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DETERMINING KINETIC PARAMETERS OF THIAMINE DEGRADATION IN THREE NASA SPACEFLIGHT FOODS IN THERMAL PROCESSING AND LONG-TERM STORAGE AND METHODS FOR ANALYZING MICROSTRUCTURE AND PRECIPITANT DEVELOPMENT IN REAL AND MODEL WINES

A Dissertation Presented

by

TIMOTHY R. GOULETTE

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2019

Food Science
DETERMINING KINETIC PARAMETERS OF THIAMINE DEGRADATION IN THREE NASA SPACEFLIGHT FOODS IN THERMAL PROCESSING AND LONG-TERM STORAGE AND METHODS FOR ANALYZING MICROSTRUCTURE AND PRECIPITANT DEVELOPMENT IN REAL AND MODEL WINES

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Eric Decker, Department Head
Food Science Department
DEDICATION

This work is dedicated to my parents, for pushing me to strive toward success and happiness, and to my two brothers for their unconditional friendship and support. They know I can be quite prickly.
ACKNOWLEDGMENTS

Support comes from U.S NASA Grant NNX14AP32G and the civil servants in the Advanced Food Technology Project, without whom this work would not be possible. Collaboration with Dr. Micha Peleg and Mark Normand from UMass were central in these works.

Support comes in equal part from E. & J. Gallo Winery, and the Research Chemistry Department, Modesto, CA 95354.

Special acknowledgement also goes to the UMass Hotel Catering staff, pilot plant manager David Prodanas, Dr. Eric Decker, Dr. D. Julian McClements and their wonderful students for their constant support and advice, and of course, Dr. Hang Xiao and all of the research lab members. Guidance from Dr. Anna Liu was greatly appreciated and critical for developing and selecting our models used in this work.
ABSTRACT

DETERMINING KINETIC PARAMETERS OF THIAMINE DEGRADATION IN THREE NASA SPACEFLIGHT FOODS IN THERMAL PROCESSING AND LONG-TERM STORAGE AND METHODS FOR ANALYZING MICROSTRUCTURE AND PRECIPITANT DEVELOPMENT IN REAL AND MODEL WINES

MAY 2019

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Directed by: Professor Hang Xiao

The current NASA spaceflight food program encompasses a wide variety of commercial and custom-made edible products. These foods are designed to ensure each crewmember receives the Recommended Dietary Allowance (RDA) of vitamins and minerals to maintain adequate health during the entire spaceflight mission. With the desire for long-duration and exploration-class missions, such as the impending mission to Mars, the retention of important nutrients such as labile vitamins in spaceflight foods is critically important. For this reason, multiple studies were conducted to determine the vitamin degradation behavior of thiamine (vitamin B₁) within three spaceflight foods which demonstrated both significant presence and instability of this compound during shelf-stabilization and in storage for two years. All foods were produced based on NASA’s recipes and were either retort sterilized to an equivalent lethality (F₀ of 6) or cooked and freeze-dried, then stored anaerobically in aluminized retort pouches. All food products were then stored at -80°C, -20°C, 4°C, 20°C, and 37°C. Vitamin retention was measured in all foods prior to and following thermal processing as well as at regular
intervals in storage to discern the degradation kinetics of thiamine, which are assumed to follow a first-order, temperature-dependent trend.

A novel and interactive kinetic modeling program, produced by our research group, was utilized to identify the two degradation parameters: decay rate constant at a given reference temperature or $k_{T_{ref}}$, and temperature sensitivity term or $c_{est}$, for vitamin B$_1$ in each food matrix during thermal processing as well as during storage. In our studies, we revealed the degree of degradation of vitamin B$_1$ in each food following various thermal processes and during two years in storage, including degradation observed in freeze-dried versions of each food product. These kinetic parameters can be utilized to track vitamin degradation over time in mission food storage and to produce optimum thermal processing conditions, preservation techniques, and storage parameters to achieve maximum stability of vitamins B$_1$ in these food products.

AND

The term “microstructure” refers to the sum total physical forms of soluble or near-soluble aggregates in wine that contribute to the viscosity, mouthfeel, stability, and perceived quality. Substances such as tannins, proteins, polysaccharides, and pigments are present in abundant amounts in wine and have been suggested in literature to contribute significantly to the formation of microstructure in wine both upon immediate proximally-driven interaction and over longer spans of time by various dynamic processes.

Two primary studies were conducted to elucidate the participating grape and wine-derived compounds that interact to form microstructure. In the first study, several novel methods were tested for consistency of results to conclusively represent a valid
interaction effect between pair-wise component additions. In the second study, a novel method for determining wine stability was tested for the explanation of precipitant formation in ageing wines. It was revealed that isothermal titration calorimetry, turbidimetry, zeta potentiometry, and transmission electron microscopy, were able to substantiate certain wine-related compounds interacting with one another, and that zeta potential mirrored the evolution of wine precipitant formation and growth, measured by turbidity. The methods proven in these works could be used to more efficiently control desired wine attributes and monitor ageing stability.
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LIST OF COMMON ABBREVIATIONS AND VARIABLES

NASA: National Aeronautics and Space Administration

B1: thiamine

\( k \): rate constant

\( T \): temperature

\( t \): time

\( n \): reaction order

\( c \): temperature sensitivity constant

\( C(t) \): momentary concentration

\( C_0 \): initial concentration

\( T_{\text{ref}} \): reference temperature

\( E_A \): activation energy

Conc.: concentration

PS: grape polysaccharide

MP: mannoprotein

TH: thaumatin

SK: grape skin tannin

SE: grape seed tannin

OC: oenin chloride

ITC: isothermal titration calorimetry

TEM: transmission electron microscopy

CS: Cabernet Sauvignon
CHAPTER 1
INTRODUCTION

DETERMINING KINETIC PARAMETERS OF THIAMINE DEGRADATION IN THREE NASA SPACEFLIGHT FOODS IN THERMAL PROCESSING AND LONG-TERM STORAGE

AND

METHODS FOR ANALYZING MICROSTRUCTURE AND PRECIPITANT DEVELOPMENT IN REAL AND MODEL WINES

1.1 NASA food systems

The current NASA spaceflight food program encompasses a wide array of commercial and custom-made edible products, including both standard foods and freeze-dried meals which require rehydration and sometimes reheating. These foods are carefully chosen and coordinated to develop a menu for each crewmember such that they receive the Recommended Dietary Allowance (RDA) of micro- and macronutrients to maintain adequate health during the entire spaceflight mission. The unique environment of spaceflight generate several challenges for providing adequate nutrition. The Advanced Food Technology project leaders and related programs from NASA carefully tailor these food systems and menus with the intent to stimulate appetite and avoid food fatigue of astronauts, as well as provide a sense of familiarity and psychological grounding in menus whose creation crewmembers are allowed to participate in (Perchonok & Bourland, 2002).

Providing such foods are currently part of a semi-routine shuttling schedule which are stowed alongside ISS-bound crewmembers. The scheduling considerations for food resupply weigh heavily on the side of crew movement and pantry depletion, and as such do not take into much consideration the degree of nutritional value in stored foods at any given time. For missions aboard the International Space Station (ISS) which generally
last no longer than 6 months and whose base of operation orbits only 400km above the surface of the Earth, this does not tend to be of concern, as food resupply can be performed relatively reliably. However, as NASA intends to embark on more long-duration and long-distance/interplanetary missions, considerations surrounding food processing and storage optimizations for food quality and nutritional value as well as monitoring of nutritional content decay over time, and lastly food safety, have become increasingly important (Cooper, Douglas, & Perchonok, 2011).

Although there are efforts being made to design a hydroponics processing stream for long-duration missions for the production of fresh food with correspondingly high nutritional content (Brief, 2015; Cooper et al., 2011; Perchonok & Bourland, 2002), resource utilization still requires that a significant portion of food biomass be supplied in pre-packaged form. Whether these products be ready-to-eat commercial options or formulated in-house, shelf stable menu items must maintain acceptable quality and nutritional content for the duration of the mission. Additionally, it is vital that these factors’ transformations over mission-specific time and temperature ranges be understood to allow for resupply scheduling or execution of other food system mitigation strategies.

Following data collection of nutritional status of Apollo crewmembers, it was revealed that certain nutrients were either lacking in the food system provided, or that the resulting bioavailability of these nutrients appeared poor. The “anti-neuritic” vitamin thiamine, or vitamin B₁, was among the lowest available nutrients in that food system (Rambaut, Smith, & Wheeler, 1975). Thus, there is particular interest in maximizing its presence in representative foods deemed high in initial thiamine and/or the food matrix renders thiamine unstable temporally during stabilization processes or during storage.
1.2 Modelling nutrient degradation

Degradation or decay behavior of nutrients of interest within chosen food systems may, in theory, be expressed mathematically and graphically as models to capture the relationship between main effects and degradation rate, such that predictions of nutrient content can be estimated following various process and during reasonable storage conditions. Such models generally must define a degradation rate \( k \), and the change in this rate \( k \) according to fluctuations in the main effects involved, as well as summate the absolute nutrient loss that occurred over a select timescale.

1.2.1 Temperature-dependence of kinetic rate

Traditionally, the temperature-dependence of the rate constant \( k \), expressed as \( k(T) \), has been described by the Arrhenius equation:

\[
k(T) = k(T_{ref}) \exp \left( \frac{E_A}{R} \left( \frac{1}{T_{ref}} - \frac{1}{T} \right) \right)
\]  

(1)

where \( k(T) \) and \( k(T_{ref}) \) are the rate constants at absolute temperatures \( T \) and a chosen reference temperature \( T_{ref} \) in degrees K, \( E_a \) the “energy of activation” in J or kJ (or cal or kcal) per mole and \( R \) the Universal gas constant in the corresponding units. This relationship has enjoyed frequent application in both model and real food systems for the determination of kinetic degradation parameters, degradation rate \( k(T) \) and assumed temperature-independent energy of activation \( E_A \), for individual compounds of interest (Farrer, 1955; Ramaswamy, Ghazala, & van de Voort, 1990).

However, in many instances within complex food matrices, multiple confounding sources of degradation from main instability factors and chemical modulators of kinetic rate, depending on the compounds unique reactivity chemistry, put into question the applicability of the Arrhenius equation. The assumption of a single, predominating
reaction leading to a compound’s decay with a single corresponding $E_A$ would not be well advised in complex systems, particularly in instances where the compound of interest has more than one vulnerable moiety, each susceptible to attack by different functional groups present in such a system (Peleg, Normand, & Corradini, 2012; Van Boekel, 2008). Rather, a more food-applicable general case could be used, which does not sacrifice the goodness of fit between kinetic rate and temperature as offered by the Arrhenius equation. This simpler exponential model is defined as:

$$k(T) = k(T_{ref})Exp[c(T - T_{ref})] \quad (2)$$

where $c$ is a temperature sensitivity term related to the energy of activation by:

$$c \approx \frac{E_A}{R(T_{ref} + 273.16)^2} \quad (3.a)$$

$$E_A \approx cR(T_{ref} + 273.16)^2 \quad (3.b)$$

This replacement model does not compress and invert the temperature scale and does not require the assumption that there is temperature-independent energy of activation of a single predominating chemical reaction within the degradation paradigm, replacing it by the temperature sensitivity parameter $c$ (Peleg et al., 2012; Peleg, Normand, & Kim, 2014).

1.2.2 Kinetic order considerations

A compound’s degradation throughout a modellable timeframe or for significant portions of the degradations scheme could be considered to follow fixed-order kinetics, defined by:

$$\frac{dC(t)}{dt} = -k[T(t)]C(t)^n \quad (4)$$
where \( C(t) \) is the vitamin’s concentration at time \( t \), \( k[T(t)] \) is the momentary rate constant at the momentary temperature \( T(t) \) and \( n \) the reaction order. The boundary condition is that at \( t = 0 \), \( C(0) = C_0 \), the initial concentration (Van Boekel, 2008).

### 1.2.3 First-order kinetics

The solution of Eq. 4 for isothermal degradation and first order kinetics, \( T = \) constant, and \( n = 1 \) is the familiar exponential decay:

\[
C(t) = \exp[-k(T)t] \cdot C_0
\]

(5)


### 1.2.4 Non-first and zero-order kinetics

Alternatively, the solution of Eq. 4 for isothermal degradation and non-first order kinetics, \( T = \) constant, and \( n \neq 1 \) is the momentary concentration defined by:

\[
C(t) = [C_0^{1-n} + k(T)(n-1)t]^{\frac{1}{1-n}}
\]

(6)

and it must be stated that in such cases where \( 0 < n < 1 \) and \( t > (C_0/k(T))^{1-n} \), \( C(t) \) defines a complex value for the content of the compound of interest. This would not be physically applicable and in this case, \( C(t) \) should be expressed as zero at this time (Peleg et al., 2016, 2014).

### 1.3 Thiamine reactivity in food

Literature detailing the degradation behavior of thiamine in simple and complex systems concludes that thiamine loss follows fixed, first-order kinetics (Farrer, 1955; Kwok et al., 1998; Peleg et al., 2016; Ramaswamy et al., 1990), suggesting that appropriate modelling would utilize the loss function described in Eq. 5 and the
temperature relationship to kinetic degradation rate defined by Eq. 2. The justification for using the replacement to the Arrhenius equation in this particular case is uniquely strong, given the myriad of chemical and physical factors that generate non-functional byproducts of thiamine from its destruction, rather than a single predominating reaction. Food matrices in this context also harbor chemical constituents that have been shown to protect thiamine from loss, or otherwise reconvert non-functional byproducts back to bioavailable thiamine.

1.3.1 Main factors in instability

Almost immediately following its discovery and isolation, thiamine was demonstrated to be susceptible to decay by high temperatures and alkali conditions particularly (Dwivedi & Arnold, 1973). These main factors allow for the differential destruction of thiamine, depending on the chemical compounds available or by simple hydrolytic cleavage or oxidation by available hydroxyl groups. Thiamine breakage typically generates its thiazole and pyrimidine constituents, supposedly by fractionation at the CH bridge that connects these constituents. An even more susceptible moiety lies in the thiazole ring, which has been shown to break in alkali conditions to eventually produce hydrogen sulfide, and in some cases, elemental sulfur and resulting thiazolone, or alternatively, a disulfide of thiamine (Dwivedi & Arnold, 1973). Even in acidic conditions, thiamine can undergo gradual CH bridge cleavage into various thiazoles and pyrimidines or replacement of amino groups by free hydroxyl groups to produce oxythiamine (Pachapurkar & Bell, 2005). Gamma and ultraviolet radiation have also been shown to lead to thiamine loss but can be mitigated by glutathione presence in the
matrix. This factor would be particularly relevant in spaceflight, and should be mitigated by some form of shielding, whether it be physical or chemical.

1.3.2 Chemical modulators of kinetic rate

    Other various food-bound compounds that factor into thiamine degradation in one way or another. In storage, the presence of acetate and/or organic aldehydes have been shown to degrade thiamine into various 2-acylthiazoles. Dissolved oxygen or oxygenating species generated in a packaged food product have been shown to accelerate thiamine loss into oxythiamine, thiochrome, or thiamine dimers by formation of a disulfide bond, and was more rapid with increased pH and when para-aminobenzoic acid is present, which is commonly found in both meats and grains (Dwivedi & Arnold, 1973). Allicin, found in onions and garlic, was shown to react with thiamine to form an allyl disulfide known as allithiamine. Additionally, while cysteine was demonstrated to participate in thiamine loss through the formation of mixed disulfides, other α-amino acids and peptides protected thiamine from decay compared to systems that did not include these amino acids. Benzaldehyde reduces thiochrome, a degradation product of oxidized thiamine, back into bioavailable thiamine, but the reaction is suspected to require a transferring base by a sort of Cannizzaro reaction (Dwivedi & Arnold, 1973).

    Thiaminase enzymes and hemin-related complex catalysts can also act upon thiamine to lead to its destruction but would generally become inactivated after sufficient thermal processing or rendered non-functional by other stabilizing techniques such as freeze-drying (which may also remove volatile substances participating in the loss or protection of thiamine). It was also revealed that Maillard-like reactions between thiamine and glucose occurred during high-temperature exposure to produce 2-
glucothiamines, although fructose involvement offered a protective effect (Dwivedi & Arnold, 1973). This phenomenon was observed even in acidic food products, which are generally thought to offer higher thiamine stability. Such preservation techniques would therefore be expected to drastically alter the degradation behavior of thiamine in a given food product, depending on the content of such reactants or protectants in the product, their transformation or loss during processing, and resulting mobility within the matrix.

1.4 Choices of kinetic model utilization

Degradation behavior can be thought of to result from several different paradigms, stemming from multiple fundamental, physical phenomena. Chemical reactivity, as a function of entropy-driven, enthalpy-deterministic transition state theory are most often applied when discussing the Arrhenius equation (Eq. 1), our suggested replacement by the double-exponential model (as shown in Eq. 2) as well as other modellable relationships such as the Eyring-Polanyi or Ball equations.

It has also been posited that chemical degradation, and deterioration of several other attributes in food and like systems arrive from a movement away from the glass-transition state temperature of the net material, resulting in matrix susceptibility to various sources of decay. However, the application of such an approach is questionable due to the insistence on a constant glass-transition temperature $T_g$ which has not been independently confirmed to exist in food matrices throughout time as other chemical and physical shifts take place (Peleg et al., 2012).

