and T_g. Differences were determined using Tukey's post hoc test for multiple comparisons at a significance level of $\alpha = 0.05$.

7.4 Results and Discussion

7.4.1 Stability of amorphous sucrose in RH-controlled desiccators

All sucrose and sucrose:additive lyophiles were initially amorphous, based on PXRD, FT-IR, and PLM analyses (as illustrated in Figure 7.1). Lyophiles stored in 11% and 23% RH-controlled desiccators all remained amorphous throughout the 4-week duration of the desiccator study, but all lyophiles crystallized by day 14 when stored in a 33% RH environment (Table 7.2). At 33% RH and 25ºC, all additives caused either no change or a delay in sucrose crystallization

when compared to the control except for blue agave syrup, in which the sucrose:blue agave syrup lyophile began to crystallize after only 2 days compared to 4-7 days for the control. At 33% RH, sucrose:xanthan gum, sucrose:HM pectin, and sucrose:corn syrup solids lyophiles did not crystallize until day 14, which was a substantial improvement over the sucrose control. Previous studies have shown more effective stabilization of amorphous sucrose by guar gum and corn syrup solids (up to 8x compared to the control) using higher weight percentages, 23.1% and 10-75% *w/w*, respectively, than the 1% *w/w* additive used here (Gabarra & Hartel, 1998; Islesias & Chirife, 1978) , indicating that as proportion of additive is increased, amorphous sucrose stability is increased. Based on all desiccator studies of sucrose lyophiles containing 1% *w/w* of additive, the stabilizing trend of the additives was: blue agave syrup < **sucrose control** ≈ inulin ≈ gum Arabic ≈ β-lactoglobulin ≈ gelatin ≈ maltodextrin DE 18 < guar gum ≈ locust bean gum ≈ LM pectin ≈ polydextrose ≈ maltodextrin DE 10 ≈ glucose syrup DE 47 ≈ honey < xanthan gum ≈ HM pectin ≈ corn syrup solids. Thus, even the addition of a small amount (1% *w/w*) of a compound can alter the crystallization of amorphous sucrose.

7.4.2 Moisture content of sucrose lyophiles

Moisture content is an important factor for initiating crystallization of amorphous materials, including sucrose (Makower & Dye, 1956; Mathlouthi, 1995), due to the plasticization by water, decrease in T_g, and increase of molecular mobility (Leinen & Labuza, 2006). This study measured the moisture contents of the initial amorphous lyophiles and lyophiles that remained amorphous after 4 weeks of storage in 11% and 23% RH desiccators using Karl Fischer titration to better understand moisture sorption in desiccators in conditions in which no sucrose crystallization was found. Sucrose lyophiles were prepared in two separate trials, due to capacity limitations, with a sucrose control in each trial. Differences in the initial moisture contents of the controls between the trials (0.7% vs. 1.4%, Table 7.3), attributed to potential variations introduced by sample handling and relative humidity of the environment on day of preparation, led to the decision to only compare moisture content differences between lyophiles and the control within a trial.

Although most initial lyophiles did not have significant ($p < 0.05$) differences in moisture content when compared to the control, a higher moisture content was found in sucrose:maltodextrin DE 18 and sucrose:honey lyophiles, which indicated that the addition of these specific additives may have altered the diffusion of moisture during lyophilization. As

anticipated, sucrose lyophiles gained significant moisture ($p < 0.05$) after 4 weeks of storage in both 11% and 23% RH conditions, with samples stored at the higher RH sorbing significantly more moisture than at the lower RH. After 4 weeks of storage at 11% RH, no significant differences in moisture content were found between any of the lyophiles compared to the control. After 4 weeks of storage at 23% RH, the only significant differences in moisture content compared to the control were found in sucrose:gelatin, sucrose:glucose syrup DE 47, and sucrose:blue agave syrup (all increased compared to the control). These results indicated that the additives often did not alter the hygroscopicity of lyophiles exposed to low RHs. Although the moisture content of the lyophiles increased by up to 4.7% moisture when stored at 23% RH, the moisture contents of the lyophiles stored in desiccators at 33% RH were not measured due to their crystallization, which is known to expel water (Makower & Dye, 1956). Additional dynamic moisture sorption studies were conducted to better understand moisture uptake preceding, during, and after crystallization.

7.4.3 Moisture sorption profiles and sucrose crystallization

To document the moisture sorption trends before, during, and after crystallization, a series of three moisture sorption profiles of all lyophiles was collected (Figure 7.2). Crystallization was indicated by a sharp mass loss due to expulsion of water (Makower & Dye, 1956). A drying step was used prior to collection of each moisture sorption profile wherein the lyophiles were held at 0% RH for 48 h, which was intended to remove variances in initial moisture content between the 2 trials and sample handling during loading into the instrument. The first set of moisture sorption profiles exposed the lyophiles to increasing RHs from 0-80% RH in 5% RH steps (Figure 7.2.A). All additives delayed sucrose crystallization compared to the control, which crystallized between 35-40% RH (evident as a lower moisture content at 40% RH than at 35% RH), and all co-formulated lyophiles crystallized at 40% RH. The percent weight change prior to crystallization varied between samples, indicating differences in moisture sorption and/or amounts of water needed to induce crystallization. For example, while the control gained ~6% weight before crystallizing, sucrose:HM pectin and sucrose:xanthan gum gained ~8.5% weight, sucrose:locust bean gum gained 8% weight, sucrose:guar gum gained 7.5%, and sucrose:honey gained only 5.5% weight before crystallizing at 40% RH. There were also differences in weight loss following crystallization. Since sucrose does not form hydrates (Carstensen & van Scoik, 1990), it is likely that the samples that did not lose as much weight on crystallization as they gained prior to

crystallizing, such as sucrose:glucose syrup DE 47, were still partially amorphous, had formed a crystalline shell that prevented further moisture loss, and/or had an altered mechanism of water diffusion through the crystal matrix.

A second set of moisture sorption studies was done at a constant 40% RH to understand sucrose crystallization patterns in an iso-RH environment known to support sucrose crystallization in a relatively short timeframe (Figure 7.2.B). The time at which each lyophile crystallized in this 40% RH environment is recorded in Table 7.2. Although all crystallization times found in this experiment were greater than for that of the control, sucrose:gum Arabic, sucrose:gelatin, sucrose:maltodextrin DE 10, sucrose:maltodextrin DE 18, and sucrose:blue agave syrup did not significantly ($p < 0.05$) delay crystallization compared to the control. Percent weight change of some samples prior to crystallization were both higher and lower than the control. While the control gained ~6.5% weight before crystallizing, sucrose:HM pectin and sucrose:xanthan gum gained ~8%, sucrose:guar gum gained ~ 7.5%, sucrose:LM pectin and sucrose:locust bean gum gained ~7%, and sucrose:β-lactoglobulin gained only 6% weight prior to crystallization. The trend of weight gain before crystallization compared to the control closely follows that found in the 0-80% RH sorption profile. Based on the 40% RH SPS experiment (Figure 7.2.B, Table 7.2), the stabilizing trend of the additives was as follows: **sucrose control** ≈ maltodextrin DE 18 ≈ gum Arabic ≈ blue agave syrup ≈ maltodextrin DE 10 ≈ gelatin < honey ≈ xanthan gum ≈ locust bean gum ≈ HM pectin < corn syrup solids < glucose syrup DE 47 < guar gum < β-lactoglobulin < inulin ≈ polydextrose < LM pectin. This trend differs from that found in the 33% RH desiccator studies in a few places. Most notably, inulin and β-lactoglobulin delayed sucrose crystallization by ~10 h (1.7x) in the SPS experiment, while in the desiccator studies these samples crystallized at the same time as the control. Additionally, inulin, polydextrose, β-lactoglobulin, and LM pectin most successfully delayed crystallization the 40% RH SPS experiments, while xanthan gum, HM pectin, and corn syrup solids were the only additives to delay crystallization until week 2 in the 33% RH desiccator.

The differences in crystallization tendencies found in the 33% RH desiccator studies compared to the 40% RH SPS studies may have been caused by differences in moisture sorption trends between the different RHs, the drying step done prior to the SPS experiment, and/or the passive headspace in the desiccator compared to the active headspace in the SPS. To better understand if the differences were due to increased RH or method of storage, a third SPS study

was completed at 33% RH after a 48 h drying step (Figure 7.2.C, Table 7.2). The stabilizing trend of the additives in this experiment was: gelatin ≈ glucose syrup DE 47 ≈ **sucrose control** ≈ gum Arabic ≈ guar gum ≈ locust bean gum ≈ blue agave syrup ≈ maltodextrin DE 10 ≈ honey < polydextrose < xanthan gum ≈ LM pectin < β-lactoglobulin < maltodextrin DE 18 < corn syrup solids < inulin ≈ HM pectin. The differences between samples in weight gain prior to crystallization was not as considerable as in the previous SPS experiments, with all samples crystallizing after gaining 5-6% weight. Aside from blue agave syrup and inulin, the lyophiles in the 33% RH SPS experiment behaved more similarly to the 33% RH desiccator experiment than the 40% RH SPS experiment. This indicated that the difference in RH was likely the major cause for the differences in stability mentioned previously, in agreement with what was shown in a similar study (Voelker et al., 2019) and the well-known dependence of crystallization on RH and moisture content (Makower & Dye, 1956; Mathlouthi, 1995). The HM pectin-containing lyophile was the most sensitive to change in RH from 33 to 40% RH. In both 33% RH experiments (SPS and desiccator), HM pectin was one of the most effective additives at delaying crystallization, but in the 40% RH experiment, the enhancement compared to the control was only 1.4x.

7.4.4 Effect of glass transition temperature on amorphous sucrose stability

Materials with high T_gs are often added to amorphous small molecule systems, including sucrose matrices, to stabilize the material by increasing the overall T_g of the matrix, which, in turn, delays/prevents recrystallization of the small molecule (Labuza & Labuza, 2004; Roe & Labuza, 2005; Roos & Karel, 1991b; van Hook, 1961). The stabilization occurs when the T_g is increased such that the T_g is above the environmental temperature, and thus, the amorphous matrix is in the glassy state. The limited molecular mobility in the glassy state delays and/or inhibits crystallization. To investigate the influence of T_g on crystallization in this study, the 'dry' T_gs of all sucrose lyophiles were measured (Table 7.3) and compared to sucrose crystallization trends. Examples of DSC thermograms of select sucrose lyophiles that were used to determine T_g can be found in the appendix (Figure A.7.1). The T_gs of the initial lyophiles ranged from ~62-71ºC, with amorphous sucrose having a T_g of 67ºC, similar to values previously reported (52-76ºC) (Allan et al., 2018; Shamblin & Zografi, 1999; Slade & Levine, 1991). The pure additives prior to lyophilization tended to have higher dry T_gs than sucrose, excluding honey (Table 7.1), which is expected in materials with high molecular weights (Slade & Levine, 1991). The dry T_gs of the additive-

containing lyophiles, however, did not vary much from the control lyophile, with only sucrose:HM pectin having a significantly ($p < 0.05$) higher T_g than the control but sucrose:glucose syrup DE 47, sucrose:honey, and sucrose:blue agave syrup having significantly ($p < 0.05$) lower T_gs than the control. Dry T_gs of lyophiles in the current study were not correlated to crystallization time in the SPS at 33% or 40% RH (R^2 = 0.0015 and 0.0016, respectively). Additionally, there was no correlation between dry T_g of the pure additives before lyophilization and the crystallization time in the SPS at 33% or 40% RH (R^2 = 0.012 and 0.0071, respectively). The lack of correlation between dry T_g of the lyophile or of the individual additive and crystallization time is presumably related to the very weak correlation between lyophile T_g and additive T_g (R^2 = 0.1515). Theoretically, the lyophiles that were prepared with additives that had the highest individual T_gs should have had the highest lyophile dry T_gs; however, since additives were in only 1% weight fractions and T_g of a matrix is influenced by the weight fractions of the individual components, the absence of correlation between lyophile T_g and additive T_g was expected, consistent with what has been seen previously in sucrose systems with low amounts of additive (Leinen & Labuza, 2006; Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker et al., 2021; Voelker et al., 2019).

Additionally, moisture content has a significant effect on T_g (Gordon & Taylor, 1953), so although dry T_gs were reported in this study, differences in moisture diffusion between lyophiles may have limited the extent to which the lyophiles were 'dried' in the DSC. All lyophile T_gs were well above the 25ºC storage temperature used in this study, so the initial lyophiles were in the glassy state at this storage condition. However, increasing the storage RH also increased the moisture contents of the samples (Table 7.3), and although the current study only measured dry T_gs and not T_gs immediately prior to crystallization, it could be assumed from moisture contents measured in the SPS studies that all samples were in the supercooled liquid state immediately prior to crystallization. Theoretically, the lyophiles with the lowest moisture contents prior to crystallization should have had the highest lyophile T_gs. However, there was only weak correlation between moisture sorbed prior to crystallization at 33% or 40% RH in the SPS and dry T_g of the lyophile (R^2 = 0.2434 and 0.2533, respectively). It is also of consequence that solids of both large and small molecular weight can have significant molecular mobility even at temperatures well below their T_gs (Hancock, Shamblin, & Zografi, 1995; Ismail & Mauer, 2019). Thus, in agreement with similar studies (Gabarra & Hartel, 1998; Leinen & Labuza, 2006; Shamblin et al., 1996;

Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker et al., 2021; Voelker et al., 2019), it was determined that T_g was not the major mechanism for determining amorphous sucrose stability.

7.4.5 Effect of structural properties of co-formulated additives on amorphous sucrose stability

Aside from T_g, several previous studies report another theory on delay of sucrose crystallization. For example, many studies have shown that weight fractions from 10-75% (*w/w*) of corn syrup solids significantly delayed or even prevented sucrose crystallization, and although T_g, and in effect viscosity and molecular mobility, did play a role in inhibiting crystallization of sucrose in these studies, results indicated that surface incorporation of the corn syrup solids and other co-lyophilized polymers on the crystal face of sucrose, due to specific interactions based on chemical similarities and molecular size, played a more predominant role in inhibiting nucleation and crystal growth (Gabarra & Hartel, 1998; Shamblin et al., 1996). This is especially notable since low weight fractions of co-lyophilized polymers, like 1-10% used in the studies by Gabarra and Hartel (1998) and Shamblin et al. (1996) and 1% used in the current study did not significantly increase T_g but did significantly delay crystallization. Thus, delay in crystallization found in the current study is presumably due to structural effects of the additives rather than T_g. This is also in agreement with a studies done by Thorat et al. (2018); (Voelker et al., 2021; Voelker et al., 2019) on the effects of co-lyophilizing 1% of saccharides, emulsifiers, and polyphenols with sucrose, in which it was found that structural properties of the saccharides, emulsifiers, and polyphenols had a more significant effect on time of crystallization than moisture sorption or T_g properties.

Role of structurally similar monomers to sucrose

According to Smythe (1967), the most effective oligosaccharides at inhibiting sucrose crystal growth are those derived from sucrose by substitution on the primary hydroxyl group attached to carbon 6 on the glucose moiety. For this reason, it was presumed that additives which could structurally overlap with sucrose, and thus, disrupt the sucrose-sucrose intermolecular interactions, would be most effective at delaying crystallization. This is in agreement with the conclusion by Thorat et al. (2018) and Voelker et al. (2021) in which a region of structural similarity along with a region of structural dissimilarity on a saccharide or polyphenol gave the most effective crystallization delay. Structural properties of the additives used in this study can be

found in Table 7.1. According to this theory, inulin, xanthan gum, polydextrose, corn syrup solids, maltodextrins, glucose syrup, honey, and blue agave syrup should have been the most effective additives in this study at delaying crystallization due to the presence of glucose and/or fructose units. While some of these predictions were seen, including the efficacy of inulin, xanthan gum, and corn syrup solids at delaying sucrose crystallization, some trends were not expected. Specifically, HM pectin and β-lactoglobulin were two of the best additives in this study at delaying crystallization and the maltodextrins and glucose syrup were not as effective as anticipated.

However, structural relationships can explain a large majority of the trends in this study. Locust bean gum and guar gum are made up of galactose and mannose, while xanthan gum has a glucose backbone (BeMiller & Huber, 2008). This explains why xanthan gum was more effective in inhibiting sucrose crystallization than were locust bean and guar gums, according to the 33% RH desiccator and SPS studies even though neither the T_gs of the three gum-containing lyophiles nor the moisture contents prior to or after storage of the lyophiles were statistically different from one another or the control ($p > 0.05$) (Tables 7.2, 7.3). Blue agave syrup and honey were expected to more successfully delay crystallization based on their abundant compositions of glucose and fructose monomers (Igoe, 2011). However, both additives were liquid in their original state, and since all additives were added on a weight basis, it is likely that the water in the material contributed to the weight. Therefore, after freeze-drying, impurities imposed by these two lyophiles were less abundant than the other additives studied, which is presumably why their enhancement of amorphous sucrose was only 1.4x for honey and 1.2x for blue agave syrup compared to the control at 40% RH. Additionally, gelatin and gum Arabic are high in polypeptide units (BeMiller & Huber, 2008; Damodaran, 2008), so structural similarities with sucrose are scarce, which is why they did not delay crystallization to a large extent. Although inulin, corn syrup solids, maltodextrins, and glucose syrup have structurally similar saccharide units to sucrose, their chain arrangement also plays a role in their efficacy at delaying sucrose crystallization.

Role of additive chain properties

Since maltodextrins and glucose syrup are long glucose chains, they were expected to perform very well in delaying sucrose crystallization due to their structural similarity to sucrose. However, the time of sucrose crystallization of maltodextrin or glucose syrup-containing lyophiles was not significantly different than the control in most cases (Table 7.2). This was presumably

caused by the tendency for maltodextrins and glucose syrup to form helices due to the glycosidic linkages in the chains (BeMiller & Huber, 2008). It is likely that only the glucose end points of the chains were able to interact with the glucose moiety of sucrose to disrupt crystallization, leading to the limited efficacy seen in this study.

However, it is also worth considering structural differences between the different DEs of maltodextrins and glucose syrup. Since the maltodextrins and glucose syrup were added on a weight basis and assuming that only the endpoints of the glucose chains are able to interact with sucrose, theoretically, short chain saccharides (high DE) should be more effective at interfering with incorporation of sucrose molecules into the crystal lattice. Shorter chains lead to more glucose endpoints that can interact with sucrose than on the longer chains (low DE) when added on a weight basis. Even though the T_gs of the maltodextrin/glucose syrup-containing lyophiles were not significantly different from one another ($p > 0.05$), sucrose:glucose syrup DE 47 crystallized significantly ($p < 0.05$) later than sucrose:maltodextrin DE 10 or 18 (1.6x, 1.2x, and 1.2x enhancement, respectively, in 40% RH SPS experiments) (Tables 7.2, 7.3), which indicates that structure of the maltodextrins and glucose syrup was more important than T_g in delaying sucrose crystallization. Although sucrose:glucose syrup DE 47 crystallized significantly faster than sucrose:maltodextrin DE 18 in the 33% RH SPS experiment, this trend reversal compared to the 40% RH SPS experiment was not observed in the 33% RH desiccator. Maltodextrins and glucose syrups can be produced by either enzyme- or acid-hydrolysis, or a combination of both. The method of hydrolysis used in the enzyme-hydrolyzed DE 47 glucose syrup compared to the acid-hydrolyzed DE 10 and 18 maltodextrins (Kearsley & Dziedzic, 1995) most likely did not play as large of a role in the difference in efficacy of crystallization inhibition as did the DE.

Corn syrup solids and polydextrose are also long glucose chains that are structurally compatible with sucrose (BeMiller & Huber, 2008), leading to successful delay in crystallization (1.5x/2.3x and 1.7x/1.8x enhancement in the 40/33% RH SPS experiments, respectively) even though neither the T_gs of the two lyophiles nor the moisture contents prior to or after storage of the lyophiles were statistically different from the control ($p < 0.05$) (Tables 7.2, 7.3). Although polydextrose has the ability to helix like the maltodextrins, it includes some sorbitol and citric acid (Lindsay, 2008), which may disrupt the helix, leading to more exposed glucose units that are able to interact with sucrose, and in effect, the increased prevention of sucrose crystallization seen in this study compared to the maltodextrins and glucose syrups. Inulin is a fructooligosaccharide, a

linear polysaccharide of fructose units linked to a terminal sucrose (BeMiller & Huber, 2008). Again, structural compatibility of inulin with sucrose was a probable cause for its ability to delay sucrose crystallization compared to the control (1.7x and 2.5x in the 40% and 33% RH SPS experiments, respectively) since neither the T_g nor the moisture contents prior to or after storage of the sucrose:inulin lyophiles were significantly different than the control ($p < 0.05$).

Role of intermolecular interactions in the absence of structurally similar monomers to sucrose

Although many polymers in this study do not have glucose or fructose units, some were still found to cause a significant delay in sucrose crystallization. Extent of hydrogen bonding is a major factor in preventing self-association of the sucrose molecules (Taylor & Zografi, 1998; Thorat et al., 2018), and therefore a molecule that has an energetic advantage to hydrogen bond with sucrose will be more effective in preventing crystal growth. Steric factors such as size may also come into play in the adsorption of the additive to the crystal face. Large molecules may not be as specific as small molecules due to the large number of sites that can adsorb to a crystal face (Cabrera & Vermilyea, 1958), but the more sites available that can interact with sucrose, the higher the likelihood of delaying crystal growth.

Guar gum, which does not contain glucose or fructose monomers, was found to significantly ($p < 0.05$) delay sucrose crystallization compared to the control in the 40% RH SPS experiment (1.6x) despite the fact that the T_gs and moisture contents were not significantly different from one another. A study done by Islesias and Chirife (1978) also showed that guar gum delayed sucrose crystallization compared to the control, citing intermolecular interactions between sucrose and guar gum as a major cause for stabilization. Thus, the delay in crystallization by guar gum in the current study was then also presumably due to intermolecular interactions between guar gum and sucrose. Guar gum may be found in a low proportion compared to sucrose, such as that used in the current study, in many powdered products, such as sauce, soup, or pudding mixes.

Additionally, HM and LM pectin are primarily made up of galacturonic acid units, not glucose or fructose units (BeMiller & Huber, 2008), but lyophiles containing both additives significantly ($p < 0.05$) delayed crystallization compared to the control in both SPS experiments (33% and 40% RH) and the 33% RH desiccator experiment (HM: 1.4x and 2.5x enhancement; LM: 1.8x and 1.8x enhancement in the 40% and 33% SPS experiments, respectively) (Table 7.2) even though there was no significant difference in moisture content of either pectin-containing

lyophile compared to sucrose prior to or following desiccator storage (Table 7.3). While the T_g of sucrose:HM pectin was significantly ($p < 0.05$) higher than the control (71 and 67ºC, respectively), it was only incrementally higher, and there was no significant difference between the T_g of sucrose:LM pectin and the control. Rather, structure of the pectins was the major cause for delay of crystallization. Their many available hydroxyl and carboxylic acid groups presumably increased ability to hydrogen bond with sucrose, adsorb to the crystal surface, and disrupt crystallization, similar to what was suggested by Thorat et al. (2018). Similarly, although gelatin and gum Arabic are high in polypeptide units and did not delay crystallization substantially, β-lactoglobulin is also high in polypeptide units but delayed crystallization by 1.7x and 2.1x in the 40% and 33% RH SPS experiments, respectively, even though neither the T_g nor the moisture contents prior to or following desiccator storage of the β-lactoglobulin-containing lyophile were significantly different than sucrose:gum Arabic, sucrose:gelatin, or the sucrose control. This delay of crystallization by β-lactoglobulin was instead presumably due to the large ratio of hydrophilic residues to surface non-polar groups (Damodaran, 2008), increasing its ability to hydrogen bond with sucrose.

7.4.6 Efficacy of additives at delaying crystallization compared to other food ingredients

Overall, structural similarities with sucrose, including glucose and fructose units and ability to hydrogen bond, were the main factors in delaying sucrose crystallization. Structural similarities allowed for intermolecular interactions between the additive and the sucrose so that the additive could adsorb to the sucrose crystal face and delay sucrose crystallization by preventing sucrose molecules from being incorporated into the lattice. However, while structural similarities can be used to describe the trends seen in this study, it is important to note that none of the additives had as substantial of an effect as was seen in previous studies using similar methods and percent additives (Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker et al., 2021; Voelker et al., 2019) (Figure 7.3). While the most effective additives from the current study in the 33% RH desiccators (xanthan gum, HM pectin, and corn syrup solids) only delayed crystallization by about 2x that of the control, similar conditions at 5-10% additive found that some di- and tri-valent cations, tri-saccharides, and polyphenols delayed crystallization >4x that of the control (remained amorphous for 30 day duration of experiment) (Thorat, Forny, et al., 2017; Thorat et al., 2018; Voelker et al., 2021), and some emulsifiers at both 1 and 5% additive induced almost immediate crystallization (Voelker et al., 2019). While additives in the previous studies were also added on a low weight

basis, on a molar basis, the high molecular weight polymers used in this study were not as high as other additives in the previous studies, and thus, there was not a large enough proportion of structurally similar groups compared to sucrose molecules present to play a substantial role in increasing the physical stability of amorphous sucrose. However, although these results indicate that polymers cannot be added in small weight fractions to food products to stabilize amorphous sucrose, polymers are very common food ingredients and are likely to be found in much higher weight fractions compared to sucrose in food products than in the current study. As was shown by Gabarra and Hartel (1998) and Islesias and Chirife (1978), increasing the weight fraction of polymers will increase the success of the polymers at delaying sucrose crystallization. When added in larger weight fractions, the current study indicates that structural properties of the polymers, not only the impact on T_g of the matrix, can be largely considered to predict effectiveness of amorphous sucrose stabilization.

7.5 Conclusion

When co-lyophilized with sucrose, all additives studied either successfully delayed crystallization of amorphous sucrose or did not change the time of sucrose crystallization. All lyophiles, including the control, remained amorphous for the 4-week duration of the experiment when stored at 25ºC in 11% and 23% RH conditions. As RH increased to 33% and 40% RH, crystallization rates increased, wherein additives delayed crystallization with varying efficacy. It was determined that although T_g is commonly reported to affect crystallization rates, T_g was not increased enough by the small weight fraction (1%) of additive used in this study to cause a stabilization of amorphous sucrose. Instead, structural compatibility of the additive with sucrose was the main factor that correlated to a successful delay in sucrose crystallization. Specifically, additives that were made up of glucose or fructose units or could effectively hydrogen bond with sucrose were thought to inhibit sucrose crystal growth by adsorbing to the sucrose crystal face and preventing other sucrose molecules from incorporating into the lattice. The information presented in this study about food-relevant polymers and other additives will benefit the food industry by better understanding mechanisms in which crystallization of amorphous sucrose is delayed in low-moisture products, such as many confectionary products and dry beverage mixes. By preventing sucrose crystallization with the addition of suitable polymers, the integrity of the product shelf-life, texture, flavor profile, aromas, and dissolution rate, among other factors, will be protected.

7.6 Tables and Figures

Table 7.1 Properties of sucrose and additives used in lyophiles. Superscript letters denote statistical significance between experimentally determined T_gs.

Component	Average MW (g/mol)	General Shape	Structural Makeup	Onset T_g (ºC) *
Sucrose [1]	342.3	Disaccharide	Glucose, Fructose	67 ± 1 [H] (freeze-dried)
Inulin [1]	3500 – 6000	Linear	Fructose, terminated with sucrose unit	99 ± 1 [E]
Guar Gum [1]	50,000 – 8,000,000	Linear with single unit branches	Mannose, galactose	-
Xanthan Gum [1]	400,000 – 15,000,000	Linear with trisaccharide unit; branches on every other main chain unit	Mannose, glucose	-
Locust Bean Gum [1]	50,000 – 3,000,000	Linear with single unit branches	Mannose, galactose	-
HM Pectin [1]	79,000 – 200,000	Linear	Mainly galacturonic acid	-
LM Pectin [1]	55,000 – 380,000	Linear	Mainly galacturonic acid	-
Gum Arabic [1]	250,000	Highly branched	Contains polypeptides	-
β-lactoglobulin [2]	18,400		Protein, polypeptides	170.8 ± 0.5 [C]
Polydextrose [3]	2000 – 5000	Branched	Glucose, small amounts of sorbitol and citric acid	97 ± 2 [EF]
Gelatin [2]	19,000 – 100,000		Protein, polypeptides	186.6 ± 0.3 [B]
Corn Syrup Solids [1]	700 – 10,000	Linear	Glucose	91 ± 1 [F]
Maltodextrin DE 10 [1]	15,000 – 20,000	Linear	Glucose (acid-hydrolyzed)	> 250 [A]
Maltodextrin DE 18 [1]	1000 – 9000	Linear	Glucose (acid-hydrolyzed)	183.8 ± 0.8 [B]
Glucose Syrup DE 47 [1]	400 – 700	Linear	Glucose (enzyme-hydrolyzed)	108 ± 4 [D]
Honey [4]	180 – 200	Mainly monosaccharides	Fructose, Glucose	12 ± 1 [I]
Blue Agave Syrup [4]	180 – 200	Mainly monosaccharides	Predominately Fructose	83 ± 1 [G]

* Materials without T_g values listed were not able to be determined by DSC for reasons that included: too high of a T_g in which the sample degraded before or during the glass transition event, and materials being too polydispersed to measure an overall T_g

[1] BeMiller and Huber (2008)
[2] Damodaran (2008)
[3] Lindsay (2008)
[4] Igoe (2011)

Table 7.2 Physical stability of amorphous sucrose lyophiles in controlled RH desiccators measured by a combination of PXRD, FTIR, and PLM, time of crystallization on exposure to iso-33% and iso-40% RH, and RH of crystallization as RH was increased 0-80% RH in the SPS instrument. Superscript letters denote statistical significance between times and RHs of crystallization. Grayscale indicates timeframe of crystallization.

	Co-formulated Additive	Crystallization in Desiccators at			Crystallization at 40% RH		Crystallization at 33% RH		Crystallization at increasing RH
		11% RH*	23% RH*	33% RH*	Crystallization time (h)	Enhancement Compared to Control	Crystallization time (h)	Enhancement Compared to Control	Crystallization RH
Sucrose	-	A	A	Day 4-7	14 ± 2^{A}	1x	85 ± 7^{A}	1x	38 ± 4^{A}
Sucrose	Inulin	A	A	Day 4 (PC)	24 ± 1^{GH}	1.7x	210 ± 40^{G}	2.5x	40 ± 0^{A}
Sucrose	Guar Gum	A	A	Day 7	22.1 ± 0.2^{EFGH}	1.6x	92.1 ± 0.4^{ABC}	1.1x	40 ± 0^{A}
Sucrose	Xanthan Gum	A	A	Day 14	19.6 ± 0.6^{BCDEF}	1.4x	155 ± 6^{CDEFG}	1.8x	40 ± 0^{A}
Sucrose	Locust Bean Gum	A	A	Day 7	20.3 ± 0.6^{BCDEFG}	1.5x	112 ± 8^{ABCD}	1.3x	40 ± 0^{A}
Sucrose	HM Pectin	A	A	Day 14	20 ± 2^{BCDEFG}	1.4x	210 ± 10^{G}	2.5x	40 ± 0^{A}
Sucrose	LM Pectin	A	A	Day 7	25.15 ± 0.07^{H}	1.8x	150 ± 20^{CDEFG}	1.8x	40 ± 0^{A}
Sucrose	Gum Arabic	A	A	Day 4 (PC)	17.0 ± 0.1^{AB}	1.2x	90 ± 20^{AB}	1x	40 ± 0^{A}
Sucrose	β-lactoglobulin	A	A	Day 4 (PC)	23.6 ± 0.4^{FGH}	1.7x	170 ± 20^{DEFG}	2.1x	40 ± 0^{A}
Sucrose	Polydextrose	A	A	Day 7	24 ± 2^{GH}	1.7x	150 ± 10^{BCDEFG}	1.8x	40 ± 0^{A}
Sucrose	Gelatin	A	A	Day 4	17.9 ± 0.6^{ABCD}	1.3x	74 ± 5^{A}	0.9x	40 ± 0^{A}
Sucrose	Corn Syrup Solids	A	A	Day 14	21.2 ± 0.4^{CDEFGH}	1.5x	190 ± 40^{FG}	2.3x	40 ± 0^{A}
Sucrose	Maltodextrin DE 10	A	A	Day 7	17.3 ± 0.1^{ABC}	1.2x	130 ± 10^{ABCDE}	1.5x	40 ± 0^{A}
Sucrose	Maltodextrin DE 18	A	A	Day 4 (PC)	17 ± 1^{AB}	1.2x	184 ± 8^{EFG}	2.2x	40 ± 0^{A}
Sucrose	Glucose Syrup DE 47	A	A	Day 7	21.8 ± 0.6^{DEFGH}	1.6x	74 ± 2^{A}	0.9x	40 ± 0^{A}
Sucrose	Honey	A	A	Day 7	19.3 ± 0.9^{BCDE}	1.4x	130 ± 20^{ABCDEF}	1.5x	40 ± 0^{A}
Sucrose	Blue Agave Syrup	A	A	Day 2 (PC)	17.0 ± 0.6^{AB}	1.2x	114.9 ± 0.4^{ABCD}	1.4x	40 ± 0^{A}

* Samples that remained amorphous for the entire 4 week desiccator study are marked "A"; length of time prior to evidence of crystallization is indicated otherwise. PC indicates the onset of crystallization before sample was largely crystalline.

Table 7.3 Percent moisture content (*wb*) of amorphous sucrose lyophiles prior to desiccator storage (Day 0) and samples that remained amorphous after 4 weeks of storage at 11% and 23% RH, and onset T_gs of initial (dry) amorphous lyophiles. Samples were prepared and analyzed in two separate trials, indicated by a break in the table. Uppercase superscript letters denote statistical significance between percent moisture of all lyophiles at the specified timepoint or storage RH (down columns), and lowercase superscript letters denote statistical significance between percent moisture of the specified lyophile at all timepoints or storage RHs (across rows). Statistical analysis was run separately for each trial. Superscript letters on T_g data denote statistical significance between T_gs only.

	Co-formulated Additive	**Week 0**	**Week 4 11% RH**	**Week 4 23% RH**	**Dry T_g (°C)**
Sucrose	-	0.7 ± 0.1% ^{ABCa}	2.83 ± 0.06% ^{ABb}	4.69 ± 0.04% ^{Bc}	67 ± 1 ^{BC}
Sucrose	Inulin	0.81 ± 0.08% ^{ABCa}	2.82 ± 0.08% ^{Bb}	4.79 ± 0.08% ^{Bc}	66 ± 1 ^{BCD}
Sucrose	Guar Gum	0.85 ± 0.06% ^{ABCa}	2.97 ± 0.08% ^{ABb}	4.69 ± 0.03% ^{Bc}	66 ± 2 ^{BCD}
Sucrose	Xanthan Gum	0.85 ± 0.03% ^{ABCa}	2.99 ± 0.03% ^{ABb}	4.75 ± 0.08% ^{Bc}	65 ± 1 ^{BCDE}
Sucrose	Locust Bean Gum	0.715 ± 0.007% ^{ABCa}	2.9 ± 0.1% ^{ABb}	4.82 ± 0.04% ^{ABc}	65.2 ± 0.4 ^{BCDE}
Sucrose	HM Pectin	0.64 ± 0.05% ^{BCa}	2.9 ± 0.2% ^{ABb}	4.9 ± 0.2% ^{ABc}	71 ± 0.6 ^{A}
Sucrose	LM Pectin	0.9 ± 0.1% ^{Aa}	2.78 ± 0.08% ^{Bb}	4.97 ± 0.06% ^{ABc}	67 ± 1 ^{BC}
Sucrose	Gum Arabic	0.60 ± 0.06% ^{Ca}	2.90 ± 0.04% ^{ABb}	4.77 ± 0.06% ^{Bc}	68.2 ± 0.7 ^{AB}
Sucrose	β-lactoglobulin	0.76 ± 0.03% ^{ABCa}	2.86 ± 0.02% ^{ABb}	4.9 ± 0.1% ^{ABc}	65.6 ± 0.6 ^{BCD}
Sucrose	Polydextrose	0.61 ± 0.06% ^{Ca}	2.90 ± 0.03% ^{ABb}	4.8 ± 0.1% ^{Bc}	65.1 ± 0.4 ^{BCDE}
Sucrose	Gelatin	0.71 ± 0.03% ^{ABCa}	3.2 ± 0.2% ^{Ab}	5.2 ± 0.1% ^{Ac}	66.3 ± 0.3 ^{BCD}
Sucrose	Corn Syrup Solids	0.875 ± 0.007% ^{ABa}	2.925 ± 0.007% ^{ABb}	4.9 ± 0.1% ^{ABc}	65.3 ± 0.7 ^{BCDE}
Sucrose	-	1.45 ± 0.07% ^{Ba}	4.3 ± 0.3% ^{Ab}	4.71 ± 0.08% ^{Bb}	67 ± 1 ^{BC}
Sucrose	Maltodextrin DE 10	1.48 ± 0.2% ^{Ba}	4.22 ± 0.03% ^{ABb}	5.4 ± 0.6% ^{ABb}	66 ± 1 ^{BCD}
Sucrose	Maltodextrin DE 18	1.73 ± 0.03% ^{Aa}	3.6 ± 0.1% ^{BCb}	5.62 ± 0.04% ^{ABc}	63.9 ± 0.6 ^{CDE}
Sucrose	Glucose Syrup DE 47	1.475 ± 0.007% ^{Ba}	3.98 ± 0.04% ^{ABCb}	6.15 ± 0.02% ^{Ac}	62.6 ± 0.8 ^{DE}
Sucrose	Honey	1.81 ± 0.08% ^{Aa}	3.4 ± 0.2% ^{Cb}	5.1 ± 0.3% ^{ABc}	61.6 ± 0.5 ^{E}
Sucrose	Blue Agave Syrup	1.40 ± 0.04% ^{Ba}	3.7 ± 0.1% ^{ABCb}	5.9 ± 0.2% ^{Ac}	63.6 ± 0.2 ^{CDE}

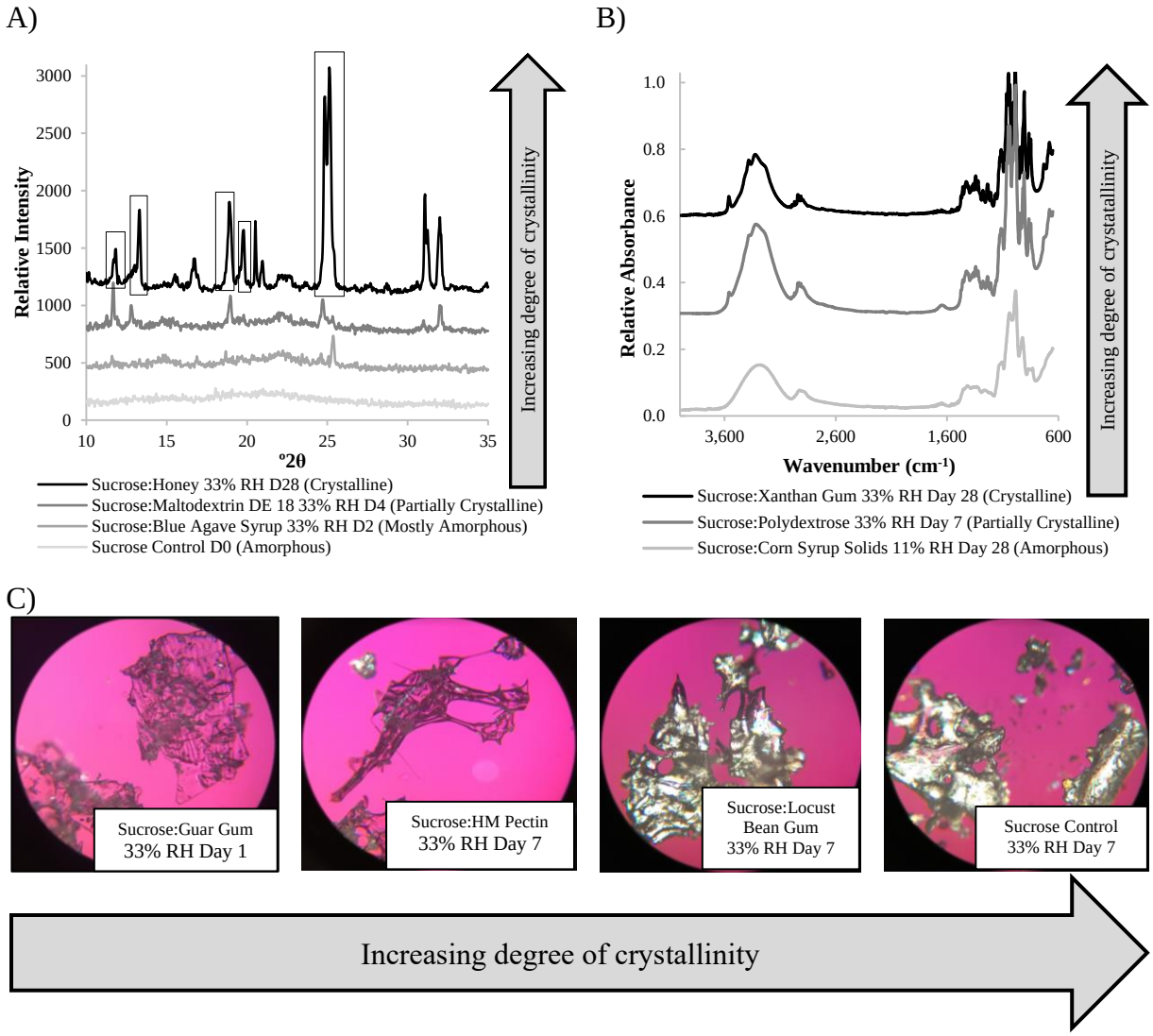
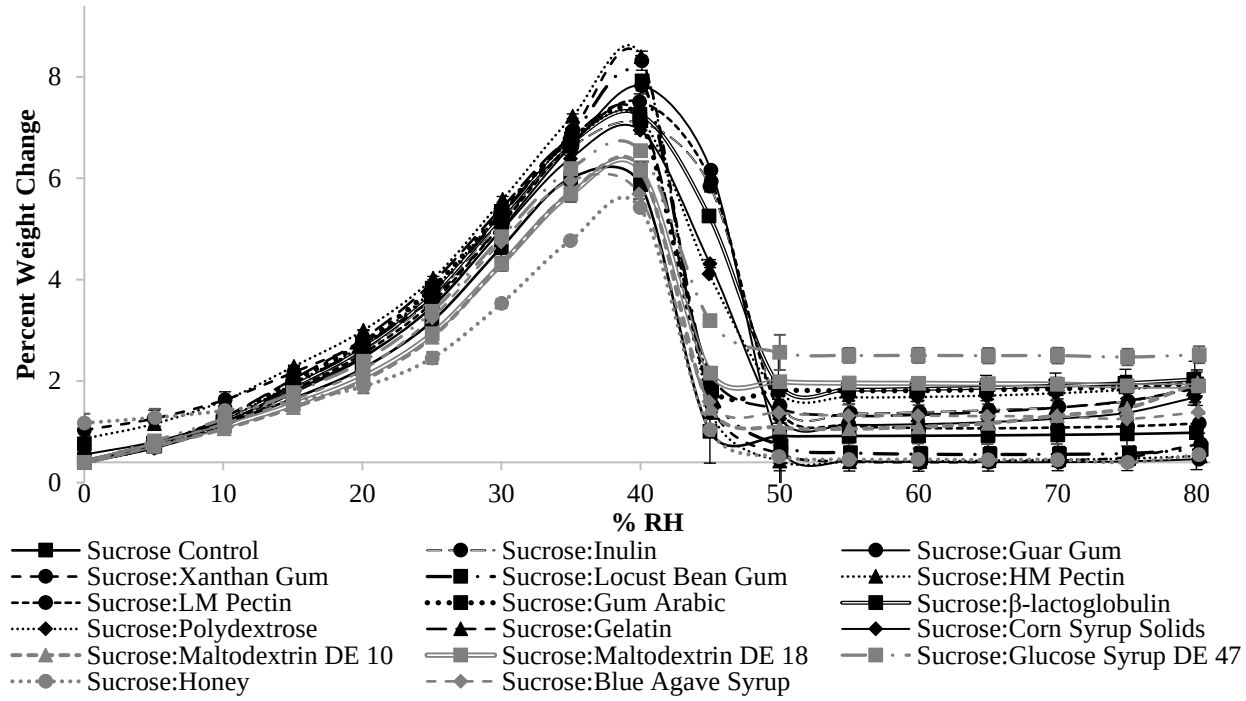


Figure 7.1 Increasing degree of crystallinity of select sucrose lyophiles over time using A) powder x-ray diffraction, where boxed in peaks are the well-defined crystalline sucrose peaks (Leinen & Labuza, 2006), B) FTIR spectra, where crystallinity was evaluated by the characteristic absorption peaks of crystalline sucrose in the region of 2800-3800 cm^{-1} wavenumbers (Lescure, 1995; Mathlouthi, 1995), and C) PLM images, where birefringence indicates crystallinity.

A)



B)

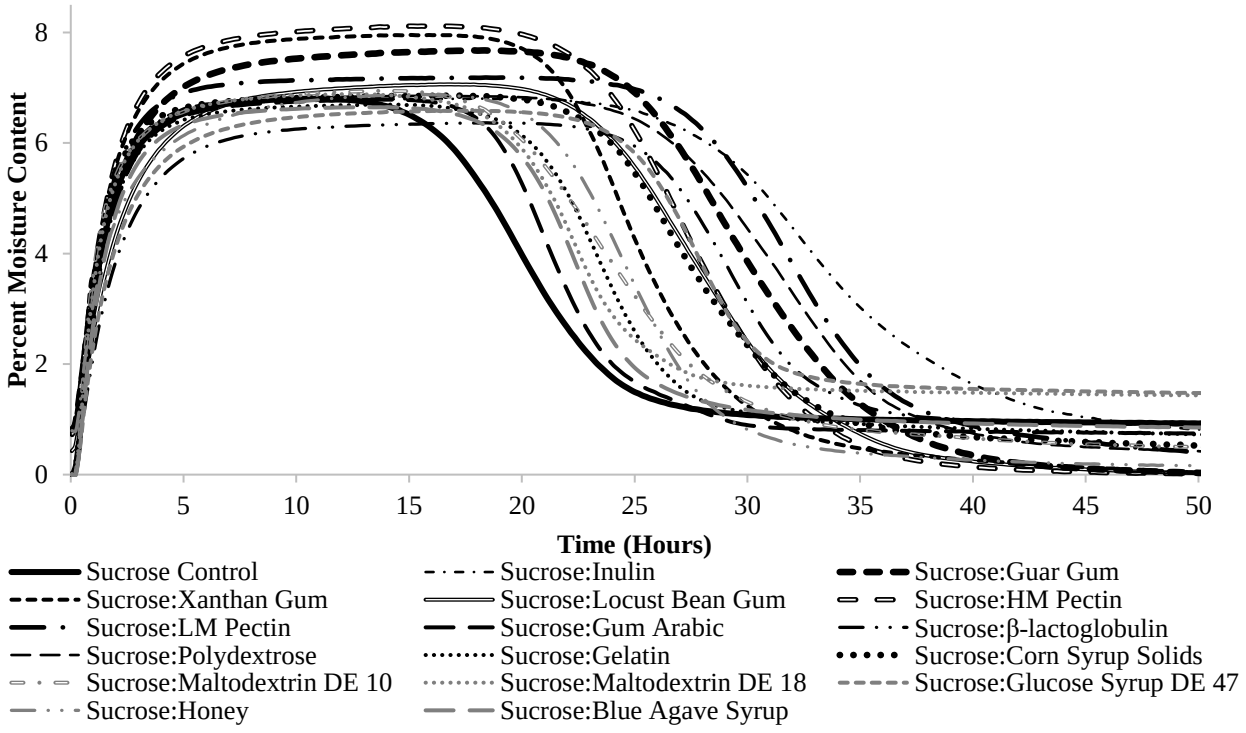
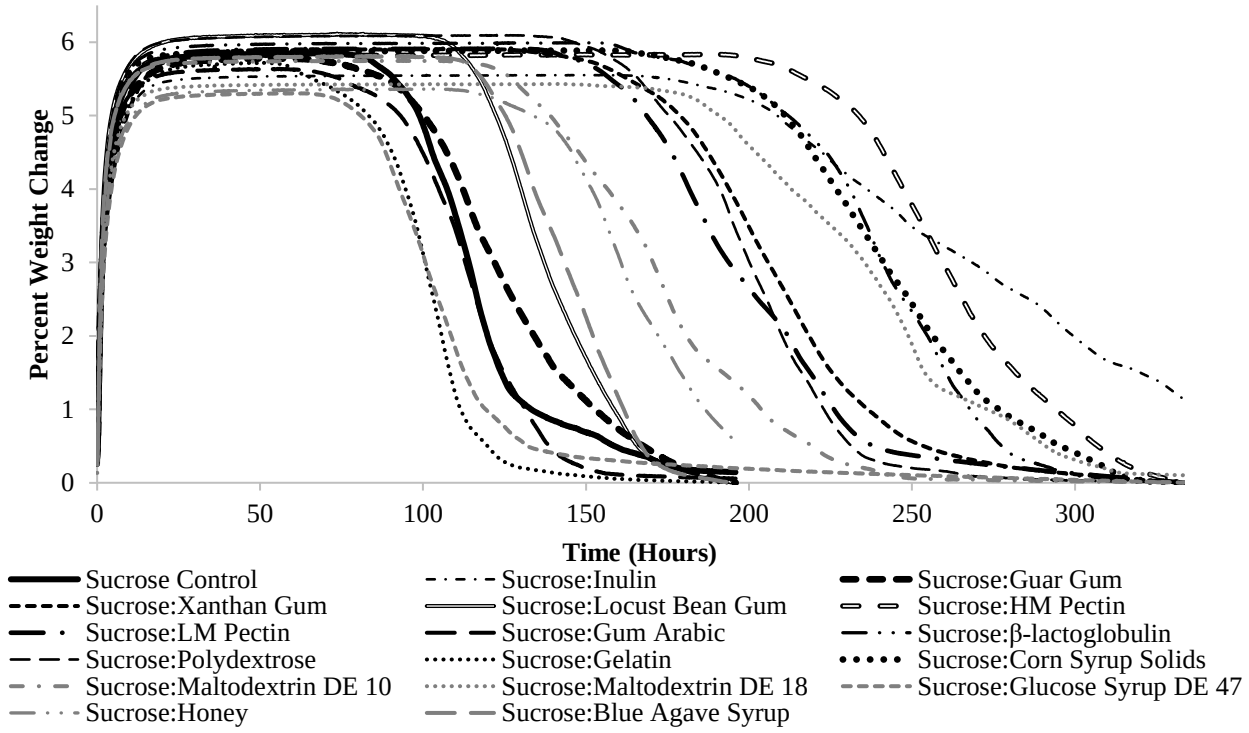


Figure 7.2 Gravimetric moisture sorption profiles of sucrose lyophiles A) from 0-80% RH, B) held at 40% RH, and C) held at 33% RH.

Figure 7.2 continued



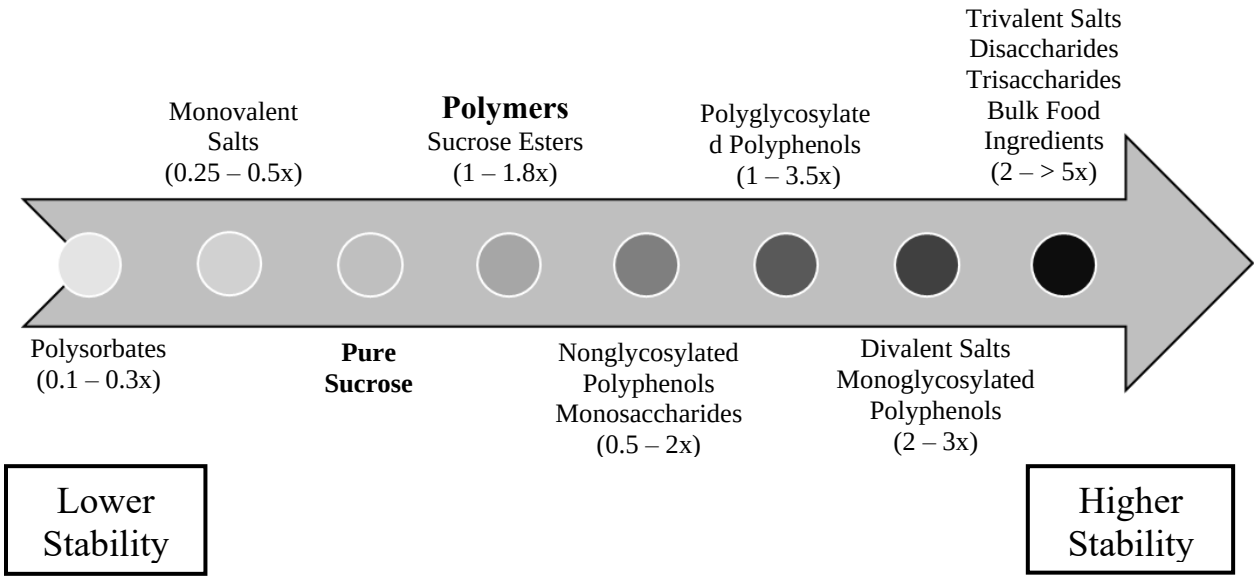


Figure 7.3 Physical stability of amorphous sucrose lyophiles prepared with various additives at similar weight proportions, adapted from Voelker, Felten, Taylor, and Mauer (2020) to include impact of polymers, in which polymer-containing lyophiles are only slightly more stable than pure sucrose at the concentration studied.

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CHAPTER 8. MOISTURE SORPTION BEHAVIORS, WATER ACTIVITY-TEMPERATURE RELATIONSHIPS, AND PHYSICAL STABILITY TRAITS OF SPICES, HERBS, AND SEASONING BLENDS CONTAINING CRYSTALLINE AND AMORPHOUS INGREDIENTS

Voelker, A. L., Sommer, A. A., & Mauer, L. J. (2020). Moisture sorption behaviors, water activity-temperature relationships, and physical stability traits of spices, herbs, and seasoning blends containing crystalline and amorphous ingredients. *Food Research International, 136*, 109608. doi:https://doi.org/10.1016/j.foodres.2020.109608

8.1 Abstract

Spices, herbs, and seasoning blends containing both crystalline and amorphous ingredients are common throughout the food industry but may exhibit unwanted clumping or caking during storage. Crystalline and amorphous ingredients are known to respond differently to increases in relative humidity (RH) and temperature. The aim of this study was to better characterize what happens to moisture sorption behaviors, water-solid interactions, and physical stability when crystalline and amorphous ingredients are co-formulated in seasoning blends. Spices, herbs, and seasoning blends, 25 in total, were studied individually and in blends of increasing complexity (binary, ternary, and quaternary) with sucrose, salt, and maltodextrin. The effects of increasing temperature and RH on moisture content, moisture sorption profiles, water activity (a_w), glass transition temperature (T_g), including Gordon-Taylor modeling, physical appearance, and degree of clumping were measured. Crossover points, the temperature at which the a_w of the amorphous ingredient(s) and the deliquescence RH of the crystalline ingredient(s) in a blend intersect, were also calculated. Caking was observed when storage conditions (RH and/or temperature) exceeded the T_g of a blend or the deliquescence RH of a crystalline ingredient in the blend. When amorphous and crystalline ingredients were blended, synergistic moisture sorption and increased caking was observed. When multiple crystalline ingredients were present, mutual deliquescence further increased the sensitivity of the blend to moisture. When environmental conditions exceeded the crossover temperature, degree of caking increased, and physical appearance was altered due to the induced deliquescence of the crystalline ingredient(s) by the a_w of the amorphous ingredient(s). In general, as complexity of blends increased, sensitivity to moisture also increased, and physical

stability of the blends decreased. The results of this study provide valuable information for increasing the physical stability of complex seasoning blends based on moisture sorption behaviors.

8.2 Introduction

Spices and herbs have played a prominent role in society, ranging from ancient civilizations to a wide variety of present-day industries. Spices have been used for their medicinal properties, such as anti-inflammatory, stimulant, digestive, and stress relief benefits; for the perfume and cosmetic industries; and in the food industry (Peter & Shylaja, 2012). Spices and herbs are used in the food industry for their natural colors, aromas, and flavors as well as for antimicrobial and antioxidant properties. Herbs are defined as the dried leaves of aromatic plants, whereas spices are the dried form of all remaining parts of an aromatic plant (Peter & Shylaja, 2012). These different plant parts may interact with water differently. Seasoning blends have increased in popularity in recent years, which may contain both spices and herbs as well as crystalline and/or amorphous additional ingredients (e.g., salts or sugars and maltodextrins, respectively). Crystalline and amorphous solids are known to interact with water differently, varying in adsorption on the surface of the solid, capillary condensation, deliquescence, crystal hydrate formation, and absorption into the bulk of the matrix (Mauer & Taylor, 2010b; Zografi, 1988).

The quality of seasonings is related to microbial load, safety, and spoilage, as well as stability of flavor, aroma, color, and physical structure. Due to the hygroscopic nature of many seasonings, the effect of water (or humidity) on physical stability is a major area of concern (Zafar, Vivacqua, Calvert, Ghadiri, & Cleaver, 2017). The presence of water in low-moisture powdered food products and seasonings can cause stickiness, agglomeration, caking, clumping, crystallization of amorphous materials, and degradation of components, among more undesirable changes for both ingredient functionality and processing of powdered seasonings (Aguilera et al., 1995; Ahlneck & Zografi, 1990; Hartmann & Palzer, 2011; K. Kwok et al., 2010).

Caking between deliquescent crystalline particles begins when water condenses due to capillary condensation and forms liquid bridges between particles, followed by physical changes that proceed through agglomeration, compaction, and liquefaction as the crystals deliquesce at a relative humidity (RH) characteristic of the specific crystalline material, known as its deliquescence point (RH_0) (Aguilera et al., 1995; Lipasek et al., 2013). Mutual deliquescence occurs when multiple deliquescent ingredients are blended together (e.g,, salts and sugars), and the

deliquescence RH of the mixture ($RH_{0,mix}$) becomes lower than the RH_0 of any individual ingredient, thereby increasing the moisture sensitivity of the crystalline blend (Hiatt et al., 2008; Mauer & Taylor, 2010a; Salameh et al., 2006). This phenomenon can be predicted using the Ross equation, wherein $RH_{0,mix} = (RH_{0,1})*(RH_{0,2})*(RH_{0,3})$ (Ross, 1975). For example, when sucrose (RH_0 = 85% (Ghorab, Toth, et al., 2014)) and NaCl (RH_0 = 75% (Ghorab, Marrs, et al., 2014)) are in physical contact with one another, $RH_{0,mix} = 0.85*0.75 = 64\%$ RH, which is lower than the RH_0 of either individual ingredient.

Amorphous materials are also prone to caking and clumping but via a different mechanism. When amorphous materials are exposed to increasing RH, moisture is absorbed into the bulk of the matrix. Water plasticizes the amorphous solid and may lower the glass transition temperature (T_g) below the environmental temperature, which leads to a transformation from a glassy, solid-like state to a less viscous, rubbery, supercooled liquid state (Slade & Levine, 1988; Zografi, 1988). The increased molecular mobility of the supercooled liquid can lead to sintering, a process in which molecules move into the gap between two neighboring powder particles to close the gap and create a sinter bridge (Feeney & Fitzpatrick, 2011; Palzer, 2005). If materials are exposed to RH cycling, water from the liquid or sintered bridges between crystalline or amorphous materials, respectively, can evaporate as RH is lowered, leading to solid bridge formation and therefore hard cakes (Feeney & Fitzpatrick, 2011; Salameh & Taylor, 2006a).

When amorphous and crystalline materials are mixed together, as is the case in many seasoning blends, additional mechanisms of caking are possible. Blending crystalline and amorphous ingredients can promote synergistic moisture sorption, which may induce dissolution of a crystalline ingredient at RHs lower than its RH_0 and/or cause a lowering of both the RH_0 of the crystalline ingredient and the T_g of the amorphous ingredient (Ghorab, Marrs, et al., 2014; Ghorab, Toth, et al., 2014; Thorat, Marrs, et al., 2017). Closed (packaged) systems containing crystalline-amorphous ingredient blends that are exposed to increasing temperatures may exhibit an increase of the a_w of an amorphous solid and a decrease of the RH_0 of a crystalline solid even in the absence of increased amounts of water (Gorling, 1958; Greenspan, 1977; Thorat, Marrs, et al., 2017). In these closed systems, there can be a temperature at which the a_w and RH_0 intersect, called the "crossover point", above which the elevated a_w of the amorphous ingredient(s) induces the deliquescence of the crystalline ingredient(s) (Thorat, Marrs, et al., 2017) (Figure 8.1). Therefore, not only are amorphous-crystalline blends more sensitive to environmental moisture

than their individual ingredients, but they also may exhibit decreased physical stability in closed packaged systems.

Spices, herbs, and seasoning blends including both crystalline and amorphous ingredients are widely used in the food industry, with the current annual global trade valued at $3-3.5 billion USD (Peter & Shylaja, 2012). However, the differences in moisture sorption behaviors between the seasonings and blends, and relevance thereof to physical and other stability traits, are not well documented. Therefore, the objectives of this study were to document and better understand the effects of formulation and storage conditions on the moisture sorption behaviors, water-solid interactions, and physical stabilities of spices, herbs, and seasoning blends by: 1) monitoring RH-controlled moisture sorption, glass transition temperature, water activity, deliquescence, and caking/clumping of a wide range of spices, herbs, and seasoning blends; and 2) investigating the effects on physical stability of co-formulation of the spices, herbs, and seasoning blends with common crystalline and amorphous ingredients, using onion powder as an example.