The Weibullian kinetics approach as informed by a survival probability function, or rather, the intrinsic inability for a chosen biological or chemical entity to remain intact, has been used for the kinetic loss modelling of several chemical constituents as well as
bacteria within foods (Corradini, Normand, & Peleg, 2008; Corradini & Peleg, 2004). For the purposes of this work however, we argue that our general-form replacement for the Arrhenius equation (Eq. 2) to be the most appropriate and consistent model for nutrient loss in cases where the rate order is fixed and the value is known. According to this model, the kinetic parameters that describe the temperature-dependence and degradation rate at a given temperature, \(k(T_{ref})\) and \(c\), can be extracted from two concentrations ratios \(C_1(t_1)\) and \(C_2(t_2)\), determined after storage times \(t_1\) and \(t_2\), at two constant temperatures, \(T_1\) and \(T_2\), or through use of two recorded time-temperature profiles, by solving the following two simultaneous equations:

\[
C_1(t_1) = \text{Exp}\{-k(T_{ref})\text{Exp}\left[c\left(T_1 - T_{ref}\right)\right]t_1\} \quad (7)
\]

and

\[
C_2(t_2) = \text{Exp}\{-k(T_{ref})\text{Exp}\left[c\left(T_2 - T_{ref}\right)\right]t_2\} \quad (8)
\]

for the two unknowns \(k(T_{ref})\) and \(c\).

AND

1.5 Wine microstructure chemistry

Wine stability to precipitate and haze formation, as well as improvement in sensory attributes during ageing is a major concern for wine producers. In production, the levels of select components are monitored and often selected for through several winemaking strategies to ensure a consistent product. Chemically speaking, there is a swathe of dynamic molecular interactions occurring, beyond micro-oxygenation and bitartrate deposition, which can affect the end product to a significant degree. Understanding which wine-derived components are at play, the relative strength of these
interactions, and their resulting morphology are critical for the industry to better control sensory attributes.

Great attention is being paid to the contribution of select wine components and extract fractions to mouthfeel in the finished product, related in part to interactions between chemical components in wine as well as their interactions with oral tissue upon ingestion. Work has been done investigating the mouthfeel effects of grape and wine-derived tannins, anthocyanins, proteins, and polysaccharides as single-components (Escot, Feuillat, Dulau, & Charpentier, 2001; Fontoin, Saucier, Teissedre, & Glories, 2008; Vidal, Francis, et al., 2004) as well as the mouthfeel impact of limited pair-wise component interactions, which typically focuses on the astringency or drying influence (Diako, McMahon, Mattinson, Evans, & Ross, 2016; Fontoin et al., 2008; Quijada-Morín, Williams, Rivas-Gonzalo, Doco, & Escribano-Bailón, 2014; Ramos-pineda, García-estévez, & Escribano-bailón, 2018; Vidal, Courcoux, et al., 2004).

Independent from this, work has been done to explain the mechanisms of certain wine-component interactions. Components of interest typically center around tannin-tannin or protein-tannin interactions, and polysaccharides that may hinder their aggregation and deposition by disruption or component surface-stabilization (Carvalho, Mateus, et al., 2006; Carvalho, Póvoas, Mateus, & De Freitas, 2006; De Freitas, Carvalho, & Mateus, 2003; Erranti, Ametti, & Onomi, 2010; Carine Le Bourvellec, Watrelot, Ginies, Imberty, & Renard, 2012; Mateus, Carvalho, Luís, & De Freitas, 2004; Mekoue Nguela, Poncet-Lerand, Sieczkowski, & Vernhet, 2016; Poncet-Lerand, Doco, Williams, & Vernhet, 2007; Riou, Vernhet, Doco, & Moutounet, 2002; Watrelot, Schulz, & Kennedy, 2017).
1.6 Analytical methods

Aggregations or soluble complexes formed by component interactions are typically determined by turbidometry, Isothermal Titration Calorimetry (ITC) and less commonly, Nuclear Magnetic Resonance (NMR) spectroscopy, among other methods (C. Le Bourvellec & Renard, 2012). To explain these aggregates, physical and chemical characteristics of several of these ubiquitous wine components and wine aggregates have been analyzed (K. Bindon et al., 2014; K. A. Bindon et al., 2016; Vernhet, Pellerin, Prieur, Osmianski, & Moutounet, 1996). This body of work has shown that entropy-driven hydrophobic forces, hydrogen bonding, and steric stabilization are the main drivers of aggregation and soluble complex formation, depending on the specific components involved. Aside of tannin interactions, anthocyanins also have been shown to play a role in interacting with polysaccharides, which change sensory attributes of finished wines (Fernandes, Bras, Mateus, & de Freitas, 2015; Trouillas et al., 2016). Individual works have not yet provided a holistic analysis of the nature, strength, preferentiality, stoichiometry of interactions between major wine macromolecules and major flavonoid classes in wine, or such interactions that can be demonstrated across methods.
CHAPTER 2

KINETIC PARAMETERS OF THIAMINE DEGRADATION IN NASA SPACEFLIGHT FOODS DETERMINED BY THE ENDPOINTS METHOD FOR LONG-TERM STORAGE

2.1 Abstract

Retention of labile vitamins such as thiamine (vitamin B1) in spaceflight foods intended for extended-duration missions is critical for the health of the spaceflight crew. In this study, the degradation kinetics of thiamine in three NASA spaceflight foods (brown rice, split pea soup, BBQ beef brisket) during storage was determined for the first time, using an interactive isothermal model and program developed by our group. Spaceflight foods were manufactured according to NASA’s specification. Thiamine content in these foods was monitored for up to 720 days at three storage temperatures, 4°C, 20°C, and 37°C, in order to produce a predictive model that estimates thiamine content at any time and temperature of storage. Results showed that brown rice and split pea soup demonstrated resistance to thiamine degradation, while thiamine in beef brisket was less stable. Assuming thiamine degradation follows first-order kinetics, model-predicted thiamine retention in brown rice stored at 20°C for 720 days was 55% of the original thiamine content after thermal processing, 42% for split pea soup, and 3% for beef brisket. Water activity, moisture content, and pH differences did not sufficiently explain the variation in the degradation kinetics of thiamine among these foods.

2.2 Introduction

The current NASA spaceflight food program encompasses a wide variety of commercial and custom-made edible products. These foods are designed to ensure each
crewmember receives the Recommended Dietary Allowance (RDA) of vitamins and minerals to maintain adequate health during the entire spaceflight mission. With the desire for long-duration and exploration-class missions, such as the impending mission to Mars, the retention of important nutrients such as labile vitamins (e.g. thiamine) in spaceflight foods is critically important. However, knowledge is currently lacking on the degradation kinetics of these vitamins during the long-term storage of spaceflight foods. To address this knowledge gap, in this study we aimed to establish the degradation kinetics of thiamine in three different spaceflight foods for, i.e. brown rice, split pea soup, BBQ beef brisket during long-term storage at three temperatures (4°C, 20°C, and 37°C) for the first time, utilizing a novel degradation modelling program and “Endpoints Method” we have developed previously (Peleg et al., 2016, 2014).

2.2.1 Theoretical Background

The kinetics of thiamine degradation during storage, like that of most vitamins of interest, has been traditionally determined from sets of experimental isothermal concentration or concentration ratio vs. time relationship determined at 3-5 different temperatures. It has been established that it follows first order kinetics, i.e., described by the rate equation (Van Boekel, 2008):

\[
\frac{dC(t)}{dt} = -k[T(t)]C(t)^n \quad (1)
\]

where \( C(t) \) is the vitamin’s concentration at time \( t \), \( k[T(t)] \) is the momentary rate constant at the momentary temperature \( T(t) \) and \( n \) the reaction order in our case \( n = 1 \). The boundary condition is that at \( t = 0 \), \( C(0) = C_0 \), the initial concentration.

The solution of Eq. 1 for isothermal degradation and first order kinetics, \( T = \) constant, and \( n = 1 \) is the familiar exponential decay:
\[ \text{Exp} \left[ \frac{C(t)}{C_0} \right] = -k(T)t \]  

where \( C(t)/C_0 \) is the vitamin’s concentration ratio, \( 0 < C(t)/C_0 \leq 1 \). (Arabshahi & Lund, 1988; E.A. Mulley & C.R. Stumbo, 1975; Kamman, Labuza & Warthesen, 1981; Kwok, Shiu, Yeung, & Niranjan, 1998; Pachapurkar & Bell, 2005; Peleg, Normand, & Goulette, 2016; Ramaswamy, Ghazala, & van de Voort, 1990).

### 2.2.2 The temperature dependence of \( k(T) \)

Traditionally, the temperature-dependence of the rate constant \( k(T) \) has been described by the Arrhenius equation:

\[ k(T) = k(T_{\text{ref}}) \text{Exp} \left[ \frac{E_A}{R} \left( \frac{1}{T_{\text{ref}}} - \frac{1}{T} \right) \right] \]  

where \( k(T) \) and \( k(T_{\text{ref}}) \) are the rate constants at absolute temperatures \( T \) and a chosen reference temperature \( T_{\text{ref}} \) in degrees K, \( E_a \) the “energy of activation” in J or kJ (or cal or kcal) per mole and \( R \) the Universal gas constant in the corresponding units. Concerns regarding the applicability of the Arrhenius equation to food science exist, and while the conventional method does produce meaningful data, a simpler and more appropriate equivalent exists (Peleg et al., 2012; Van Boekel, 2008):

\[ k(T) = k(T_{\text{ref}}) \text{Exp}[c(T - T_{\text{ref}})] \]  

where \( c \) is a constant and related to the energy of activation by:

\[ c \approx \frac{E_A}{R(T_{\text{ref}}+273.16)^2} \]  \hspace{1cm} (5.a)

\[ E_A \approx cR(T_{\text{ref}} + 273.16)^2 \]  \hspace{1cm} (5.b)

This replacement model does not compress and invert the temperature scale and does not require the assumption that there is temperature-independent energy of
activation of a single predominating chemical reaction within the degradation paradigm, replacing it by the temperature sensitivity parameter \( c \) (Peleg et al., 2012, 2014).

According to this model, the kinetic parameters \( k(T_{\text{ref}}) \) and \( c \) can be extracted from two concentrations ratios \( C_1(t_1) \) and \( C_2(t_2) \), determined after storage times \( t_1 \) and \( t_2 \), at two constant temperatures, \( T_1 \) and \( T_2 \) by solving the following two simultaneous equations:

\[
C_1(t_1) = \exp\{-k(T_{\text{ref}})\exp[c(T_1 - T_{\text{ref}})]t_1\} \quad (6)
\]

and

\[
C_2(t_2) = \exp\{-k(T_{\text{ref}})\exp[c(T_2 - T_{\text{ref}})]t_2\} \quad (7)
\]

where \( k(T_{\text{ref}}) \) and \( c \) are the two unknowns.

### 2.2.3 Estimating future concentration ratios during storage

Once \( k(T_{\text{ref}}) \) and \( c \) have been estimated from two experimentally determined concentration ratios, they can be used to estimate the concentration ratio at any other temperature \( T_3 \), \( C_3(t_3) \), in a pertinent temperature range. The predicted value can then be compared with a corresponding experimental value to validate the kinetic model and the assumptions on which it is based.

Solving for two unknowns is facilitated by the NDSolve function of Mathematica® (Wolfram Research, Champaign IL, USA), the program utilized in model construction and its use. The procedure to extract \( k(T_{\text{ref}}) \) and \( c \) from two entered experimental data points has been automated in the form of a freely downloadable interactive Wolfram Demonstration:

http://demonstrations.wolfram.com/PredictionOfIsothermalDegradationByTheEndpoints

Method/ shown in Figures 1-2. [The Demonstration has instructions and examples. The
free CDF Player which runs the Demonstration, and over 11,000 other demonstrations to date, can be loaded following instructions on the screen. As shown in Figure 1, after entering $T_1$, $t_1$ & $C_1$ and $T_2$, $t_2$ & $C_2$ the user matches the two reconstructed degradation curves with the data points by moving the $k(T_{ref})$ and $c$ sliders. When matched, the user can generate the curve for any third temperature $T_3$ and calculate the vitamin retention at any time $t_3$ at this temperature as well as at $T_1$ and $T_2$.

Figure 1. Kinetic parameter, $k(T_{ref})$ and $c$, estimation by Endpoints Method. Left: the two experimental points with the reconstructed degradation curves produced with the default values and a manually entered $T_{ref}$. Right: the matched reconstructed curves. Notice that the matching is done by manually moving the $k(T_{ref})$ and $c$ sliders.
This study sought to utilize the Endpoints Method of the isothermal model to determine the kinetic parameters of thiamine degradation in three NASA-provided formulations (or ‘recipes’ in NASA’s terminology), and to offer insight on the optimum experimental parameters ($T$ and $t$) to perform, minimizing analytical inventory, sampling, and waste. From these data, the degradation of the labile vitamin thiamine can be estimated in these foods for use in NASA diet construction and resupply scheduling considerations.

2.3 Materials and Methods

2.3.1 NASA’s specifications for brown rice, split pea soup, and BBQ beef brisket

The spaceflight food composition and manufacturing specifications were provided by NASA. Specifications detail the precise formulation for each food product, quality inspection procedure, package testing, and storage instructions. These specific products
were chosen jointly by NASA and our group as representative of un-enriched foods with a potential high thiamine instability (data not shown). As the specifics of these recipes are confidential, the exact details are withheld in this work.

2.3.2 Ingredient acquisition and food production

Bulk brown rice, low-sodium vegetable broth, green split peas, boneless ham, raw deckle-off beef brisket, BBQ sauce, and dry BBQ marinade were purchased from Performance Foodservice (1 Performance Blvd, Springfield, MA, USA). Liquid smoke flavoring, ham base, mono-sodium glutamate (MSG), salt, pepper, whole milk, corn starch, red wine vinegar, and bullion were purchased from a local grocery store (175 University Drive, Amherst, MA, USA). All foods were produced abiding by the precise instruction of the NASA specifications.

The brown rice product consisted of raw brown rice and low-sodium vegetable broth, such that the thermal process to obtain shelf-stabilization also cooks the rice thoroughly. Split pea soup was produced after initial rinsing of the dried split peas. Beef brisket was produced by trimming excess fat and coating in BBQ dry rub, wrapping in plastic wrap, and marinating for 24 hrs in refrigerator.

2.3.3 Packaging

The prepared spaceflight foods were filled into flexible aluminized pouches [12.065 cm x 20.48 cm (4.75” x 8.0625”); Tan PE/.0007Foil/3mil Coex Sealant] that were purchased from Heritage Packaging (441 Market St, Lawrence, MA, USA). Approx. 25 kg of brown rice product, including broth, was produced and distributed into pouches to a final weight of 121 g in each pouch, producing approx. 200 units. 200 pouches of split pea soup were produced in total and filled to a weight of 150 g per
pouch, and another 200 units of beef brisket product were produced at a fill weight of 120 g per pouch. Pouch opening was cleaned and dried prior to vacuum-sealing which was done using VacMaster SVP 20 (Overland Park, KS, USA) to remove excess air. Pouches were then aliquoted into batches of 44 pouches for shelf-stabilization.

2.3.4 Shelf-stabilization

Thermal processing was performed using Allpax Multimode R&D 2402 Series Retort, running a hot-water spray thermal process. The temperature probes used were C-4 flexible wire thermocouples, acquired from Ecklund-Harrison (11000 Metro Pkwy, Ste. 40, Fort Meyers, FL, USA) and affixed to the pouch. The end of the flexible wire was positioned into the center of the pouch with food material placed at the end of the tip. Each thermal-process temperature profile was recorded using HH378 data logger (OMEGA Engineering, INC., 800 Connecticut Ave., Suite 5N01, Norwalk, CT, USA). Temperature-time plots were produced from these recordings using Se379 software (Cetani Corporation, 11495 N Pennsylvania St Suite 240, Carmel, IN, USA) and used to calculate the equivalent lethality of each batch. The target thermal process lethality was that of the NASA-provided value of $F_0 = 6.0$ from the General Method, by processing at a cook temperature of 121°C for a sufficient time. Pouches were cooled under pressure to 110°C or cooler prior to entry into storage.

2.3.5 Long-term storage

Food products were placed in varying storage vessels at: 37°C, 20°C, and 4°C for routine sampling and analysis for use in the model, and regularly inspected for swelling or other signs of spoilage or pouch leaks. Sampling occurred in regular 90-day intervals.
up to 720 days for products stored at 37°C and 20°C and 120 days intervals up to 720 days for 4°C.

2.3.6 Thiamine extraction

Vitamin extraction followed a modified version of the Association of Official Agricultural Chemists (AOAC) International official method 942.23 Thiamine (Vitamin B₁) in Human and Pet Foods (AOAC 942.23, 2005; Ma & Wyatt, 1983). Modifications were made to better suit the process of thiamine extraction and detection for these three spaceflight foods. The phosphorolytic enzyme used was taka-diastase (Pfaltz & Bauer, 172 E. Aurora St., Waterbury, CT, USA) due to proven equivalent activity to the suggested enzymes. Chromatographic columns used for purification were Kimble™ Kontes™ FlexColumns™ at 1.0 cm x 20 cm, 16 mL capacity using the Three-way Luer Stopcock of the same brand. Sodium acetate, sodium hydroxide, potassium chloride, hydrochloric acid, potassium ferricyanide, isobutanol, thiamine hydrochloride as well as thiamine pyrophosphate were all purchased from Fisher Scientific (168 Third Avenue, Waltham, MA, USA). Quinine sulfate and the purification resin utilized in these columns, Amberlite CG-50 (Type I), were both purchased from Sigma-Aldrich (1 Strathmore Rd, Natick, MA, USA).