8.3 Materials and Methods

8.3.1 Materials

Twenty-five seasoning powders and dried herb leaves were analyzed for moisture sorption properties in this study, obtained from seasoning suppliers in the Midwest and commercial sources (McCormick & Company (Baltimore, MD) and Penzeys Spices (Wauwatosa, WI)): allspice, basil powder, black pepper, cayenne, chili powder, cinnamon, clove, coriander, cumin, garlic, ginger, mace, mustard, nutmeg, onion, oregano powder, paprika, rosemary powder, thyme, turmeric, basil leaves, oregano leaves, parsley leaves, curry powder, and garam masala. Seasoning blends containing additional crystalline and/or amorphous ingredients were prepared using sucrose (Mallinckrodt Chemicals, Phillipsburg, NJ), NaCl (Sigma-Aldrich, St. Louis, MO), and Glucidex maltodextrin DE 40 (Roquette, Lestrum, France).

RH-controlled desiccators were maintained using the following saturated salt solutions: potassium acetate, magnesium chloride, potassium carbonate, sodium bromide, potassium iodide, sodium chloride, and potassium chloride obtained from Fisher Scientific (Fair Lawn, NJ). The RHs created by these salt solutions at different storage temperatures are reported in Table 8.1. Phosphorus pentoxide (P_2O_5) was used to maintain a RH of ~0% (Fisher Scientific). For

volumetric one-component Karl Fischer titrations, the reagents HYDRANAL-Composite 2 (titrant), HYDRANAL-Methanol Rapid (working medium), and HYDRANAL-Water Standard 10 were purchased from Sigma-Aldrich (St. Louis, MO).

8.3.2 Storage treatments

For some studies, samples were analyzed 'as is'. However, prior to other studies, samples were stored in controlled RH and temperature environments. For these, approximately 1 g of an individual seasoning or 1:1 (*w/w*) physical blends of the seasoning and sucrose, NaCl, or maltodextrin were weighed into pans and stored in desiccators containing P_2O_5 (~0% RH) or saturated salt solutions (as shown in Table 8.1). The desiccators were stored at 20°C, 25°C, 30°C, 40°C, and 50°C using temperature-controlled rooms and water-jacketed incubators until the samples had reached equilibrium.

8.3.3 Moisture content analysis

The moisture contents of seasonings were determined using three methods: a one-component volumetric Karl Fischer titration (V20S Volumetric KF Titrator, Mettler-Toledo, LLC, Columbus, OH), vacuum oven drying, and co-distillation with toluene, which is the official AOAC method for moisture in spices (AOAC 986.21). Moisture content analyses are notoriously difficult assays from which to derive accurate results, with different methods often giving very different results (Mauer & Bradley, 2017). These three methods were compared to identify and discuss the most accurate, precise, and efficient method for determining moisture content in the context of this study. For the Karl Fischer titration, approximately 50 mg of seasoning was added to HYDRANAL-Methanol Rapid working medium to extract water. The sample was titrated using HYDRANAL-Composite 2 titrant to measure the moisture content in % moisture wet basis (*wb*). The system was calibrated using a HYDRANAL-Water Standard 10.0 (10 mg/g = 1% water content) prior to each day of analysis. The vacuum oven method, adapted from AOAC method 979.12, consisted of heating 1-2 g of seasoning in aluminum pans and drying under vacuum in a vacuum/vent valve Across International vacuum oven (Livingston, NJ) at 70°C for 6 h, at which point moisture content was calculated using weight loss on drying. Preliminary experiments determined that approximately constant weight had been reached at this point, indicating drying

was complete. Co-distillation with toluene was used according to AOAC method 986.21. Comparison of the three methods was completed for determining the initial moisture contents of 8 seasonings, in triplicate.

Subsequent moisture content analyses were also completed to determine the effects of controlled-RH storage in desiccators on moisture contents of samples, using onion powder as an example. The initial moisture content was measured by Karl Fischer titration. Samples were then stored in desiccators for 3 weeks. Based on results from the comparisons of moisture content analysis methods, at the end of storage, two approaches were used to determine the final moisture contents of onion powder: Karl Fischer titration and a gravimetric technique using the initial moisture content of seasonings determined by Karl Fischer. All moisture contents were measured in at least duplicate.

8.3.4 Moisture sorption analysis

Moisture sorption profiles of all seasonings were collected at 25ºC using a SPSx-1μ Dynamic Vapor Sorption Analyzer (Projekt Messtechnik, Ulm, Germany). Seasonings, sucrose, NaCl, and maltodextrin, as well as binary, ternary, and quaternary blends of the ingredients, were prepared for moisture sorption analysis. Approximately 1 g of each sample was placed in a pan that was then placed in a 23-ring sample holder in the instrument and equilibrated at 0% RH for 12 h in the instrument. Samples were then analyzed from 0-95% RH in 5% RH increments, with an end-point criterion of < 0.01% weight change within 30 min and a maximum residence time of 6 h. The moisture sorption profile (moisture gain vs. RH) of each sample was plotted using the percent change in mass at the end of each RH step as the equilibration moisture gain at that RH.

A second approach was also used to monitor moisture sorption of samples that were stored in static RH desiccators (with RH controlled by saturated solutions of potassium carbonate, sodium bromide, and NaCl) in controlled temperature (20, 30, 40, 50ºC) environments The RHs of these salt solutions at these temperatures are reported in Table 8.1. Gravimetric weight change over a 3-week period was documented.

8.3.5 Water activity measurements

Six powdered seasonings (clove, garlic, ginger, nutmeg, onion, and rosemary) were placed in glass jars with minimal headspace and equilibrated at 20, 25, 30, 35, 40, 45 and 50ºC in incubators overnight. RH was not controlled in the glass jars. The a_ws of the temperature equilibrated samples were measured using both an AquaLab 4TE water activity meter (chilled mirror dewpoint) and an Aqualab TDL water activity meter (tunable diode laser sensor to allow measurement of volatile materials) (METER Group, Inc., Pullman, WA), both set to the same temperature at which the samples had been equilibrated. Samples were measured on each instrument in triplicate to compare the two methods of measurement. Due to results from the 4TE and TDL comparison and ease of use, the additional seasonings were similarly equilibrated at increasing temperatures, and their water activities were measured in duplicate using the Aqualab 4TE meter to determine effect of temperature on a_w.

Four powdered seasonings (allspice, black pepper, coriander, and cumin), selected due to their differences in starting moisture contents, a_ws, and response of a_w to temperature increases, were equilibrated in RH-controlled desiccators (with RH controlled by saturated solutions of potassium acetate, potassium carbonate, potassium iodide, and NaCl, as reported in Table 8.1) at increasing temperatures (20, 25, 30, 35, 40, 45, 50ºC) for 28 days to allow for complete equilibration, after which time the a_ws at each temperature were measured using the AquaLab 4TE. The measured a_ws were then plotted with RH_0s of fructose, sucrose, NaCl, and blends calculated by the Ross equation, including fructose:NaCl, sucrose:NaCl, fructose:sucrose, and fructose:sucrose:NaCl (Lipasek et al., 2013; Ross, 1975) to determine the "crossover points" of blends of amorphous spices and crystalline ingredients.

8.3.6 Glass transition temperature and Gordon-Taylor modeling

Onset T_gs of all seasonings and maltodextrin were measured by differential scanning calorimetry (DSC) using a DSC 4000 (PerkinElmer, Waltham, MA). The instrument was calibrated with indium and verified with the melting point of water. Dry nitrogen was used to purge the system at 20 mL/min. To determine the 'dry T_g' of seasonings, samples were weighed (5-10 mg) into 50 µL aluminum DSC pans (PerkinElmer), hermetically sealed, and punctured to create a pinhole to allow water vapor to escape. Due to the high moisture sorption trait of the onion

powder and the notoriousness of its caking in commercial or home use (Debnath, Hemavathy, & Bhat, 2002; Peleg & Mannheim, 1977), further studies were completed using onion as an example to better understand seasoning interactions with added crystalline and amorphous ingredients and resultant effects on moisture sorption. Gordon-Taylor modeling of onion powder was also completed to study the effect of increasing storage RH on T_g, which can be an indicator of physical stability. For Gordon-Taylor modeling, onion powders that had been pre-equilibrated at increasing RHs (0, 23, 33, 43, 58, 69, 75, and 84% RH) at 25ºC were analyzed for onset T_g without a pinhole to measure T_g at varying moisture contents. A heat-cool-heat protocol was used to determine onset T_g, which was defined as the temperature in which an endothermic event characterized by a baseline shift began in the second scan. Samples were equilibrated at a temperature at least 20ºC below the expected T_g, then heated at a rate of 20ºC/min to approximately 20ºC above the expected T_g to erase thermal history of the sample. Samples were then cooled to the initial temperature at a rate of 50ºC/min and held for 3-5 min to allow the temperature to equilibrate. A second scan heated samples at a rate of 20ºC/min to approximately 20ºC above the expected T_g. Temperature ranges for each sample were dependent on moisture content. All T_gs were measured in duplicate.

The Gordon-Taylor equation (Gordon & Taylor, 1953) was used to model the onset T_g vs. moisture content relationship of onion powder using Matlab (Mathworks, Natick, MA):

$$T_{g(mix)} = \frac{(w_1 T_{g1}) + (k w_2 T_{g2})}{w_1 + (k w_2)} \quad (1)$$

where w_1 is the weight fraction of water, w_2 is the weight fraction of solid, T_{g1} is the T_g of water (136 K), T_{g2} is the 'dry T_g' of onion powder in Kelvin, and k is a fitting constant. The Gordon-Taylor equation is used to describe the T_g of an amorphous blend as a function of weight fraction of all amorphous ingredients, where the fitting constant k is considered a ratio of the free volumes of the two components (Hancock & Zografi, 1994).

8.3.7 Sample photography and physical assessment

Nine seasonings (allspice, basil powder, black pepper, coriander, cumin, ginger, onion, oregano powder, and turmeric) were geometrically blended with sucrose, NaCl, or maltodextrin DE 40 in a 1:1 *w/w* ratio. Blended and individual seasonings were stored in RH-controlled desiccators (with RH controlled by saturated solutions of potassium carbonate, sodium bromide, and NaCl) at increasing temperature (20, 30, 40, 50ºC) conditions. Samples were photographed in

an Elviros light box and analyzed qualitatively for degree of clumping over a 3-week period. Degree of clumping was determined using a 1-5 ranked scale adapted from Grant and Bell (2012). Samples that moved freely with no visible clumping were considered "free flowing" and ranked '1'. Samples that contained some clumps but still some free flowing powder were considered "partially caked" and ranked '2'. Samples that had no free flowing powder but rather hard cakes that were fully attached to the sample cup were considered "fully caked" and ranked '3'. Samples that had both solid and liquid components were considered a "slurry" and ranked '4'. Completely liquefied samples were considered "liquid" and ranked '5'. Numerical rankings were recorded incrementally over time for up to 21 days.

To visualize the effect of blend complexity on moisture sorption behaviors, polarized light microscope (Omano, China) time lapse videos of onion powder, NaCl, and sucrose in a RH-controlled microscope stage (GenRH, Allentown, PA) were taken using an iPhone 6s camera attached to the microscope eyepiece by an iDu LabCam adapter (Detroit, MI). The blend was observed over time at 70% RH and ambient temperature (22 ± 2ºC), an environmental condition below the RH_0 of either individual crystalline ingredient (RH_0 of sucrose: 85%, NaCl 75% RH).

8.3.8 Statistical analysis

Single-variable ANOVA using SAS 9.4 (SAS Institute, Cary, NC) was used to determine significant differences between moisture content measurements, water activity measurements, and T_gs. Differences were determined using Tukey's post hoc test for multiple comparisons at a significance level of $\alpha = 0.05$.

8.4 Results and Discussion

8.4.1 Moisture content determination

A comparison of the initial moisture contents of eight spices determined by three methods (Karl Fischer titration, toluene distillation, and vacuum oven drying) is provided in Figure 8.2.A. Differences in initial moisture content were found between the different spices (ranging between ~5% to 9% *wb*), with allspice and black pepper generally having higher initial moisture contents than the other spices. Some significant differences were found between the different moisture determination techniques for five of the eight spices (allspice, cayenne, chili powder, coriander,

and onion powder). For three of these spices (allspice, coriander, and onion powder), the vacuum oven drying resulted in the lowest determined moisture content, by ~1-3%, which could indicate incomplete moisture loss in the oven technique. For the other two spices (cayenne and chili powder), no differences were found between the moisture contents determined by Karl Fisher titration and vacuum oven drying, but the distillation with toluene resulted in the highest moisture content determination for both spices. Loss of volatiles (aromatic compounds in seasonings) and heat-induced changes in samples often result in over-estimation of moisture content in vacuum oven analyses (Mauer & Bradley, 2017). However, in no cases were results from the vacuum oven drying greater than those from the other techniques, potentially indicating that loss of volatiles and/or heat-induced changes in the samples did not significantly affect weight loss during vacuum heating. The official AOAC method for moisture content determination of spices is co-distillation with toluene (AOAC 986.21), for which the water is volumetrically measured; however, the method is often not adaptable to routine testing and is prone to human error in reading the volume of water in a receiving tube (Mauer & Bradley, 2017). Given the convenience and lower sample size necessary for Karl Fischer analyses compared to toluene distillations and the statistically similar results between the Karl Fisher analysis and toluene distillation for the majority of spices analyzed, Karl Fischer analyses were used for the remainder of the study to determine sample moisture contents.

Following 3 weeks of RH-controlled storage in desiccators, it was shown that increasing the storage RH increased the moisture content of the onion powder samples, as expected, from < 5% *wb* at 0% RH to > 25% *wb* at 85% RH (Figure 8.2.B). Samples stored in 0% RH desiccators resulted in decreased in moisture content compared to the initial value (~6% *wb*), while storage at 23% and 33% RH resulted in approximately unchanged moisture contents (6-7% *wb*), presumably due to relative proximity of these RHs to that of the environment. Samples stored in desiccators with RHs > 33% RH resulted in increased moisture content. The moisture contents determined gravimetrically and by Karl Fisher analysis were generally similar, being within 0.7% of each other, although some statistically significant differences were found at the intermediate storage RHs (Figure 8.2.B). Following exposure to the highest RHs, 75% and 85% RH, moisture content determination by Karl Fischer had a much higher standard deviation than gravimetric measurements, presumably due to the small sample size measured by Karl Fischer (Tainter & Grenis, 2001).

8.4.2 Moisture sorption behaviors

Dynamic moisture sorption profiles at 25ºC were measured for all 25 seasonings (Figure 8.3.A). Differences in moisture sorption patterns were found between the different seasonings, varying by ~40% weight change at the highest RHs. Considering the percent weight gain following the 95% RH equilibration step as a point of comparison, the seasonings were divided into categories of low, medium, and high moisture sorption. In this categorization, low was defined as < 25% weight gain, medium was defined as between 25 and 40% weight gain, and high was defined as > 40% weight gain. Eleven seasonings (allspice, black pepper, cinnamon, clove, coriander, curry powder, garam masala, mace, mustard, nutmeg, and thyme), more spices than herbs, were considered low moisture sorption seasonings (Figure 8.3.A.i). Nine seasonings (cayenne, cumin, garlic, ginger, oregano leaves, oregano powder, paprika, rosemary powder, and turmeric) were considered medium moisture sorption seasonings (Figure 8.3.A.ii). And five seasonings (basil leaves, basil powder, chili powder, onion, and parsley) were considered high moisture sorption seasonings (Figure 8.3.A.iii), with parsley sorbing the most moisture at 91% RH (51% *w/w*). Parsley, and other leafy herbs, have been used as flavor carrier vehicles in foods, for which the high moisture sorption tendency could be problematic.

Particle density, morphology, and composition may be contributing factors to these differences in moisture sorption patterns between the seasonings. For example, using the USDA FoodData Central nutrient database (U.S. Department of Agriculture, 2019), a slight positive correlation was found between moisture sorption and percent nutrient content for sodium (R^2 = 0.115) and sugar (R^2 = 0.1606), which may be due to the deliquescence points of NaCl and sucrose (75% and 85% RH, respectively). A more considerable positive correlation was found between moisture sorption and protein content (R^2 = 0.5505), presumably due to the water-holding capacity of proteins. On the other hand, a slight negative correlation was found between moisture sorption and lipid content (R^2 = 0.1962), likely due to the hydrophobic nature of lipids. More than just nutrient composition influences the moisture sorption profiles of the spices and herbs. For example, basil leaves and basil powder have the same composition, but basil leaves gained 47% moisture (*w/w*) at 95% RH, while basil powder gained only 41% moisture (*w/w*). Similarly, oregano leaves gained 37% moisture (*w/w*) at 95% RH, while oregano powder gained only 34% moisture (*w/w*). This indicates that particle density and morphology also play a role in moisture sorption behaviors,

with the less dense and larger herb leaves, which may have had a more porous structure, sorbing more moisture than the powders.

A dynamic moisture sorption study documented the effects of co-formulating onion powder in binary, ternary, and quaternary blends with sucrose, NaCl, and/or maltodextrin (Figure 8.3.B). The moisture sorption profiles of sucrose, NaCl, and this maltodextrin have been published previously (Ghorab, Marrs, et al., 2014; Ghorab, Toth, et al., 2014; Thorat, Marrs, et al., 2017), documenting the RH_0 of sucrose at 85% RH at 25ºC and the RH_0 of NaCl at 75% RH at 25ºC. The amorphous maltodextrin exhibits a type II moisture sorption profile, sorbing more water at lower RHs but less water than the crystalline ingredients at RHs exceeding their RH_0s (Ghorab, Marrs, et al., 2014; Ghorab, Toth, et al., 2014; Thorat, Marrs, et al., 2017). Blends containing NaCl gained significantly more weight than the other samples at RHs above 70% RH. Although it would be expected that significant weight gain would begin occurring above the RH_0 of salt (as seen in the enhanced moisture sorption of salt-containing samples at RHs $\geq$ 75% RH in Figure 8.3.B), an inflection in the moisture sorption profiles of the blends containing NaCl occurred between 65 and 70% RH (Figure 8.3.B.i), thus lower than the RH_0 of salt. Physically mixing amorphous and crystalline ingredients is known to induce synergistic moisture sorption below the RH_0 with the potential to also lower the RH_0 (Ghorab, Marrs, et al., 2014; Ghorab, Toth, et al., 2014; Hiatt, Taylor, & Mauer, 2011; Thorat, Marrs, et al., 2017). This was also seen in the onion:sucrose:maltodextrin blend, which had an increased rate of moisture sorption also between 65 and 70% RH, well below the RH_0 of sucrose (85% RH at that temperature) (Ghorab, Toth, et al., 2014). The binary, ternary, and quaternary blends were prepared for all 25 seasonings in this study. All seasonings were found to exhibit increased moisture sorption with increasing complexity of the blends, with an inflection point between 65 and 70% RH for blends containing salt (similar to Figure 8.3.B.i).

The effects of temperature on the moisture sorption of onion powder and binary blends of onion powder with sucrose, salt, or maltodextrin were monitored over time in desiccators at multiple RHs and temperatures (Figure 8.4). As expected, increasing RH increased moisture sorption in all blends. Increasing temperature led to an increase in moisture sorption most notably at 75% RH in binary blends with sucrose or NaCl. Onion:NaCl samples sorbed significantly more moisture at 75% RH than other blends, attributed to the lower RH_0 of NaCl compared to sucrose (75% and 85% RH, respectively). In onion powder alone and the binary blend of onion powder

with the amorphous maltodextrin, the majority of moisture sorption occurred within the first 4 days of storage, with a leveling off in mass following day 4 (Figure 8.4.A and 8.4.D). The onion powder sorbed a maximum of ~20% water at 75% RH, with little difference between the storage temperatures (Figure 8.4.A). Combining onion powder with maltodextrin decreased the total moisture sorption of the blend to ~15% water at 75% RH, again with little difference between the different storage temperatures. The binary blends of onion powder with the crystalline ingredients behaved differently (Figure 8.4.B and 8.4.C). These onion:crystal blends continued to sorb water over time at the highest storage RH and exhibited differences in moisture sorption at the different temperatures, with the largest differences occurring in the onion:salt blends which sorbed ~140% water at 75% RH and 50ºC over the 3-week storage period. Thus, it can be concluded that both formulation and storage conditions influence the moisture sorption patterns of seasoning blends.

8.4.3 Effect of seasoning volatiles on water activity measurements

While a common approach for measuring a_w uses a chilled mirror dewpoint method, such as that housed in the Aqualab 4TE instrument, volatiles, such as those found in spices, can often disrupt this measurement (Peter & Shylaja, 2012). A chilled mirror dewpoint determines the a_w of a sample by measuring the temperature of the sample and the temperature of the mirror when condensation from the headspace occurs on the chilled mirror. However, volatiles may condense on the mirror, interfering with this measurement (Mauer & Bradley, 2017). An alternate a_w measurement technique using a tunable diode laser, such as that housed in the Aqualab TDL instrument, may be used to measure a_w without the interference of volatiles (Allan & Mauer, 2017a). A comparative study to document the effects of temperature on the initial 'as is' a_ws of six seasonings (clove, garlic, ginger, nutmeg, onion, and rosemary) measured at temperatures ranging from 20 to 50ºC was conducted to determine if differences would be found between the measurement techniques. Significant differences were found between the a_ws of different types of seasonings; however, no significant differences ($p < 0.05$) in a_w were found between the two a_w measurements for any spice at any temperature. The a_ws of the seasonings at 25ºC were as follows: ginger (0.69) > clove (0.54) > nutmeg (0.53) > rosemary (0.47) > garlic (0.31) > onion (0.29). Increasing temperature was found to affect a_ws; however, both instruments measured the same response. Due to ease of calibration and use, the Aqualab 4TE was used for all subsequent a_w measurements.

8.4.4 Effect of temperature, water activity, and deliquescence on seasoning blend stability

Increasing the temperature of amorphous solids in a closed system is often known to increase the a_w of the amorphous system (Gorling, 1958). To determine whether or not the seasonings followed this general trend, the effects of increasing temperature on the a_ws of the 25 seasonings were documented (Figure 8.5). Ten of the seasonings exhibited increasing a_ws with increasing temperature (Figure 8.5.A). Of these ten seasonings, coriander exhibited the largest increase in a_w with temperature, significantly ($p < 0.05$) increasing from 0.44 to 0.52 a_w as temperature increased from 20 to 50ºC. R^2 values of the trendlines with positive slopes suggested high correlation in most cases, with all seasonings excluding ginger significantly ($p < 0.05$) increasing in a_w as temperature increased. Nine seasonings had a_w vs. temperature trendline slopes that were near to zero (Figure 8.5.B). Five seasonings exhibited negative slopes (Figure 8.5.C), with a_w significantly ($p < 0.05$) decreasing as temperature increased; however, the very low R^2 values of the trendlines with negative slopes suggested limited correlation. Seasonings with negative or close to zero trendline slopes for the a_w-temperature relationship tended to have higher sugar contents than those with positive slopes. The deliquescence points of sugars decrease as temperature increases (Lipasek et al., 2013), which was likely a contributing factor for the a_w-temperature relationship for seasonings with high sugar concentrations.

Going beyond the initial 'as is' a_ws of the seasonings and to further analyze the effect of temperature on their physical stability, four seasonings (allspice, black pepper, coriander, and cumin), selected because they had different starting moisture contents, a_ws, and response of a_w to temperature increases, were pre-equilibrated in controlled RH and temperature environments, and then the effects of temperature on their a_ws were determined (Figure 8.6). The initial a_ws of these samples were consistent with the RHs at which they had been stored. Trendline slopes for the a_w-temperature relationship in this experiment were positive for all seasonings and RHs studied. This is consistent with the initial 'as is' findings for allspice and black pepper; however, coriander and cumin had close to zero 'as is' trendline slopes. This could be taken to indicate that starting moisture contents, and thus a_ws, alter the a_w-temperature relationship for some seasonings.

In addition to the increase of a_w with increasing temperature for many amorphous solids and some seasonings, RH_0 is known to decrease with increasing temperature due to increased solubility (Greenspan, 1977; Lipasek et al., 2013). When the temperature-dependent a_w plot of an amorphous ingredient is superimposed with the temperature-dependent RH_0 plot of a crystalline

ingredient, a "crossover point" between the two plots can indicate the temperature above which deliquescence of the crystalline ingredient will occur due to the a_w of the amorphous ingredient in an amorphous-crystalline blend (shaded area in Figure 8.1) (Thorat, Marrs, et al., 2017). The a_ws of the four seasonings were plotted with the RH_0s of sucrose, fructose, and NaCl, and the $RH_{0,mix}$s calculated using the Ross equation for fructose:NaCl, sucrose:NaCl, fructose:sucrose, and fructose:sucrose:NaCl to determine crossover points (Tables 8.2, 8.3). Due to mutual deliquescence lowering, i.e. the lowered $RH_{0,mix}$ compared to the RH_0s of the individual crystalline ingredients, it can be shown that a blend of multiple crystalline ingredients with the amorphous ingredient will result in a lower crossover point and therefore lower physical stability than less complex blends (Figure 8.1).

In the case of binary blends of a seasoning (allspice, black pepper, coriander, or cumin) with an individual crystalline ingredient (fructose, NaCl, or sucrose), the crossover point occurred when the a_w (or RH) of the seasoning exceeded the RH_0 of the crystalline component at a given temperature. Because the RH_0 of fructose is 63% RH at 25ºC (Lipasek et al., 2013), seasonings stored in desiccators at RHs exceeding the RH_0 (e.g., the 68 and 75% RH desiccators) were already above the crossover point. The crossover at 25ºC would have occurred at higher RHs for NaCl and sucrose, which have RH_0s of 75% RH and 85% RH, respectively, at this temperature (Ghorab, Marrs, et al., 2014; Ghorab, Toth, et al., 2014). Two situations create greater potential problems by lowering the temperature of the crossover point: 1) when multiple deliquescent crystalline ingredients are present, which lowers the $RH_{0,mix}$, and 2) when increasing temperature increases the a_w of the amorphous ingredient and/or decreases the RH_0 of the crystalline component in a closed system (Table 8.2, Figures 8.1, 8.6). When looking at seasonings blended with two or three crystalline ingredients (Table 8.3), all spices stored at 43, 70, and 75% RH (initial RHs at 20ºC) had crossover points with fructose:NaCl, fructose:sucrose, and fructose:sucrose:NaCl below 50ºC. All spices stored at 70 and 75% RH blended with sucrose:NaCl had crossover points below 50ºC. Additionally, increasing the number of crystalline ingredients in the blend decreased the crossover temperatures, due to mutual deliquescence causing the lowered $RH_{0,mix}$ of the blend compared to the RH_0s of the individual crystalline ingredients. For example, while the crossover point of coriander stored at 70% RH blended with sucrose was 101ºC, the crossover points when blended with sucrose:NaCl or fructose:sucrose:NaCl were 22ºC and -24ºC, respectively. While the crossover point of cumin stored at 43% RH blended with sucrose was 224ºC, the crossover points

when blended with sucrose:NaCl or fructose:sucrose:NaCl were 126ºC and 23ºC, respectively (Table 8.3). From the calculated crossover points, it was shown that as number of ingredients in a blend was increased, physical stability of the blend was decreased (Table 8.3).

When the crossover point drops below the storage temperature of a seasoning blend, undesirable physical changes are likely to occur, including clumping and caking; thus, it is desirable for blends to have crossover temperatures well above the storage temperature. Theoretically, calculated crossover points could be used to set maximum initial a_w or moisture content guidelines in the food industry to avoid potential for crossover in closed packaged products that may be exposed to temperature fluctuations during transportation or storage. Additionally, some thermal analyses, such as DSC, may also benefit from higher crossover temperatures (Thorat, Marrs, et al., 2017). Most T_g measurements using DSC employ a heat-cool-heat protocol as described in section 2.6. Specifically, amorphous:crystalline blends may be exposed to elevated temperatures in a closed system that could exceed the crossover temperature. In these cases, the crystal may deliquesce, and when the sample is rapidly cooled in the DSC, the initially crystalline component may solidify in the amorphous state, leading to a lower T_g in the second heating scan (Thorat, Marrs, et al., 2017). Thus, caution should be used in analyzing the data from thermal analyses that may exceed crossover temperatures.

8.4.5 Glass transition and Gordon-Taylor model of onion powder

The T_g of an amorphous material relative to moisture content and environmental conditions can dictate physical stability due to the transition from the glassy state, when the environmental temperature is below the T_g, to the supercooled liquid state, when the environmental temperature is above the T_g (Zografi, 1988). The 'dry' T_gs of seasonings identified using the DSC technique ranged from 63 to 138ºC (Table 8.4). The T_gs of multiple seasonings, including herb leaves and powders, were unable to be clearly identified using the DSC experimental approach, possibly attributed to heterogeneity or complex composition of the systems (Bell & Touma, 1996); however, the free flowing powder trait of all seasonings could be taken to indicate all dry and initial T_gs were above ambient temperature. When amorphous materials are in the supercooled liquid state, increased mobility can cause sintering, which can lead to caking of the powder (Hartmann & Palzer, 2011; Palzer, 2005). It is well-known that as moisture content is increased, T_g is decreased due to the low T_g of water (-137ºC), which can be modelled by the Gordon-Taylor equation (Gordon &

Taylor, 1953; Slade & Levine, 1991). Thus, the more effective measure of physical stability was the change in T_g as storage RH was increased, resulting in moisture content increase.

Using onion powder as an example, the decrease in T_g with the increase in storage RH was measured (Figure 8.3.B), and the data were fitted to the Gordon-Taylor model with a k value of 0.19 (Figure 8.7). According to the model, the T_g decreased to below 25ºC when the moisture content exceeded 10% (*wb*), which occurred following storage at 58% RH. Because environmental RH conditions often exceed 58% RH during food production, transportation, and storage, prolonged exposure of onion powder to this RH in open containers may cause caking and clumping as moisture sorption induces a lowering of T_g to below common ambient temperatures (as observed in Figure 8.8). Additionally, it is of interest to note that the T_g of amorphous ingredients has the potential to decrease in physical blends with crystalline ingredients (Ghorab, Marrs, et al., 2014; Ghorab, Toth, et al., 2014; Thorat, Marrs, et al., 2017). Thus, the physical stability of seasoning blends may be increasingly sensitive to moisture sorption.

8.4.6 Humidity- and temperature-induced physical responses of onion powder blends

Physical stability of onion powder and its binary blends with sucrose, NaCl, or maltodextrin was monitored visually and using a scaled caking test (Figure 8.8). As can be seen by the photographs and the caking rank in Figure 8.8, as RH and temperature increased, caking of onion powdered also increased. Interestingly, there were only minor changes in degree of caking from day 3 to day 11, indicating that most caking occurred quickly. It was hypothesized that caking of pure onion powder, which was shown by powder x-ray diffraction to be completely amorphous, would result from the environmental temperature exceeding the T_g at a specified moisture content (Hartmann & Palzer, 2011). Additionally, as $T-T_g$ increased, the extent of caking was also expected to increase. Using the Gordon-Taylor model and moisture sorption isotherm of onion powder (Figure 8.7), it was predicted that the transition from glassy to supercooled liquid state, resulting in caking, would occur when the onion powder stored at 43% RH reached 50ºC, when that stored at 58% RH reached 30ºC, and at all temperatures at which the 75% RH samples were held. While visual darkening of the samples seemed to follow this trend, the ranked caking scale indicated fully caked samples at temperatures lower than predicted. This was presumably due to the heterogenous composition of pure onion powder, which contains lipids, proteins, and sugars, including sucrose, glucose, and fructose (U.S. Department of Agriculture, 2019).