2.3.6.1 Acid and enzyme digestion

Digestions were conducted in quadruplicate for all foods and temperature treatment, plus the thiamine hydrochloride and thiamine pyrophosphate standards. Foods were ‘stomached’ (crudely mashed) in pouch prior to opening, and again stirred to homogenize upon opening. 0.1 M HCl was used as the extraction buffer. Acidified samples were digested at 100°C for 30 minutes, cooled to <50°C and aliquoted. Aliquots
were diluted with 0.1 M HCl and readjusted to pH 4.0-4.5 with double-normal sodium acetate. 5 mL 1% aqueous taka-diastase solution was then added, and the sample incubated at 45°C for 3 hrs. Aliquoted samples were then acidified with 2 M HCl to pH 3.5 and refrigerated overnight.

2.3.6.2 Filtration and purification of thiamine

Samples were then re-mixed and aliquoted evenly into centrifuge tubes and centrifuged at 3220 g, 4°C, for 5 minutes. Supernatant was filtered through Whatman™ Quantitative Grade 40 Filter Paper (Fisher Scientific) using vacuum pump assistance to collect crude extract. Crude extract was fed through chromatographic columns containing Amberlite CG-50 resin adjusted to pH 5. Columns were washed with near-boiling H₂O and thiamine was eluted with near-boiling 0.1 M HCl solution containing 25% KCl.

2.6.3 Oxidation of thiamine for detection

Two 5 mL portions from the final eluate from each sample were aliquoted into reaction tubes. The remaining solution was stored at -80°C in case of need to re-analyze. Tubes were wrapped in aluminum foil and the remaining steps were conducted in a dark chemical hood to minimize photodegradation. 3 grams solid KCl were added to each reaction tube, along with 3 mL oxidizing reagent (0.04% potassium ferricyanide in 15% aqueous NaOH) for one of the two reaction tubes, while 3 mL 15% aqueous NaOH was added to the other, to serve as the blank. Immediately following the addition of oxidizing or blank solution, 4 mL isobutanol was added to the reaction tubes and shaken. Both thiamine standards were treated identically to the above samples. All samples were then shaken again and allowed to settle before continuing.
The isobutanol layer was collected (top layer) and aliquoted into 8 wells per sample (oxidized or blank) of a 96-well flat-black-bottom plates (Fisher Scientific). Plates were protected from light using aluminum foil until read using a BioTek Synergy 2 plate reader (100 Tigan St., Winooski, VT, USA) running a fluorometric protocol at input filter 365 nm and output filter 435 nm, and fluorometric data collected using Gen5 software version 1.09 (BioTek).

2.3.6.4 Modifications from the original method

Modifications made to the original AOAC method 942.23 included decreasing original sample volume and dilution factor, re-concentration in less isobutanol during thiochrome solubilization, and detection using a fluorometric plate reader. Modifications did not produce any significant decrease in overall extraction efficiency. Plate reader was calibrated against quinine sulfate solution. Taka-diastase phosphorolytic efficiency was measured by a pure thiamine pyrophosphate digestion. Recovery of >85% free thiamine was deemed suitable.

2.3.7 Thiamine concentration determination

Thiamine content in the reaction solution prior to oxidation is calculated by comparison to the results of a thiochrome standard curve made from a separate thiamine hydrochloride standard and dilution with NaOH prior to oxidation and measurement. Appropriate volumetric dilution and mass fraction was calculated back to express the original thiamine content in each food. The mean of each quadruplicate set was calculated as well as the standard deviation. Thiamine content was expressed as mean μg/g ± SD of a given food at that time point of storage and μg thiamine per pouch ± SD at that time point in storage. Thiamine content was then compared to the initial value of
each food \((t = 0)\), taken immediately following retort processing and expressed as a concentration ratio \((C_t / C_0)\).

2.3.8 Application of the model to experimental data

Momentary concentration, time duration in storage and temperature of that storage were recorded for use in the modelling program. Any pair of momentary data sets were input into the program entitled Prediction of Isothermal Degradation by the Endpoints Method and as the model requires, parameters \(k(T_{ref})\) and \(c\) are adjusted in tandem to produce estimated degradation trajectories that pass through both endpoints. The parameters that produce a satisfactory fit for the model are then recorded. Theoretically, these parameters describe the entire temperature-dependence and time-effect of degradation. However, in this work, parameters are averaged from four discrete blocks of three momentary data sets (90, 180, 270, 360 days, see Tables 1-3) from like times up to 360 days, across the temperature range: 4°C, 20°C, and 37°C to produce an improved interpolation effect and diminish the effect of sampling error. Though any and all combinations of time-temperature data may included and averaged for a more holistic picture, our approach sought to minimize data handling and improve model accuracy. Average \(k(T_{ref})\) and \(c\) values were then used to produce predictive trajectories for thiamine degradation in its respective food medium at typical ambient storage temperature, for NASA’s purposes this would be 20°C, all within the same module.

2.3.9 Moisture determination

Contents from pouches from each food were prepared for water activity, moisture content, and pH analysis. Water activity \((a_w)\) was measured using AquaLab Dew Point Water Activity Meter 4TE (2365 NE Hopkins Ct., Pullman, WA, USA). Moisture content
was measured based on 1 g sample using A&D MX-50 moisture analyzer (2100 Landmeier Rd., Elk Grove, IL, USA). Approximate pH was measured in homogenized samples using Metrohm 827 pH Lab meter (9250 Camden Field Parkway, Riverview, FL, USA).

2.4 Results and Discussion

2.4.1 Thiamine content by food, storage time, and storage temperature

After storage in the aforementioned constant temperatures for up to 480 days, thiamine content is reported in Figure 3 in terms of thiamine content per serving size, and therefore per pouch. Brown rice demonstrated the highest overall stability of thiamine across all temperature and time ranges, with split pea soup performing slightly worse and beef brisket even more so. Brown rice pouches contained on average a thiamine content of 77 µg per serving, compared to split pea soup pouches which contained 110 µg per serving on average. It was also observed that beef brisket contained dramatically less thiamine per serving and per gram of food originally compared to either brown rice or split pea soup, at 15 µg per serving. Additionally, beef brisket stored at 37℃ revealed such degradation after 270 days of storage that thiamine content was not detectable in the samples and a corresponding retention of 0% thiamine was used in the modeling.
Figure 3. Experimental thiamine loss in three spaceflight foods during storage at three constant temperatures.

Brown rice and split pea soup stored at the ambient condition of 20°C at 720 days demonstrated a similar thiamine retention, at final content of 45 µg and 52 µg per serving, respectively. Refrigerated (4°C) brown rice experienced a marked improvement in thiamine retention and thereby slower degradation, while this effect was seemingly diminished in split pea soup and absent in beef brisket.

The thiamine content in the foods tested prior to and during storage cannot be summarily attributed to the thiamine content in the raw material, although raw brown rice is documented to have approx. 10 times more thiamine per 100 g than raw beef on
average across food product labels that were available for comparison. Rather, pre-
processing prior to shelf-stabilization and storage may have also contributed to the
decline in thiamine content of the beef, as the aggressive roasting for multiple hours in a
moderate-pH environment can lead to a marked loss of thiamine (B. W. Beadle, D. A.
indeed receive the least thermal exposure prior to retort, and it also happens that brown
rice demonstrated higher thiamine retention than beef brisket and comparable retention
after retorting (per gram basis).

2.4.2 Kinetic parameter determination and prediction of degradation at 20C

The program and embedded model utilized in the determination of kinetic
parameters of thiamine degradation in each food is shown in Figure 1. An example of
two sets of temperatures, times, and endpoint concentration ratios are shown as inputs
into the model in the top left of both module. The right panel demonstrates a fit produced
by adjusting parameters $k(T_{ref})$ and $c$, and therefore a mathematical description of the
degradation experienced in both cases of storage via Eqs. 6 and 7, and in theory a
description of the temperature-dependence of thiamine degradation in that food via Eq. 4.
In this work, averaged $k(T_{ref})$ and $c$ parameters were determined using the three possible
combinations of temperatures, times, and resulting concentration ratios from four distinct
time points of analyses, as seen in Tables 1-3.

Average $k(T_{ref})$ and $c$ values were gathered from separate analytical
groupings/blocks and subsequent averaging of their three possible model constructions
after: 90 days of storage for 20°C and 37°C and 120 days for 4°C (90 days), 240 days of
storage for 20°C and 37°C and 360 days for 4°C (180 days), 270 days of storage for 20°C
and 37°C and 360 days for 4°C (270 days), 360 days of storage for 20°C and 37°C and 360 days for 4°C (360 days). Time is reported in days for granularity in modelling.

### Parameter estimates

<table>
<thead>
<tr>
<th>BR Blocks</th>
<th>( T_1 ) (°C)</th>
<th>( t_1 ) (days)</th>
<th>( k_{Tref} )</th>
<th>( c )</th>
<th>( k_{Tref} )</th>
<th>( c )</th>
<th>( k_{Tref} )</th>
<th>( c )</th>
</tr>
</thead>
<tbody>
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<td>90 days</td>
<td>4</td>
<td>120</td>
<td>20</td>
<td>90</td>
<td>25</td>
<td>0.00175</td>
<td>0.0490</td>
<td>0.00177 ± 0.0003</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>90</td>
<td>37</td>
<td>90</td>
<td>25</td>
<td>0.00210</td>
<td>0.0800</td>
<td>0.00084 ± 0.0004</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>90</td>
<td>4</td>
<td>120</td>
<td>25</td>
<td>0.00145</td>
<td>0.0650</td>
<td>0.00111 ± 0.0001</td>
</tr>
<tr>
<td>180 days</td>
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<td>20</td>
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<td>4</td>
<td>240</td>
<td>25</td>
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<td>0.0380</td>
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</tr>
<tr>
<td>270 days</td>
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<td>20</td>
<td>270</td>
<td>25</td>
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<td>0.0500</td>
<td>0.00114 ± 0.0004</td>
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<td></td>
<td>20</td>
<td>270</td>
<td>37</td>
<td>270</td>
<td>25</td>
<td>0.00108</td>
<td>0.0650</td>
<td>0.00119 ± 0.0004</td>
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<tr>
<td></td>
<td>37</td>
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<td>4</td>
<td>360</td>
<td>25</td>
<td>0.00110</td>
<td>0.0530</td>
<td>0.00114 ± 0.0004</td>
</tr>
</tbody>
</table>

Table 1. Kinetic parameters produced from best match in corresponding model constructed for brown rice (BR), along with averaged kinetic parameters across temperature range which are then used to describe the estimated degradation.

### Parameter estimates

<table>
<thead>
<tr>
<th>PS Blocks</th>
<th>( T_1 ) (°C)</th>
<th>( t_1 ) (days)</th>
<th>( k_{Tref} )</th>
<th>( c )</th>
<th>( k_{Tref} )</th>
<th>( c )</th>
<th>( k_{Tref} )</th>
<th>( c )</th>
</tr>
</thead>
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<td>120</td>
<td>25</td>
<td>0.00190</td>
<td>0.0500</td>
<td>0.00152 ± 0.0001</td>
</tr>
<tr>
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<td>20</td>
<td>180</td>
<td>25</td>
<td>0.000800</td>
<td>0.0200</td>
<td>0.00152 ± 0.0001</td>
</tr>
<tr>
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<td>37</td>
<td>180</td>
<td>25</td>
<td>0.00106</td>
<td>0.0800</td>
<td>0.00152 ± 0.0001</td>
</tr>
<tr>
<td></td>
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<td>4</td>
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<td>0.0470</td>
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<tr>
<td>270 days</td>
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<td>360</td>
<td>20</td>
<td>270</td>
<td>25</td>
<td>0.00145</td>
<td>0.0400</td>
<td>0.00152 ± 0.0001</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>270</td>
<td>37</td>
<td>270</td>
<td>25</td>
<td>0.00152</td>
<td>0.0500</td>
<td>0.00152 ± 0.0001</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>270</td>
<td>4</td>
<td>360</td>
<td>25</td>
<td>0.00160</td>
<td>0.0450</td>
<td>0.00152 ± 0.0001</td>
</tr>
<tr>
<td>360 days</td>
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<td>360</td>
<td>20</td>
<td>360</td>
<td>25</td>
<td>0.00133</td>
<td>0.0350</td>
<td>0.00155 ± 0.0002</td>
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<tr>
<td></td>
<td>20</td>
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<td>37</td>
<td>360</td>
<td>25</td>
<td>0.00152</td>
<td>0.0650</td>
<td>0.00155 ± 0.0002</td>
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<td></td>
<td>37</td>
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<td>4</td>
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<td>0.00180</td>
<td>0.0500</td>
<td>0.00155 ± 0.0002</td>
</tr>
</tbody>
</table>

Table 2. Kinetic parameters obtained from the best match of the data with reconstructed degradation curves for split pea soup (PS), along with averaged kinetic parameters across temperature range.
Table 3. Kinetic parameters produced from best match in corresponding model constructed for beef brisket (BB), along with averaged kinetic parameters across temperature range which are then used to describe the estimated degradation.

Considering that the scope of some experiments is more limited, it is important to note that the model is capable of making predictions regarding compound degradation using one set of parameters, \( k(T_{\text{ref}}) \) and \( c \), without any averaging, within the same program. An example of a prediction being made using the \( k(T_{\text{ref}}) \) and \( c \) from two temperatures, times, and corresponding concentration ratios is shown in Figure 2. Additionally, once average \( k(T_{\text{ref}}) \) and \( c \) values are produced through the three-way pairing mentioned above, those values instead can be input into the model to make predictions about the degradation at any appropriate storage temperature and time. The program is made relatively easy to learn and use by allowing all approximate kinetic parameter determinations to be made visually with the use of sliders, as shown in Figs. 2 and 3.

### 2.4.3 Estimation of thiamine retention

Four distinct predictive models were produced using four independent analyses across the storage temperatures used. Predictive models were produced within the module.
shown in Figure 2 using averaged kinetic parameters for each time block, and the estimated thiamine content following successive 30-day intervals at 20°C was plotted against the experimental values at the same storage temperature to evaluate precision of the kinetic parameters and therefore the precision of the model. Figure 4 shows the resulting degradation estimation from the model, using the corresponding $k(T_{ref})$ and $c$ values from Tables 1-3.
Figure 4. Thiamine degradation in three NASA-provided foods in 20°C storage, including four separate predictive models built from each block of narrow endpoint sets.

Aside from the “90 day” model construct for brown rice and other models after 360 days for beef brisket, there does not exist another predictive model that deviates from the experimental thiamine content by more than ~10%. It is possible that in the case of brown rice, confounding sources of thiamine degradation persisted for the first hundred
days of storage, or there were deviations in heat distribution during the retort process sufficient to over-process some samples in certain regions, leading to a higher than expected degradation when those over-processed samples were analyzed. In the case of beef brisket, low levels of thiamine created a problem with accurate quantification. Although, care was taken to randomize batching and sampling from the retort loads. Despite these specific models, all other predictive models tested revealed high precision compared to experimental values. The model with the lowest deviation from the experimental values across all foods appeared to be the model constructed with data from analyses block “270 days,” 270 days of storage for 20°C and 37°C and 360 days for 4°C. This model explained degradation sufficiently in time already passed and in future thiamine measures for up to 360 days. For this reason, the “270 days” model was used to extrapolate thiamine degradation in each food out to 720 days to assess long-term stability.

The comparison between the thiamine content predictions made using the “270 days” model and the experimental values at each time-point is shown quantitatively in Table 4. At 720 days of 20°C storage, the model prediction was 5.45% off from the experimental values. However, the optimal time allotted for storage prior to analysis for subsequent modelling should be considered on a case-by-case basis.
<table>
<thead>
<tr>
<th>Time (days)</th>
<th>PS Experimental thiamine content (µg/150g)</th>
<th>$k_{ref}$ (270 day average)</th>
<th>c (270 day average)</th>
<th>PS Predicted thiamine content (µg/150g)</th>
<th>Mean difference (µg)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>110.877 ± 5.168</td>
<td></td>
<td></td>
<td>110.877 ± 0.000</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>90</td>
<td>99.670 ± 3.461</td>
<td>0.00152 ± 0.00008</td>
<td>0.045 ± 0.005</td>
<td>99.380 ± 0.300</td>
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<td>0.26%</td>
</tr>
<tr>
<td>180</td>
<td>97.711 ± 2.824</td>
<td></td>
<td></td>
<td>89.074 ± 0.538</td>
<td>8.64</td>
<td>7.79%</td>
</tr>
<tr>
<td>270</td>
<td>80.609 ± 7.635</td>
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<td></td>
<td>79.838 ± 0.724</td>
<td>0.77</td>
<td>0.70%</td>
</tr>
<tr>
<td>360</td>
<td>74.861 ± 0.688</td>
<td></td>
<td></td>
<td>71.559 ± 0.866</td>
<td>3.30</td>
<td>2.98%</td>
</tr>
<tr>
<td>450</td>
<td>68.861 ± 3.461</td>
<td></td>
<td></td>
<td>64.138 ± 0.970</td>
<td>4.72</td>
<td>4.26%</td>
</tr>
<tr>
<td>540</td>
<td>64.213 ± 2.544</td>
<td></td>
<td></td>
<td>57.487 ± 0.547</td>
<td>6.72</td>
<td>6.06%</td>
</tr>
<tr>
<td>630</td>
<td>55.897 ± 1.193</td>
<td></td>
<td></td>
<td>51.526 ± 0.233</td>
<td>4.36</td>
<td>3.94%</td>
</tr>
<tr>
<td>720</td>
<td>52.234 ± 4.750</td>
<td></td>
<td></td>
<td>46.183 ± 0.749</td>
<td>6.05</td>
<td>5.45%</td>
</tr>
</tbody>
</table>

Table 4. Comparison of model prediction vs. experimental thiamine values in split pea soup (PS) stored at 20℃ using averaged kinetic parameters from “270 day” data for up to 720 days. Less than 10% difference was demonstrated at all time points.

2.4.4 Prediction of thiamine content by 720 days

In Figure 5 it is shown what would be expected for thiamine degradation if 20℃ storage persisted for 720 days for each food, using the chosen model. In brown rice, a thiamine retention of 55% from the original content is expected. In split pea soup, a retention of 42% is expected. In beef brisket, a retention of 3% is predicted by the model. By this we can assess generally the relative thiamine stability of each food. Additionally, given the determined kinetic parameters, thiamine retention can be predicted at any time or appropriate temperature. While brown rice and split pea soup could be deemed comparable for thiamine stability for long-term storage, beef brisket performed poorly in this regard. In fact, the thiamine content dropped below 20% in beef brisket after 360 days of storage.
Figure 5. Thiamine degradation estimation up to 720 days in 20°C storage from the kinetic parameters estimation from Block “270 days” data compared to experimental values. The corresponding parameter’s numerical values are listed in Tables 1-3.

Since it has been widely reported that thiamine stability, and food vitamin stability generally, is lower at increased water activity ($a_w$) and pH (B. W. Beadle, D. A. Greenwood, 1943; Dennison, Kirk, Bach, Kokoczka, & Heldman, 1977; Dwivedi & Arnold, 1973; Farrer, 1955), $a_w$ and moisture content as well as pH were analyzed in each
food following packaging. The results are shown in Table 5. Contrary to the relationship expressed by Dennison et al. and Farer et al., it was revealed that beef brisket had the lowest \( a_w \) and pH of the three foods, as well as the lowest moisture content. As reported by Arabshahi & Lund, 1988, proteinaceous food products such as beef or pork demonstrated the opposite effect of \( a_w \) at lower values.

<table>
<thead>
<tr>
<th></th>
<th>Brown rice</th>
<th>Split pea soup</th>
<th>Beef brisket</th>
</tr>
</thead>
<tbody>
<tr>
<td>( a_w )</td>
<td>0.9988</td>
<td>0.9971</td>
<td>0.9653</td>
</tr>
<tr>
<td>Moisture content (%)</td>
<td>61.97</td>
<td>75.72</td>
<td>54.26</td>
</tr>
<tr>
<td>pH</td>
<td>6.25</td>
<td>6.11</td>
<td>5.04</td>
</tr>
</tbody>
</table>

Table 5. Measured \( a_w \), water content, and pH in each food product following packaging.

Additionally, brown rice, which demonstrated the highest overall thiamine stability at the temperature modelled, also contained the highest \( a_w \) of the three foods, at 0.998. It did however, contain a lower moisture content at 62.0% compared to split pea soup, which contained 75.7% moisture by weight. This may be due to the production and thermal processing of the rice grain, which is performed on raw rice. In the retort, liquid broth is intended to be absorbed by the rice to soften it. It is possible that this liquid does not penetrate the entire grain, and leaves a significant portion of the kernel dry and crystallized, and therefore resistant to temporal degradation in storage. It would not be expected then that brown rice and the relationship that it shares with \( a_w \), in regards to thiamine degradation rate, follows the same rule as suggested by some research (Arabshahi & Lund, 1988). Other food matrix considerations such as the presence of sulfur derivatives from protein degradation, peroxide radicals formed from lipid oxidation, the ratio of free to bound thiamine (as pyrophosphates), among other
attributes, may contribute to the differences between food products (Dwivedi & Arnold, 1973) and will require further investigation.

Thiamine degradation of NASA spaceflight foods have been examined and modelled for the first time. Previous works that have demonstrated thiamine degradation (at and around the chosen storage temperatures) follows fixed, first-order kinetics have allowed for the implementation of interactive modelling. With these assumptions, a model has been produced that is capable of reducing much of the work previously necessary to determine kinetic parameters for any compound known to follow fixed, first-order kinetics, for use in multiple industrial applications. The Endpoints Method implemented herein requires the determination of the initial concentration of a given compound in a medium, followed by two separate analyses of that compound’s retention following two dissimilar storage conditions over time to produce kinetic parameters $k(T_{ref})$ and $c$, which relate the temperature to degradation rate, and is an adequate and simpler alternative to the Arrhenius method. Once these parameters are determined, precise predictions of the compound’s concentration can be made at any appropriate time and temperature in a given food.

The spaceflight foods produced using NASA specifications contained varying amounts of thiamine, with varying degrees and rates of degradation. Brown rice and split pea soup contained the highest levels of thiamine and resisted degradation up to 720 days of storage at lower storage temperatures. Beef brisket, however, bore lower levels of thiamine originally and experienced more rapid degradation, offering 25% of the original content into 450 days. The models produced from the experimental thiamine concentrations demonstrated high precision along the current storage timeframe,
particularly for the model constructed with data from 270 days and 360 days of storage under 4°C, 20°C, and 37°C. From using this model to predict thiamine retention in all foods at 20°C for up to 720 days of storage, it was shown that brown rice and split pea soup continued to resist degradation to about the same degree. However, predictions regarding vitamin retention can be conducted at any time or appropriate temperature. Beef brisket contained 3% of the original thiamine content after 720 days of storage according to the model. To minimize sampling and analytical errors, and to improve the estimated degradation long-term, kinetic parameters can also be estimated using endpoints from drastically different times and temperatures of storage. In this way, slight deviations observed in the compound’s degradation at any one point in food storage can be balanced against another.

To potentially explain the differences in thiamine degradation rates among foods, the water activity, moisture content, and pH was investigated, but did not align with the conventional knowledge surrounding the effect of $a_w$ or pH on degradation rate and thiamine stability. Further work needs to be done to discern the critical food matrix properties that significantly contribute to the variation in thiamine degradation among foods. There is interest to produce a robust model that can take into account these food matrix properties and adjust degradation kinetics accordingly.
CHAPTER 3
FREEZE DRYING ALTERS DEGRADATION KINETIC RATE AND TEMPERATURE DEPENDENCE OF THIAMINE IN NASA SPACEFLIGHT FOODS

3.1 Abstract

Freeze-drying is an advanced and energy-intensive preservation technique that is often employed to ensure a much longer shelf-life and nutritional stability profile of suitable food products. In this study, the degradation kinetics of thiamine in three freeze-dried versions of NASA spaceflight foods (brown rice, split pea soup, BBQ beef brisket) during storage was determined for the first time, using an interactive isothermal model and program developed by our group. Spaceflight foods were manufactured according to NASA’s specification, with the replacement of thermal processing by freeze-dry stabilization. Thiamine content in these freeze-dry foods was monitored for up to 720 days at three storage temperatures, 4°C, 20°C, and 37°C, in order to produce a predictive model that estimates thiamine content at any time and temperature of storage. Results showed that our isothermal model was effective at predicting vitamin content up to 720 days for each food. The degradation across freeze-dried foods demonstrated low temperature sensitivity, but not an insignificant intrinsic degradation rate, such that at 20°C, there was a retention of 46% thiamine was observed in brown rice, 62% was preserved in split pea soup, and 11% was preserved in BBQ beef brisket.

3.2 Introduction

The Advanced Food Technology project from NASA aims to produce a robust nutritional program for astronauts with the intent to sustain current short-duration
missions (i.e. research and operations onboard the International Space Station) and to ensure optimal health and logistical controls onboard long-duration missions that are being planned, such as the anticipated manned Mars-bound mission in the coming years. Given that long-duration missions outside of near-earth orbit produce unique obstacles for both procuring fresh and nutritious food sources as well as protecting those food sources from quality and nutritional decay, extra consideration needs to be taken in investing in food preservation techniques that reduce the need to resupply food stores and to protect food nutrients from thermal or temporal degradation.

Thermal stabilization is the most common shelf stabilization technique in the food industry by a large margin. Thermal stabilization provides superior protection to bacterial spoilage over refrigerated or otherwise unstabilized food products. While thermal stabilization provides a superior mouthfeel and flavor profile overall compared to freeze drying, it has been shown that reducing the water activity ($a_w$) in a food matrix significantly improves the retention of essential nutrients and lowers the temperature sensitivity of those nutrients in regards to their degradation and loss (Dibazar, Ghanem, & Brooks, 2016; Gaiek, Lange, & Weber, 1994; Sharma & Le Maguer, 1996; Uddin, Hawlader, Ding, & Mujumdar, 2002). To elucidate the absolute retention, degradation rate, and temperature sensitivity of nutrients of interest to NASA following freeze drying, an experiment was conducted in which the Isothermal Version of the Endpoints Method was utilized (Peleg et al., 2016, 2014).

### 3.2.1 Theoretical Background

The kinetics of thiamine degradation during storage, like that of most vitamins of interest, has been traditionally determined from sets of experimental isothermal
concentration or concentration ratio vs. time relationship determined at 3-5 different temperatures. It has been established that it follows first order kinetics, i.e., described by the rate equation (Van Boekel, 2008):

$$\frac{dC(t)}{dt} = -k[T(t)]C(t)^n \quad (1)$$

where $C(t)$ is the vitamin’s concentration at time $t$, $k[T(t)]$ is the momentary rate constant at the momentary temperature $T(t)$ and $n$ the reaction order in our case $n = 1$. The boundary condition is that at $t = 0$, $C(0) = C_0$, the initial concentration.

The solution of Eq. 1 for isothermal degradation and first order kinetics, $T =$ constant, and $n = 1$ is the familiar exponential decay:

$$\text{Exp} \left[ \frac{C(t)}{C_0} \right] = -k[T]t \quad (2)$$

where $C(t)/C_0$ is the vitamin’s concentration ratio, $0 < C(t)/C_0 \leq 1$. (Arabshahi & Lund, 1988; E.A. Mulley & C.R. Stumbo, 1975; Kamman, Labuza & Warthesen, 1981; Kwok, Shiu, Yeung, & Niranjan, 1998; Pachapurkar & Bell, 2005; Peleg, Normand, & Goulette, 2016; Ramaswamy, Ghazala, & van de Voort, 1990).

### 3.2.2 The temperature dependence of $k(T)$

Traditionally, the temperature-dependence of the rate constant $k(T)$ has been described by the Arrhenius equation:

$$k(T) = k(T_{\text{ref}})\text{Exp} \left[ \frac{E_a}{R} \left( \frac{1}{T} - \frac{1}{T_{\text{ref}}} \right) \right] \quad (3)$$

where $k(T)$ and $k(T_{\text{ref}})$ are the rate constants at absolute temperatures $T$ and a chosen reference temperature $T_{\text{ref}}$ in degrees K, $E_a$ the “energy of activation” in J or kJ (or cal or kcal) per mole and $R$ the Universal gas constant in the corresponding units. Concerns regarding the applicability of the Arrhenius equation to food science exist, and while the
conventional method does produce meaningful data, a simpler and more appropriate
equivalent exists (Peleg et al., 2012; Van Boeckel, 2008):

\[ k(T) = k(T_{ref}) \exp[c(T - T_{ref})] \]  \hspace{1cm} (4)

where \( c \) is a constant and related to the energy of activation by:

\[ c \approx \frac{E_A}{R(T_{ref}+273.16)^2} \]  \hspace{1cm} (5.a)

\[ E_A \approx cR(T_{ref} + 273.16)^2 \]  \hspace{1cm} (5.b)

This replacement model does not compress and invert the temperature scale and
does not require the assumption that there is temperature-independent energy of
activation of a single predominating chemical reaction leading to the loss kinetics under
investigation, replacing it by the temperature sensitivity parameter \( c \) (Peleg et al., 2012,
2014).

According to this model, the kinetic parameters \( k(T_{ref}) \) and \( c \) can be extracted
from two concentrations ratios \( C_1(t_1) \) and \( C_2(t_2) \), determined after storage times \( t_1 \) and \( t_2 \),
at two constant temperatures, \( T_1 \) and \( T_2 \) by solving the following two simultaneous
equations:

\[ C_1(t_1) = \exp\{-k(T_{ref})\exp[c(T_1 - T_{ref})]t_1\} \]  \hspace{1cm} (6)

\[ C_2(t_2) = \exp\{-k(T_{ref})\exp[c(T_2 - T_{ref})]t_2\} \]  \hspace{1cm} (7)

where \( k(T_{ref}) \) and \( c \) are the two unknowns.

3.2.3 Estimating future concentration ratios during storage

Once \( k(T_{ref}) \) and \( c \) have been estimated from two experimentally determined
concentration ratios, they can be used to estimate the concentration ratio at any other
temperature \( T_3 \), \( C_3(t_3) \), in a pertinent temperature range. The predicted value can then be
compared with a corresponding experimental value to validate the kinetic model and the assumptions on which it is based.

Solving for two unknowns is facilitated by the NDSolve function of Mathematica® (Wolfram Research, Champaign IL, USA), the program utilized in model construction and its use. **Figure 6** shows a plot of experimental endpoints consisting of final storage temperature, final analysis time, and the residual concentration of the target compound, expressed as a concentration ratio. When parameters $k(T_{ref})$ and $c$ are identified through visual assisted selection and the NDSolve function, degradation curves are generated in the right modules of **Figure 6**.

![Figure 6. Demonstration of the Endpoints Method graphical interface. Left: the two experimental endpoints corresponding to the momentary and concentration coordinates. Right: the endpoints are met with corresponding degradation curves that are defined by degradation parameters $k(T_{ref})$ and $c$.](image)

This study sought to utilize the isothermal version of the Endpoints Method to determine the kinetic parameters of thiamine degradation in three NASA-provided
formulations (or ‘recipes’ in NASA’s terminology) which are then freeze-dried, and to offer insight on the optimum experimental parameters ($T$ and $t$) to perform, minimizing analytical inventory, sampling, and waste. From these data, the degradation of the labile vitamin thiamine can be estimated in these foods for use in NASA food program process considerations.

3.3 Materials and Methods

3.3.1 NASA’s specifications for brown rice, split pea soup, and BBQ beef brisket

Food products were initially produced according to the food composition and manufacturing specifications provided by NASA. Specifications detailed the precise formulation for each food product, quality inspection procedure, package testing, and storage instructions. These specific products were chosen jointly by NASA and our group as representative of un-enriched foods with a potential high thiamine instability (data not shown). As the specifics of these recipes are confidential, the exact details are withheld in this work.

3.3.2 Ingredient acquisition and food production

Bulk brown rice, low-sodium vegetable broth, green split peas, boneless ham, raw deckle-off beef brisket, BBQ sauce, and dry BBQ marinade were purchased from Performance Foodservice (1 Performance Blvd, Springfield, MA, USA). Liquid smoke flavoring, ham base, mono-sodium glutamate (MSG), salt, pepper, whole milk, corn starch, red wine vinegar, and bullion were purchased from a local grocery store (175 University Drive, Amherst, MA, USA). All foods were produced abiding by the precise instruction of the NASA specifications.
The brown rice product consisted of raw brown rice and low-sodium vegetable broth, such that the thermal process to obtain shelf-stabilization also cooks the rice thoroughly. Split pea soup was produced after initial rinsing of the dried split peas. Beef brisket was produced by trimming excess fat and coating in BBQ dry rub, wrapping in plastic wrap, and marinating for 24 hrs in refrigerator.

### 3.3.3 Packaging

The prepared spaceflight foods were filled into flexible aluminized pouches [12.065 cm x 20.48 cm (4.75” x 8.0625”); Tan PE/.0007Foil/3mil Coex Sealant] that were purchased from Heritage Packaging (441 Market St, Lawrence, MA, USA). Approx. 25 kg of brown rice product, including broth, was produced and distributed into pouches to a final weight of 121 g in each pouch, producing approx. 200 units. 200 pouches of split pea soup were produced in total and filled to a weight of 150 g per pouch, and another 200 units of beef brisket product were produced at a fill weight of 120 g per pouch. Pouch opening was cleaned and dried prior to vacuum-sealing which was done using VacMaster SVP 20 (Overland Park, KS, USA) to remove excess air. Pouches were then aliquoted into batches of 44 pouches for shelf-stabilization.

### 3.3.4 Shelf-stabilization

Prior to freeze-dry processing, all packaged food samples were placed in a -40°C for at least 24 hours to fully freeze. Samples were individually removed from the freezer and comminuted by hand or mallet to increase surface area for moisture transmission. Samples were indexed and placed inside Glad-brand sandwich sized (6-5/8” x 5-7/8” / 16.8 x 14.9 cm) double zipper bags (The Glad Products Co., 1221 Broadway, Oakland, CA 94612) and placed directly into Genesis Pilot Lyophilizer (SP Scientific, 3538 Main
Each freeze-dried batch was gradually ramped from -40°C to 25°C for the primary process (See Supp. 1). Following a total process time of ca. 3.5 days, all samples were removed and re-packaged by a double enclosure system consisting of: a clear primary MB 225L pouch (5” x 8.75” / 12.7 cm x 22.225 cm, 225 micron nylon/EVOH/enhanced linear low density polyethylene) purchased from Winpak (100 Saulteaux Crescent, Winnipeg, MB R3J 3T3, Canada), and an opaque white aluminized secondary pouch (7” x 12” OD / 17.78 cm x 30.48 cm, Seal depth: 3/8” / 0.9525 cm; 1/48 gauge PET/98 gauge White OPP/.00035 Foil/2mil LLDPE with tear notches at 3/4” / 1.905 cm) purchased from Technipaq (975 Lutter Dr, Crystal Lake, IL 60014, USA).

VacMaster SVP 20 (Overland Park, KS, USA) at 1.016 bar (~30 in. Hg) was used to seal pouches.

### 3.3.5 Long-term storage

Food products were placed in varying storage vessels at: 37°C, 20°C, and 4°C for routine sampling and analysis for use in the model, and regularly inspected for swelling or other signs of spoilage or pouch leaks. Sampling occurred in regular 90-day intervals up to 720 days for products stored at 37°C and 20°C and 120 days intervals up to 720 days for 4°C.