As has been noted, increasing the complexity of seasoning blends alters moisture sorption, which has the potential to decrease physical stability. In all cases, darkening of the samples and caking rank increased as both temperature and RH were increased, with the 75% RH samples changing the most significantly in physical appearance and some differences noted between the different blends (Figure 8.8). When NaCl was blended with onion powder and stored at 75% RH (RH_0 of NaCl), sample darkening and caking rank increased compared to pure onion powder. When maltodextrin was present, the extent of color change was limited compared to other blends, presumably due to the high T_g of that maltodextrin (Ghorab, Marrs, et al., 2014). This storage and observation experiment was also completed with 8 other seasonings (allspice, basil powder, black pepper, coriander, cumin, ginger, oregano powder, and turmeric), and similar trends were found. In all cases, seasonings blended with crystals had more visual darkening and increase in caking than seasonings blended with maltodextrin or no additional ingredient, and seasonings blended with NaCl had the most caking and darkening, especially at 75% RH, presumably due to increased moisture sorption. Overall, blends had more caking and change in physical appearance than single seasonings.

To visualize the effects of storage at 70% RH and ambient temperature on the moisture sorption of a blend containing onion powder, NaCl, and sucrose, a time lapse video was taken of the sample stored in a RH controlled microscope stage and monitored by a polarized light microscope, in which one second of the video corresponds to approximately one hour in real time (Figure 8.9). While the reported RH_0s of sucrose and NaCl are 85% and 75% RH, respectively (Ghorab, Marrs, et al., 2014; Ghorab, Toth, et al., 2014), the time lapse video shows the mutual deliquescence of sucrose and NaCl at an RH below either of the individual RH_0s (70% RH) when the crystals were in contact with one another. The $RH_{0,mix}$ of sucrose and NaCl is ~64% RH at ambient temperature. This deliquescence, in turn, contributed to a physical change, including visible liquid bridge formation between the onion powder particles. Additionally, at 70% RH, onion powder had a moisture content of approximately 13% (*wb*), resulting in a T_g of 5ºC, according to the Gordon-Taylor model (Figure 8.7), and thus, the supercooled liquid state of onion powder. Since there are visible signs of plasticization of the onion powder even where it is not in contact with any crystals, the water absorbed by the onion powder may have also contributed to the dissolution of the crystals. Regardless, this video demonstrates the increased sensitivity of ingredient blends to environmental moisture.

8.5 Conclusion

Spices, herbs, and seasonings have a wide variation in moisture contents, moisture sorption behaviors, and a_ws. Blending multiple ingredients together altered moisture sorption behaviors. Synergistic moisture sorption occurred when an amorphous seasoning was blended with crystalline ingredients, and caking was observed when storage RH and temperature exceeded the T_g of a seasoning and/or RH_0 of a crystalline ingredient. An increase in sensitivity to moisture, including caking, was found when amorphous and crystalline ingredients were blended, with even greater sensitivity when multiple crystalline ingredients were present, attributed to mutual deliquescence, increased water uptake, and lowered T_g. When the environmental temperature exceeded the crossover temperature at which the a_w of an amorphous ingredient and the RH_0 of a crystalline ingredient intersect, deliquescence and caking occurred. In general, it was found that as seasoning blend complexity was increased, sensitivity to moisture increased and physical stability decreased. The quantitative moisture sorption isotherms, documented temperature effects on the a_ws of seasonings, and crossover points of seasoning blends with commonly co-formulated crystalline ingredients provide valuable resources for understanding moisture uptake in seasoning blends and for formulating more physically stable seasoning blends.

8.6 Tables and Figures

Table 8.1 Change in equilibrium RH of saturated salt solutions with increasing temperature.

Temperature (°C)	% RH [1]				
	Potassium Acetate	**Potassium Carbonate**	**Sodium Bromide**	**Potassium Iodide**	**Sodium Chloride**
20	23.1 ± 0.3	43.2 ± 0.3	59.1 ± 0.4	69.9 ± 0.3	75.5 ± 0.1
25	22.5 ± 0.3	43.2 ± 0.4	57.6 ± 0.4	68.9 ± 0.2	75.3 ± 0.1
30	21.6 ± 0.5	43.2 ± 0.5	56.0 ± 0.4	67.9 ± 0.2	75.1 ± 0.1
35			54.6 ± 0.4	67.0 ± 0.2	74.9 ± 0.1
40			53.2 ± 0.4	66.1 ± 0.2	74.7 ± 0.1
45			52.0 ± 0.5	65.3 ± 0.2	74.5 ± 0.2
50			50.9 ± 0.6	64.5 ± 0.3	74.4 ± 0.2

[1] Greenspan (1977)

Table 8.2 Effects of temperature on deliquescence points of individual ingredients and blends.

Temperature (ºC)	RH_0 and $RH_{0,mix}$ (% RH)						
	Fructose [1]	Sucrose [1]	NaCl [1]	Fructose:NaCl [2]	Sucrose:NaCl [2]	Fructose:Sucrose [2]	Fructose:Sucrose:NaCl [2]
20	65.3	86.1	76.0	49.6	65.4	56.2	42.7
25	63.1	85.7	75.8	47.9	65.0	54.1	41.0
30	61.0	85.3	75.6	46.1	64.5	52.0	39.3
35	58.8	84.9	75.4	44.4	64.0	50.0	37.7
40	56.7	84.5	75.2	42.6	63.5	47.9	36.0
45	54.6	84.1	75.0	40.9	63.1	45.9	34.4
50	52.4	83.7	74.8	39.2	62.6	43.9	32.8

[1] Lipasek et al. (2013)
[2] Calculated from RH_0s using the Ross equation

Table 8.3 Effects of initial storage RH of a seasoning on the crossover points when the pre-equilibrated seasoning was blended with single or multiple deliquescent crystalline ingredients. Shading indicates a crossover point below 50ºC, the highest temperature used in this study.

	Crossover Points (ºC)															
	Allspice				Black Pepper				Coriander				Cumin			
	23% RH *	43% RH *	70% RH *	75% RH *	23% RH *	43% RH *	70% RH *	75% RH *	23% RH *	43% RH *	70% RH *	75% RH *	23% RH *	43% RH *	70% RH *	75% RH *
Fructose	86	54	17	7	88	55	18	6	87	56	20	7	89	62	17	2
Sucrose	224	143	96	19	232	155	119	94	225	149	101	87	229	224	196	135
NaCl	217	127	63	39	226	139	80	44	218	134	70	41	223	214	152	56
Fructose:NaCl	69	34	-14	-27	70	33	-16	-31	69	36	-9	-29	71	37	-25	-43
Sucrose:NaCl	156	86	14	-11	161	91	16	-18	156	90	22	-14	160	126	10	-46
Fructose:Sucrose	75	42	1	-10	76	42	1	-12	75	44	5	-11	77	47	-4	-19
Fructose:Sucrose:NaCl	58	23	-29	-44	60	22	-33	-50	59	25	-24	-47	61	23	-47	-66

* Initial RH of saturated salt solution at 20ºC; RHs at other temperatures are reported in Table 8.1
** Values were calculated using trendline slopes of a_w-temperature and RH_0-temperature relationships and rounded to the nearest whole degree

Table 8.4 'Dry' onset T_gs of seasonings containing amorphous material. Superscript letters indicate statistical significance between T_gs.

Seasoning	'Dry' T_g
Allspice	68 ± 6ºC ^{FG}
Black Pepper	86 ± 3ºC ^{DE}
Cayenne	94 ± 3ºC ^{CDE}
Clove	103 ± 3ºC ^{BC}
Coriander	138 ± 2ºC ^{A}
Cumin	80. ± 4ºC ^{EF}
Garlic	127 ± 5ºC ^{A}
Ginger	63 ± 4ºC ^{G}
Mace	94 ± 7ºC ^{CDE}
Onion	110.9 ± 0.8ºC ^{B}
Turmeric	101 ± 4ºC ^{BCD}

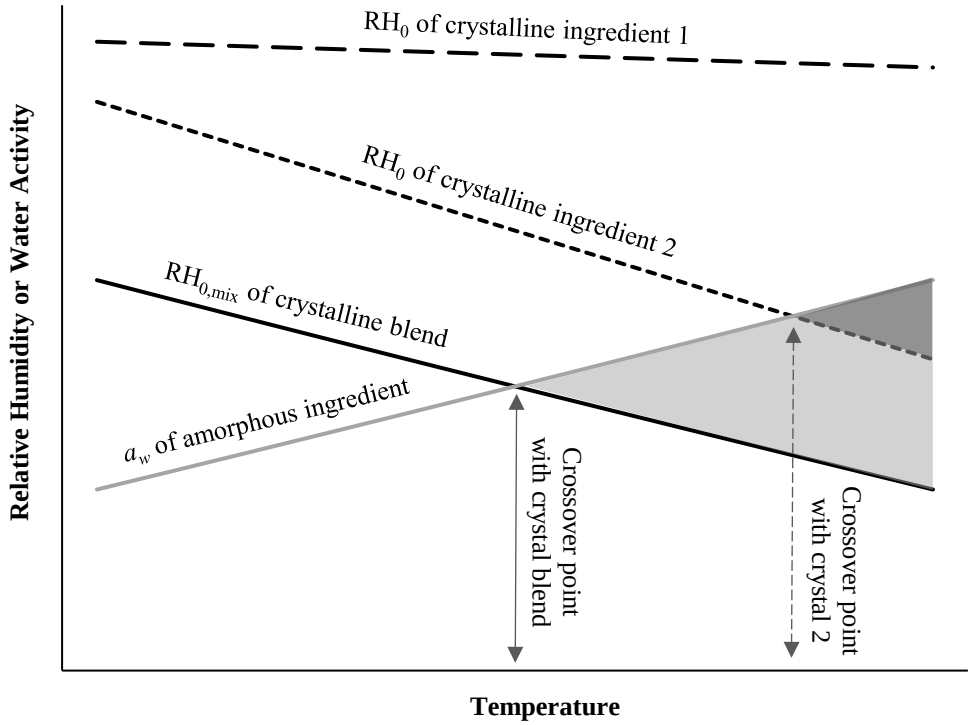
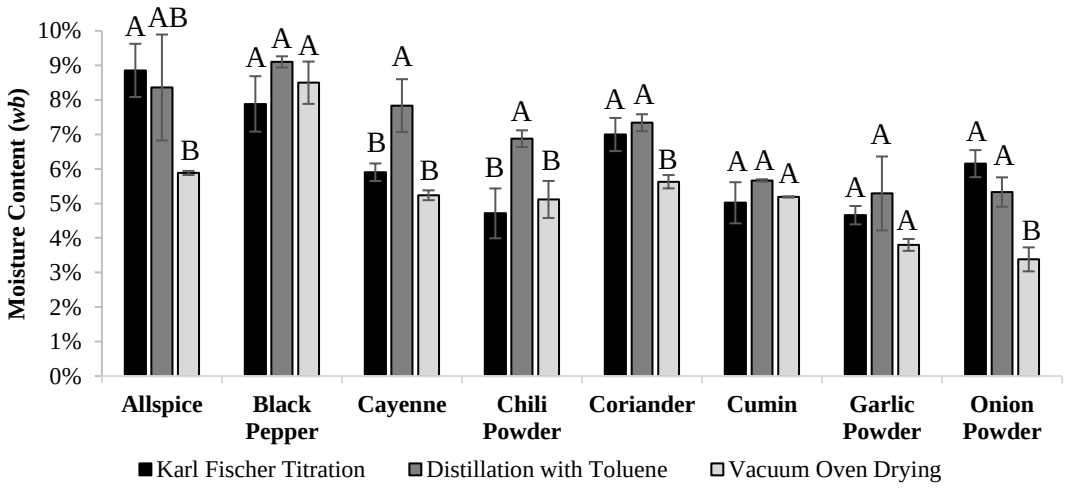


Figure 8.1 Schematic of the determination of crossover points in amorphous-crystalline ingredient blends. The crossover point occurs when the a_w of the amorphous ingredient equals the RH_0 of the crystalline ingredient(s). When this temperature is exceeded, the a_w of the system caused by the amorphous ingredient induces deliquescence of the crystalline ingredient. The shaded regions indicate the occurrence of deliquescence, wherein the dark gray is deliquescence when only crystalline ingredient 2 is present, and the lighter gray is deliquescence when both crystalline ingredient 1 and 2 are present.

A)



B)

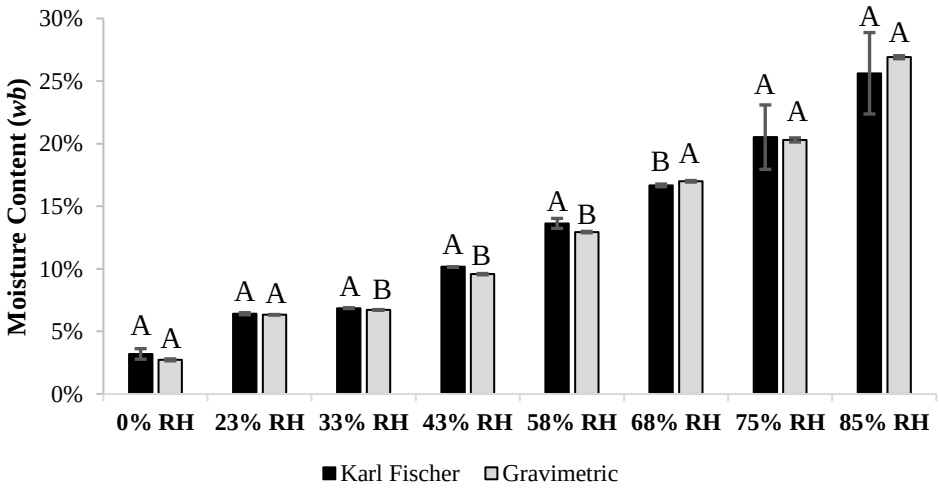


Figure 8.2 Comparison of moisture content measurements of seasonings. A) Initial moisture contents of 8 seasonings measured by Karl Fischer, toluene distillation, and gravimetric vacuum oven. B) Moisture content of onion powder following storage in controlled RH desiccators measured by Karl Fischer and gravimetric weight change based on an initial Karl Fischer measurement. Uppercase letters indicate statistical significance between the measurements of each spice or each storage condition.

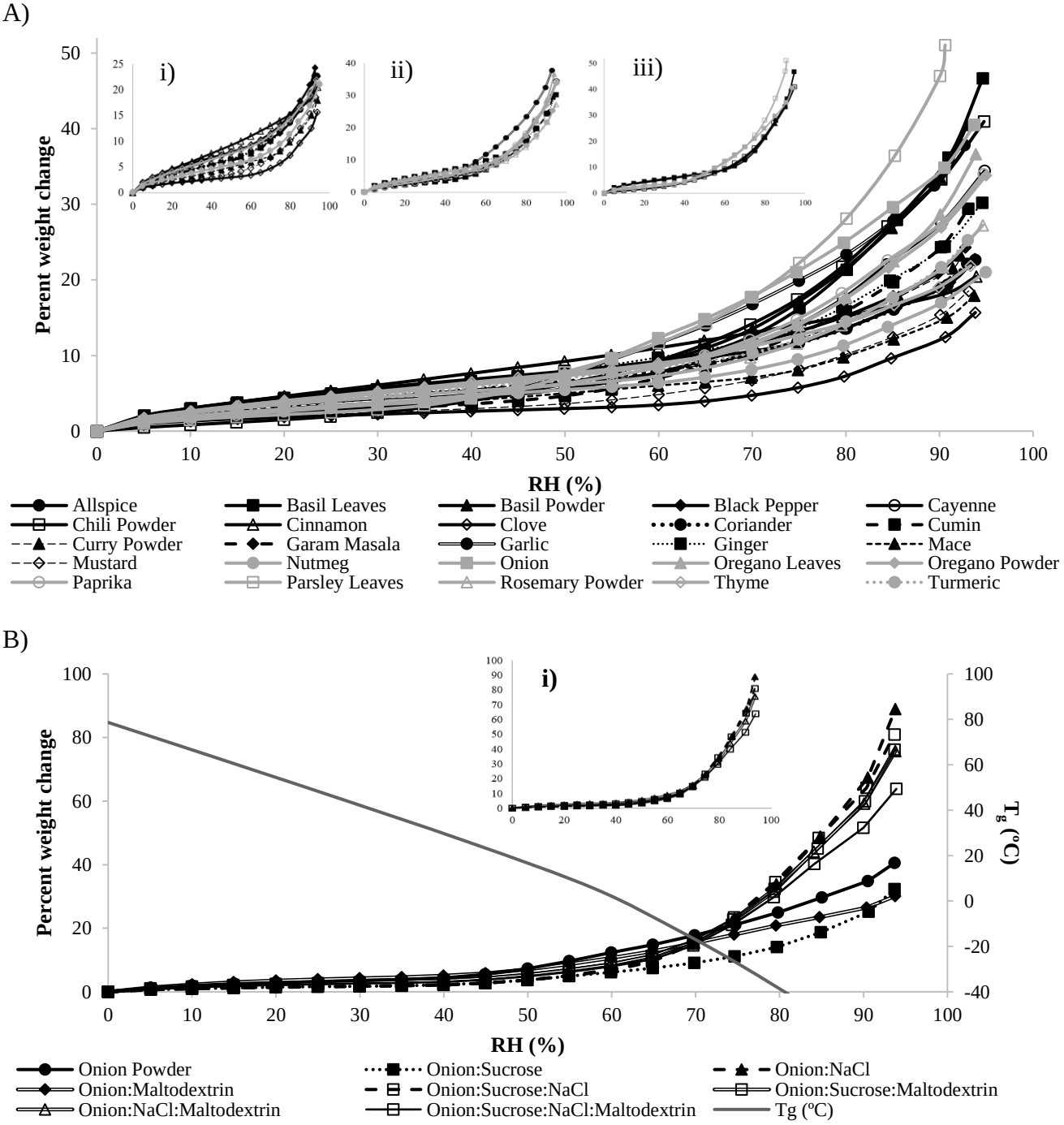


Figure 8.3 Moisture sorption profiles (at 25ºC) of: A) 25 individual seasonings, in which the insert graphs indicate i) low moisture sorption, ii) medium moisture sorption, and iii) high moisture sorption, and B) onion powder and binary, ternary, and quaternary blends with sucrose, NaCl, and maltodextrin crossed over with onion powder T_g, indicating how increasing moisture as environmental RH is increased causes a decrease in T_g, in which the insert graph i) includes moisture sorption of blends containing NaCl.

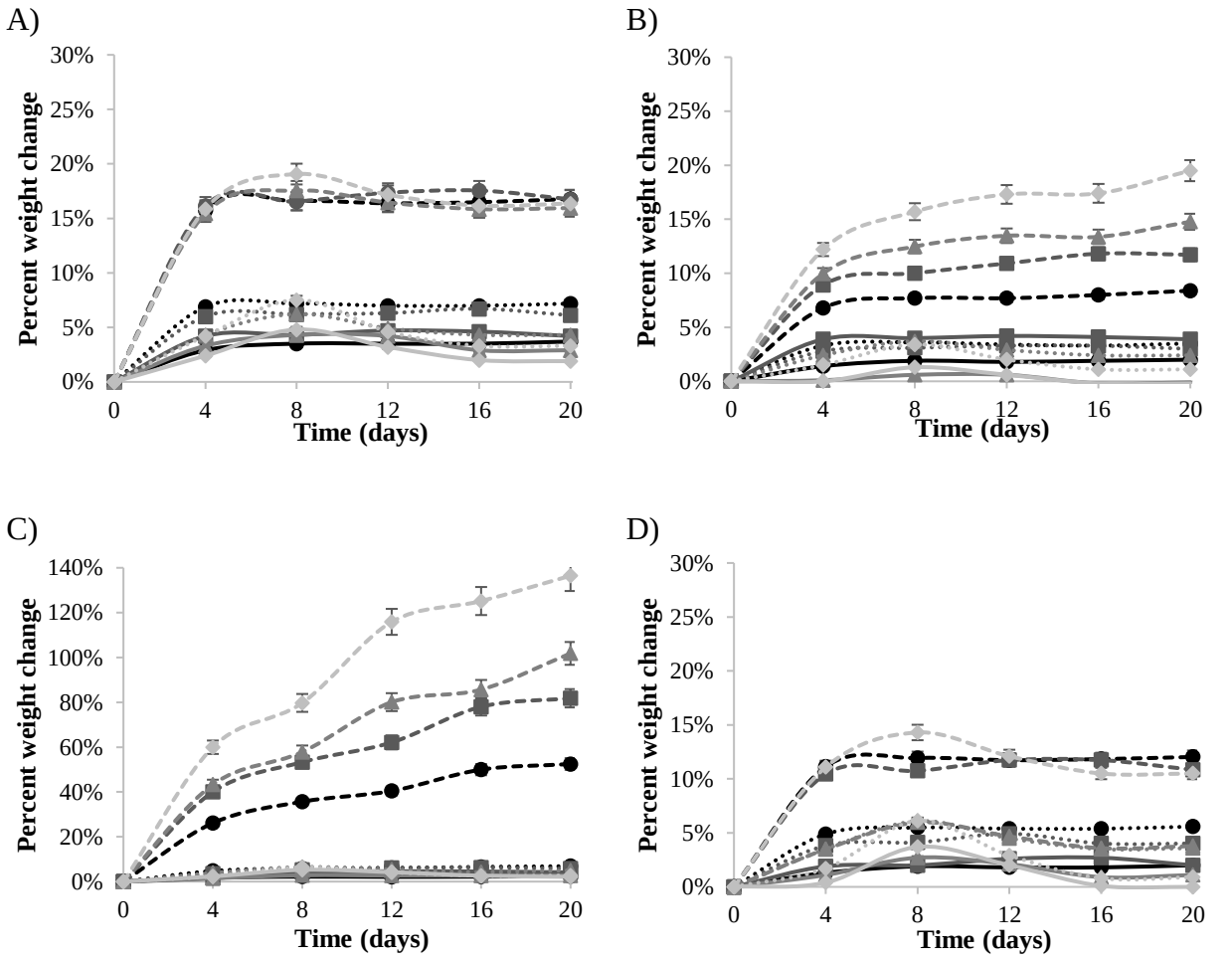


Figure 8.4 Moisture sorption profiles over time following storage in increasing RH and temperature desiccators, in which change in RH with temperature is indicated, of A) onion powder, and binary blends of onion powder with B) sucrose, C) NaCl, and D) maltodextrin. Though there is a lack of reported data on RH of potassium carbonate above 30ºC (Table 8.1; Greenspan, 1977), it was supposed that the RH remained 43% at 40 and 50ºC.

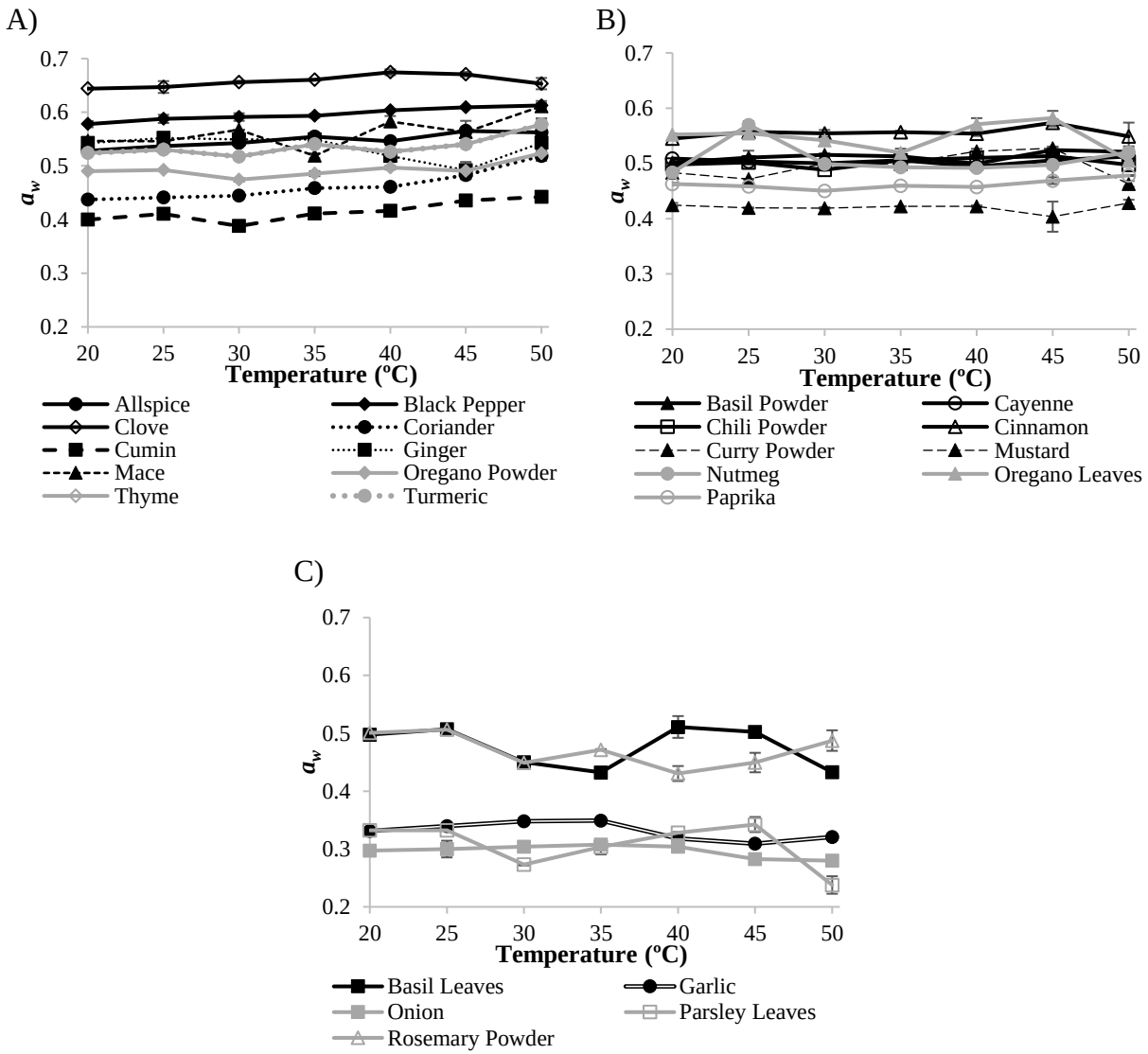


Figure 8.5 Water activities of seasonings at increasing temperatures: A) increased a_w (slope > 0.0005), B) no change in a_w (-0.0005 < slope < 0.0005), and C) decreased a_w (slope < -0.0005).

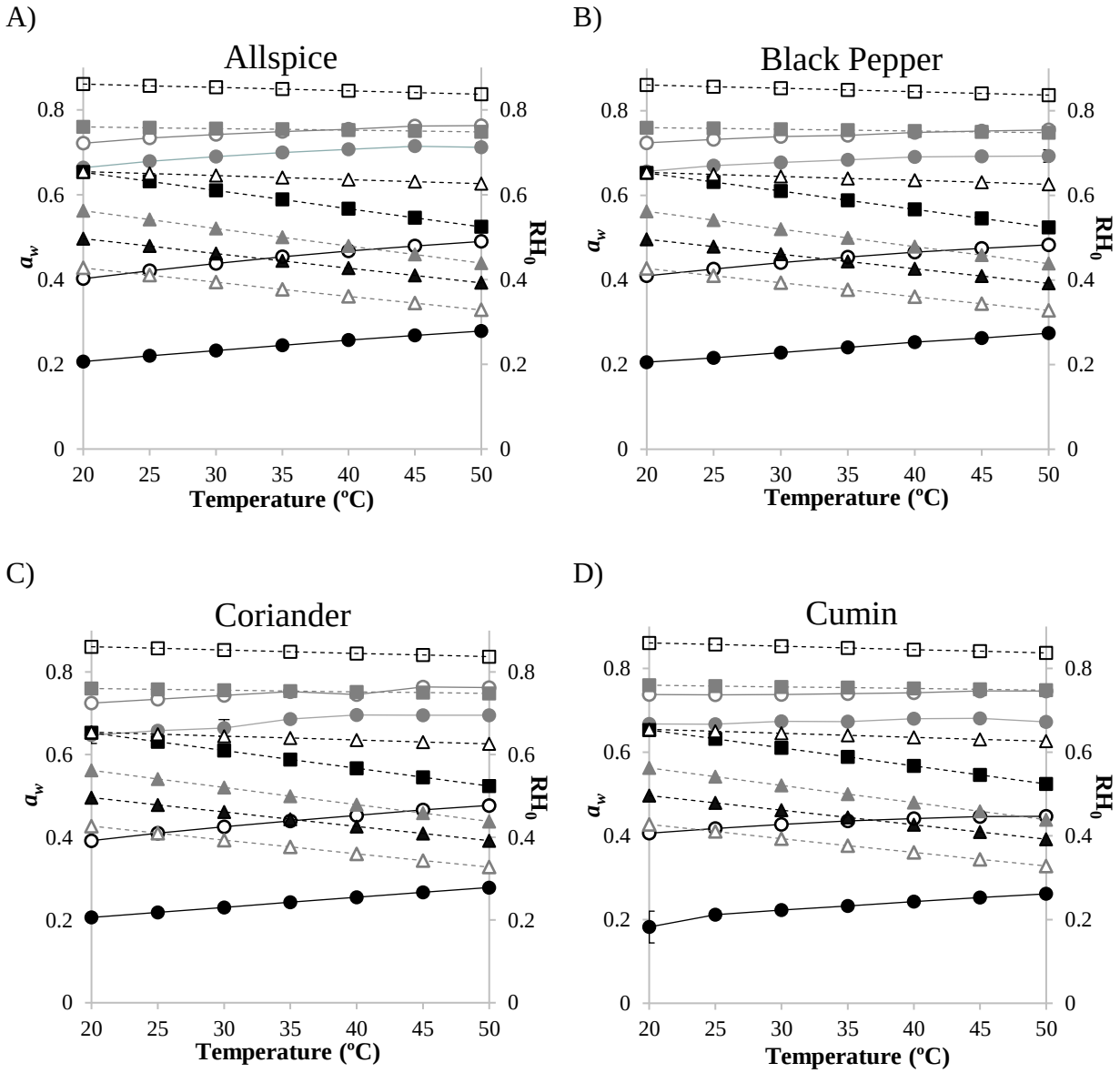


Figure 8.6 Water activities of 4 seasonings at increasing temperatures following storage at increasing RHs using the saturated salt solutions indicated in the legend (potassium acetate, potassium carbonate, potassium iodide, and sodium chloride): A) allspice, B) black pepper, C) coriander, and D) cumin. The RHs of the saturated salt solutions at storage temperatures are also reported in Table 8.1. All seasonings were plotted with deliquescence points (RH_0) of sucrose, fructose, NaCl, fructose:NaCl, sucrose:NaCl, fructose:sucrose, and fructose:sucrose:NaCl to determine crossover points to monitor physical stability of a system containing both amorphous and crystalline ingredients. Water activities are indicated by solid lines, and RH_0s are indicated by dashed lines.