### 3.3.6 Thiamine extraction

Vitamin extraction followed a modified version of the Association of Official Agricultural Chemists (AOAC) International official method 942.23 Thiamine (Vitamin B\textsubscript{1}) in Human and Pet Foods (AOAC 942.23, 2005; Ma & Wyatt, 1983). Modifications were made to better suit the process of thiamine extraction and detection for these three spaceflight foods. The phosphorolytic enzyme used was taka-diastase (Pfaltz & Bauer,
172 E. Aurora St., Waterbury, CT, USA) due to proven equivalent activity to the suggested enzymes. Chromatographic columns used for purification were Kimble™ Kontes™ FlexColumns™ at 1.0 cm x 20 cm, 16 mL capacity using the Three-way Luer Stopcock of the same brand. Sodium acetate, sodium hydroxide, potassium chloride, hydrochloric acid, potassium ferricyanide, isobutanol, thiamine hydrochloride as well as thiamine pyrophosphate were all purchased from Fisher Scientific (168 Third Avenue, Waltham, MA, USA). Quinine sulfate and the purification resin utilized in these columns, Amberlite CG-50 (Type I), were both purchased from Sigma-Aldrich (1 Strathmore Rd, Natick, MA, USA).

3.3.6.1 Acid and enzyme digestion

Digestions were conducted in quadruplicate for all foods and temperature treatment, plus the thiamine hydrochloride and thiamine pyrophosphate standards. Freeze dried foods were first re-diluted with sterile DI water to obtain original sample weight prior to freeze-drying. Brown rice products lost an average of 61% of their original weight in lyophilization, while split pea soup and beef brisket lost 77% and 54% of their original weight in moisture, accordingly. Rehydrated products were then ‘stomached’ and added to a kitchen-scale blender to homogenize fully. 0.1 M HCl was used as the extraction buffer. Acidified samples were digested at 100°C for 30 minutes, cooled to <50°C and aliquoted. Aliquots were diluted with 0.1 M HCl and readjusted to pH 4.0-4.5 with double-normal sodium acetate. 5 mL 1% aqueous taka-diastase solution was then added, and the sample incubated at 45°C for 3 hrs. Aliquoted samples were then acidified with 2 M HCl to pH 3.5 and refrigerated overnight.
3.3.6.2 Filtration and purification of thiamine

Samples were then re-mixed and aliquoted evenly into centrifuge tubes and centrifuged at 3220 g, 4°C, for 5 minutes. Supernatant was filtered through Whatman™ Quantitative Grade 40 Filter Paper (Fisher Scientific) using vacuum pump assistance to collect crude extract. Crude extract was fed through chromatographic columns containing Amberlite CG-50 resin adjusted to pH 5. Columns were washed with near-boiling H₂O and thiamine was eluted with near-boiling 0.1 M HCl solution containing 25% KCl.

3.3.6.3 Oxidation of thiamine for detection

Two 5 mL portions from the final eluate from each sample were aliquoted into reaction tubes. The remaining solution was stored at -80°C in case of need to re-analyze. Tubes were wrapped in aluminum foil and the remaining steps were conducted in a dark chemical hood to minimize photodegradation. 3 grams solid KCl were added to each reaction tube, along with 3 mL oxidizing reagent (0.04% potassium ferricyanide in 15% aqueous NaOH) for one of the two reaction tubes, while 3 mL 15% aqueous NaOH was added to the other, to serve as the blank. Immediately following the addition of oxidizing or blank solution, 4 mL isobutanol was added to the reaction tubes and shaken. Both thiamine standards were treated identically to the above samples. All samples were then shaken again and allowed to settle before continuing.

The isobutanol layer was collected (top layer) and aliquoted into 8 wells per sample (oxidized or blank) of a 96-well flat-black-bottom plates (Fisher Scientific). Plates were protected from light using aluminum foil until read using a BioTek Synergy 2 plate reader (100 Tigan St., Winooski, VT, USA) running a fluorometric protocol at input
filter 365 nm and output filter 435 nm, and fluorometric data collected using Gen5 software version 1.09 (BioTek).

3.3.6.4 Modifications from the original method

Modifications made to the original AOAC method 942.23 included decreasing original sample volume and dilution factor, re-concentration in less isobutanol during thiochrome solubilization, and detection using a fluorometric plate reader. Modifications did not produce any significant decrease in overall extraction efficiency. Plate reader was calibrated against quinine sulfate solution. Taka-diastase phosphorolytic efficiency was measured by a pure thiamine pyrophosphate digestion. Recovery of >85% free thiamine was deemed suitable.

3.3.7 Thiamine concentration determination

Thiamine content in the reaction solution prior to oxidation is calculated by comparison to the results of a thiochrome standard curve made from a separate thiamine hydrochloride standard and dilution with NaOH prior to oxidation and measurement. Appropriate volumetric dilution and mass fraction was calculated back to express the original thiamine content in each food. The mean of each quadruplicate set was calculated as well as the standard deviation. Thiamine content was expressed as mean μg/g ± SD of a given food at that time point of storage and μg thiamine per pouch ± SD at that time point in storage. Thiamine content was then compared to the initial value of each food (t = 0), taken immediately following retort processing and expressed as a concentration ratio (C_t / C_0).
3.3.8 Application of the model to experimental data

Momentary concentration, time duration in storage and temperature of that storage were recorded for use in the modelling program. Any pair of momentary data sets were input into the program entitled Prediction of Isothermal Degradation by the Endpoints Method and as the model requires, parameters $k(T_{ref})$ and $c$ are adjusted in tandem to produce estimated degradation trajectories that pass through both endpoints. The parameters that produce a satisfactory fit for the model are then recorded. Theoretically, these parameters describe the entire temperature-dependence and time-effect of degradation. However, in this work, parameters are averaged from four discrete blocks of three momentary data sets (90, 180, 270, 360 days, see Tables 1-3) from like times up to 360 days, across the temperature range: 4°C, 20°C, and 37°C to produce an improved interpolation effect and diminish the effect of sampling error. Though any and all combinations of time-temperature data may in included and averaged for a more holistic picture, our approach sought to minimize data handling and improve model accuracy. Average $k(T_{ref})$ and $c$ values were then used to produce predictive trajectories for thiamine degradation in its respective food medium at typical ambient storage temperature, for NASA’s purposes this would be 20°C, all within the same module.

3.3.9 Moisture loss determination

Contents from pouches were weighed prior to and following freeze-dry processing to assess moisture loss. It was assumed that weight lost was entirely from moisture due to water and the mass of lost volatiles was insignificant.
3.4 Results and discussion

3.4.1 Thiamine content by food, storage time, and storage temperature

Freeze-dried brown rice (BR), split pea soup (PS), and BBQ beef brisket (BB) stored at 4°C, 20°C, and 37°C were periodically pulled and analyzed for their thiamine content (Figure 7). Thiamine concentration within each food was expressed as μg per serving mass, in grams. It was observed that in freeze-dried (FD) brown rice, temperature served to accelerate degradation of thiamine such that at identical or similar time points of analysis, foods stored at lower temperatures would exhibit higher thiamine content. This temperature dependence of degradation rate was less affectual in FD PS samples across the temperature range tested throughout the storage lifetime, similar to FD BB samples.
Figure 7. Experimental thiamine loss in three freeze-dried spaceflight foods during storage at three constant temperatures for 720 days.

It was observed that initial thiamine contents were similar and relatively high within PS and BR products at ca. 115 μg per serving. In absolute abundance, the FD BR product was the richest in thiamine initially. FD BB demonstrated much less thiamine overall and a much lower stability in regard to temporal degradation such that in 37°C storage, thiamine was undetectable at 540 days of storage and on.

Given that the purpose for freeze-dry processing is generally to protect food nutrients from degradation and transformation at high temperatures as opposed to thermal stabilization processes, these data were compared to thiamine measures from thermally
processed versions of these same spaceflight foods which were also stored in the same conditions for the same storage lifetime, and analyzed simultaneously (Chapter 2, Figure 3). Improvement in thiamine retention by freeze-drying as opposed to thermal processing was calculated and is shown in Figures 8-10.

Figure 8. Freeze-dried foods stored at 4°C were measured for their thiamine content and compared to thermally processed versions that were measured for their thiamine content simultaneously. Percent difference, increase or decrease, of thiamine in these versions were compared at each point of analysis for up to 720 days.
Figure 9. Freeze-dried foods stored at 20°C were measured for their thiamine content and compared to thermally processed versions that were measured for their thiamine content simultaneously. Percent difference, increase or decrease, of thiamine in these versions were compared at each point of analysis for up to 720 days.

Retention of thiamine immediately following stabilization processing was improved by a large margin in brown rice that was freeze-dried (47% increase in initial thiamine on a per serving basis). Although, the same improvement was not observed in the other two foods. Despite the lack of initial improvement in thiamine retention in PS and BB samples, at subsequent points of analysis, thiamine retention was generally improved by freeze-drying. Additionally, the degree to which freeze-drying improved thiamine retention at points of analysis in freeze-dried foods was dependent on storage temperature.

Freeze-dried foods stored at 4°C and 20°C experienced improvements in thiamine retention compared to their thermally processed version by no more than 50% on
average. By the end of the storage time frame of 720 days in each respective temperature, thiamine retention was higher in the freeze-dried versions. It was observed that thiamine retention, or rather, the protection against thiamine degradation, was particularly effective at 37°C, where thiamine within BR was >125% more abundant at 720 days of storage compared to thermally processed BR, and thiamine within PS was 200% more abundant than in its thermally processed counterpart at 720 days. The same improvement was found in FD BB samples stored in this condition, until the point where thiamine was no longer detectable within freeze-dried samples (Figure 5). These data suggest an alteration in the temperature sensitivity of thiamine and its degradation in foods with reduced moisture content, which was analyzed further by comparisons of the degradation parameters determined by the Endpoints Method in this work.
Figure 10. Freeze-dried foods stored at 37°C were measured for their thiamine content and compared to thermally processed versions that were measured for their thiamine content simultaneously. Percent difference, increase or decrease, of thiamine in these versions were compared at each point of analysis for up to 720 days.

3.4.2 Kinetic parameter determination and prediction of degradation at 20C

The program and embedded model utilized in the determination of kinetic parameters of thiamine degradation in each freeze-dried food is shown in Figure 6. An example of two sets of temperatures, times, and endpoint concentration ratios are shown as inputs into the model in the top left of both module. The right panel demonstrates a fit produced by adjusting parameters $k(T_{ref})$ and $c$, and therefore a mathematical description of the degradation experienced in both cases of storage via Eqs. 6 and 7, and in theory, a description of the temperature-dependence of thiamine degradation in that food via Eq. 4. In this work, averaged $k(T_{ref})$ and $c$ parameters were determined using the three possible
combinations of temperatures, times, and resulting concentration ratios from four distinct time points of analyses, as seen in Tables 6-8.

Average $k(T_{\text{ref}})$ and $c$ values were gathered from separate analytical groupings/blocks and subsequent averaging of their three possible model constructions after: 90 days of storage for 20°C and 37°C and 120 days for 4°C (90 days), 240 days of storage for 20°C and 37°C and 360 days for 4°C (180 days), 270 days of storage for 20°C and 37°C and 360 days for 4°C (270 days), 360 days of storage for 20°C and 37°C and 360 days for 4°C (360 days). Time is reported in days for granularity in modelling.

<table>
<thead>
<tr>
<th>FD BR Prediction set</th>
<th>Single model</th>
<th>Average model</th>
</tr>
</thead>
<tbody>
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<td>$T_1$ (°C)</td>
<td>$t_1$ (days)</td>
<td>$T_2$ (°C)</td>
</tr>
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<td>20</td>
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Table 6. Freeze-dried (FD) brown rice (BR) degradation parameters determination using four time-blocked data sets, corresponding to endpoint times, temperatures, and resulting thiamine concentration measures. Degradation parameters $k_{\text{ref}}$ and $c$ are used to describe the degradation rate in parts per unit time, and the temperature dependence of degradation rate, respectively, and models can be constructed through substitution of novel times and temperatures within our proposed rate equation.
Table 7. Freeze-dried (FD) split pea soup (PS) degradation parameters determination using four time-blocked data sets, corresponding to endpoint times, temperatures, and resulting thiamine concentration measures. Degradation parameters $k_{T_{ref}}$ and $c$ are used to describe the degradation rate in parts per unit time, and the temperature dependence of degradation rate, respectively, and models can be constructed through substitution of novel times and temperatures within our proposed rate equation.

Parameters were determined for each pair of temperatures and storage times, with corresponding thiamine concentration measures, and subsequently averaged by groups of
90-day time intervals (ca. every 3 months) for up to 360 days. Each resulting set of degradation parameters \( k_{T_{\text{ref}}} \) and \( c \) define a model described by a general case of either Eq. 6 or 7 such that any pertinent times and temperatures of storage can be entered, along with the reference temperature originally chosen to determine \( k_{T_{\text{ref}}} \) and \( c \) for an estimation of thiamine content, or theoretically, any compound that decays following fixed first-order kinetics (Peleg et al., 2016, 2014).

3.4.3 Estimation of thiamine retention in freeze-dried foods

Following the estimation of kinetic parameters \( k_{T_{\text{ref}}} \) and \( c \), and thereby establishing a sort of predictive model for thiamine retention at select times and temperatures, experimental data was compared to model-predicted thiamine content at the same time intervals at 20°C to gauge model fitness (Figure 11). 20°C was selected as the test condition due to the fact that this condition represents the likely and approximate food storage situation on both short and long-duration missions for NASA.
Figure 11. Thiamine degradation in three NASA-provided foods in 20°C storage, including four separate predictive models built from each block of endpoint sets: 90, 180, 270, and 360 days.

In FD BR and FD PS experimental vs. predicted thiamine retention, the “90 day” model overpredicted thiamine degradation over time, which improved in later models. Residuals analysis was conducted for each freeze-dried food and final error (experimental vs. predicted concentration retention of original thiamine amount) at 720 days, and average model error, was determined (Tables 9-11).
FD BR 20°C Experimental vs. Model Residuals

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>90 Day Residuals</th>
<th>180 Day Residuals</th>
<th>270 Day Residuals</th>
<th>360 Day Residuals</th>
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<td><strong>6.7%</strong></td>
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Residual Avg | 9.1% | 0.3% | -2.2% | 1.5%

Overestimates | 8 | 4 | 1 | 5

Underestimates | 0 | 3 | 7 | 3

Table 9. Freeze-dried (FD) brown rice (BR) experimental vs predicted retention residual values for 20°C storage in 90-day time increments created using the average degradation parameters from 90, 180, 270, and 360 day models. Cases of model overestimation or underestimation are presented, and the majority case is listed as Avg Prediction. In bold are the residual values at the conclusion of the storage lifetime and the average residual value for the entire course of experimental analysis and corresponding model.

FD PS 20°C Experimental vs. Model Residuals

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<th>Time (days)</th>
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Residual Avg | **13.9%** | **10.3%** | **5.2%** | **2.2%**

Overestimates | 7 | 6 | 6 | 5

Underestimates | 1 | 2 | 2 | 3

Avg Prediction | Overestimate | Overestimate | Overestimate | Overestimate |

Table 10. Freeze-dried (FD) split pea soup (PS) experimental vs predicted retention residual values for 20°C storage in 90-day time increments created using the average degradation parameters from 90, 180, 270, and 360 day models. Cases of model overestimation or underestimation are presented, and the majority case is listed as Avg Prediction. In bold are the residual values at the conclusion of the storage lifetime and the average residual value for the entire course of experimental analysis and corresponding model.
FD BB 20°C Experimental vs. Model Residuals

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<tr>
<td>0.5%</td>
<td>6</td>
<td>2</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 11. Freeze-dried (FD) BBQ beef brisket (BB) experimental vs predicted retention residual values for 20°C storage in 90-day time increments created using the average degradation parameters from 90, 180, 270, and 360 day models. Cases of model overestimation or underestimation are presented, and the majority case is listed as Avg Prediction. In bold are the residual values at the conclusion of the storage lifetime and the average residual value for the entire course of experimental analysis and corresponding model.

Residuals analysis revealed that “270 day” and “360 day” models offered the lowest final residuals at 720 days, and generally the lowest average residual values compared to the models produced with earlier storage data. The “360 day” model produced predictions that were within 10% of the experimental value in each case, with correspondingly low average residuals overall. Overestimations of thiamine loss in predictive models were nearly universal, with only one exception in FD BR (Table 9). This result is most likely due to the temperature dependence of thiamine degradation such that the 37°C storage condition produced a much greater effect on thiamine loss than either 4°C or 20°C that could not be explained purely by the temperature dependence relationship assumed in either the traditionally-used Arrhenius relationship (Eq. 3) or our replacement (Eq. 4). Since degradation parameters used in modelling were produced for each pair of temperatures and times and then averaged, the dramatic loss observed at
37°C resulted in an anticipation of greater thiamine loss than what was experimentally observed. However, it should be noted that a bias towards overestimation of thiamine loss is somewhat preferable to underestimation or undetermined or random bias, in the sense that endeavors by NASA to resupply the food system of astronauts would benefit from cautious or preemptive action. Overconfidence in the nutritional quality of foods stored onboard could produce dangerous situations for the health of the crew.

Alterations in thiamine degradation rate and temperature sensitivity are to be expected upon major food matrix property changes, particular those including moisture content and pH (Dwivedi & Arnold, 1973). This is due to both chemical and mechanistic considerations of thiamine conversion and breakage, as well as molecular water acting as a general carrier and medium for reactive species. As such, the kinetic parameters of thiamine within freeze-dried foods generated through our modelling procedure were compared to those established in their thermally processed counterparts shown in Chapter 2, Tables 1-3 (Table 12).

<table>
<thead>
<tr>
<th>Predictive Model</th>
<th>Food</th>
<th>Avg $k_{25\degree C}$</th>
<th>Avg $c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>270 Day</td>
<td>Brown Rice</td>
<td>0.001110</td>
<td>0.05667</td>
</tr>
<tr>
<td></td>
<td>FD Brown Rice</td>
<td>0.001222</td>
<td>0.01600</td>
</tr>
<tr>
<td></td>
<td>Split Pea Soup</td>
<td>0.001523</td>
<td>0.04500</td>
</tr>
<tr>
<td></td>
<td>FD Split Pea Soup</td>
<td>0.001083</td>
<td>0.01267</td>
</tr>
<tr>
<td></td>
<td>Beef Brisket</td>
<td>0.006100</td>
<td>0.03833</td>
</tr>
<tr>
<td></td>
<td>FD Beef Brisket</td>
<td>0.002573</td>
<td>0.02483</td>
</tr>
</tbody>
</table>

Table 12. Kinetic parameter average from “270 day” model produced for each food.

Freeze-drying, as suggested by degradation parameters associated with the “270 day” model, had similar effects on both BR and PS products. It was observed that the degradation rate of thiamine at the chosen reference temperature of 25°C remained relatively low in BR and PS, but there was a stark decrease in the temperature sensitivity
in both products. This is to say that that relationship between storage temperature and estimated degradation rate was made less affectual following freeze-drying. In other words, freeze-dried versions of BR or PS would experience a lowered protective effect from refrigeration on thiamine loss, and also a lesser effect in temperature abuse conditions. FD BB experienced a similar decline in temperature sensitivity, with an additional decrease in intrinsic degradation rate compared to its thermally processed version.

Overall, freeze-drying offered improvements in initial thiamine retention compared to thermal processing. Thiamine was apparently more stable in freeze-dried versions of foods across time and the temperatures tested in this work. Our approach in determining kinetic parameters that explain thiamine degradation as it relates to time and temperature was successful and produced models with <10% deviation in their prediction compared to experimental values. Modelling explained superior resistance to temperature-related degradation in freeze-dried products compared to thermally processed products, but degradation parameters were altered in inconsistent ways, seemingly due to differences in food matrix composition, chemistry and structure.

The integral constituents in these foods that contribute to such alterations in degradation parameters on thiamine are yet to be conclusively determined, and merits further investigation. Developing a model than can incorporate these food matrix properties in its kinetic parameters’ estimation can eliminate the need to perform independent experiments on each food when dealing with one compound of interest, and rather, these properties can simply be measured and entered into the program, and their individual weight of effect on \( k_{Tref} \) and \( c \) can be instantaneously applied.
CHAPTER 4

NON-ISOTHERMAL ENDPOINTS METHOD AS A NOVEL TECHNIQUE FOR
THE PREDICTION OF THIAMINE LOSS DURING THERMAL PROCESSING

4.1 Abstract

Thermal processing is a common and effective food preservation technique for
the prevention of enzymatic deterioration and bacterial spoilage. However, the exposure
of food products to high temperatures and pressures in this way invariably leads to
degradation of both the food matrix and the nutrients contained within that matrix.
NASA’s current food program for astronauts includes many commercial and produced
food products that are preserved in this way. In order to elucidate the real effects of
thermal processing on nutrient losses in their food products, and to create optimum
thermal processes that minimize the degradation of nutrients while achieving the targeted
microbial kill, an experiment was conducted based on the utilization of a novel Non-
Isothermal Version of the Endpoints Method, which was shown to produce reliable
predictions of the loss of thiamine present in three NASA-identical food products (brown
rice, split pea soup, and BBQ beef brisket) following a thermal process that would be
common in food process applications. The predictive model generated by the Endpoints
Method, produced thiamine retention predictions within the measurement variation of the
experimental data for all foods.

4.2 Introduction

Although the investigation of vitamin degradation kinetics would be considered
an exhausted field of study in the realm of food science by most researchers at this time,
interest has been recently renewed by The National Aeronautics and Space
Administration (NASA) to research the nature of vitamin degradation and its relationship to processing conditions undergone to render their spaceflight food safe for storage and consumption by the astronauts aboard short-duration and long-duration missions. Due to this renewed research interest, an experiment was devised for the construction of predictive models (defined simply by a set of degradation parameters) that would: 1. Discern the relationship between a compound of interest, its intrinsic instability to deterioration, and process temperature and time and 2. Quantify and manipulate that relationship in such a way that process temperature and time could be optimized during processing for the minimization of vitamin loss in spaceflight foods.

Thiamine (vitamin B₁) was selected for investigation because of its prevalence in common astronaut eating programs, its essential nature for human health, and for its documented heat instability (data now shown). Thiamine in food has been long demonstrated to become oxidized into oxythiamine (Pachapurkar & Bell, 2005), degrade into its constituent thiazole and pyrimidine moieties when subject to high temperature at slightly acidic pH values, as well as decay into volatiles such as hydrogen sulfide, 2-methylfuran, 2-methylthiophene, and 2-methyl-4-5-dihydrothiophene, and in some cases, elemental sulfur (Dwivedi & Arnold, 1973). In any case, it has been well supported that thiamine degradation into its constituent byproducts follows fixed, first-order kinetics (E.A. Mulley, C.R. Stumbo, 1975; Kwok et al., 1998; Peleg et al., 2016; Ramaswamy et al., 1990). The model utilized herein assumes that the degradation rate order does not change within the temperature or time ranges tested. Given this assumption (that \( n = 1 \) and does not change), a decay equation can be written:

\[
\frac{dC(t)}{dt} = -k[T(t)]C(t) \quad (1)
\]
where \( C(t) \) is the concentration at time \( t \), and \( k \) is the momentary rate constant at a specified temperature for a duration of time \( t \) (Van Boekel, 2008).

For an isothermal case where there exists a constant temperature \( T(t) = T \), and for the boundary condition of initial concentration of a compound \( C(0) = C_0 \), the Eq. 1 has an analytical solution described by:

\[
\frac{C(t)}{C_0} = \text{Exp}\left[-k(T)t\right] \quad (2)
\]

With the same terms as annotated in Eq. 1. In order to determine the momentary rate constant \( k \) as it pertains to a given process temperature at a given time, a temperature dependence must be established, assuming such a relationship exists. A temperature sensitivity term can be related to the change in degradation rate \( k \) by the following relationship (Corradini & Peleg, 2004; Peleg et al., 2012, 2016, 2014):

\[
k(T) = k(T_{\text{ref}}) \text{Exp}\left[c(T-T_{\text{ref}})\right] \quad (3)
\]

where the rate constant \( k \) is defined at a reference temperature \( (T_{\text{ref}}) \) and is applied to the temperature sensitivity term \( c \) as it relates to the temperature of interest \( T \). This relationship is based on the Arrhenius equation in that the temperature sensitivity term \( c \) is related to the activation energy \( E_a \) by the equation:

\[
E_A \approx cR(T_{\text{ref}} + 273.16)^2 \quad (4)
\]

Where \( R \) is the universal gas constant in appropriate units. This relationship can be exploited for not just isothermal degradation but non-isothermal degradation over the course of a given thermal process by constructing an ordinary differential equation outlined below.
4.2.1 The non-isothermal case

In every thermal process, temperature varies over time to (generally) achieve a short come-up ramp in temperature to the desired hold temperature where most of the microbial kill is achieved, known as the cook step. Once the desired microbial inactivation/kill is obtained, the food products are then cooled to a lower temperature that will not further degrade the food matrix and quality. Throughout this process, the degradation rate of important micronutrients is constantly being altered, making direct analysis and determination of degradation rate more difficult. However, when the degradation rate is related to temperature as described in Eq. 1 and temperature varies over time, the momentary loss of a compound of interest can be described by the equation:

\[
\frac{dC(t)}{dt} = -k_{Tref} \exp[c(T(t) - T_{ref})]C(t) \quad (5)
\]

Where \( C(0) = 1 \) as the boundary condition. This differential equation allows us to develop a method for summating the loss of thiamine during a thermal process, given we can establish likely solutions for \( k_{Tref} \) and \( c \).

4.3 Materials and Methods

4.3.1 NASA’s specifications for brown rice, split pea soup, and BBQ beef brisket

The spaceflight food composition and manufacturing specifications were provided by NASA. Specifications detail the precise formulation for each food product, quality inspection procedure, package testing, and storage instructions. These specific products were chosen jointly by NASA and our group as representative of un-enriched foods with a potential high thiamine instability (data not shown). As the specifics of these recipes are confidential, the exact details are withheld in this work.
4.3.2 Ingredient acquisition and food production

Bulk brown rice, low-sodium vegetable broth, green split peas, boneless ham, raw deckle-off beef brisket, BBQ sauce, and dry BBQ marinade were purchased from Performance Foodservice (1 Performance Blvd, Springfield, MA, USA). Liquid smoke flavoring, ham base, mono-sodium glutamate (MSG), salt, pepper, whole milk, corn starch, red wine vinegar, and bullion were purchased from a local grocery store (175 University Drive, Amherst, MA, USA). All foods were produced abiding by the precise instruction of the NASA specifications.

The brown rice product consisted of raw brown rice and low-sodium vegetable broth, such that the thermal process to obtain shelf-stabilization also cooks the rice thoroughly. Split pea soup was produced after initial rinsing of the dried split peas. Beef brisket was produced by trimming excess fat and coating in BBQ dry rub, wrapping in plastic wrap, and marinating for 24 hrs in refrigerator.

4.3.3 Packaging

The prepared spaceflight foods were filled into flexible aluminized pouches [12.065 cm x 20.48 cm (4.75” x 8.0625”); Tan PE/.0007Foil/3mil Coex Sealant] that were purchased from Heritage Packaging (441 Market St, Lawrence, MA, USA). Approx. 25 kg of brown rice product, including broth, was produced and distributed into pouches to a final weight of 121 g in each pouch, producing approx. 200 units. 200 pouches of split pea soup were produced in total and filled to a weight of 150 g per pouch, and another 200 units of beef brisket product were produced at a fill weight of 120 g per pouch. Pouch opening was cleaned and dried prior to vacuum-sealing which was
done using VacMaster SVP 20 (Overland Park, KS, USA) to remove excess air. Pouches were then aliquoted into batches of 44 pouches for shelf-stabilization.

### 4.3.4 Shelf-stabilization

Three architypes of thermal profiles were produced such that process time and temperature varied significantly but reasonably for food process applications. These architypes were deemed High-Temperature/Short-Time (A), Moderate-Temperature/Moderate-Time (B), and Low Temperature/Short-Time (C). Differential thermal processing was performed using Allpax Multimode R&D 2402 Series Retort, running a hot-water spray thermal process. The temperature probes used were C-4 flexible wire thermocouples, acquired from Ecklund-Harrison (11000 Metro Pkwy, Ste. 40, Fort Meyers, FL, USA) and affixed to the pouch. The end of the flexible wire was positioned into the center of the pouch with food material placed at the end of the tip. Each thermal process temperature profile was recorded using HH378 data logger (OMEGA Engineering, INC., 800 Connecticut Ave., Suite 5N01, Norwalk, CT, USA). Temperature-time plots were produced from these recordings using Se379 software (Cetani Corporation, 11495 N Pennsylvania St Suite 240, Carmel, IN, USA) and used to calculate the equivalent lethality of each batch. The target thermal process lethality was that of the NASA-provided value of $F_0 = 6.0$ from the General Method, by processing at a cook temperature of 121°C for a sufficient time. Pouches were cooled under pressure to 110°C or cooler prior to entry into storage. Each thermal process was run in triplicate batches and probed in quadruplicate and averaged.
4.3.5 Thiamine extraction

Vitamin extraction followed a modified version of the Association of Official Agricultural Chemists (AOAC) International official method 942.23 Thiamine (Vitamin B₁) in Human and Pet Foods (AOAC 942.23, 2005; Ma & Wyatt, 1983). Modifications were made to better suit the process of thiamine extraction and detection for these three spaceflight foods. The phosphorolytic enzyme used was taka-diastase (Pfaltz & Bauer, 172 E. Aurora St., Waterbury, CT, USA) due to proven equivalent activity to the suggested enzymes. Chromatographic columns used for purification were Kimble™ Kontes™ FlexColumns™ at 1.0 cm x 20 cm, 16 mL capacity using the Three-way Luer Stopcock of the same brand. Sodium acetate, sodium hydroxide, potassium chloride, hydrochloric acid, potassium ferricyanide, isobutanol, thiamine hydrochloride as well as thiamine pyrophosphate were all purchased from Fisher Scientific (168 Third Avenue, Waltham, MA, USA). Quinine sulfate and the purification resin utilized in these columns, Amberlite CG-50 (Type I), were both purchased from Sigma-Aldrich (1 Strathmore Rd, Natick, MA, USA).

4.3.5.1 Acid and enzyme digestion

Digestions were conducted in quadruplicate for all foods and temperature treatment, plus the thiamine hydrochloride and thiamine pyrophosphate standards. Foods were ‘stomached’ (crudely mashed) in pouch prior to opening, and again stirred to homogenize upon opening. 0.1 M HCl was used as the extraction buffer. Acidified samples were digested at 100°C for 30 minutes, cooled to <50°C and aliquoted. Aliquots were diluted with 0.1 M HCl and readjusted to pH 4.0-4.5 with double-normal sodium acetate. 5 mL 1% aqueous taka-diastase solution was then added, and the sample
incubated at 45°C for 3 hrs. Aliquoted samples were then acidified with 2 M HCl to pH 3.5 and refrigerated overnight.

4.3.5.2 Filtration and purification of thiamine

Samples were then re-mixed and aliquoted evenly into centrifuge tubes and centrifuged at 3220 g, 4°C, for 5 minutes. Supernatant was filtered through Whatman™ Quantitative Grade 40 Filter Paper (Fisher Scientific) using vacuum pump assistance to collect crude extract. Crude extract was fed through chromatographic columns containing Amberlite CG-50 resin adjusted to pH 5. Columns were washed with near-boiling H₂O and thiamine was eluted with near-boiling 0.1 M HCl solution containing 25% KCl.

2.6.3 Oxidation of thiamine for detection

Two 5 mL portions from the final eluate from each sample were aliquoted into reaction tubes. The remaining solution was stored at -80°C in case of need to re-analyze. Tubes were wrapped in aluminum foil and the remaining steps were conducted in a dark chemical hood to minimize photodegradation. 3 grams solid KCl were added to each reaction tube, along with 3 mL oxidizing reagent (0.04% potassium ferricyanide in 15% aqueous NaOH) for one of the two reaction tubes, while 3 mL 15% aqueous NaOH was added to the other, to serve as the blank. Immediately following the addition of oxidizing or blank solution, 4 mL isobutanol was added to the reaction tubes and shaken. Both thiamine standards were treated identically to the above samples. All samples were then shaken again and allowed to settle before continuing.

The isobutanol layer was collected (top layer) and aliquoted into 8 wells per sample (oxidized or blank) of a 96-well flat-black-bottom plates (Fisher Scientific). Plates were protected from light using aluminum foil until read using a BioTek Synergy 2
plate reader (100 Tigan St., Winooski, VT, USA) running a fluorometric protocol at input filter 365 nm and output filter 435 nm, and fluorometric data collected using Gen5 software version 1.09 (BioTek).

4.3.5.4 Modifications from the original method

Modifications made to the original AOAC method 942.23 included decreasing original sample volume and dilution factor, re-concentration in less isobutanol during thiochrome solubilization, and detection using a fluorometric plate reader. Modifications did not produce any significant decrease in overall extraction efficiency. Plate reader was calibrated against quinine sulfate solution. Taka-diastase phosphorolytic efficiency was measured by a pure thiamine pyrophosphate digestion. Recovery of >85% free thiamine was deemed suitable.

4.3.6 Thiamine concentration determination

Thiamine content in the reaction solution prior to oxidation is calculated by comparison to the results of a thiochrome standard curve made from a separate thiamine hydrochloride standard and dilution with NaOH prior to oxidation and measurement. Appropriate volumetric dilution and mass fraction was calculated back to express the original thiamine content in each food. The mean of each quadruplicate set was calculated as well as the standard deviation. Thiamine content was expressed as mean μg/g ± SD of a given food at that time point of storage and μg thiamine per pouch ± SD at that time point in storage. Thiamine content was then compared to the initial value of each food \((t = 0)\), taken immediately following retort processing and expressed as a concentration ratio \((C_t/C_0)\).
4.3.7 Application of the model to experimental data

Initial concentration of thiamine prior to processing, resulting concentration following processing, and the temperature and time of each thermal process were recorded for use in the modelling program. Any pair of thermal profiles and their corresponding resultant concentration of thiamine expressed as a concentration ratio \( (C_t/C_0) \) were input into the program and as the model requires, parameters \( k(T_{ref}) \) and \( c \) are adjusted in tandem to produce estimated degradation trajectories that pass through both endpoints utilizing the relationship described in Eq. 5. The parameters that produce a satisfactory fit for the model are then recorded. Theoretically, these parameters describe the entire temperature-dependence and time-effect of degradation. \( k(T_{ref}) \) and \( c \) values were then recorded from each pair of thermal profiles and their corresponding data and utilized to predict the retention of thiamine in the unused profile, all within the same module. This was repeated for each pairwise combination of thermal profiles within each food.

4.4 Results and discussion

4.4.1 Retort thermal processing

Thermal processes were run in triplicate for each of three foods tested in this study, those being a brown rice product (BR), a split pea soup product (PS), and a BBQ beef brisket (BB) product, and food packages were probed in quadruplicate throughout the process vessel to monitor and record the temperature within the food. Average temperature and time plots, representing “thermal profiles,” for each food are shown in Figure 12.
Figure 12. Thermal processes were conducted in triplicate and probed in quadruplicate. High, Mod, and Low refer to the maximum temperature of the respective process. Processes were constructed to expose products to varying degrees of thermal penetration. Equivalent lethality ($F_0$) was calculated as a preliminary metric by Simpson’s rules.