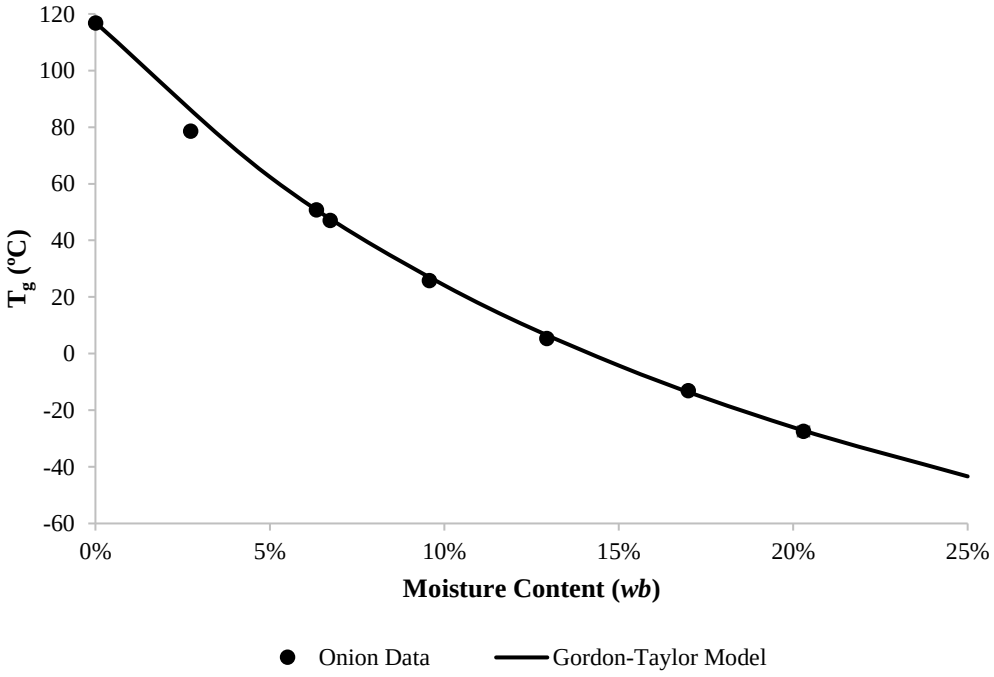


Figure 8.7 Gordon-Taylor model of onion powder, indicting the effect of moisture content on T_g.

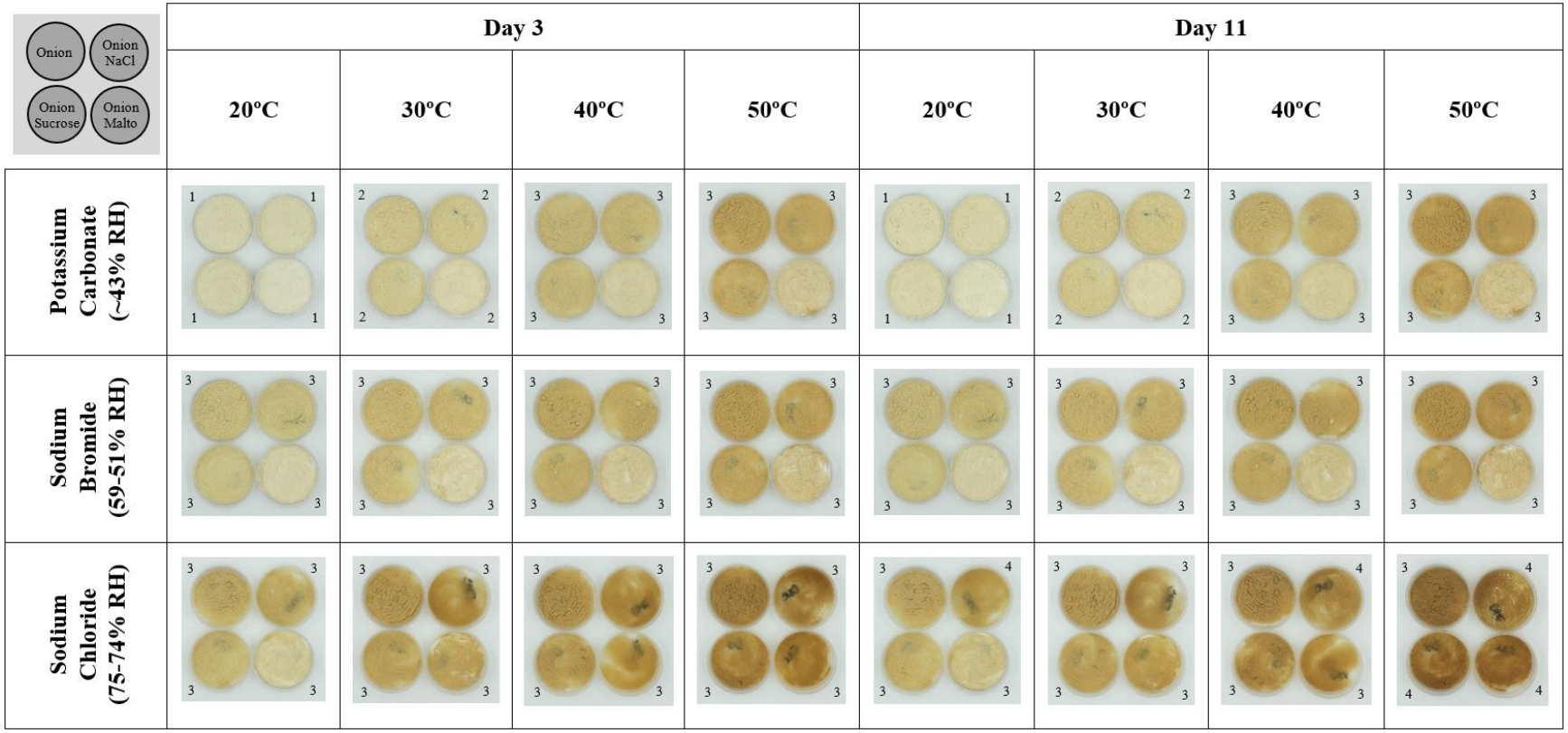


Figure 8.8 Physical assessment of onion powder and binary ingredient mixtures following exposure to increasing RH and temperature over time. Equilibrium RHs of saturated salt solutions are dependent on temperature (Table 8.1). Physical assessment of caking was ranked using the following scale: 1-free flowing; 2-partially caked; 3-fully caked; 4-slurry; 5-liquid.

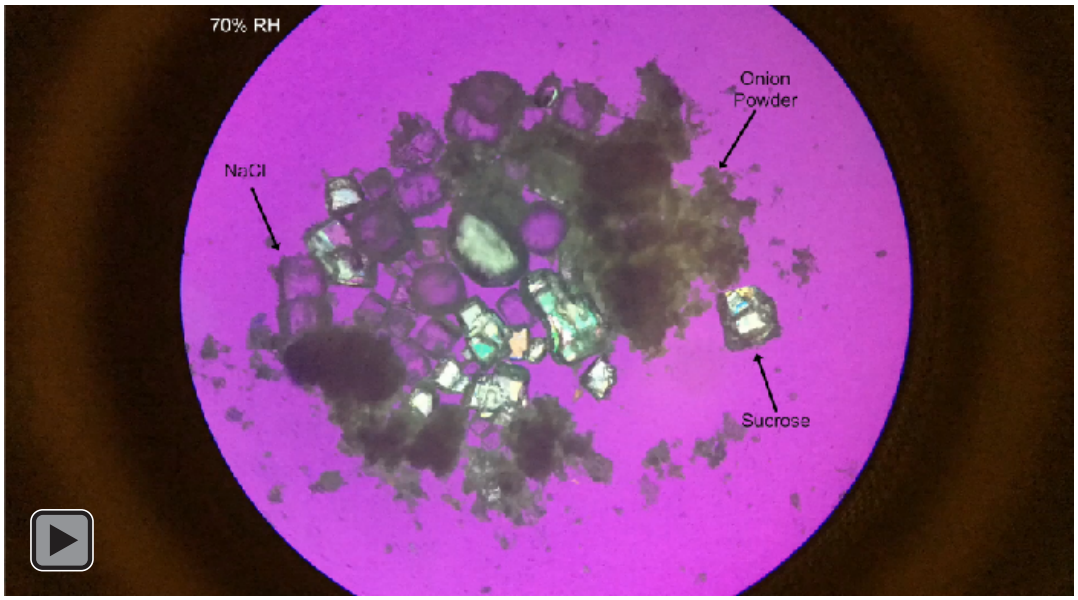


Figure 8.9 Time lapse video of onion powder, NaCl, and sucrose at 70% RH. One second of the video corresponds to approximately one hour in real time with 55 frames per second of video.

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CHAPTER 9. SUMMARY AND CONCLUSIONS

The research in this dissertation explored two major food chemistry topics: vitamin stability and water-solid interactions. The chemical stability of thiamine (vitamin B_1) was studied in solution as a function of vitamin salt form, vitamin concentration, pH, ions in solution, and food formulation, specifically in a model bread dough system. Several factors were found to significantly contribute to the reaction rate and activation energy of thiamine degradation, with specific emphasis on pH and vitamin concentration. Thiamine degradation followed approximately first-order reaction kinetics in most cases; however, the pH of the thiamine system significantly affected exact reaction order (~1.3 in pH 6 solutions compared to ~1.0 in pH 3 solutions), degradation pathway, degradation products, and sensory properties. While thiamine was found to be more stable in an acidic environment, the degradation pathway at this pH led to degradation products with much stronger odor and color properties, which are often seen as undesirable. Additionally, thiamine stability in the aqueous phase of bread dough was found to significantly differ from that in simple solutions of similar pH, specifically as a result of intermolecular interactions and the protective effect from starch and gluten. This indicated that food matrix, in addition to pH, must be considered to predict thiamine stability in foods and supplements. The reaction kinetics of thiamine degradation presented in this dissertation demonstrated how thiamine delivery can be improved and/or optimized in a variety of foods and dietary supplements containing thiamine.

Water-solid interactions were also explored in this dissertation, covering two key topics: 1) the effects of food formulation on the crystallization tendency of amorphous sucrose; and 2) the moisture sorption behaviors of spices and seasoning blends as a function of blend formulation. The crystallization tendency of amorphous sucrose was significantly affected by the structure of the co-formulated additive. When an additive contained both a structurally similar region to sucrose, which was able to form intermolecular interactions at the crystal interface, and a structurally dissimilar region to sucrose, which was able to prevent further incorporation into the crystal lattice, as in glycosylated polyphenols, sucrose esters, and some polymers, the additive led to a significant delay or prevention of sucrose crystallization. Alternatively, when sucrose was co-formulated with polysorbates, which had an increased propensity for hydrogen bonding with sucrose, a templating effect led to an increase in nucleation and thus, almost immediate sucrose crystallization. In spices

and seasoning blends, moisture sorption and physical stability properties were altered as a function of blend complexity. As blend complexity increased, sensitivity to moisture increased and physical stability decreased. Specifically, when both amorphous and crystalline ingredients and/or multiple deliquescent crystals were present in the same blend, caking occurred at much lower temperatures and RHs. The physical stability of amorphous sucrose, spices, and seasoning blends presented in this dissertation demonstrated how food structure and composition can be optimized to improve product quality and functionality in many food products.

While this dissertation included multiple areas of food chemistry, including the chemical stability of thiamine, crystallization of amorphous sucrose, and moisture sorption properties of spices and seasoning blends, these topics provide valuable information on the chemical and physical stability of ingredient systems and how they can be controlled for optimal ingredient functionality.

APPENDIX

Chapter 2

Table A.2.1 Percent TMN remaining after storage at the specified conditions over time: A) 25ºC, B) 40ºC, C) 60ºC, D) 70ºC, and E) 80ºC. Uppercase superscript letters on values for each concentration denote statistical significance within that concentration (down columns). Lowercase superscript letters denote statistical significance between concentrations for each day (across rows).

A)

TMN 25ºC

Percent Vitamin Remaining

Days	1 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL	27 mg/mL
0	97.0 ± 0.5%^{Cab}	98 ± 1%^{ABCab}	96.7 ± 0.5%^{ABCDab}	95 ± 3%^{BCb}	100 ± 2%^{Aa}
7	98.9 ± 0.3%^{Ba}	99.6 ± 0.2%^{ABa}	99 ± 1%^{ABa}	99 ± 3%^{Aa}	97.9 ± 0.7%^{ABa}
14	100.8 ± 0.2%^{Aa}	99.7 ± 0.2%^{ABab}	100.6 ± 0.3%^{Aa}	97 ± 1%^{ABCb}	97 ± 1%^{ABb}
21	98.5 ± 0.2%^{Bab}	99.1 ± 0.9%^{ABa}	98 ± 2%^{ABCab}	98.2 ± 0.2%^{ABab}	96.3 ± 0.5%^{ABCb}
28	100.3 ± 0.3%^{Aab}	100.7 ± 0.3%^{Aa}	100.5 ± 0.8%^{Aab}	99.4 ± 0.2%^{Ab}	96.5 ± 0.2%^{ABCc}
35	98.9 ± 0.4%^{Ba}	99 ± 2%^{ABCa}	97.9 ± 0.6%^{ABCDab}	98.1 ± 0.8%^{ABa}	95.5 ± 0.3%^{BCb}
63	96.9 ± 0.5%^{Ca}	96.1 ± 0.3%^{BCDa}	93 ± 3%^{BCDEab}	96 ± 1%^{ABCa}	91 ± 2%^{DEb}
91	97.0 ± 0.5%^{Ca}	95 ± 1%^{CDabc}	92 ± 3%^{DEc}	96.1 ± 0.7%^{ABCab}	93.8 ± 0.5%^{BCDbc}
119	96.7 ± 0.3%^{Ca}	93 ± 3%^{DEa}	93 ± 3%^{CDEa}	95.4 ± 0.6%^{BCa}	93 ± 2%^{CDEa}
147	95.0 ± 0.72%^{Da}	91 ± 2%^{Eabc}	88 ± 3%^{Ec}	93.7 ± 0.4%^{DCab}	90 ± 2%^{DEbc}
174	94.1 ± 0.4%^{Da}	89 ± 1%^{Eb}	74 ± 2%^{Ec}	91 ± 1%^{Dab}	89 ± 2%^{Eb}

B)

TMN 40ºC

Percent Vitamin Remaining

Days	1 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL	27 mg/mL
0	97.2 ± 0.2%^{ABCa}	97.5 ± 0.9%^{Aa}	97 ± 1%^{Aa}	97.4 ± 0.2%^{Aa}	96.3 ± 0.6%^{Aa}
7	97.4 ± 0.2%^{ABa}	99 ± 1%^{Aa}	98.7 ± 0.5%^{Aa}	98 ± 1%^{Aa}	91.6 ± 0.3%^{BCb}
14	98 ± 1%^{Aab}	100 ± 2%^{Aa}	98.96 ± 0.04%^{Aab}	96.9 ± 0.1%^{Aab}	95.22 ± 0.03%^{Ab}
21	94.7 ± 0.6%^{CDab}	96.1 ± 0.7%^{Aa}	96 ± 1%^{Aa}	94.2 ± 0.4%^{Aab}	93.3 ± 0.1%^{ABb}
28	95.1 ± 0.7%^{BCDa}	97 ± 3%^{Aa}	94.5 ± 0.8%^{Aa}	95.3 ± 0.9%^{Aa}	93.3 ± 0.6%^{ABa}
35	92.8 ± 0.2%^{Da}	94 ± 4%^{Aa}	89 ± 2%^{Ba}	92 ± 2%^{Aa}	88.9 ± 0.1%^{Ca}
63	87 ± 1%^{Ea}	80 ± 4%^{Ba}	61.0 ± 0.3%^{Cb}	58 ± 4%^{Bb}	44 ± 2%^{Dc}
91	84.2 ± 0.9%^{Fa}	77 ± 8%^{BCa}	55 ± 3%^{Db}	42 ± 1%^{Cc}	35.9 ± 0.1%^{Ec}
119	81 ± 2%^{Ga}	66 ± 2%^{CDb}	51 ± 2%^{DEc}	39.5 ± 0.9%^{Cd}	34.1 ± 0.1%^{Ee}
147	77.5 ± 0.9%^{Ha}	62 ± 2%^{Db}	46.7 ± 0.8%^{Ec}	35 ± 4%^{Cd}	33.8 ± 0.1%^{EFd}
174	75.1 ± 0.6%^{Ha}	59 ± 1%^{Db}	46.3 ± 0.8%^{Ec}	39 ± 4%^{Cd}	30 ± 3%^{Fe}

Table A.2.1 continued

C)

TMN 60ºC

Percent Vitamin Remaining

Days	1 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL	27 mg/mL
0	100.9 ± 0.5%^{Aab}	97 ± 1%^{Ac}	98.5 ± 0.2%^{Ac}	98.6 ± 0.4%^{Abc}	101 ± 1%^{Aa}
1	99.5 ± 0.8%^{Aa}	96.5 ± 0.1%^{Ac}	96.3 ± 0.2%^{Ac}	97.0 ± 0.7%^{Bbc}	99 ± 1%^{Bab}
4	93.0 ± 0.9%^{Ba}	88.9 ± 0.4%^{Bab}	86 ± 4%^{Bb}	90.0 ± 0.7%^{Cab}	89.9 ± 0.2%^{Cab}
7	89.2 ± 0.9%^{Ca}	84.5 ± 0.8%^{Cb}	81 ± 2%^{Bc}	77.3 ± 0.3%^{Dd}	58.9 ± 0.2%^{Ce}
11	84.9 ± 0.8%^{Da}	76.1 ± 0.4%^{Db}	66 ± 3%^{Cc}	45.9 ± 0.1%^{Ed}	38.58 ± 0.04%^{De}
14	76.7 ± 0.8%^{Ea}	67.1 ± 0.1%^{Eb}	55 ± 1%^{Dc}	39.5 ± 0.1%^{Fd}	34.1 ± 0.3%^{Ee}

D)

TMN 70ºC

Percent Vitamin Remaining

Days	1 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL	27 mg/mL
0	96.7 ± 0.3%^{Aa}	95.3 ± 0.6%^{Aa}	95.6 ± 0.6%^{Aa}	99 ± 3%^{Aa}	99 ± 3%^{Aa}
1	95.8 ± 0.9%^{Aa}	93.7 ± 0.8%^{Bab}	93 ± 2%^{Aab}	92 ± 1%^{Ab}	93.5 ± 0.9%^{Aab}
2	88.1 ± 0.5%^{Ba}	85.0 ± 0.4%^{Ca}	83 ± 1%^{Bab}	79 ± 5%^{Bb}	64 ± 2%^{Bc}
3	90.5 ± 0.5%^{ABa}	84.7 ± 0.6%^{Cab}	78.5 ± 0.9%^{Bb}	55.7 ± 0.4%^{Cc}	52 ± 8%^{Cc}
5	77 ± 4%^{Ca}	66.1 ± 0.5%^{Db}	59 ± 2%^{Cc}	44 ± 1%^{Dd}	40.8 ± 0.3%^{CDd}
7	73 ± 4%^{Ca}	62.1 ± 0.3%^{Eb}	55 ± 2%^{Dc}	41 ± 1%^{Dd}	39 ± 4%^{Dd}

E)

TMN 80ºC

Percent Vitamin Remaining

Days	1 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL	27 mg/mL
0	97.1 ± 0.1%^{Aab}	95.5 ± 0.2%^{Ab}	95.0 ± 0.5%^{Ab}	99 ± 2%^{Aa}	96 ± 2%^{Aab}
1	87 ± 1%^{Ba}	81.8 ± 0.7%^{Bb}	73 ± 1%^{Bc}	55.5 ± 0.9%^{Bd}	46 ± 1%^{Be}
2	76 ± 1%^{Ca}	66 ± 1%^{Cb}	57.2 ± 0.8%^{Cc}	42.0 ± 0.7%^{Cd}	36.1 ± 0.4%^{Ce}
3	73.1 ± 0.1%^{Da}	62 ± 1%^{Db}	52 ± 1%^{Dc}	39 ± 1%^{Dd}	36 ± 1%^{Cd}
5	55.41 ± 0.07%^{Ea}	47.5 ± 0.8%^{Eb}	41 ± 3%^{Ec}	31.2 ± 0.7%^{Ed}	32 ± 2%^{Cd}
7	48.0 ± 0.3%^{Fa}	42 ± 1%^{Fab}	38 ± 2%^{Eb}	30.3 ± 0.3%^{Ec}	31 ± 4%^{Cc}

Table A.2.2 Percent TMN remaining after storage at the specified conditions over time: A) 25ºC, B) 40ºC, C) 60ºC, D) 70ºC, and E) 80ºC. Uppercase superscript letters on values for each concentration denote statistical significance within that concentration (down columns). Lowercase superscript letters denote statistical significance between concentrations for each day (across rows).

A)

TClHCl 25ºC
Percent Vitamin Remaining

Days	1 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL	27 mg/mL	100 mg/mL	300 mg/mL	500 mg/mL
0	102.8 ± 0.4%^{BCDa}	102.3 ± 0.1%^{ABab}	102 ± 1%^{Acb}	98.6 ± 0.4%^{DEe}	100.5 ± 0.2%^{Acd}	97.6 ± 0.2%^{ABe}	99.9 ± 0.3%^{Ad}	94.1 ± 0.1%^{ABf}
7	101.1 ± 0.6%^{Ea}	100.5 ± 0.5%^{Ba}	100 ± 1%^{Aab}	97.9 ± 0.4%^{Ebc}	97.3 ± 0.5%^{Bc}	97.0 ± 0.6%^{ABcd}	99.1 ± 0.4%^{Aabc}	95 ± 2%^{ABd}
14	103.04 ± 0.09%^{BCDa}	102.1 ± 0.5%^{ABab}	101.3 ± 0.5%^{Ab}	100.6 ± 0.4%^{BCbc}	101.1 ± 0.1%^{Ab}	99 ± 1%^{ABc}	100.6 ± 0.4%^{Abc}	95.1 ± 0.5%^{ABd}
21	103.6 ± 0.4%^{BCa}	102.7 ± 0.7%^{ABab}	102 ± 1%^{Aabc}	102 ± 1%^{ABbcd}	101.0 ± 0.7%^{Abcd}	99.9 ± 0.6%^{Ad}	100.5 ± 0.2%^{Acd}	95.2 ± 0.4%^{ABe}
28	102.1 ± 0.5%^{DEa}	101 ± 2%^{ABa}	100.7 ± 0.6%^{Aab}	99.9 ± 0.3%^{BCDab}	99.1 ± 0.1%^{ABab}	97 ± 2%^{ABb}	99.9 ± 0.7%^{Aab}	92 ± 2%^{ABc}
63	102.3 ± 0.5%^{CDEa}	101.3 ± 0.1%^{ABa}	100 ± 3%^{Aa}	100.2 ± 0.4%^{BCDa}	100 ± 3%^{ABa}	98 ± 2%^{ABa}	99.4 ± 0.4%^{Aa}	89 ± 4%^{Bb}
88	103.3 ± 0.3%^{BCDa}	101 ± 1%^{ABab}	101 ± 2%^{Aab}	101.1 ± 0.5%^{BCab}	100.7 ± 0.6%^{Aab}	98.6 ± 0.6%^{ABb}	100 ± 1%^{Ab}	93 ± 1%^{ABc}
119	103.3 ± 0.5%^{BCDa}	101 ± 2%^{ABa}	100 ± 2%^{Aa}	101 ± 0.7%^{BCa}	99.8 ± 0.9%^{ABa}	98.3 ± 0.8%^{ABa}	99.5 ± 0.4%^{Aa}	90 ± 4%^{Bb}
147	105.7 ± 0.9%^{Aa}	103.6 ± 0.4%^{Aab}	101 ± 3%^{Aabc}	99.5 ± 0.6%^{CDEabc}	99.2 ± 0.7%^{ABbc}	95 ± 4%^{Bc}	98 ± 3%^{Abc}	98 ± 2%^{Abc}
174	104.1 ± 0.6%^{Ba}	102 ± 1%^{ABa}	104 ± 3%^{Aa}	103.0 ± 0.9%^{Aa}	101.1 ± 0.3%^{Aab}	99.2 ± 0.8%^{ABab}	98 ± 4%^{Aab}	93 ± 6%^{ABb}

B)

TClHCl 40ºC
Percent Vitamin Remaining

Days	1 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL	27 mg/mL	100 mg/mL	300 mg/mL	500 mg/mL
0	102.2 ± 0.2%^{ABa}	101.7 ± 0.4%^{Aab}	101.4 ± 0.7%^{Aab}	98.5 ± 0.3%^{Cde}	100.7 ± 0.2%^{Abc}	97.6 ± 0.4%^{Aef}	99.4 ± 0.4%^{Adc}	96.5 ± 0.9%^{Af}
7	102 ± 1%^{ABCa}	101 ± 1%^{ABab}	97 ± 4%^{Aab}	98.9 ± 0.3%^{BCab}	99.5 ± 0.1%^{ABCab}	96 ± 3%^{Ab}	100.4 ± 0.6%^{Aab}	95 ± 1%^{Ab}
14	102.5 ± 0.3%^{Aa}	101 ± 1%^{ABa}	97 ± 3%^{Abc}	99.1 ± 0.4%^{BCab}	100 ± 1%^{ABab}	99.1 ± 0.5%^{Aab}	100.8 ± 0.4%^{Aa}	93.5 ± 0.8%^{Ac}
21	101.9 ± 0.3%^{ABCa}	100.9 ± 0.5%^{ABab}	98 ± 8%^{Aab}	99.3 ± 0.1%^{ABCab}	100.5 ± 0.6%^{ABab}	99 ± 1%^{Aab}	101.6 ± 0.8%^{Aa}	94.5 ± 0.3%^{Ab}
28	100.5 ± 0.6%^{BCDa}	100 ± 1%^{ABa}	99 ± 3%^{Aa}	99.2 ± 0.6%^{BCa}	98.90 ± 0.5%^{Ca}	98 ± 1%^{Aa}	99.7 ± 0.8%^{Aa}	93.1 ± 0.9%^{Ab}
63	99.7 ± 0.2%^{Da}	99.3 ± 0.1%^{ABa}	96 ± 2%^{Abc}	98.7 ± 0.1%^{Cab}	98.9 ± 0.5%^{ABCab}	97.4 ± 0.7%^{Aab}	99.0 ± 0.6%^{Aab}	94.0 ± 0.7%^{Ac}
88	100.0 ± 0.3%^{CDa}	98.3 ± 0.7%^{ABa}	98 ± 5%^{Aa}	100.0 ± 0.2%^{ABCa}	99.6 ± 0.4%^{ABCa}	97 ± 1%^{Aa}	101 ± 1%^{Aa}	92 ± 3%^{Ab}
119	99.5 ± 0.1%^{Da}	97.1 ± 0.7%^{Ba}	97 ± 7%^{Aa}	99.4 ± 0.2%^{ABCa}	98.7 ± 0.6%^{BCa}	94 ± 3%^{Aa}	94 ± 7%^{Aa}	93 ± 6%^{Aa}
147	98.9 ± 0.4%^{Da}	99.6 ± 0.9%^{ABa}	100 ± 2%^{Aa}	101 ± 1%^{Aa}	99.6 ± 0.2%^{ABCa}	96 ± 4%^{Aa}	95 ± 1%^{Aa}	99 ± 4%^{Aa}
174	99 ± 1%^{Da}	100 ± 3%^{ABa}	95 ± 8%^{Aa}	100 ± 1%^{ABa}	99.3 ± 0.5%^{ABCa}	90 ± 10%^{Aa}	100.1 ± 0.2%^{Aa}	97 ± 2%^{Aa}

Table A.2.2 continued

C)

TClHCl 60ºC
Percent Vitamin Remaining

Days	1 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL	27 mg/mL	100 mg/mL	300 mg/mL	500 mg/mL
0	99.4 ± 0.2%^{Aa}	97.6 ± 0.5%^{Aa}	97.0 ± 0.4%^{Aa}	97.9 ± 0.8%^{ABa}	97.0 ± 0.9%^{Aa}	97 ± 2%^{ABCa}	98.7 ± 0.3%^{Aa}	96.9 ± 0.4%^{Aa}
1	98 ± 2%^{ABab}	95.4 ± 0.5%^{ABb}	95 ± 2%^{Ab}	97.3 ± 0.3%^{ABab}	97.1 ± 0.5%^{Aab}	95 ± 2%^{BCDb}	101 ± 3%^{Aa}	94.4 ± 0.7%^{ABb}
4	98 ± 1%^{ABa}	96.5 ± 0.4%^{ABab}	94 ± 1%^{Abc}	98.9 ± 0.1%^{Aa}	97.7 ± 0.4%^{Aa}	99 ± 2%^{Aa}	99.1 ± 0.2%^{Aa}	91 ± 1%^{BCc}
7	97 ± 3%^{ABCa}	96 ± 2%^{ABa}	93 ± 4%^{Aa}	97.5 ± 0.8%^{AB}	97 ± 2%^{Aa}	97.0 ± 0.2%^{ABCa}	98 ± 2%^{ABa}	94 ± 1%^{ABa}
11	95 ± 2%^{ABCDab}	97 ± 4%^{Aab}	95.4 ± 0.9%^{Aab}	99 ± 2%^{Aa}	98 ± 2%^{Aab}	96.6 ± 0.7%^{ABCab}	98.5 ± 0.1%^{ABab}	93.7 ± 0.4%^{ABb}
14	90.5 ± 0.2%^{DEbc}	95.2 ± 0.2%^{ABabc}	90 ± 5%^{Ac}	96.4 ± 0.4%^{ABab}	97 ± 3%^{Aa}	94.3 ± 0.7%^{CDabc}	96.0 ± 0.4%^{ABabc}	90.2 ± 0.9%^{BCc}
18	93 ± 1%^{BCDEbc}	93 ± 3%^{ABbc}	95 ± 2%^{Aabc}	98.5 ± 0.7%^{ABa}	96.9 ± 0.1%^{Aab}	98 ± 1%^{ABa}	98.7 ± 0.5%^{Aa}	90 ± 2%^{BCc}
21	93 ± 2%^{BCDEbc}	94.2 ± 0.9%^{ABabc}	95.3 ± 0.7%^{Aab}	98 ± 1%^{ABa}	95.8 ± 0.3%^{Aab}	97 ± 1%^{ABCa}	96 ± 1%^{ABab}	90.7 ± 0.7%^{BCc}
25	92 ± 2%^{CDEab}	94 ± 2%^{ABab}	93 ± 2%^{Aab}	97 ± 1%^{ABa}	96.7 ± 0.1%^{Aa}	94.9 ± 0.6%^{BCDab}	97.0 ± 0.7%^{ABa}	90 ± 3%^{BCb}
28	93 ± 2%^{BCDEab}	93 ± 2%^{ABab}	94 ± 2%^{Aab}	96.5 ± 0.4%^{ABa}	95.5 ± 0.1%^{Aa}	97.1 ± 0.7%^{ABCa}	96.5 ± 0.1%^{ABa}	91 ± 1%^{BCb}
56	88 ± 2%^{Ea}	90 ± 2%^{Ba}	89 ± 6%^{Aa}	93 ± 1%^{Ba}	94 ± 4%^{Aa}	92.3 ± 0.8%^{DEa}	93 ± 1%^{Ba}	87.5 ± 0.6%^{Ca}
100	81 ± 1%^{Fb}	82 ± 3%^{Cb}	86 ± 4%^{ABab}	85.2 ± 0.3%^{Cab}	85 ± 3%^{Bab}	89.8 ± 0.9%^{Ea}	86.7 ± 0.5%^{Cab}	82 ± 1%^{Db}
157	73.8 ± 0.9%^{Ga}	75 ± 3%^{Ca}	76 ± 7%^{Ba}	80 ± 5%^{Ca}	78 ± 4%^{Ba}	79.3 ± 0.3%^{Fa}	78 ± 6%^{Da}	72 ± 3%^{Ea}

Table A.2.2 continued

D)

TClHCl 70ºC
Percent Vitamin Remaining

Days	1 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL	27 mg/mL	100 mg/mL	300 mg/mL	500 mg/mL
0	98.8 ± 0.1%^{Aab}	98.2 ± 0.8%^{ABab}	98 ± 2%^{Aab}	100.1 ± 0.3%^{ABab}	96 ± 3%^{Aa}	97 ± 1%^{Aab}	97.00 ± 1%^{Aab}	96 ± 2%^{Ab}
1	99 ± 4%^{Aa}	97 ± 2%^{ABab}	95 ± 2%^{ABab}	96.7 ± 0.5%^{ABCab}	97.1 ± 0.1%^{BCab}	93.6 ± 0.2%^{ABCb}	96.60 ± 0.2%^{Aab}	94.55 ± 0.08%^{ABab}
2	99 ± 3%^{Aabc}	99 ± 2%^{Aabc}	96 ± 2%^{Aabcd}	99.7 ± 0.4%^{Aab}	100.1 ± 0.4%^{Aa}	94.3 ± 0.1%^{ABd}	95.9 ± 0.4%^{Abcd}	95 ± 1%^{ABcd}
3	95 ± 0.9%^{Aabc}	96.4 ± 0.3%^{ABab}	95 ± 1%^{ABabc}	97.4 ± 0.4%^{ABa}	97.00 ± 1%^{BCa}	94 ± 1%^{ABCbc}	96.8 ± 0.6%^{Aa}	92 ± 1%^{ABCc}
5	93 ± 1%^{ABcd}	95.2 ± 0.1%^{ABCbc}	95.5 ± 0.5%^{ABabc}	95 ± 1%^{BCDbc}	98.0 ± 0.8%^{ABa}	92.4 ± 0.2%^{BCd}	96 ± 1%^{Aab}	92.1 ± 0.1%^{BCd}
7	92 ± 3%^{ABab}	94.6 ± 0.6%^{ABCab}	94 ± 2%^{ABab}	94.6 ± 0.6%^{BCDab}	97 ± 1%^{BCa}	91.6 ± 0.7%^{BCDab}	95 ± 3%^{Aab}	91 ± 2%^{CDb}
12	85.7 ± 0.6%^{ABe}	90.9 ± 0.1%^{BCcd}	94.1 ± 0.5%^{ABab}	96 ± 1%^{BCDa}	95.4 ± 0.9%^{CDab}	90.9 ± 0.4%^{CDcd}	92 ± 2%^{ABbc}	88 ± 2%^{Dde}
17	86 ± 8%^{ABab}	89 ± 1%^{CDab}	83 ± 3%^{Cb}	93.7 ± 0.7%^{CDa}	93.1 ± 0.6%^{DEab}	88.7 ± 0.5%^{DEab}	91 ± 4%^{ABab}	83.9 ± 0.4%^{Eab}
21	69 ± 1%^{BCe}	83 ± 1%^{Dcd}	87 ± 2%^{BCb}	92.6 ± 0.4%^{Da}	92 ± 1%^{Ea}	85 ± 2%^{EFbc}	87 ± 2%^{Bb}	80.7 ± 0.6%^{Ed}
31	66 ± 2%^{Cb}	82 ± 6%^{Da}	81 ± 6%^{Ca}	82 ± 3%^{Ea}	85.9 ± 0.4%^{Fa}	83 ± 2%^{Fa}	75 ± 4%^{Cab}	74 ± 1%^{Fab}

E)

TClHCl 80ºC
Percent Vitamin Remaining

Days	1 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL	27 mg/mL	100 mg/mL	300 mg/mL	500 mg/mL
0	98.5 ± 0.1%^{Aa}	98.0 ± 0.4%^{Aa}	97 ± 2%^{Aa}	97.5 ± 0.3%^{Aa}	97.4 ± 0.7%^{Aa}	94 ± 3%^{ABb}	97.8 ± 0.2%^{Aa}	93.3 ± 0.6%^{Ab}
1	95.1 ± 0.7%^{Aa}	98 ± 3%^{ABa}	95.4 ± 0.3%^{Aa}	96 ± 2%^{ABa}	98 ± 2%^{Aa}	96 ± 2%^{Aa}	96.3 ± 0.8%^{Aa}	93 ± 2%^{Aa}
2	96 ± 2%^{Aa}	96.8 ± 0.3%^{ABa}	94.6 ± 0.6%^{Aa}	95.4 ± 0.2%^{ABa}	97 ± 2%^{ABa}	97 ± 4%^{Aa}	95.0 ± 0.6%^{Aa}	93.3 ± 0.8%^{Aa}
3	92 ± 3%^{ABa}	93 ± 1%^{ABCa}	93.6 ± 0.6%^{Aa}	93.7 ± 0.4%^{ABa}	95 ± 3%^{ABa}	96 ± 5%^{Aa}	95 ± 2%^{Aa}	92.3 ± 0.8%^{Aa}
5	88 ± 4%^{ABa}	92 ± 1%^{ABCa}	93 ± 1%^{ABa}	93.4 ± 0.6%^{ABa}	94 ± 4%^{ABa}	92 ± 4%^{ABa}	91 ± 3%^{ABa}	91.0 ± 0.4%^{Aa}
7	83 ± 4%^{Ba}	90 ± 4%^{ABCa}	91 ± 3%^{ABa}	90.6 ± 0.6%^{BCa}	90 ± 3%^{Ba}	89 ± 5%^{ABa}	86 ± 4%^{ABa}	89.5 ± 0.8%^{Aa}
12	77 ± 4%^{Cb}	89 ± 4%^{BCa}	87 ± 3%^{ABa}	87.2 ± 0.3%^{Ca}	82 ± 2%^{Cab}	84 ± 5%^{Bab}	81 ± 8%^{Bab}	86 ± 2%^{ABa}
17	55 ± 4%^{Dd}	86 ± 4%^{Ca}	81 ± 7%^{Bab}	64 ± 4%^{Dcd}	72 ± 1%^{Dbc}	71 ± 1%^{Cbc}	68 ± 2%^{Cc}	80 ± 5%^{BCab}
21	51 ± 4%^{Dde}	70.2 ± 0.3%^{Dab}	50 ± 4%^{Ce}	59 ± 2%^{Dcde}	65 ± 1%^{Dabc}	64 ± 1%^{Cabc}	61 ± 7%^{Cbcd}	73 ± 4%^{CDa}
31	40 ± 1%^{Ed}	60.3 ± 0.7%^{Eab}	48 ± 3%^{Ccd}	47 ± 1%^{Ecd}	56 ± 3%^{Eabc}	52 ± 3%^{Dbc}	45 ± 8%^{Dcd}	66 ± 6%^{Da}

Table A.2.3 Words used by sensory panelists when asked to describe the sample they found to have the strongest aroma and their frequencies. Words used generally indicated that the aroma was unfavorable.

Words Used to Describe Stronger Smelling Sample	Word Count
Rubber/Plastic	16
Medicine/Vitamins	13
Balloon/Latex	8
Musty/Chalky/Dusty/Stale/Rotten	8
Woody	6
Unpleasant/Stinky/Foul/Offensive/Rotten	6
Pungent/Sharp	5
Bitter	4
Chemical	4
Meaty/Sulfur	2
Iron	2
Leather	1
Lingering	1

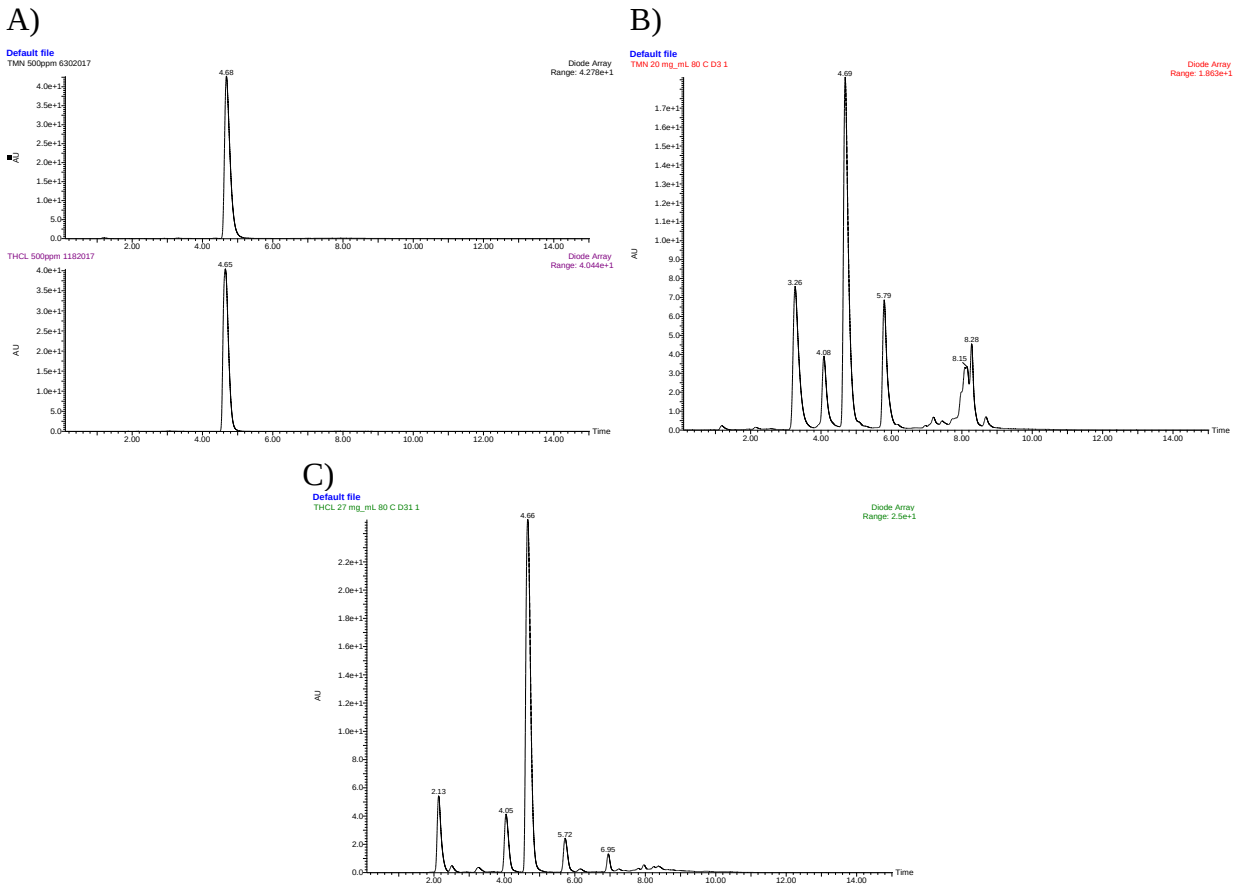

Figure A.2.1 Example of HPLC chromatograms of A) TMN and TClHCl at day 0, B) TMN with degradation products, and C) TClHCl with degradation products.

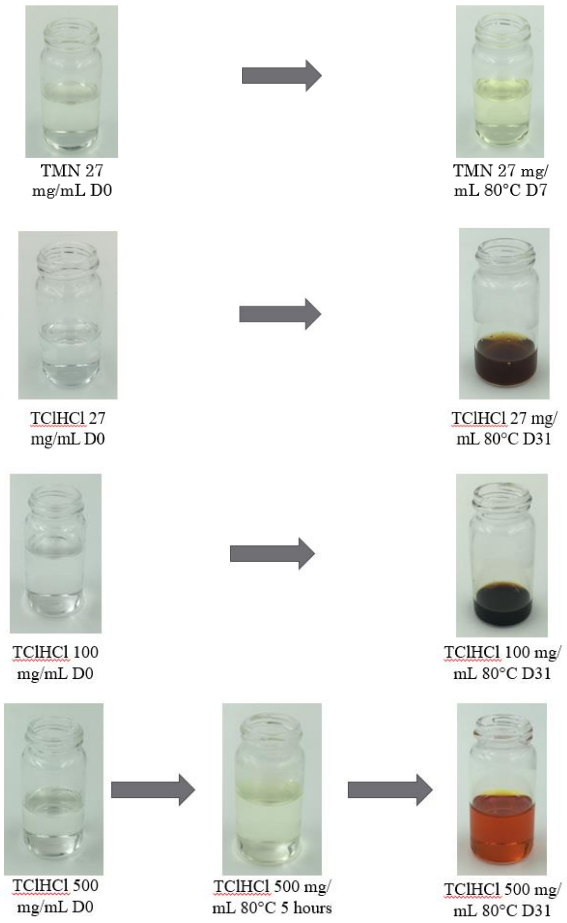

Figure A.2.2 Photographs of selected TMN and TClHCl samples used to determine LAB values indicating differences in degradation patterns based on color change.

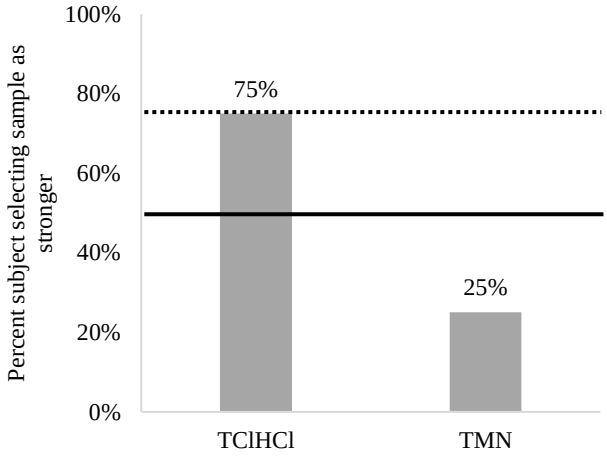

Figure A.2.3 75% of the sensory panelists chose TClHCl as the stronger smelling sample when compared to TMN. The solid line indicates chance performance at 50%, and the dotted line indicates the percent identifications required to reach significance (75%).

Table A.3.1 Percent TMN remaining after storage at the specified conditions over time: A) 25ºC, B) 40ºC, C) 60ºC, D) 70ºC, and E) 80ºC. Uppercase superscript letters on values for each sample type denote statistical significance within that sample type (down columns). Lowercase superscript letters denote statistical significance between sample types for each day (across rows).

A)

TMN 25ºC
Percent Vitamin Remaining

Days	pH 3				pH 6			
	TMN with HNO_3		TMN with HCl		TMN with HNO_3		TMN with HCl	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	100.1 ± 0.3% ^{Aa}	100. ± 2% ^{Aa}	100.1 ± 0.3% ^{Aa}	100.0 ± 0.7% ^{Aa}	100.1 ± 0.8% ^{Aa}	100. ± 2% ^{Aa}	100.0 ± 0.4% ^{Aa}	100.1 ± 0.5% ^{Aa}
7	99.1 ± 0.6% ^{ABabc}	98.7 ± 0.4% ^{ABabc}	98 ± 1% ^{Ac}	100.3 ± 0.4% ^{Aa}	98.5 ± 0.4% ^{Abc}	99.3 ± 0.1% ^{Aabc}	98.1 ± 0.2% ^{ABc}	99.9 ± 0.4% ^{Aab}
28	99.1 ± 0.6% ^{ABa}	98.0 ± 0.3% ^{BCa}	98.2 ± 0.4% ^{Aa}	99.2 ± 0.2% ^{Aa}	96.5 ± 0.6% ^{Aa}	99 ± 4% ^{Aa}	95.3 ± 0.6% ^{Ba}	100. ± 3% ^{Aa}
63	97 ± 2% ^{Bab}	97.6 ± 0.9% ^{BCab}	96 ± 3% ^{Aab}	99.6 ± 0.2% ^{Aa}	90. ± 3% ^{Bc}	93 ± 1% ^{Bbc}	89 ± 1% ^{CDc}	93.1 ± 0.7% ^{Bbc}
91	99.3 ± 0.7% ^{Aa}	96.5 ± 0.9% ^{Ca}	99.7 ± 0.7% ^{Aa}	99 ± 1% ^{Aa}	89.6 ± 0.9% ^{Bb}	65 ± 3% ^{Cc}	90.0 ± 0.9% ^{Cb}	64 ± 2% ^{Cc}
119	99.3 ± 0.7% ^{Aa}	96.8 ± 0.4% ^{BCa}	98.3 ± 0.4% ^{Aa}	100. ± 1% ^{Aa}	87 ± 1% ^{BCb}	50.1 ± 0.1% ^{Dc}	88 ± 3% ^{CDb}	50. ± 1% ^{Dc}
140	99.5 ± 0.7% ^{Aa}	96.6 ± 0.7% ^{BCa}	99.3 ± 0.9% ^{Aa}	100. ± 1% ^{Aa}	85 ± 2% ^{BCb}	46.1 ± 0.5% ^{DEc}	86 ± 1% ^{DEb}	46.5 ± 0.6% ^{DEc}
161	99 ± 1% ^{ABa}	96.4 ± 0.1% ^{Cb}	99 ± 1% ^{Aab}	98.6 ± 0.9% ^{Aab}	83 ± 1% ^{Cc}	43.8 ± 0.2% ^{Ed}	85 ± 1% ^{Ec}	44.3 ± 0.4% ^{Ed}
392	98.0 ± 0.5% ^{ABa}	97.29 ± 0.06% ^{BCa}	91 ± 2% ^{Bb}	99.6 ± 0.2% ^{Aa}	64 ± 4% ^{Dd}	38.48 ± 0.06% ^{Fe}	76.1 ± 0.7% ^{Fc}	39.1 ± 0.2% ^{Fe}

B)

TMN 40ºC
Percent Vitamin Remaining

Days	pH 3				pH 6			
	TMN with HNO_3		TMN with HCl		TMN with HNO_3		TMN with HCl	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	100.1 ± 0.3% ^{Aa}	100. ± 2% ^{Aa}	100.1 ± 0.3% ^{ABCa}	100.0 ± 0.7% ^{Aa}	100.1 ± 0.8% ^{Aa}	100. ± 2% ^{Aa}	100.0 ± 0.4% ^{Aa}	100.1 ± 0.5% ^{Aa}
7	98.7 ± 0.6% ^{ABa}	97.2 ± 0.5% ^{Abc}	98.6 ± 0.5% ^{ABCa}	98.4 ± 0.2% ^{Aab}	96.8 ± 0.8% ^{ABc}	96.1 ± 0.2% ^{Bcd}	95.3 ± 0.2% ^{Bd}	96.5 ± 0.2% ^{Bcd}
28	97.9 ± 0.2% ^{ABa}	95.6 ± 0.4% ^{Aab}	97.4 ± 0.1% ^{ABCa}	99 ± 4% ^{Aa}	91.5 ± 0.2% ^{Bbc}	47 ± 2% ^{Cd}	89.1 ± 0.4% ^{Cc}	47.3 ± 0.1% ^{Cd}
42	-	-	-	-	84 ± 4% ^{Ca}	42.3 ± 0.1% ^{Db}	82 ± 1% ^{Da}	42.6 ± 0.5% ^{Db}
63	96.3 ± 0.9% ^{Ba}	95.0 ± 0.5% ^{Aa}	96 ± 1% ^{BCa}	96.9 ± 0.7% ^{Aa}	84.3 ± 0.9% ^{Cb}	38.9 ± 0.1% ^{Ed}	80. ± 2% ^{DEc}	39.00 ± 0.02 ^{Ed}
91	98.4 ± 0.4% ^{ABa}	99 ± 4% ^{Aa}	103 ± 3% ^{Aa}	99 ± 1% ^{Aa}	83.4 ± 0.6% ^{CDb}	37.1 ± 0.3% ^{EFc}	81 ± 1% ^{Db}	38.1 ± 0.5% ^{Ec}
119	98.2 ± 0.7% ^{ABa}	95 ± 1% ^{Ab}	97 ± 2% ^{ABCab}	97 ± 1% ^{Aab}	79.3 ± 0.9% ^{CDEc}	36.1 ± 0.3% ^{FGd}	77.0 ± 0.9% ^{EFc}	36.8 ± 0.3% ^{Fd}
140	100. ± 2% ^{Aa}	98 ± 5% ^{Aa}	101 ± 4% ^{ABa}	96.9 ± 0.9% ^{Aa}	78 ± 1% ^{DEb}	35.5 ± 0.1% ^{FGc}	75 ± 1% ^{Fb}	35.9 ± 0.3% ^{Fc}
161	98 ± 1% ^{ABab}	95 ± 1% ^{Ab}	99 ± 3% ^{ABCa}	95.9 ± 0.7% ^{Aab}	75.5 ± 0.9% ^{Ec}	34.6 ± 0.2% ^{Gd}	74.2 ± 0.8% ^{Fc}	34.8 ± 0.2% ^{Gd}
392	92 ± 2% ^{Ca}	93.8 ± 0.3% ^{Aa}	95 ± 2% ^{Ca}	96 ± 4% ^{Aa}	54 ± 5% ^{Fb}	31.2 ± 0.8% ^{Hc}	56 ± 1% ^{Gb}	30.73 ± 0.02% ^{Hc}

Table A.3.1 continued

C)

TMN 60ºC
Percent Vitamin Remaining

Days	pH 3				pH 6			
	TMN with HNO_3		TMN with HCl		TMN with HNO_3		TMN with HCl	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	100.1 ± 0.3% ^{Aa}	100. ± 2% ^{Aa}	100.1 ± 0.3% ^{Aa}	100.0 ± 0.7% ^{Aa}	99.9 ± 0.4% ^{Aa}	100. ± 1% ^{Aa}	99.9 ± 0.6% ^{Aa}	100.1 ± 0.7% ^{Aa}
1	-	-	-	-	98.2 ± 0.4% ^{Aa}	94.4 ± 0.1% ^{Bc}	98.1 ± 0.2% ^{Ba}	95.2 ± 0.1% ^{Bb}
2	-	-	-	-	-	59.2 ± 0.1% ^{Cb}	-	64.2 ± 0.5% ^{Ca}
3	-	-	-	-	85.1 ± 0.5% ^{Bb}	41.5 ± 0.2% ^{Dd}	90.9 ± 0.1% ^{Ca}	43.9 ± 0.2% ^{Dc}
7	97.5 ± 0.8% ^{Aa}	98 ± 3% ^{Aba}	97.3 ± 0.9% ^{Aba}	97.9 ± 0.3% ^{Aba}	78.6 ± 0.4% ^{Cc}	36.9 ± 0.3% ^{Ed}	85 ± 1% ^{Db}	39.3 ± 0.2% ^{Ed}
12	-	-	-	-	71 ± 2% ^{Db}	34.9 ± 0.1% ^{Fc}	75.2 ± 0.2% ^{Ea}	36.83 ± 0.08% ^{Fc}
16	-	-	-	-	66 ± 2% ^{Eb}	32.3 ± 0.4% ^{Gc}	70.5 ± 0.2% ^{Fa}	34.9 ± 0.8% ^{Gc}
21	-	-	-	-	63 ± 2% ^{Eb}	31.7 ± 0.5% ^{Gd}	67.2 ± 0.6% ^{Ga}	34.2 ± 0.6% ^{Gc}
28	94 ± 1% ^{Aa}	92 ± 1% ^{Ba}	94 ± 3% ^{Aba}	95 ± 1% ^{Aba}	-	-	-	-
63	84.5 ± 0.9% ^{Ba}	92 ± 3% ^{Ba}	87 ± 8% ^{Bca}	94 ± 2% ^{Ba}	-	-	-	-
91	81 ± 4% ^{Ba}	82 ± 3% ^{Ca}	80. ± 6% ^{Cda}	83 ± 3% ^{Ca}	-	-	-	-
119	74 ± 3% ^{Ca}	75 ± 2% ^{Da}	70.6 ± 0.2% ^{Dea}	75 ± 4% ^{Da}	-	-	-	-
140	71 ± 3% ^{Cdab}	72 ± 2% ^{Deab}	67.2 ± 0.1% ^{Eb}	72 ± 2% ^{Dea}	-	-	-	-
161	67 ± 3% ^{Da}	66.2 ± 0.9% ^{Ea}	65 ± 2% ^{Ea}	67 ± 2% ^{Ea}	-	-	-	-
392	39 ± 2% ^{Ea}	38 ± 2% ^{Fa}	38 ± 5% ^{Fa}	39 ± 2% ^{Fa}	-	-	-	-

D)

TMN 70ºC
Percent Vitamin Remaining

Days	pH 3				pH 6			
	TMN with HNO_3		TMN with HCl		TMN with HNO_3		TMN with HCl	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	100.1 ± 0.1% ^{Aa}	100. ± 1% ^{Aa}	100.1 ± 0.3% ^{Aa}	99.9 ± 0.5% ^{Aa}	99.9 ± 0.3% ^{Aa}	100. ± 1% ^{Aa}	99.9 ± 0.6% ^{Aa}	100.1 ± 0.7% ^{Aa}
1	91.9 ± 0.7% ^{Bcd}	95.0 ± 0.7% ^{Bcab}	97 ± 2% ^{Aa}	93.65 ± 0.08% ^{Bbc}	85 ± 1% ^{Be}	50. ± 1% ^{Bg}	89.8 ± 0.7% ^{Bd}	53.5 ± 0.9% ^{Bf}
2	91.2 ± 0.4% ^{Bc}	95.6 ± 0.5% ^{Bcab}	97.8 ± 0.9% ^{Aa}	93 ± 2% ^{Bbc}	81 ± 1% ^{Ce}	42.19 ± 0.02% ^{Cf}	87 ± 1% ^{Cd}	44.8 ± 0.2% ^{Cf}
4	93 ± 2% ^{Bc}	95.9 ± 0.3% ^{BCb}	100. ± 2% ^{Aa}	94.3 ± 0.4% ^{Bbc}	75.6 ± 0.4% ^{De}	38.2 ± 0.5% ^{Df}	80.2 ± 0.2% ^{Dd}	40.3 ± 0.2% ^{Df}
7	92 ± 3% ^{Bb}	97.4 ± 0.3% ^{Aba}	97.9 ± 0.1% ^{Aa}	93.4 ± 0.7% ^{Bb}	67.8 ± 0.7% ^{Ed}	34.8 ± 0.3% ^{Ee}	72.2 ± 0.7% ^{Ec}	37.3 ± 0.3% ^{Ee}
13	88 ± 3% ^{Bb}	94.6 ± 0.7% ^{Ca}	93.0 ± 0.5% ^{Ba}	91 ± 1% ^{Ba}	53.6 ± 0.9% ^{Fd}	30.6 ± 0.1% ^{Fe}	57.2 ± 0.7% ^{Fc}	32.3 ± 0.5% ^{Fe}
29	76 ± 3% ^{Cc}	84.2 ± 0.8% ^{Da}	79.8 ± 0.5% ^{Cbc}	80.6 ± 0.3% ^{Cab}	-	-	-	-
42	68 ± 2% ^{Db}	75 ± 2% ^{Ea}	70. ± 2% ^{Dab}	71 ± 3% ^{Dab}	-	-	-	-

Table A.3.1 continued

E)

TMN 80ºC
Percent Vitamin Remaining

Days	pH 3				pH 6			
	TMN with HNO_3		TMN with HCl		TMN with HNO_3		TMN with HCl	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	99.0 ± 0.5% ^{Ab}	99 ± 1% ^{Ab}	100.1 ± 0.6% ^{Ab}	101.9 ± 0.5% ^{Aa}	98.3 ± 0.6% ^{Ab}	98.9 ± 0.1% ^{Ab}	102.5 ± 0.6% ^{Aa}	102.5 ± 0.2% ^{Aa}
1	98 ± 2% ^{Aa}	98 ± 1% ^{Aa}	95 ± 2% ^{Ba}	97.9 ± 0.6% ^{Ba}	90.8 ± 0.7% ^{Bb}	51.0 ± 0.5% ^{Bd}	85 ± 2% ^{Bc}	50. ± 1% ^{Bd}
2	97 ± 2% ^{Aa}	97.8 ± 0.9% ^{Aa}	97.7 ± 0.2% ^{ABa}	97.1 ± 0.6% ^{Ba}	74 ± 3% ^{Cb}	38 ± 2% ^{Cc}	75 ± 3% ^{Cb}	42 ± 3% ^{Cc}
4	93 ± 3% ^{Aa}	96 ± 3% ^{Aa}	88 ± 2% ^{Cb}	93.1 ± 0.4% ^{Cab}	58.9 ± 0.8% ^{Dc}	37.7 ± 0.3% ^{Cd}	54.7 ± 0.6% ^{Dc}	37 ± 2% ^{CDd}
7	87 ± 3% ^{Ba}	90. ± 3% ^{Ba}	81.9 ± 0.5% ^{Db}	89 ± 1% ^{Da}	42.5 ± 0.6% ^{Ec}	32.17 ± 0.04% ^{Dd}	41.4 ± 0.5% ^{Ec}	32 ± 2% ^{Dd}
13	73 ± 2% ^{Cab}	74 ± 3% ^{Cab}	71 ± 1% ^{Eb}	78 ± 2% ^{Ea}	37.3 ± 0.7% ^{Fc}	24.9 ± 0.3% ^{Ed}	25.2 ± 0.4% ^{Fd}	26 ± 3% ^{Ed}
29	48.8 ± 0.1% ^{Db}	56 ± 2% ^{Da}	47.9 ± 0.7% ^{Fb}	55 ± 1% ^{Fa}	-	-	-	-

Table A.3.2 Percent TClHCl remaining after storage at the specified conditions over time: A) 25ºC, B) 40ºC, C) 60ºC, D) 70ºC, and E) 80ºC. Uppercase superscript letters on values for each sample type denote statistical significance within that sample type (down columns). Lowercase superscript letters denote statistical significance between sample types for each day (across rows).