The three processes produced were deemed “High Temperature” or “High,” “Moderate Temperature” or “Mod,” and “Low Temperature” or “Low.” High and Mod processes were developed with the same come-up time, as if reflected in the thermal profiles shown. The Mod process remained at cook temperature for 10 minutes, as did the Low process, albeit with an extended come-up time. The cool step was programmed to be equal in time, temperature and pressure gradient among all three processes.

Equivalent lethality, according to Simpson’s rules of integration, for the deterioration of *Clostridium botulinum* spores to render foods shelf stable and safe for human consumption were calculated and tagged to each thermal process in Figure 12. The phenomena of bacterial population loss by thermal processing has been demonstrated to follow similar kinetics to nutrient loss (Corradini et al., 2008), and was used as a quasi-measure of thermal penetration. The High process invoked an $F_0$ of 5.641. NASA
requires their foods to experience an equivalent lethality of at least 6 to be considered in-
spec. The Mod process, which was designed to reflect the most common thermal process
collection, obtained an $F_0$ of 14.465, while the Low process achieved an $F_0$ of 3.753.
Note that although the High and Low process differ significantly in their thermal profile
composition, their calculated lethality reflected similar thermal penetration overall.

4.4.2 Thiamine content by food and process

Following each thermal process, the resulting BR, PS, or BB samples were stored
at -80°C until ready for thiamine analysis. This was also done for food samples that were
packaged but not thermally processed, providing an initial thiamine content in the foods
prior to thermal processing. Thiamine was measured and reported in μg/serving for each
food (Figure 13).
Figure 13. Brown rice, split pea soup, and BBQ beef brisket were analyzed for their thiamine content prior to (Initial) and following three thermal processes varying in their time-temperature profile. Each food’s thiamine content resulting from thermal processing was statistically analyzed pairwise by Tukey’s HSD. Significantly different thiamine retentions are indicated by letters a, b, and c. Values in each food sharing the same letter are not statistically different at p > 0.05.

Thiamine amounted to ca. 85 μg per serving in BR prior to thermal processing, ca. 130 μg per serving in PS, and ca. 19 μg per serving in BB samples. Each thermal
process led to significant loss in thiamine content, but not equally, as determined by Tukey’s HSD test of all pair-wise comparisons at p < 0.05. The effect of process type on thiamine loss by percent was similar between BR and PS, but not the same in BB samples. This could potentially be due to the drastic food matrix differences between the largely carbohydrate-comprised BR and PS samples, compared to the largely fatty and proteinaceous BB product.

These differences can be expressed in kinetic parameters that describe thiamine’s kinetic degradation rate and the temperature sensitivity to decay within a particular food formulation given the unique chemical species present, pH, and moisture content, which can then be used to produce predictive models which estimate thiamine loss during other thermal processes for that same food.

4.4.2 Kinetic parameter determination

Estimation of kinetic parameters $k_{T_{ref}}$ and $c$ was performed through use of the novel non-isothermal modelling program produced by our lab group, as shown in Figure 14. The following procedure is performed independently for each food formulation. Upon importation of two select thermal profiles, and entry of the resulting measured thiamine content (expressed as a concentration ratio compared to the thiamine content of the product prior to processing), the values for $k_{T_{ref}}$ and $c$ can be estimated through use of sliders or manual entry, such that degradation trajectories/curves are drawn through the bottom module seen in Figure 14. Upon determination of a $k_{T_{ref}}$ and $c$ pair that satisfies both concentration ratio endpoints, these parameters are recorded and left unchanged while one of the two thermal profiles are replaced with the third, unused process, and a estimation of thiamine retention is subsequently produced.
Figure 14. Screenshot of the non-isothermal program with estimated degradation parameter sliders for rapid acquisition of non-isothermal kinetic model construction. Once kinetic parameters $k_{T_{\text{ref}}}$ and $c$ are estimated, either thermal profile can be replaced with a theoretical or suggested profile for concentration retention estimations.

4.4.3 Prediction of thiamine retention following processing

The construction of a predictive model, as defined by degradation parameters $k_{T_{\text{ref}}}$ and $c$, was performed using each pairwise combination of processes for each food at a select reference temperature, $T_{\text{ref}}$. Estimations of thiamine retention following a given process was compared to the observed values for that thermal process not used in the model construction process and is reported in Table 13.
<table>
<thead>
<tr>
<th>Foods</th>
<th>Tref (°C)</th>
<th>Process Pair</th>
<th>$k_{\text{ref}}$</th>
<th>$c$</th>
<th>Predicting</th>
<th>Observed retention (%)</th>
<th>Predicted retention (%)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown Rice</td>
<td>119</td>
<td>High-Mod</td>
<td>0.0180</td>
<td>0.180</td>
<td>Low</td>
<td>86.4%</td>
<td>86.4%</td>
<td>0.0%</td>
</tr>
<tr>
<td></td>
<td>119</td>
<td>High-Low</td>
<td>0.0181</td>
<td>0.184</td>
<td>Mod</td>
<td>69.6%</td>
<td>68.2%</td>
<td>1.4%</td>
</tr>
<tr>
<td></td>
<td>119</td>
<td>Mod-Low</td>
<td>0.0180</td>
<td>0.172</td>
<td>High</td>
<td>84.1%</td>
<td>84.9%</td>
<td>0.8%</td>
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<tr>
<td>Split Pea Soup</td>
<td>119</td>
<td>High-Mod</td>
<td>0.0170</td>
<td>0.260</td>
<td>Low</td>
<td>89.2%</td>
<td>87.2%</td>
<td>2.0%</td>
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<tr>
<td></td>
<td>119</td>
<td>High-Low</td>
<td>0.0120</td>
<td>0.200</td>
<td>Mod</td>
<td>82.4%</td>
<td>86.5%</td>
<td>4.1%</td>
</tr>
<tr>
<td></td>
<td>119</td>
<td>Mod-Low</td>
<td>0.0170</td>
<td>0.390</td>
<td>High</td>
<td>92.9%</td>
<td>94.0%</td>
<td>1.1%</td>
</tr>
<tr>
<td>BBQ Beef Brisket</td>
<td>119</td>
<td>High-Mod</td>
<td>0.0085</td>
<td>0.131</td>
<td>Low</td>
<td>87.5%</td>
<td>88%</td>
<td>0.5%</td>
</tr>
<tr>
<td></td>
<td>119</td>
<td>High-Low</td>
<td>0.0085</td>
<td>0.131</td>
<td>Mod</td>
<td>92.5%</td>
<td>92.1%</td>
<td>0.4%</td>
</tr>
<tr>
<td></td>
<td>119</td>
<td>Mod-Low</td>
<td>0.0120</td>
<td>0.300</td>
<td>High</td>
<td>95.3%</td>
<td>94.0%</td>
<td>1.3%</td>
</tr>
</tbody>
</table>

Table 13. Kinetic parameters obtained within the non-isothermal model from pairs of processes’ temperature profiles and resulting thiamine retention data are used to predict thiamine concentration resulting from the third, unused process.

The kinetic parameters established suggest that thiamine within BR, PS, and BB product experience degradation differently in regard to intrinsic degradation rate and temperature sensitivity, and that the combination of these factors are extracted differently depending on the thermal profiles imported into the program. Regardless, these degradation parameters obtained established models which produced accurate estimations of thiamine compared to what was observed. Non-isothermal models in this experiment produced thiamine retention predictions that differed from the observed values by less than 5% in terms of percent original retention. This difference accounted for approximately the same degree of variation as was seen in the measurement variation of thiamine in each analysis (Figures 15-17).
Figure 15. Experimental thiamine retention following each process for brown rice (bars) compared to estimations of thiamine content from the non-isothermal model produced using the other two thermal processes and corresponding thiamine measures (line).

Figure 16. Experimental thiamine retention following each process for split pea soup (bars) compared to estimations of thiamine content from the non-isothermal model produced using the other two thermal processes and corresponding thiamine measures (line).
Figure 17. Experimental thiamine retention following each process for BBQ beef brisket (bars) compared to estimations of thiamine content from the non-isothermal model produced using the other two thermal processes and corresponding thiamine measures (line).

Thiamine was successfully quantified within three NASA spaceflight foods prior to and following three varying retort thermal processes by our modified AOAC method. Thiamine loss by thermal processing was observed, and thiamine degradation was dependent on the kind of thermal process applied. However, the effect of process conditions on the degree of thiamine loss was not universal among foods and was a likely result of food chemistry and matrix variations and unique chemical lability of thiamine to such variations. The variation of thermal process effects on thiamine loss in each food was successfully represented by degradation parameters $k_{Tref}$ and $c$ and used in modelling thiamine loss during thermal processing with low error (<5% difference). Non-isothermal modelling performed by our method resulted in predictions of thiamine content within measurement variation. This approach offers promising results for the rapid estimation of
degradation parameters that not only describe the behavior of nutrient loss in process foods, but can then be used to construct predictive models and estimations of nutrient loss in process not yet performed for the optimization of nutrient preservation and microbial kill.
CHAPTER 5

PHYSICOCHEMICAL METHODS FOR DETERMINING MICROSTRUCTURE DEVELOPMENT IN MODEL WINE VIA PAIR-WISE COMPONENT INTERACTIONS

5.1 Abstract

Various chemical interactions occur in wine during both production and post-ferment ageing that can influence wine quality and consumer preference. In this study, the nature, strength, and binding stoichiometry of interactions between wine components were evaluated pair-wise by isothermal titration calorimetry (ITC), resulting morphology was identified in a model system by transmission electron microscopy (TEM), and turbidity and zeta potential changes during and as a result of these component interactions were measured. Grape polysaccharide extract, wine-yeast mannoprotein extract, and a select model protein, thaumatin, were chosen due to their ubiquity in finished wines. These macromolecules were reacted with tannin extracts from grape seed and skin, as well as the model anthocyanin malvidin-3-O-glucoside. Using ITC, we identified a strong exothermic reaction between seed tannins and both mannoprotein and thaumatin, and a weaker exothermic reaction between skin tannins and thaumatin, while mannoproteins and grape polysaccharides reacted in a endothermic manner with skin tannins. Malvidin-3-O-glucoside demonstrated a weak initial interaction with thaumatin only, which may still result in slower, long-term aggregation during wine ageing. It was suggested in several ITC results that aggregate formation was occurring, which were supported by changes in turbidity and by zeta potential in the case of thaumatin and seed tannin. TEM was able to confirm microstructure features of mannoprotein and grape
polysaccharides in model wine buffer, as well as the structure produced by pair-wise component interactions between thaumatin and seed tannin.

5.1.1 Practical Application

Determining the key constituents from grapes and yeast that can interact to form aggregates during wine production and ageing and understanding their potential preferentiality and strength to one another will allow for specific monitoring and control of these components to produce a more consistent product for the consumer, as well as allow for the supplementation of specific components to achieve a desired microstructure shall they confer different sensory attributes. This work reveals a swathe of methods that can be employed to determine the development of wine microstructure development from pair-wise component interactions.

5.2 Introduction

Wine stability to precipitate and haze formation, as well as improvement in sensory attributes during ageing is a major concern for wine producers. In production, the levels of select components are monitored and often selected for through several winemaking strategies to ensure a consistent product. Chemically speaking, there is a swathe of dynamic molecular interactions occurring, beyond micro-oxygenation and bitartrate deposition, which can affect the end product to a significant degree. Understanding which wine-derived components are at play, the relative strength of these interactions, and their resulting morphology are critical for the industry to better control sensory attributes.

Great attention is being paid to the contribution of select wine components and extract fractions to mouthfeel in the finished product, related in part to interactions
between chemical components in wine as well as their interactions with oral tissue upon ingestion. Work has been done investigating the mouthfeel effects of grape and wine-derived tannins, anthocyanins, proteins, and polysaccharides as single-components (Escot, Feuillat, Dulau, & Charpentier, 2001; Fontoin, Saucier, Teissedre, & Glories, 2008; Vidal, Francis, et al., 2004) as well as the mouthfeel impact of limited pair-wise component interactions, which typically focuses on the astringency or drying influence (Diako, McMahon, Mattinson, Evans, & Ross, 2016; Fontoin et al., 2008; Quijada-Morín, Williams, Rivas-Gonzalo, Doco, & Escribano-Bailón, 2014; Ramos-pineda, García-estévez, & Escribano-bailón, 2018; Vidal, Courcoux, et al., 2004). Independent from this, work has been done to explain the mechanisms of certain wine-component interactions. Components of interest typically center around tannin-tannin or protein-tannin interactions, and polysaccharides that may hinder their aggregation and deposition by disruption or component surface-stabilization (Carvalho, Mateus, et al., 2006; Carvalho, Póvoas, Mateus, & De Freitas, 2006; De Freitas, Carvalho, & Mateus, 2003; Erranti, Ametti, & Onomi, 2010; Carine Le Bourvellec, Watrelot, Ginies, Imberty, & Renard, 2012; Mateus, Carvalho, Luís, & De Freitas, 2004; Mekoue Nguela, Poncet-Legrand, Sieczkowski, & Vernhet, 2016; Poncet-Legrand, Doco, Williams, & Vernhet, 2007; Riou, Vernhet, Doco, & Moutounet, 2002; Watrelot, Schulz, & Kennedy, 2017).

Aggregations or soluble complexes formed by component interactions are typically determined by turbidometry, Isothermal Titration Calorimetry (ITC) and less commonly, Nuclear Magnetic Resonance (NMR) spectroscopy, among other methods (C. Le Bourvellec & Renard, 2012). To explain these aggregates, physical and chemical characteristics of several of these ubiquitous wine components and wine aggregates have
been analyzed (K. Bindon et al., 2014; K. A. Bindon et al., 2016; Vernhet, Pellerin, Prieur, Osmianski, & Moutounet, 1996). This body of work has shown that entropy-driven hydrophobic forces, hydrogen bonding, and steric stabilization are the main drivers of aggregation and soluble complex formation, depending on the specific components involved. Aside of tannin interactions, anthocyanins also have been shown to play a role in interacting with polysaccharides, which change sensory attributes of finished wines (Fernandes, Bras, Mateus, & de Freitas, 2015; Trouillas et al., 2016).

Individual works have not yet provided a holistic analysis of the nature, strength, preferentiality, stoichiometry of interactions between major wine macromolecules and major flavonoid classes in wine, or such interactions that can be demonstrated across methods. In this work, major grape and wine macromolecules were reacted with tannin extracts and a model anthocyanin by ITC to determine the aforementioned characteristics. Aggregate size was measured by turbidity and TEM. A potential explanation of component aggregation by surface-potential neutralization and stabilization was investigated by zeta potential measurement.

5.3 Materials and Methods

5.3.1 Model wine buffer selection

All experiments carried out were performed in model wine buffer, prepared as follows: 12% v/v ethanol in deionized water, 5 g/L tartaric acid (Fisher Scientific, 168 Third Avenue, Waltham, MA, USA), pH adjusted to 3.2 with 5N NaOH. The ionic strength was adjusted to 70 mM using NaCl, as it was suggested that increases in ionic strength correspond to hindered protein-tannin aggregation (De Freitas et al., 2003). 0.01% v/v TFA was added to the model wine buffer to inhibit protease activity in case of
contamination. Buffer conditions were chosen to generalize both red and white wine chemical conditions. The solution was stored in the refrigerator when not in use.

5.3.2 Individual wine components

Prior to preparation, each extract provided was extensively desalted against DI water, using a 2 kDa dialysis membrane, and lyophilized. Individual wine components were prepared in model wine buffer and readjusted to pH 3.2 using NaOH or HCl. Common grape and wine macromolecules used were: a neutral and acidic grape polysaccharide extract (PS) and a mannoprotein extract (MP) sourced from Laffort as MANNOSTAB® LIQUIDE 200 and provided by E&J Gallo Research Chemistry Department as a lyophilized powder, and a mixture of thaumatin I and thaumatin II proteins (TH) (Fisher Scientific). For all downstream analysis, each macromolecule solution was prepared to 300 µM. The tannin components used were: a grape skin tannin extract (SK) and grape seed tannin extract (SE) provided by E&J Gallo, and the model anthocyanin used was malvidin-3-O-glucoside, also known as oenin chloride (OC), purchased from Alkemist Labs (12661 Hoover St, Garden Grove, CA, USA). See Table 14. Each flavonoid component was prepared to 1.5 mM. All solutions to be tested were then centrifuged for 5 minutes at 3220 g at 4ºC, and the supernatants were collected in fresh tubes, which were purged with gaseous nitrogen and sealed. Samples were prepared fresh each day and stored at room temperature prior to each analysis.
<table>
<thead>
<tr>
<th>Component</th>
<th>Molecular weight (Da)</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grape polysaccharides</td>
<td>50000-150000*</td>
<td>A combination of linear and branched polysaccharides, rich in acidic residues</td>
<td>E. &amp; J. Gallo Winery</td>
</tr>
<tr>
<td>Mannoprotein</td>
<td>~40000</td>
<td>A yeast-derived glycoprotein extract, containing branching mannan chains and a small protein domain</td>
<td>Laffort</td>
</tr>
<tr>
<td>Thaumatin</td>
<td>~22000</td>
<td>A mixture of globular thaumatin I and II</td>
<td>Alfa Aesar</td>
</tr>
<tr>
<td>Skin tannin</td>
<td>~400</td>
<td>A grape-derived tannin extract, containing mostly monomeric quercetin glycoside</td>
<td>E. &amp; J. Gallo Winery</td>
</tr>
<tr>
<td>Seed tannin</td>
<td>~2000</td>
<td>A grape-derived polymeric tannin extract (mDP 5.5), rich in galloylated terminal subunits</td>
<td>E. &amp; J. Gallo Winery</td>
</tr>
<tr>
<td>Malvidin-3-O-glucoside</td>
<td>528.891</td>
<td>The predominant anthocyanin class found in red wine grapes</td>
<td>Alkemist Labs</td>
</tr>
</tbody>
</table>

Table 14. Summary of chemical components used in pair-wise reactions across methods, detailing their molecular weights, their physicochemical properties, and where they were acquired. * = based on hydrodynamic volume of dextran standards.