A)

TClHCl 25ºC
Percent Vitamin Remaining

Days	pH 3				pH 6			
	TClHCl with HCl		TClHCl with HNO_3		TClHCl with HCl		TClHCl with HNO_3	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	100.0 ± 0.4% ^{Aa}	100.1 ± 0.6% ^{BCa}	100.1 ± 0.3% ^{Aa}	100.0 ± 0.2% ^{Ca}	100.0 ± 0.4% ^{Aa}	100.1 ± 0.9% ^{Aa}	100.1 ± 0.5% ^{Aa}	100.1 ± 0.4% ^{Aa}
7	99.0 ± 0.2% ^{Aab}	96 ± 3% ^{Dab}	99.1 ± 0.5% ^{Aab}	95 ± 4% ^{Db}	99.1 ± 0.2% ^{Aab}	99.0 ± 0.4% ^{Aab}	99.4 ± 0.5% ^{ABab}	101.0 ± 0.2% ^{Aa}
28	99.9 ± 0.1% ^{Aab}	102.1 ± 0.2% ^{ABCa}	95 ± 1% ^{Bb}	98.3 ± 0.1% ^{CDab}	94 ± 5% ^{ABb}	99.8 ± 0.2% ^{Aab}	96 ± 2% ^{ABCab}	101 ± 2% ^{Aa}
63	101 ± 2% ^{Aab}	105.5 ± 0.6% ^{Aa}	99 ± 2% ^{ABbc}	105.4 ± 0.6% ^{Aa}	94.0 ± 0.8% ^{ABc}	99 ± 1% ^{Abc}	96 ± 2% ^{ABCbc}	98 ± 5% ^{Abc}
91	100.0 ± 0.5% ^{Aab}	100.1 ± 0.5% ^{BCab}	101.9 ± 0.3% ^{Aa}	100.1 ± 0.5% ^{BCab}	93 ± 2% ^{ABb}	72 ± 6% ^{Bc}	95.2 ± 0.8% ^{BCab}	72 ± 5% ^{Bc}
119	100.9 ± 0.1% ^{Aa}	101.2 ± 0.7% ^{BCa}	101.7 ± 0.5% ^{Aa}	101.1 ± 0.2% ^{BCa}	89 ± 4% ^{BCb}	52 ± 2% ^{Cc}	93 ± 1% ^{CDb}	52 ± 3% ^{Cc}
140	99.6 ± 0.2% ^{Aa}	99.9 ± 0.5% ^{Ca}	99.7 ± 0.3% ^{Aa}	99.4 ± 0.3% ^{Ca}	84 ± 4% ^{CDc}	46.9 ± 0.9% ^{CDd}	89 ± 2% ^{Db}	47 ± 2% ^{CDd}
161	101.1 ± 0.3% ^{Aa}	100.1 ± 0.4% ^{BCa}	101.4 ± 0.4% ^{Aa}	100.1 ± 0.1% ^{BCa}	79 ± 1% ^{Dc}	42.6 ± 0.6% ^{DEd}	89 ± 2% ^{Db}	43.8 ± 0.8% ^{Dd}
392	100. ± 5% ^{Aa}	103.5 ± 0.1% ^{ABa}	101 ± 4% ^{Aa}	103.8 ± 0.1% ^{ABa}	68 ± 2% ^{Eb}	39.1 ± 0.1% ^{Ec}	73 ± 2% ^{Eb}	40.0 ± 0.2% ^{Dc}

Table A.3.2 continued

B)

TClHCl 40ºC
Percent Vitamin Remaining

Days	pH 3				pH 6			
	TClHCl with HCl		TClHCl with HNO_3		TClHCl with HCl		TClHCl with HNO_3	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	100.0 ± 0.4% ^{ABa}	100.1 ± 0.6% ^{ABa}	100.1 ± 0.3% ^{ABCa}	100.0 ± 0.2% ^{ABa}	100.0 ± 0.4% ^{Aa}	100.1 ± 0.9% ^{Aa}	100.1 ± 0.5% ^{Aa}	100.1 ± 0.4% ^{Aa}
7	98.6 ± 0.1% ^{ABab}	98.43 ± 0.03% ^{Bbc}	99.1 ± 0.2% ^{ABCa}	98.0 ± 0.2% ^{Bcd}	97.5 ± 0.1% ^{Ad}	95.3 ± 0.4% ^{Bf}	97.6 ± 0.2% ^{ABd}	96.8 ± 0.3% ^{Be}
28	100.5 ± 0.3% ^{ABa}	97.8 ± 0.3% ^{Bab}	95 ± 3% ^{Cbc}	99.8 ± 0.9% ^{ABa}	93 ± 2% ^{Bc}	46.0 ± 0.7% ^{Cd}	95 ± 1% ^{Bbc}	46.0 ± 0.1% ^{Cd}
42	-	-	-	-	91.2 ± 0.9% ^{Ba}	43.40 ± 0.9% ^{Db}	89 ± 3% ^{Ca}	44.2 ± 0.1% ^{Db}
63	100.5 ± 0.6% ^{ABa}	103.0 ± 0.7% ^{Aa}	100. ± 3% ^{ABCa}	104 ± 2% ^{Aa}	85 ± 3% ^{CDb}	41.42 ± 0.6% ^{Ec}	86 ± 2% ^{Cb}	42.2 ± 0.2% ^{Ec}
91	101 ± 1% ^{ABab}	99.6 ± 0.4% ^{ABb}	104 ± 3% ^{Aa}	103 ± 1% ^{Aab}	89 ± 3% ^{BCc}	38.7 ± 0.5% ^{Fd}	85.2 ± 0.5% ^{CDc}	39.2 ± 0.2% ^{Fd}
119	101 ± 2% ^{Aa}	101 ± 3% ^{ABa}	100. ± 1% ^{ABCa}	101 ± 2% ^{ABa}	82.4 ± 0.3% ^{DEb}	37.7 ± 0.2% ^{FGc}	81.5 ± 0.2% ^{DEb}	38.4 ± 0.3% ^{FGc}
140	100. ± 2% ^{ABa}	99 ± 3% ^{ABa}	100. ± 1% ^{ABCa}	101 ± 3% ^{ABa}	79.0 ± 0.3% ^{Eb}	36.7 ± 0.1% ^{Gc}	78.8 ± 0.3% ^{Eb}	37.5 ± 0.3% ^{GHc}
161	101 ± 2% ^{ABa}	99 ± 2% ^{ABa}	101 ± 1% ^{ABa}	101 ± 2% ^{ABa}	78.6 ± 0.2% ^{Eb}	36.6 ± 0.2% ^{Gc}	78.5 ± 0.1% ^{Eb}	37.4 ± 0.4% ^{Hc}
392	96 ± 3% ^{Ba}	99.8 ± 0.4% ^{ABa}	98 ± 2% ^{BCa}	101.3 ± 0.2% ^{ABa}	57 ± 3% ^{Fb}	32.1 ± 0.1% ^{Hc}	59 ± 3% ^{Fb}	33.1 ± 0.4% ^{Ic}

C)

TClHCl 60ºC
Percent Vitamin Remaining

Days	pH 3				pH 6			
	TClHCl with HCl		TClHCl with HNO_3		TClHCl with HCl		TClHCl with HNO_3	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	100.0 ± 0.4% ^{Aa}	100.1 ± 0.6% ^{Aa}	100.1 ± 0.3% ^{Aa}	100.0 ± 0.2% ^{Aa}	100.1 ± 0.4% ^{Aa}	99.9 ± 0.4% ^{Aa}	100. ± 1% ^{Aa}	100.1 ± 0.3% ^{Aa}
1	-	-	-	-	99.3 ± 0.3% ^{Aa}	96.1 ± 0.2% ^{Bb}	99.6 ± 0.2% ^{Aa}	96.3 ± 0.4% ^{Bb}
2	-	-	-	-	-	79.0 ± 0.6% ^{Ca}	-	79.3 ± 0.4% ^{Ca}
3	-	-	-	-	85.1 ± 0.4% ^{Bb}	45.6 ± 0.4% ^{Dc}	86.5 ± 0.6% ^{Ba}	45.1 ± 0.3% ^{Dc}
7	99.0 ± 0.2% ^{ABa}	100.0 ± 0.9% ^{Aa}	99.3 ± 0.5% ^{Aa}	99.1 ± 0.4% ^{Aa}	83.4 ± 0.2% ^{Cb}	42.1 ± 0.3% ^{Ec}	84.5 ± 0.3% ^{Cb}	42.0 ± 0.2% ^{Ec}
12	-	-	-	-	75.67 ± 0.09% ^{Db}	39.4 ± 0.2% ^{Fc}	76.6 ± 0.1% ^{Da}	39.0 ± 0.3% ^{Fc}
16	-	-	-	-	69.1 ± 0.1% ^{Ea}	37.80 ± 0.06% ^{Gb}	69.6 ± 0.3% ^{Ea}	38.5 ± 0.2% ^{Gb}
21	-	-	-	-	66.7 ± 0.4% ^{Fa}	36.6 ± 0.3% ^{Hb}	67.6 ± 0.1% ^{Fa}	36.0 ± 0.5% ^{Hb}
28	95.0 ± 0.6% ^{Ba}	97 ± 1% ^{Aa}	98 ± 3% ^{Aa}	95 ± 4% ^{Aa}	-	-	-	-
63	88 ± 2% ^{Cb}	97 ± 3% ^{Aa}	92 ± 2% ^{Bab}	97 ± 1% ^{Aa}	-	-	-	-
91	78 ± 2% ^{Da}	84 ± 4% ^{Aa}	83 ± 1% ^{Ca}	82 ± 1% ^{Ba}	-	-	-	-
119	71 ± 2% ^{Ea}	75 ± 5% ^{Ca}	74.2 ± 0.4% ^{Da}	74 ± 2% ^{Ca}	-	-	-	-
140	67 ± 2% ^{EFa}	72 ± 4% ^{CDa}	71.0 ± 0.6% ^{Da}	70. ± 1% ^{CDa}	-	-	-	-
161	63 ± 2% ^{Fa}	66 ± 4% ^{Da}	67.1 ± 0.6% ^{Ea}	65.4 ± 0.3% ^{Da}	-	-	-	-
392	35 ± 2% ^{Ga}	38 ± 3% ^{Ea}	39 ± 1% ^{Fa}	36 ± 2% ^{Ea}	-	-	-	-

Table A.3.2 continued

D)

TClHCl 70ºC
Percent Vitamin Remaining

Days	pH 3				pH 6			
	TClHCl with HCl		TClHCl with HNO_3		TClHCl with HCl		TClHCl with HNO_3	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	100.1 ± 0.4% ^{Aa}	100. ± 1% ^{Aa}	100.0 ± 0.2% ^{Aa}	99.9 ± 0.8% ^{Aa}	100.1 ± 0.4% ^{Aa}	99.9 ± 0.4% ^{Aa}	100. ± 1% ^{Aa}	100.1 ± 0.3% ^{Aa}
1	99.9 ± 0.7% ^{Aa}	100.1 ± 0.2% ^{Aa}	100. ± 3% ^{Aa}	101.1 ± 0.5% ^{Aa}	91 ± 1% ^{Bb}	55 ± 1% ^{Bc}	92 ± 1% ^{Bb}	53.6 ± 0.7% ^{Bc}
2	99.9 ± 0.2% ^{Aa}	100.3 ± 0.5% ^{Aa}	100. ± 1% ^{Aa}	101.2 ± 0.2% ^{Aa}	87.2 ± 0.8% ^{Cb}	46.4 ± 0.3% ^{Cc}	87.8 ± 0.7% ^{Cb}	46.1 ± 0.1% ^{Cc}
4	99 ± 2% ^{Aa}	99 ± 1% ^{Aa}	100.3 ± 0.4% ^{Aa}	100.2 ± 0.4% ^{Aa}	78.5 ± 0.7% ^{Db}	41.6 ± 0.1% ^{Dc}	79.0 ± 0.7% ^{Db}	41.5 ± 0.1% ^{Dc}
7	98 ± 2% ^{ABa}	98.4 ± 0.6% ^{Aa}	99.4 ± 0.7% ^{Aa}	100. ± 1% ^{Aa}	68.7 ± 0.8% ^{Eb}	38.1 ± 0.2% ^{Ec}	69.1 ± 0.1% ^{Eb}	37.88 ± 0.07% ^{Ec}
13	94 ± 2% ^{Bb}	95.3 ± 0.8% ^{Bab}	98 ± 2% ^{Aa}	97 ± 1% ^{Bab}	53.4 ± 0.7% ^{Fc}	32.6 ± 0.3% ^{Fd}	53.8 ± 0.2% ^{Fc}	32.5 ± 0.3% ^{Fd}
29	81 ± 4% ^{Ca}	86 ± 1% ^{Ca}	85 ± 3% ^{Ba}	85 ± 1% ^{Ca}	-	-	-	-
42	69.0 ± 0.8% ^{Db}	75 ± 1% ^{Da}	75.4 ± 0.3% ^{Ca}	76 ± 1% ^{Da}	-	-	-	-

E)

TClHCl 80ºC
Percent Vitamin Remaining

Days	pH 3				pH 6			
	TClHCl with HCl		TClHCl with HNO_3		TClHCl with HCl		TClHCl with HNO_3	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	98.8 ± 0.5% ^{Abc}	96.84 ± 0.05% ^{Ad}	99.7 ± 0.6% ^{Aab}	99.7 ± 0.8% ^{Aab}	100.04 ± 0.08% ^{Aab}	97.8 ± 0.4% ^{Acd}	100.6 ± 0.5% ^{Aa}	99.2 ± 0.7% ^{Aabc}
1	98.0 ± 0.8% ^{Aa}	98.8 ± 0.2% ^{Aa}	96.9 ± 0.8% ^{ABa}	97.5 ± 0.1% ^{Aa}	86.5 ± 0.3% ^{Bb}	49 ± 1% ^{Bc}	85 ± 2% ^{Bb}	46.1 ± 0.8% ^{Bc}
2	-	-	-	-	74.4 ± 0.6% ^{Cb}	42 ± 3% ^{Bc}	81 ± 2% ^{Ba}	42 ± 2% ^{Cc}
4	89.7 ± 0.7% ^{Bb}	89 ± 2% ^{Bb}	93 ± 2% ^{BCab}	94.3 ± 0.1% ^{Ba}	57.7 ± 0.7% ^{Dc}	35 ± 3% ^{Cd}	58.1 ± 0.6% ^{Cc}	35 ± 2% ^{Dd}
7	86 ± 4% ^{Ba}	89 ± 1% ^{Ba}	89 ± 4% ^{Ca}	91.8 ± 0.8% ^{Ba}	42.8 ± 0.9% ^{Ec}	30. ± 3% ^{CDd}	51 ± 4% ^{Db}	29 ± 2% ^{Ed}
13	73 ± 2% ^{Cc}	75 ± 1% ^{Cbc}	79.3 ± 0.4% ^{Dab}	81 ± 1% ^{Ca}	24.6 ± 0.6% ^{Fe}	25 ± 4% ^{De}	41 ± 2% ^{Ed}	23 ± 1% ^{Fe}
29	48.9 ± 0.4% ^{Dc}	56 ± 2% ^{Db}	60. ± 3% ^{Eab}	63 ± 2% ^{Da}	-	-	-	-

Table A.3.3 pH of TMN solutions after storage at the specified conditions over time: A) 25ºC, B) 40ºC, C) 60ºC, D) 70ºC, and E) 80ºC. Uppercase superscript letters on values for each sample type denote statistical significance within that sample type (down columns). Lowercase superscript letters denote statistical significance between sample types for each day (across rows).

A)

TMN 25ºC
pH Over Time

Days	pH 3				pH 6			
	TMN with HNO_3		TMN with HCl		TMN with HNO_3		TMN with HCl	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	3^{BCb}	3^{Bb}	3^{Db}	3^{Ab}	6^{Aa}	6^{Ba}	6^{Aa}	6^{Ba}
7	3.042 ± 0.003^{Ad}	3.008 ± 0.003^{Be}	3.036 ± 0.004^{Cd}	2.999 ± 0.003^{Ae}	5.913 ± 0.004^{Ac}	6.033 ± 0.004^{Aa}	5.965 ± 0.004^{Bb}	6.020 ± 0.003^{Aa}
63	2.98 ± 0.02^{Ce}	2.966 ± 0.004^{Ce}	3.024 ± 0.005^{Cd}	2.992 ± 0.004^{ABde}	5.7105 ± 0.0007^{Bb}	5.444 ± 0.006^{Cc}	5.753 ± 0.004^{Ca}	5.468 ± 0.004^{Cc}
161	3.033 ± 0.003^{ABf}	3.033 ± 0.002^{Af}	3.096 ± 0.004^{Be}	2.997 ± 0.008^{Ag}	5.315 ± 0.004^{Ca}	4.963 ± 0.004^{Dc}	5.285 ± 0.004^{Db}	4.905 ± 0.003^{Dd}
392	3.035 ± 0.004^{ABd}	2.998 ± 0.003^{Bd}	3.144 ± 0.004^{Ac}	2.975 ± 0.005^{Bd}	4.93 ± 0.05^{Db}	4.914 ± 0.002^{Eb}	5.078 ± 0.006^{Ea}	4.911 ± 0.004^{Db}

B)

TMN 40ºC
pH Over Time

Days	pH 3				pH 6			
	TMN with HNO_3		TMN with HCl		TMN with HNO_3		TMN with HCl	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	3^{CBb}	3^{Ab}	3^{Cb}	3^{Ab}	6^{Aa}	6^{Aa}	6^{Aa}	6^{Aa}
7	3.055 ± 0.005^{Ac}	3.00 ± 0.01^{Ac}	2.99 ± 0.01^{Cc}	2.98 ± 0.01^{ABc}	5.740 ± 0.002^{Bb}	5.891 ± 0.003^{Ba}	5.76 ± 0.03^{Bb}	5.95 ± 0.05^{Aa}
63	3.02 ± 0.02^{ABc}	2.942 ± 0.004^{Bd}	3.026 ± 0.005^{Bc}	2.94 ± 0.01^{Cd}	5.220 ± 0.003^{Ca}	4.920 ± 0.004^{Cb}	5.248 ± 0.006^{Ca}	4.913 ± 0.008^{Bb}
161	3.027 ± 0.005^{ABd}	2.945 ± 0.002^{Be}	3.078 ± 0.004^{Ac}	2.959 ± 0.004^{BCe}	5.110 ± 0.004^{Da}	4.90 ± 0.01^{Cb}	5.120 ± 0.002^{Da}	4.901 ± 0.004^{Bb}
392	2.970 ± 0.005^{Cf}	2.780 ± 0.002^{Cg}	3.009 ± 0.003^{BCe}	2.749 ± 0.004^{Dh}	4.965 ± 0.003^{Eb}	4.824 ± 0.003^{Dd}	4.999 ± 0.003^{Ea}	4.88 ± 0.02^{Bc}

Table A.3.3 continued

C)

TMN 60ºC
pH Over Time

Days	pH 3				pH 6			
	TMN with HNO_3		TMN with HCl		TMN with HNO_3		TMN with HCl	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	3^{Ab}	3^{Ab}	3^{Ab}	3^{Ab}	6^{Ba}	6^{Aa}	6^{Ba}	6^{Aa}
1	-	-	-	-	6.23 ± 0.09^{Aa}	5.8445 ± 0.0007^{Bb}	6.106 ± 0.003^{Aa}	5.823 ± 0.005^{Bb}
3	-	-	-	-	5.676 ± 0.003^{Ca}	5.039 ± 0.005^{Cc}	5.654 ± 0.006^{Cb}	5.041 ± 0.004^{Cc}
7	3.02 ± 0.01^{Aa}	2.939 ± 0.008^{Ab}	3.01 ± 0.01^{Aa}	2.921 ± 0.002^{Bb}	-	-	-	-
12	-	-	-	-	5.356 ± 0.003^{Da}	4.931 ± 0.004^{Db}	5.376 ± 0.004^{Da}	4.86 ± 0.05^{Db}
21	-	-	-	-	5.270 ± 0.003^{Da}	4.920 ± 0.003^{Dc}	5.241 ± 0.004^{Eb}	4.903 ± 0.002^{Dd}
63	2.956 ± 0.004^{Aa}	2.587 ± 0.003^{Bc}	2.941 ± 0.002^{Ab}	2.573 ± 0.002^{Cd}	-	-	-	-
161	2.49 ± 0.06^{Bb}	2.19 ± 0.09^{Cc}	2.84 ± 0.04^{Ba}	2.310 ± 0.009^{Dbc}	-	-	-	-
392	2.9 ± 0.1^{Aa}	2.0 ± 0.1^{Cb}	-	2.210 ± 0.003^{Eb}	-	-	-	-

D)

TMN 70ºC
pH Over Time

Days	pH 3				pH 6			
	TMN with HNO_3		TMN with HCl		TMN with HNO_3		TMN with HCl	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	3^{Bb}	3^{Ab}	3^{ABb}	3^{Ab}	6^{Aa}	6^{Aa}	6^{Aa}	6^{Aa}
1	3.00 ± 0.01^{Bcd}	2.99 ± 0.01^{Acd}	3.034 ± 0.009^{Ac}	2.8 ± 0.1^{ABd}	5.69 ± 0.02^{Ba}	5.173 ± 0.007^{Bb}	5.7195 ± 0.0007^{Ba}	5.140 ± 0.003^{Bb}
4	3.075 ± 0.006^{Ac}	2.977 ± 0.006^{Ad}	2.997 ± 0.006^{ABd}	2.893 ± 0.005^{ABe}	5.47 ± 0.01^{Ca}	5.063 ± 0.006^{Cb}	5.460 ± 0.004^{Ca}	5.06 ± 0.02^{Cb}
13	2.930 ± 0.003^{Ce}	2.828 ± 0.003^{Bf}	2.99 ± 0.02^{Bd}	2.804 ± 0.002^{Bf}	5.066 ± 0.002^{Db}	4.816 ± 0.003^{Dc}	5.120 ± 0.004^{Da}	4.831 ± 0.004^{Dc}
29	2.895 ± 0.004^{Db}	2.562 ± 0.003^{Cc}	2.922 ± 0.003^{Ca}	2.565 ± 0.006^{Cc}	-	-	-	-
42	2.897 ± 0.004^{Db}	2.5745 ± 0.0007^{Cc}	2.9275 ± 0.0007^{Ca}	2.517 ± 0.004^{Cd}	-	-	-	-

Table A.3.3 continued

E)

TMN 80ºC
pH Over Time

Days	pH 3				pH 6			
	TMN with HNO_3		TMN with HCl		TMN with HNO_3		TMN with HCl	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	3^{BCb}	3^{Ab}	3^{Bb}	3^{Ab}	6^{Aa}	6^{Aa}	6^{Aa}	6^{Aa}
1	3.20 ± 0.02^{Ae}	3.03 ± 0.01^{Af}	2.98 ± 0.04^{BCf}	2.77 ± 0.02^{Bg}	5.18 ± 0.06^{Cb}	4.63 ± 0.02^{Dd}	5.56 ± 0.04^{Ba}	5.00 ± 0.04^{Bc}
2	3.092 ± 0.001^{ABe}	2.965 ± 0.004^{Af}	3.158 ± 0.004^{Ad}	2.960 ± 0.004^{Af}	5.699 ± 0.005^{Ba}	5.080 ± 0.004^{Bc}	5.624 ± 0.004^{Bb}	5.092 ± 0.004^{Bc}
4	3.02 ± 0.03^{ABCc}	2.70 ± 0.03^{Be}	2.92 ± 0.03^{BCd}	2.64 ± 0.01^{Ce}	5.18 ± 0.03^{Ca}	4.83 ± 0.02^{Cb}	5.26 ± 0.01^{Ca}	4.845 ± 0.007^{Cb}
7	3.09 ± 0.05^{ABc}	2.62 ± 0.03^{Bd}	2.96 ± 0.03^{BCc}	2.61 ± 0.03^{Cd}	5.11 ± 0.05^{Ca}	4.82 ± 0.03^{Cb}	5.22 ± 0.02^{Ca}	4.78 ± 0.06^{Cb}
13	2.99 ± 0.08^{BCc}	2.41 ± 0.02^{Cd}	2.94 ± 0.02^{BCc}	2.42 ± 0.03^{Dd}	4.92 ± 0.02^{Da}	4.63 ± 0.04^{Db}	4.97 ± 0.04^{Da}	4.62 ± 0.02^{Db}
29	2.89 ± 0.06^{Ca}	2.30 ± 0.03^{Db}	2.87 ± 0.04^{Ca}	2.30 ± 0.01^{Eb}	-	-	-	-

Table A.3.4 pH of TClHCl solutions after storage at the specified conditions over time: A) 25ºC, B) 40ºC, C) 60ºC, D) 70ºC, and E) 80ºC. Uppercase superscript letters on values for each sample type denote statistical significance within that sample type (down columns). Lowercase superscript letters denote statistical significance between sample types for each day (across rows).

A)

TClHCl 25ºC
pH Over Time

Days	pH 3				pH 6			
	TClHCl with HCl		TClHCl with HNO_3		TClHCl with HCl		TClHCl with HNO_3	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	3^{Cb}	3^{Cb}	3^{Bb}	3^{Db}	6^{Ba}	6^{Ba}	6^{Ca}	6^{Ba}
7	3.067 ± 0.004^{Ad}	3.036 ± 0.005^{Be}	3.003 ± 0.004^{Bf}	3.076 ± 0.005^{Ad}	6.169 ± 0.003^{Ab}	6.073 ± 0.004^{Ac}	6.245 ± 0.003^{Aa}	6.081 ± 0.004^{Ac}
63	3.050 ± 0.004^{ABe}	3.035 ± 0.005^{Bef}	3.037 ± 0.006^{Aef}	3.025 ± 0.005^{Cf}	5.965 ± 0.004^{Cb}	5.905 ± 0.005^{Cc}	6.038 ± 0.004^{Ba}	5.862 ± 0.002^{Cd}
161	3.042 ± 0.005^{Bd}	3.007 ± 0.003^{Cf}	3.038 ± 0.004^{Ad}	3.024 ± 0.004^{Ce}	5.240 ± 0.004^{Db}	5.045 ± 0.003^{Dc}	5.396 ± 0.002^{Da}	5.042 ± 0.003^{Dc}
392	3.058 ± 0.006^{ABd}	3.054 ± 0.004^{Ad}	2.998 ± 0.003^{Be}	3.049 ± 0.004^{Bd}	4.980 ± 0.004^{Ec}	5.03 ± 0.01^{Db}	5.066 ± 0.004^{Ea}	4.9875 ± 0.0007^{Ec}

Table A.3.4 continued

B)

TClHCl 40ºC
pH Over Time

Days	pH 3				pH 6			
	TClHCl with HCl		TClHCl with HNO_3		TClHCl with HCl		TClHCl with HNO_3	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	3 ^{Ab}	3 ^{Ab}	3 ^{Cb}	3 ^{Bb}	6 ^{Aa}	6 ^{Aa}	6 ^{Ba}	6 ^{Aa}
7	2.96 ± 0.07 ^{Ac}	3.02 ± 0.01 ^{Ac}	3.007 ± 0.004 ^{BCc}	3.035 ± 0.002 ^{Ac}	6.009 ± 0.003 ^{Aa}	5.926 ± 0.005 ^{Bab}	6.019 ± 0.004 ^{Aa}	5.910 ± 0.008 ^{Bb}
63	2.993 ± 0.004 ^{Ae}	2.976 ± 0.004 ^{Bf}	3.0115 ± 0.0007 ^{BCd}	2.984 ± 0.004 ^{Cef}	5.305 ± 0.003 ^{Bb}	4.987 ± 0.004 ^{Cc}	5.345 ± 0.003 ^{Ca}	4.994 ± 0.004 ^{Cc}
161	3.048 ± 0.004 ^{Ae}	2.946 ± 0.004 ^{Cg}	3.034 ± 0.004 ^{Ae}	2.983 ± 0.004 ^{Cf}	5.125 ± 0.004 ^{Cb}	4.940 ± 0.003 ^{Dc}	5.147 ± 0.009 ^{Da}	4.896 ± 0.004 ^{Dd}
392	3.046 ± 0.004 ^{Ae}	2.769 ± 0.001 ^{Dh}	3.019 ± 0.004 ^{Bf}	2.825 ± 0.002 ^{Dg}	4.942 ± 0.004 ^{Db}	4.837 ± 0.004 ^{Ed}	4.9805 ± 0.0007 ^{Ea}	4.882 ± 0.006 ^{Dc}

C)

TClHCl 60ºC
pH Over Time

Days	pH 3				pH 6			
	TClHCl with HCl		TClHCl with HNO_3		TClHCl with HCl		TClHCl with HNO_3	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	3 ^{Bb}	3 ^{Ab}	3 ^{ABb}	3 ^{Ab}	6 ^{Ba}	6 ^{Aa}	6 ^{Ba}	6 ^{Aa}
1	-	-	-	-	6.130 ± 0.003 ^{Ab}	5.904 ± 0.004 ^{Bd}	6.178 ± 0.005 ^{Aa}	5.98 ± 0.01 ^{Ac}
3	-	-	-	-	5.745 ± 0.009 ^{Ca}	5.12 ± 0.03 ^{Cb}	5.772 ± 0.004 ^{Ca}	5.104 ± 0.004 ^{Bb}
7	3.041 ± 0.004 ^{Aa}	2.985 ± 0.004 ^{Ab}	3.033 ± 0.003 ^{Aa}	2.957 ± 0.002 ^{Ac}	-	-	-	-
12	-	-	-	-	5.419 ± 0.006 ^{Db}	4.981 ± 0.005 ^{Dc}	5.458 ± 0.006 ^{Da}	4.983 ± 0.006 ^{Cc}
21	-	-	-	-	5.292 ± 0.003 ^{Eb}	4.963 ± 0.003 ^{Dd}	5.317 ± 0.001 ^{Ea}	4.980 ± 0.004 ^{Cc}
63	2.982 ± 0.004 ^{Ba}	2.782 ± 0.005 ^{Bc}	2.962 ± 0.004 ^{BCb}	2.770 ± 0.004 ^{Bd}	-	-	-	-
161	2.86 ± 0.01 ^{Ca}	2.29 ± 0.02 ^{Cb}	2.91 ± 0.04 ^{CDa}	2.29 ± 0.02 ^{Cb}	-	-	-	-
392	2.850 ± 0.004 ^{Cb}	2.258 ± 0.004 ^{Cc}	2.870 ± 0.004 ^{Da}	2.240 ± 0.004 ^{Dd}	-	-	-	-

Table A.3.4 continued

D)

TClHCl 70ºC
pH Over Time

Days	pH 3				pH 6			
	TClHCl with HCl		TClHCl with HNO_3		TClHCl with HCl		TClHCl with HNO_3	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	3^{Bb}	3^{Ab}	3^{Bb}	3^{Ab}	6^{Aa}	6^{Aa}	6^{Aa}	6^{Aa}
1	2.99 ± 0.02^{Bc}	2.99 ± 0.04^{Ac}	2.974 ± 0.006^{Cc}	3.004 ± 0.003^{Ac}	5.78 ± 0.04^{Ba}	5.27 ± 0.01^{Bb}	5.80 ± 0.02^{Ba}	5.21 ± 0.01^{Bb}
4	3.039 ± 0.004^{Ae}	2.990 ± 0.004^{Af}	3.055 ± 0.005^{Ae}	3.008 ± 0.005^{Af}	5.556 ± 0.009^{Ca}	5.052 ± 0.004^{Cd}	5.533 ± 0.001^{Cb}	5.075 ± 0.004^{Cc}
13	3.032 ± 0.004^{Ac}	2.869 ± 0.002^{Be}	2.96 ± 0.01^{Cd}	2.84 ± 0.02^{Be}	5.155 ± 0.003^{Da}	4.899 ± 0.005^{Db}	5.159 ± 0.003^{Da}	4.9075 ± 0.004^{Db}
29	2.923 ± 0.003^{Ca}	2.712 ± 0.003^{Cd}	2.887 ± 0.002^{Db}	2.775 ± 0.004^{Cc}	-	-	-	-
42	2.975 ± 0.001^{Ba}	2.70 ± 0.01^{Cd}	2.885 ± 0.004^{Db}	2.752 ± 0.004^{Cc}	-	-	-	-

E)

TClHCl 80ºC
pH Over Time

Days	pH 3				pH 6			
	TClHCl with HCl		TClHCl with HNO_3		TClHCl with HCl		TClHCl with HNO_3	
	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL	1 mg/mL	20 mg/mL
0	3^{Ab}	3^{Ab}	3^{ABb}	3^{Ab}	6^{Aa}	6^{Aa}	6^{Aa}	6^{Aa}
1	3.12 ± 0.03^{Ae}	2.93 ± 0.01^{ABf}	3.02 ± 0.02^{ABf}	2.82 ± 0.01^{Cg}	5.63 ± 0.04^{Ba}	5.01 ± 0.03^{Bc}	5.52 ± 0.03^{Bb}	4.81 ± 0.02^{Cd}
2	3.108 ± 0.008^{Ac}	2.919 ± 0.003^{ABd}	3.11 ± 0.07^{Ac}	2.892 ± 0.005^{Bd}	5.65 ± 0.01^{Ba}	5.061 ± 0.004^{Bb}	5.65 ± 0.02^{Ba}	5.022 ± 0.008^{Bb}
4	3.11 ± 0.03^{Ad}	2.82 ± 0.03^{BCe}	3.02 ± 0.03^{ABd}	2.73 ± 0.02^{De}	5.32 ± 0.03^{Ca}	4.92 ± 0.03^{Cb}	5.23 ± 0.01^{Ca}	4.82 ± 0.02^{Cc}
7	3.1 ± 0.1^{Ac}	2.75 ± 0.04^{Cd}	3.00 ± 0.04^{ABcd}	2.72 ± 0.02^{Dd}	5.13 ± 0.04^{Da}	4.80 ± 0.02^{Db}	5.20 ± 0.08^{Ca}	4.73 ± 0.02^{Db}
13	3.1 ± 0.2^{Ac}	2.52 ± 0.03^{Dd}	2.84 ± 0.02^{Ccd}	2.63 ± 0.02^{Ed}	4.94 ± 0.01^{Ea}	4.59 ± 0.01^{Eb}	5.03 ± 0.02^{Da}	4.53 ± 0.01^{Eb}
29	3.0 ± 0.1^{Aa}	2.38 ± 0.05^{Eb}	2.92 ± 0.04^{BCa}	2.51 ± 0.01^{Fb}	-	-	-	-

Table A.4.1. Percent TMN remaining in dough liquor after storage at the specified conditions over time: A) 25ºC, B) 40ºC, C) 60ºC, D) 70ºC, and E) 80ºC. Uppercase superscript letters denote statistical significance within that thiamine concentration (down columns). Lowercase superscript letters denote statistical significance between concentrations for each day (across rows).

A)

TMN in Dough Liquor 25ºC
Percent Vitamin Remaining

Days	1 mg/mL	20 mg/mL
0	100. ± 2% ^{Aa}	100. ± 4% ^{Aa}
7	102.5 ± 0.3% ^{Aa}	93.4 ± 0.8% ^{ABb}
14	100.0 ± 0.4% ^{Aa}	85.9 ± 0.9% ^{BCb}
28	99.6 ± 0.5% ^{Aa}	78 ± 1% ^{CDb}
42	99.4 ± 0.4% ^{Aa}	73 ± 1% ^{DEb}
62	99.3 ± 0.8% ^{Aa}	68 ± 3% ^{Eb}
91	99.7 ± 0.4% ^{Aa}	67 ± 2% ^{Eb}
118	99.6 ± 0.7% ^{Aa}	67 ± 4% ^{Eb}
153	100. ± 2% ^{Aa}	67 ± 4% ^{Eb}
193	103 ± 3% ^{Aa}	67 ± 6% ^{Eb}

B)

TMN in Dough Liquor 40ºC
Percent Vitamin Remaining

Days	1 mg/mL	20 mg/mL
0	100. ± 2% ^{Aa}	100. ± 4% ^{Aa}
7	100.7 ± 0.3% ^{Aa}	82 ± 2% ^{ABb}
14	97 ± 1% ^{ABa}	71 ± 5% ^{BCb}
28	95 ± 2% ^{ABa}	67 ± 9% ^{BCb}
42	92 ± 7% ^{ABa}	65 ± 8% ^{BCb}
62	88 ± 9% ^{ABCa}	66 ± 9% ^{BCb}
91	86 ± 9% ^{ABCa}	65 ± 8% ^{BCb}
118	82 ± 9% ^{BCa}	65 ± 7% ^{BCa}
153	71 ± 5% ^{Ca}	64 ± 5% ^{BCa}
193	72 ± 5% ^{Ca}	61 ± 5% ^{Ca}

C)

TMN in Dough Liquor 60ºC
Percent Vitamin Remaining

Days	1 mg/mL	20 mg/mL
0	100. ± 2% ^{Aa}	100. ± 4% ^{Aa}
1	98 ± 2% ^{Aa}	93.9 ± 0.8% ^{Bb}
2	91.0 ± 0.4% ^{Ba}	87.6 ± 0.1% ^{Cb}
4	76.0 ± 0.3% ^{Ca}	74.8 ± 0.4% ^{Db}
7	41 ± 1% ^{Db}	60.5 ± 0.4% ^{Ea}
11	36.6 ± 0.2% ^{Eb}	51.74 ± 0.04% ^{Fa}
14	35.3 ± 0.1% ^{Eb}	45.8 ± 0.2% ^{Ga}
18	35 ± 1% ^{Eb}	42.2 ± 0.1% ^{Ga}

Table A.4.1 continued

D)

TMN in Dough Liquor 70ºC
Percent Vitamin Remaining

Days	1 mg/mL	20 mg/mL
0	100. ± 2% ^{Aa}	100. ± 4% ^{Aa}
1	92.8 ± 0.4% ^{Ba}	90. ± 4% ^{Ba}
2	81.6 ± 0.4% ^{Ca}	79 ± 1% ^{Cb}
4	59.6 ± 0.3% ^{Da}	58.6 ± 0.7% ^{Da}
7	51 ± 1% ^{Ea}	46 ± 1% ^{Eb}
11	44.0 ± 0.3% ^{Fa}	40.4 ± 0.3% ^{EFb}
14	40.2 ± 0.4% ^{Ga}	36 ± 1% ^{FGb}
18	37 ± 2% ^{Ga}	32.3 ± 0.4% ^{Gb}

E)

TMN in Dough Liquor 80ºC
Percent Vitamin Remaining

Days	1 mg/mL	20 mg/mL
0	100. ± 2% ^{Aa}	100. ± 4% ^{Aa}
1	89 ± 2% ^{Ba}	79 ± 2% ^{Bb}
2	79 ± 2% ^{Ca}	57 ± 1% ^{Cb}
4	60. ± 2% ^{Da}	41.9 ± 0.5% ^{Db}
7	43 ± 1% ^{Ea}	33.6 ± 0.5% ^{Eb}
11	31.7 ± 0.7% ^{Fa}	27.5 ± 0.5% ^{Fb}
14	27 ± 1% ^{FGa}	22.9 ± 0.4% ^{Gb}
18	23.5 ± 0.9% ^{Ga}	19.2 ± 0.5% ^{Gb}

Table A.4.2. TMN and dough liquor solution pH after storage at the specified conditions over time: A) 25ºC, B) 40ºC, C) 60ºC, D) 70ºC, and E) 80ºC. Uppercase superscript letters denote statistical significance within that thiamine concentration (down columns). Lowercase superscript letters denote statistical significance between concentrations for each day (across rows).

A)

TMN in Dough Liquor 25ºC
pH Over Time

Days	1 mg/mL	20 mg/mL
0	6.167 ± 0.004 ^{Ab}	6.4975 ± 0.0007 ^{Aa}
7	3.504 ± 0.004 ^{Bb}	5.138 ± 0.006 ^{Ba}
14	3.55 ± 0.03 ^{Bb}	5.006 ± 0.006 ^{Ba}
62	3.56 ± 0.02 ^{Bb}	4.69 ± 0.01 ^{Ca}
118	3.55 ± 0.01 ^{Bb}	4.544 ± 0.005 ^{Ca}
153	3.6 ± 0.1 ^{Bb}	4.6 ± 0.1 ^{Ca}
193	3.595 ± 0.006 ^{Bb}	4.55 ± 0.07 ^{Ca}

Table A.4.2 continued

B)

TMN in Dough Liquor 40ºC

pH Over Time

Days	1 mg/mL	20 mg/mL
0	6.167 ± 0.004 ^{Ab}	6.4975 ± 0.0007 ^{Aa}
7	4.32 ± 0.02 ^{Bb}	5.551 ± 0.008 ^{Ba}
14	4.074 ± 0.001 ^{Cb}	5.381 ± 0.009 ^{Ca}
62	3.716 ± 0.009 ^{Db}	5.21 ± 0.02 ^{Da}
118	3.692 ± 0.001 ^{Db}	5.1 ± 0.1 ^{DEa}
153	3.73 ± 0.02 ^{Db}	5.1695 ± 0.0007 ^{DEa}
193	3.71 ± 0.01 ^{Db}	5.05 ± 0.02 ^{Ea}

C)

TMN in Dough Liquor 60ºC

pH Over Time

Days	1 mg/mL	20 mg/mL
0	6.167 ± 0.004 ^{Ab}	6.4975 ± 0.0007 ^{Aa}
1	6.161 ± 0.004 ^{Ab}	6.1905 ± 0.0007 ^{Ba}
7	5.48 ± 0.02 ^{Ba}	5.36 ± 0.02 ^{Cb}
11	5.207 ± 0.007 ^{Ca}	5.23 ± 0.04 ^{Da}
14	5.164 ± 0.004 ^{Da}	5.165 ± 0.002 ^{DEa}
18	5.1295 ± 0.0007 ^{Ea}	5.09 ± 0.03 ^{Ea}

D)

TMN in Dough Liquor 70ºC

pH Over Time

Days	1 mg/mL	20 mg/mL
0	6.167 ± 0.004 ^{Ab}	6.4975 ± 0.0007 ^{Aa}
1	6.047 ± 0.003 ^{Ba}	5.975 ± 0.003 ^{Bb}
4	5.618 ± 0.004 ^{Ca}	5.33 ± 0.01 ^{Cb}
11	5.52 ± 0.02 ^{Da}	5.08 ± 0.04 ^{Db}
14	5.449 ± 0.004 ^{Ea}	5.01 ± 0.03 ^{Db}
18	5.294 ± 0.004 ^{Fa}	4.85 ± 0.07 ^{Eb}

E)

TMN in Dough Liquor 80ºC

pH Over Time

Days	1 mg/mL	20 mg/mL
0	6.167 ± 0.004 ^{Ab}	6.4975 ± 0.0007 ^{Aa}
1	6.084 ± 0.004 ^{Ba}	5.796 ± 0.002 ^{Bb}
4	5.665 ± 0.009 ^{Ca}	5.172 ± 0.005 ^{Cb}
11	5.115 ± 0.004 ^{Da}	4.65 ± 0.03 ^{Db}
14	4.905 ± 0.006 ^{Ea}	4.54 ± 0.01 ^{Eb}
18	4.678 ± 0.008 ^{Fa}	4.29 ± 0.05 ^{Fb}

Table A.4.3. TMN, dough liquor, and 0.1% potassium sorbate solution pH after storage at the specified conditions over time: A) 25ºC, B) 40ºC, and C) 60ºC. Uppercase superscript letters denote statistical significance within that thiamine concentration (down columns). Lowercase superscript letters denote statistical significance between concentrations for each day (across rows).

A)

TMN in Dough Liquor 25ºC
pH Over Time

Days	1 mg/mL	20 mg/mL
0	6.22 ± 0.02^{Ab}	6.460 ± 0.008^{Aa}
7	3.77 ± 0.02^{Bb}	5.20 ± 0.04^{Ba}
13	3.83 ± 0.02^{Bb}	5.26 ± 0.06^{Ba}
63	3.496 ± 0.005^{Cb}	4.663 ± 0.004^{Ca}
104	3.54 ± 0.02^{Cb}	4.679 ± 0.002^{Ca}
144	3.50 ± 0.06^{Cb}	4.61 ± 0.01^{Ca}

B)

TMN in Dough Liquor 40ºC
pH Over Time

Days	1 mg/mL	20 mg/mL
0	6.22 ± 0.02^{Ab}	6.460 ± 0.008^{Aa}
7	4.766 ± 0.004^{Ca}	4.4 ± 0.2^{Ba}
13	4.90 ± 0.01^{Ba}	4.197 ± 0.006^{Bb}
63	3.680 ± 0.003^{Db}	4.041 ± 0.001^{Ba}
104	3.68 ± 0.02^{Db}	4.02 ± 0.01^{Ba}
144	3.653 ± 0.003^{Db}	3.954 ± 0.006^{Ba}

C)

TMN in Dough Liquor 60ºC
pH Over Time

Days	1 mg/mL	20 mg/mL
0	6.22 ± 0.02^{Ab}	6.460 ± 0.008^{Aa}
1	6.022 ± 0.004^{Bb}	6.097 ± 0.004^{Ba}
7	5.549 ± 0.001^{Cb}	5.560 ± 0.003^{Ca}
11	5.580 ± 0.005^{Ca}	5.51 ± 0.02^{Db}
13	5.56 ± 0.01^{Ca}	5.455 ± 0.006^{Eb}

Table A.4.4. Percent TMN remaining in dough liquor solutions with 0.1% potassium sorbate after storage at the specified conditions over time: A) 25ºC, B) 40ºC, and C) 60ºC. Uppercase superscript letters denote statistical significance within that thiamine concentration (down columns). Lowercase superscript letters denote statistical significance between concentrations for each day (across rows).

A)

TMN in Dough Liquor 25ºC

Percent Vitamin Remaining

Days	1 mg/mL	20 mg/mL
0	100. ± 2% ^{Aa}	100. ± 6% ^{Aa}
7	97.9 ± 0.1% ^{ABa}	92.9 ± 0.7% ^{ABb}
13	97.2 ± 0.2% ^{BCa}	91 ± 1% ^{ABCb}
28	96.5 ± 0.3% ^{BCa}	86 ± 3% ^{BCb}
42	96.4 ± 0.3% ^{BCa}	87 ± 3% ^{BCb}
63	95.3 ± 0.5% ^{Ca}	84 ± 3% ^{BCb}
104	98 ± 1% ^{BCa}	83 ± 2% ^{BCb}
144	97.2 ± 0.3% ^{BCa}	82 ± 6% ^{Cb}

B)

TMN in Dough Liquor 40ºC

Percent Vitamin Remaining

Days	1 mg/mL	20 mg/mL
0	100. ± 2% ^{Aa}	100. ± 6% ^{Aa}
7	93.7 ± 0.2% ^{Ba}	92.7 ± 0.8% ^{ABa}
13	83 ± 1% ^{Cb}	89 ± 1% ^{ABa}
28	61.6 ± 0.9% ^{Db}	84 ± 5% ^{BCa}
42	46.5 ± 0.1% ^{Eb}	82 ± 5% ^{BCDa}
63	34.4 ± 0.6% ^{Fb}	81 ± 5% ^{BCDa}
104	29.4 ± 0.3% ^{Gb}	75 ± 4% ^{CDa}
144	28 ± 2% ^{Gb}	72 ± 4% ^{Da}

C)

TMN in Dough Liquor 60ºC

Percent Vitamin Remaining

Days	1 mg/mL	20 mg/mL
0	100. ± 2% ^{Aa}	100. ± 6% ^{Aa}
1	88 ± 1% ^{Ba}	89 ± 2% ^{Ba}
2	69 ± 2% ^{Cb}	87 ± 2% ^{Ba}
4	45 ± 3% ^{Db}	76.7 ± 0.5% ^{Ca}
7	39 ± 1% ^{Eb}	72.3 ± 0.7% ^{Ca}
11	36 ± 1% ^{Eb}	62 ± 2% ^{Da}
13	35 ± 2% ^{Eb}	59.5 ± 0.3% ^{Da}

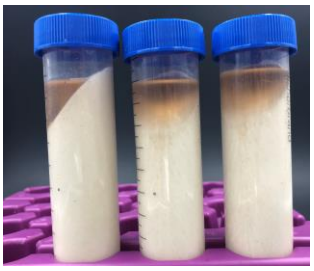

Figure A.4.1 Visual appearance of bread dough following centrifugation.

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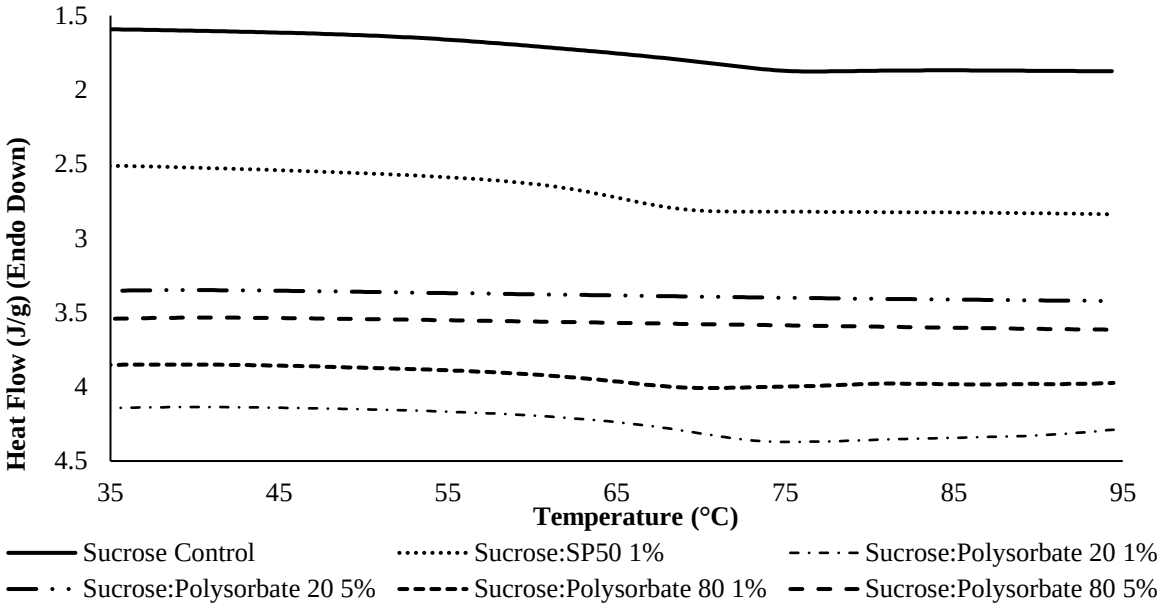

Figure A.5.1 DSC thermograms of select sucrose lyophiles. T_g is indicated by the shift in baseline. No T_g was found in sucrose lyophiles prepared with 5% polysorbates, as seen by the lack in baseline shift. A slight baseline shift (T_g event) was seen in the sucrose lyophiles prepared with 1% polysorbates.

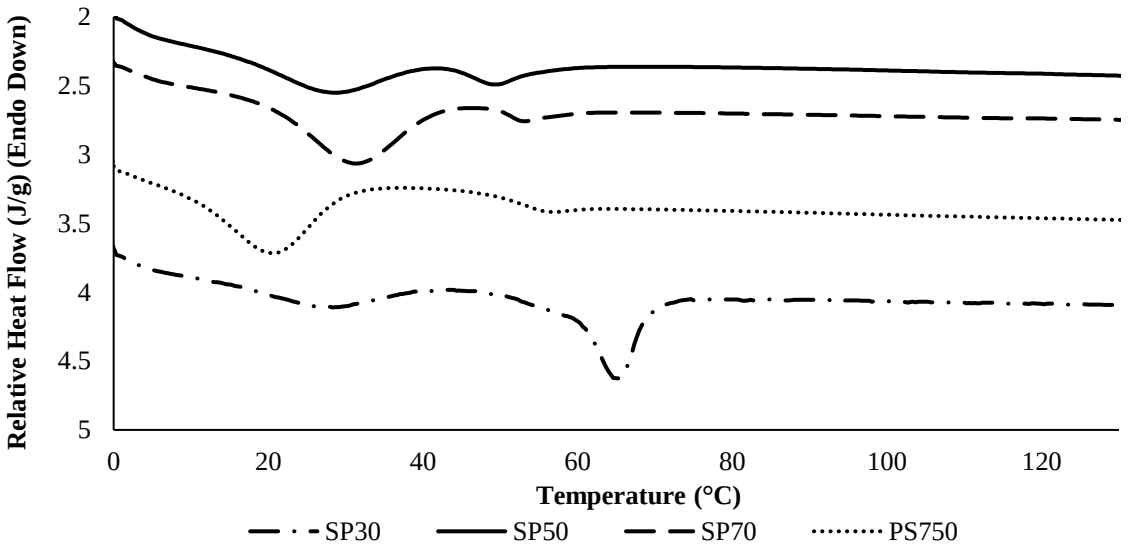

Figure A.5.2 DSC thermograms of sucrose esters, which exhibit two T_ms, indicated by a sharp endothermic peak.

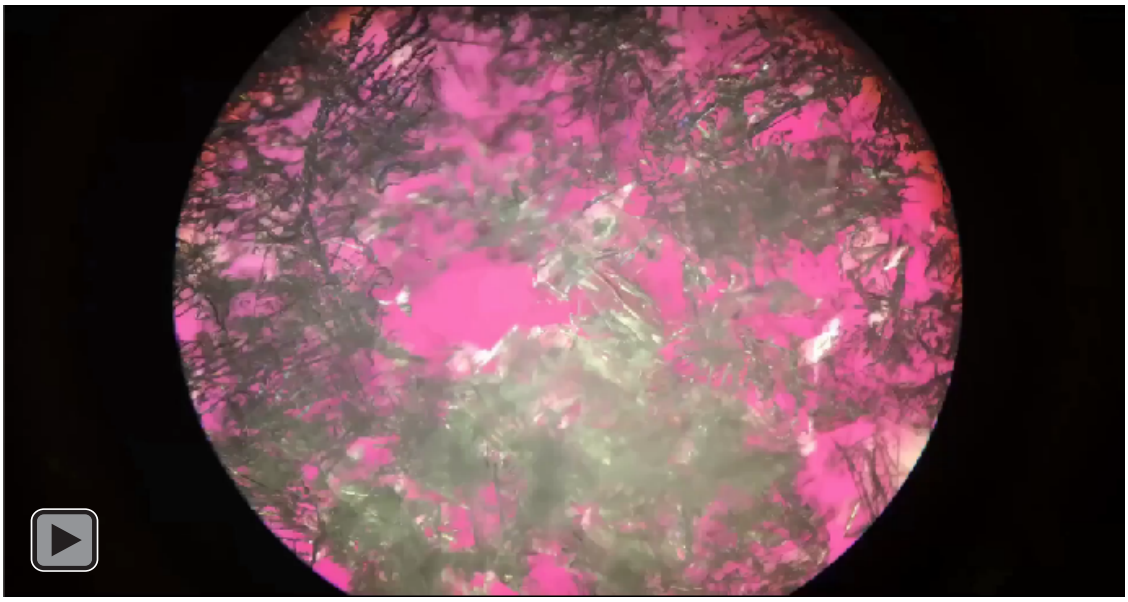

Figure A.5.3 Timelapse video of crystallization of the sucrose control at 40% RH in a RH controlled microscope stage. The video is 320 frames per second, so the actual duration of the video was approximately 29.3 hours.

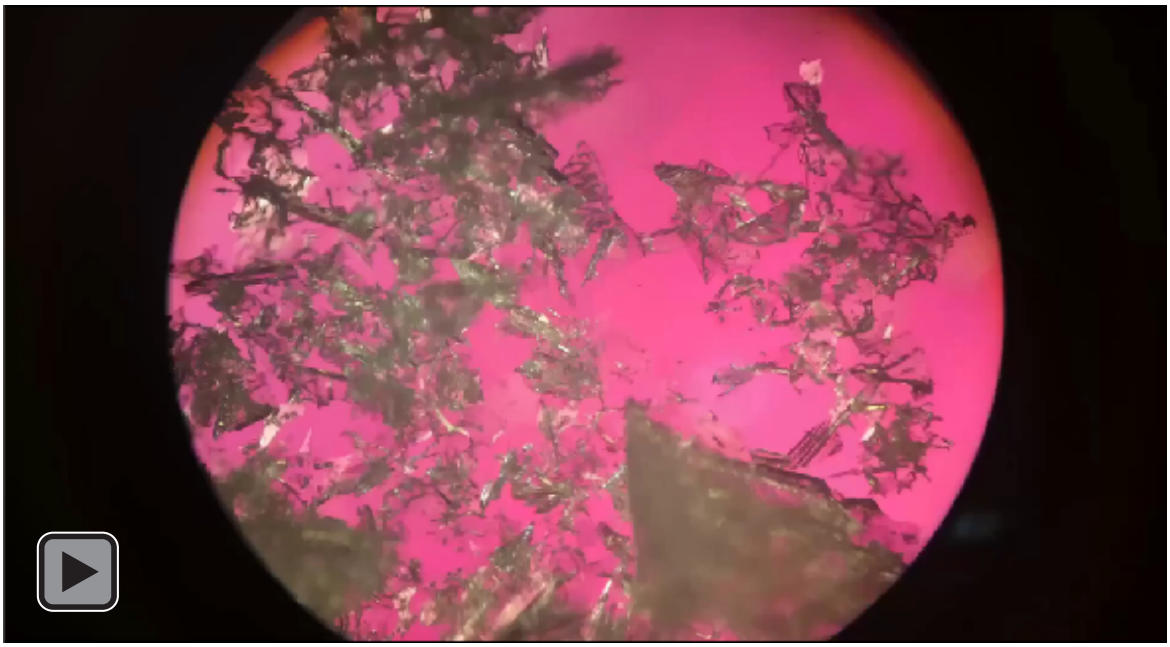

Figure A.5.4 Timelapse video of crystallization of the sucrose:polysorbate 20 1% lyophile at 40% RH in a RH controlled microscope stage. The video is 40 frames per second, so the actual duration of the video was approximately 3.3 hours.

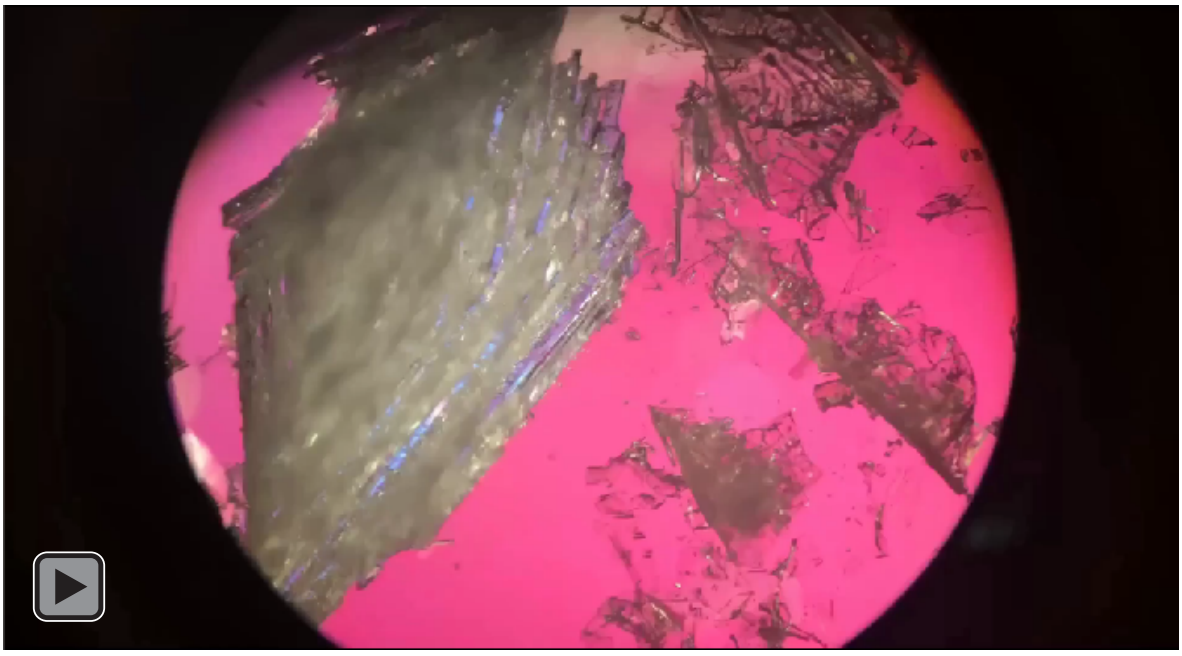

Figure A.5.5 Timelapse video of crystallization of the sucrose:polysorbate 80 1% lyophile at 40% RH in a RH controlled microscope stage. The video is 60 frames per second, so the actual duration of the video was approximately 5.5 hours.

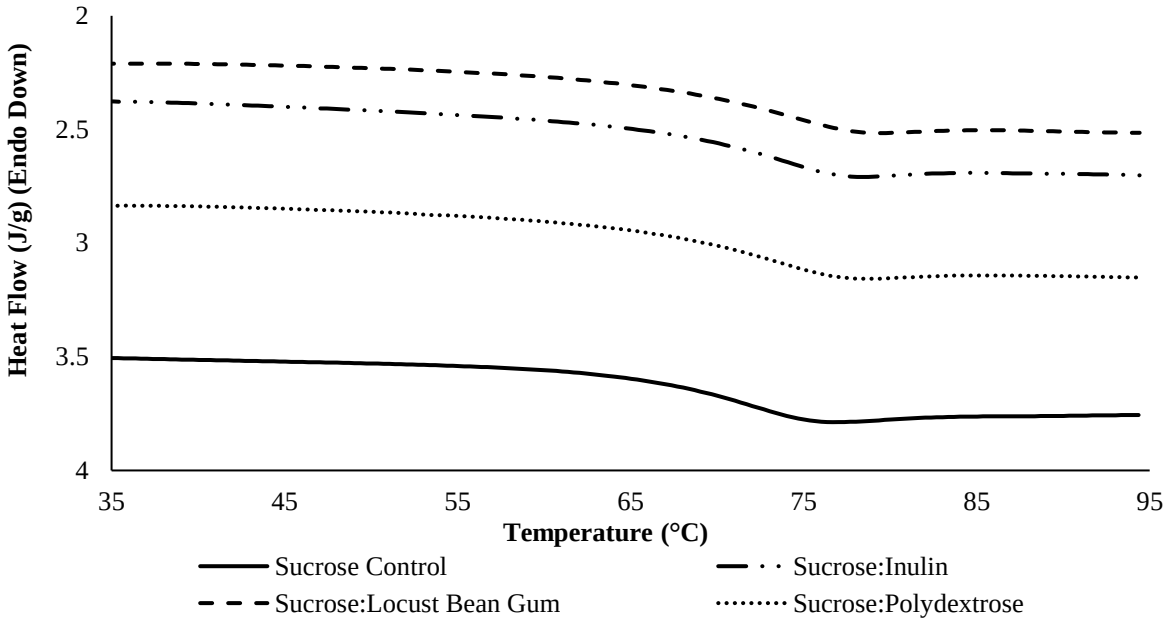

Figure A.7.1 DSC thermograms of select sucrose lyophiles. T_g is indicated by the shift in baseline.

PUBLICATIONS

Voelker, A. L., Felten, C., Taylor, L. S., & Mauer, L. J. (2021). Effects of polyphenols on crystallization of amorphous sucrose lyophiles. *Food Chemistry, 338*, 128061. doi:https://doi.org/10.1016/j.foodchem.2020.128061

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