5.3.3 Isothermal titration calorimetry

Titrations were executed using a Malvern MicroCal VP-ITC (Malvern Panalytical Ltd, Grovewood Road, Malvern, UK). Experiments were performed at 25°C. Prior to each experiment, solutions were sonicated for 20 minutes to disrupt weak self-aggregation. The 1.448 mL sample cell was filled with 300 µM PS, MP, or TH, and the syringe was filled with a select 1.5 mM SK, SE, or OC. Experiments were carried out over the course of 29 successive 6 µL injections, constantly stirred at 300 rpm. The duration of each injection was 12 seconds and the time delay between each injection was 150 seconds. These parameters were selected to achieve a final molar ratio of approximately 2:1 (flavonoid:macromolecule) in the sample well.
Heat flux from each titration was measured, recorded as peaks, integrated, normalized, and transformed and expressed as sample-well enthalpy change in calories per mol of injectant using MicroCal Origin software (Microcal Software Inc., 1 Roundhouse Plaza, Northampton, MA, USA). Control titrations of SK, SE, or OC into model wine buffer were performed and the results were subtracted from the peak integration to account for heat of dilution and potential self-association. Each experiment was carried out in triplicate. Model fitting was conducted using MicroCal Origin software, and component interaction parameters were established based on the binding model that best fit each isotherm.

5.3.4 Turbidity measurements

Turbidity experiments were carried out in the same manner as the ITC protocol. Aliquots of SK, SE, or OC were slowly added (12 seconds) to a sample vial containing PS, MP, or TH, while swirling, and measured at 25°C. The molar ratio produced by each addition mirrored that of the ITC experiments so that direct comparisons could be made. Turbidity was measured in triplicate using a Hach 2100Q Turbidimeter (Hach Company, P.O. Box 389, Loveland, CO, USA) and turbidity of relevant control solutions was subtracted from the data.

5.3.5 Structure identification from TEM

5 µL of single component (PS, MP, TH, SK, SE, and OC) and mixed component (PS+SK, PS+SE, PS+OC, MP+SK, MP+SE, MP+OC, TH+SK, TH+SE, and TH+OC) solutions were aliquoted on 200 mesh copper support grids (and imaged using Philips CM10 Electron Microscope (FEI Company/Philips Electron Optics, P.O Box 218, Eindhoven, Netherlands). 1% uranyl acetate adjusted to pH 5.0 was used as the stain and
fixing agent for the support grids and excess liquid was wicked from the grid. Abundant/common structures were identified by searching the grid area at a magnification of 46 kX and these structures were imaged in triplicate. Grids, stain, and technical support were provided by University of Massachusetts Worcester Medical School, Core Electron Microscopy Facility and staff, 55 Lake Avenue North Worcester, MA, USA.

5.3.6 Zeta potential during component addition

Dissolved components in model wine buffer were analyzed for their slipping plane charge (zeta potential) and charge neutralization which may lead to aggregation throughout flavonoid component addition in the same manner as ITC and turbidity experiments using Zetasizer Nano ZS (Malvern Panalytical Ltd, Grovewood Road, Malvern, UK). Refractive index and viscosity parameters were selected from an internal table containing values for 12% ethanol in water. Zeta potential was measured using DTS1070 disposable cuvettes (Malvern Panalytical Ltd), following 29 successive injections of SK, SE, and OC to samples of PS, MP, and TH, corresponding to the same molar ratio progression as described above. Data were averaged from 20 readings per sample and measurements were conducted in triplicate. Data of <5 mV were the product of greater than 20 readings, according to the unit’s programming.

5.4 Results and Discussion

5.4.1 Isothermal titration calorimetry

Various component interactions were observed by ITC within the concentration range tested, see Figure 18. Thaumatin demonstrated reactivity with all tannin/anthocyanin components tested, with the strongest and most favorable reactivity
existing between TH and SE. Both SK and SE additions presented an overall enthalpy-driven reaction with TH, corresponding to conformational changes of hydrogen-bonding geometry and Van der Waals interactions (Datta, Amit K., Rösgen, Jörg, Rajarathnam, 2015). Reaction between TH and OC demonstrated greater contribution by entropy rather than enthalpy, but only slightly. This result suggests that a hydrophobic process drives the reaction, possibly by disruption of tertiary protein structure by OC.

Figure 18. Isotherms for all combinations of macromolecules titrated with tannin fractions or anthocyanin using VP-ITC.

Mannoprotein demonstrated a strongly favorable, enthalpy-driven interaction with SE, see Figure 18. SK to MP demonstrated a similarly prominent yet entropy-driven interaction, with the total reaction being exhausted at a molar ratio of approx. 1:1 flavonoid to macromolecule (Table 15). Comparatively, the enthalpy, ΔH, produced by MP reacting with SE was approx. 10% of the net enthalpy produced by TH + SE.
Table 15. Enthalpy change each reaction as well as the calculated stoichiometry for each pair-wise interaction which demonstrated binding on ITC. MP corresponds to mannoprotein, TH corresponds to thaumatin, SK to skin tannin, SE to seed tannin, and OC to malvidin-3-O-glucoside.

Grape polysaccharides did not clearly demonstrate reactivity with SK, SE, or OC in these experiments. A slight, potentially entropy-driven reaction between PS and SE was calculated. However, this may simply be due to changes in heat capacity of the reaction volume as SE was added and was dissociated to the PS solution in the sample well. Isotherms were not modellable using the conventional fitting models provided by the MicroCal Origin software. This is thought to be due to the nature of the reactivities expected between these compounds. We expect to observe surface deposition of flavonoids to both thaumatin and mannoprotein and thus, a more appropriate Freundlich equation should be considered:

$$\log(q_s) = \log(K_F) + b_F \log(C_s)$$  \hspace{1cm} (1)

where $q_s$ is the mass ratio (mg/mg) of the flavonoid adsorbed to the macromolecule, $C_s$ is the equilibrium concentration of the free flavonoid following the reaction upon successive titrations, $K_F$ (L/mg) is an empirical constant representing the total adsorption capacity of the macromolecule, and $b_F$ is a dimensionless “adsorption intensity” term. The Freundlich equation would be expected to better describe the isotherms observed for both MP and TH to SK, as the ITC model fitting is based on either one, two, or multiple successive binding sites per macromolecule. Due to the nature of tannin condensing onto
protein, none such model fittings are as appropriate (Boulton, Roger B., Singleton, Vernon L., Bisson, Linda F., Kunkee, 2013; Springer, Chen, Stahlecker, Cousins, & Sacks, 2016; Springer, Sherwood, & Sacks, 2016). Table 15 details the reaction enthalpy and stoichiometry for each successful pair-wise interaction.

5.4.2 Turbidity change from component addition

Multiple pair-wise interactions that produced detectable suspended aggregates were revealed by turbidity changes throughout the titration scheme (Figure 19). Thaumatin solutions produced large instantaneous changes in turbidity when skin and seed tannins were added. As shown in Figure 20, seed tannin addition produced the largest and most rapid increases in turbidity with thaumatin, and by a large margin. A final molar ratio of 20:1 flavonoid to macromolecule was achieved to detect the total reactivity of TH to both SK and SE. The bulk of these aggregates were not soluble, though, and deposited out within minutes following completed titration. In regards to OC addition to thaumatin, it was the only combination of OC which did not see a net turbidity decrease, as the OC solution was completely transparent. This may suggest some formation of small soluble aggregates which overcome the diluent effect of OC on turbidity.
Mannoprotein experienced a more modest increase in turbidity following SK addition compared to thaumatin, however, the change was more pronounced with the SE addition. There was no such effect by OC addition, which is supported by a lack of reactivity demonstrated by ITC. MP solutions combined with SK and SE produced visible haze which did not diminish with time as was the case with thaumatin (not shown). Again, PS solutions were largely unchanged upon flavonoid addition, further suggesting that PS are largely unreactive with the components being tested.
5.4.3 Structure identification from TEM

Interactions suggested by both ITC and turbidity measurements were supported by TEM images for TH + SE (Figure 21). Upon addition of SE, thaumatin formed large globules, as visualized in image C. SK had less of an obvious effect, although structure did seem to indicate modest aggregation. The increase in hydrophobic (lighter color) particles corresponds to exposed hydrophobic patches on TH which would orient themselves to interact with SE. Due to the monomeric quercetin glycosides in SK, such a strong denaturing effect was not expected. Rather, a surface deposition and rapid consumption of TH is likely. As shown here, TEM is a promising tool for observing the conformational and final structural changes produced by protein – tannin interactions.
Figure 21. TEM images of pure thaumatin in model wine buffer (A), thaumatin reacted with skin tannin (B) and with seed tannin (C). Stain used was 1% uranyl acetate pH 5.0. Scale is 200nm taken at 46 kX.

TEM also revealed tertiary structure of both free mannoprotein and grape polysaccharides, both large and small (Figures 22 – 23). Prior knowledge regarding the composition of mannoprotein as largely a highly-branched carbohydrate polymer with small protein cores was supported visually in images D and E (Cameron, Cooper, &
Neufeld, 1988; Poncet-Legrand et al., 2007; Ramos-pineda et al., 2018; Watrelot et al., 2017). Additionally, the structure of the imaged polysaccharides coincides with other images taken of plant cellulose and other carbohydrate polymers (Fengel & Wegener, 1979). In this way, TEM can be utilized to observe the conformation of suspended wine macromolecules.

Figure 22. TEM images of pure mannoprotein in model wine buffer taken at 46 kX with a scale of 200nm (D) and at 87 kX (E). Stain used was 1% uranyl acetate pH 5.0. Mannoprotein can be identified by long carbohydrate chains emanating from a hydrophobic core.
5.4.4 Zeta potential change from component addition

Slipping plane charge neutralization can drive rapid aggregation which leads to system instability and flocculation, haze formation, or creaming over time. The effect of added flavonoids on the measured zeta potential of macromolecule solutions was tested to determine if the slipping plane of suspended material was becoming more neutral, and favorable to aggregation, as a method of determining reactivity. As shown in Figure 24, zeta potential neutralization was observed for TH + SE reactions, demonstrating that this single protein – tannin reaction was detected by all methods listed above. Initial surface charge of TH persisted as highly positive, and not liable to aggregation. Yet upon addition of SE, the measured surface charge rapidly declined, where ITC, turbidity changes, and TEM all support reactivity and subsequent aggregation.
Interestingly, MP + SE did not experience a lasting effect on the overall zeta potential measured. Rather, an initial neutralization corresponding to the same region contained in the stoichiometry determined from the ITC results was observed. At this same stage, turbidity reached its approximate maximum as well. Following successive SE addition, however, measured zeta potential decreased due to the highly-negative SE fraction. This result, combined with the observation that turbid MP + SE solutions were persistent with time, suggests that MP + SE reactivity is not liable to such rampant
aggregation as to lead to system instability. Nonetheless, an interaction was again suggested by this method in the case of MP + SE. It is important to note that a control titration was performed using a blank, where flavonoid solutions were added and measured for their zeta potential, and these curves were subtracted from the final plots. Aside from the interactions observed between TH + SE, and MP + SE, no noticeable effect of added flavonoids on macromolecule zeta potential was revealed by this method. This suggests that the method is effective at revealing strong protein – tannin interactions mechanistically.

Across all methods, it was repeatedly demonstrated that there existed an interaction between thaumatin and seed tannin, and mannoprotein and seed tannin. There existed modest interactions between these macromolecules and skin tannin at the concentrations tested, as well as a possible interaction between TH and OC. Polysaccharides were largely unreactive based on the methods employed but may still have implications for wine quality and mouthfeel. ITC experiments revealed instantaneous interactions between macromolecules and titrated flavonoids. Turbidity provided insights on how such interactions produce aggregates, supported by TEM results, particularly in the case of TH + SE. Zeta potential can be measured as a means to explain a mechanism of interaction between proteins and tannins, but not universally.

This work focused mainly on rapid, nearly instantaneous interactions that lead to the formation of aggregates on a minute-scale. These data do not necessarily predict the resultant structure produced by condensing tannins over time, deposition of tartrates, and the like. Work still must be done to determine the total array of interactions between wine
components, and improvements in methods and methodologies are always being sought after by the wine industry to ensure product quality and consumer satisfaction.
CHAPTER 6

CABERNET SAUVIGNON AGE-RELATED PRECIPITATES EXPERIENCE
ZETA POTENTIAL NEUTRALIZATION AND RECOVERY DURING
ACCELERATED MATURATION

6.1 Abstract

Potassium bitartrate (KHT) crystals are commonly referred to as the main precipitating component in cold-stabilized and aged wines. However, many other components can coprecipitate or polymerize to form insoluble complexes, which will also appear in wine sediment. While the chemical mechanisms that lead to these insoluble complexes and polymers are well documented in wine research, the physical nature of these materials and the physical mechanisms that facilitate aggregation and deposition are not often considered. We hypothesized that ageing Cabernet Sauvignon experiences precipitation due, in part, to a favorable and neutral zeta potential state of solubilized particles. In this study, a young Cabernet Sauvignon (CS) vintage was exposed to accelerated maturation conditions. The sediments were collected over time, and zeta potential of the resulting suspensions were analyzed. Turbidity and optical microscopy were conducted as a means to track the progression of precipitation. Zeta potential of young CS was shown to neutralize during accelerated ageing, but reached a minimum absolute value at 3 months in storage. Subsequently, zeta potential recovered to the original, more negative value, which directly corresponded with the measured turbidity change in the wine, suggesting that a dynamic evolution of particle slipping plane charge neutralization and subsequent stabilization occur during Cabernet Sauvignon wine ageing.
6.1.1 Practical Application

Wine-precipitate formation during ageing is generally seen as undesirable to wine consumers, and considered as a sign of low wine quality. By understanding the physical properties of these wine precipitates, the principle of their aggregation, and the rate at which they form in Cabernet Sauvignon, compliant additives or valid winemaking strategies can be employed to minimize these precipitates and improve customer satisfaction.

6.2 Introduction

Wine gets better with age. That is the adage when it comes to the coalescence of several sensory attributes of a bottled wine into a more smooth and enjoyable product with time. However, one common consumer-reported issue pertains to the presence of sediment or crystals in unopened wines, which occurs over time in both white and red varieties. This phenomenon is often seen by consumers as an indication of poor wine quality or lapses in process control. As such, a great amount of effort and research has been allocated into the development of novel process techniques or compliant additives to inhibit their formation (Bajard-sparrow, Latham, Lankhorst, & Lironde, n.d.; A Bosso, Salmaso, De Faveri, Guaita, & Franceschi, 2010; Antonella Bosso et al., 2015; Coulter et al., 2015; Greeff, Robillard, & du Toit, 2012; Lankhorst et al., 2017; Manns, Siricururatana, Padilla-Zakour, & Sacks, 2015; Soares, Geraldes, Fernandes, dos Santos, & de Pinho, 2009; Sturza & Covaci, 2015; Versari, Barbanti, Parpinello, & Galassi, 2002; Zamfir, Cotea, Luchian, Niculaua, & Odageriu, 2014). These include cold maceration controls, cold-stabilization, crystal seeding, nanofiltration, the usage of carboxymethylcellulose, mannoprotein, and metatartaric acid, among others.
To optimize the development and implementation of these methods, it has become a priority to also identify the chemical makeup of these precipitates and their constituent parts as well as the chemical kinetics of formation (Covaci, 2015; Haslam, 2007; Vernhet et al., 1999b, 1999a). Although there is much attention being paid specifically to potassium bitartrate (KHT) crystallization and deposition, research has revealed that ~20% (w/v) of ethanol-precipitated wine material corresponds to KHT crystals, while ~30% accounts for polysaccharides, 30% condensed tannins, ~7.5% protein, and the rest containing other cations or organic acids (K. A. Bindon et al., 2016). These compounds are all liable to impact and participate in sediment formation to some degree. Contending with the numerous chemical factors at play in precipitate formation in wine, a simpler physico-chemical approach was explored to understand the mechanism of precipitate formation in wine. Zeta potential of a young Cabernet Sauvignon was investigated throughout a 6-month period of accelerated ageing at 37°C.

Zeta potential (\(\zeta\)-potential) has been utilized in food and beverage systems for some time to describe the degree of aggregative stability of emulsions and the stabilizing or coagulating effect of additives to such systems (Banat, 1997; Chung, Sher, Rousset, Decker, & McClements, 2017; Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, Narváez-Cuenca, & McClements, 2014; Guzey & McClements, 2007; Mohan, McClements, & Udenigwe, 2016; Toro-Uribe, López-Giraldo, & Decker, 2018). The \(\zeta\)-potential of a particle describes the charge at the surface of a particle together with the immediately affected solution, rather than the charge at the surface of the particle itself. In the case of an aqueous system, this potential exists at the edge of an electric double layer (EDL) consisting of the real charged particle surface, a layer of corresponding ions
in solution, known as the Stern layer, and an intermediate layer known as the slipping plane, which mediates the Stern layer charge and that of the surrounding medium (Delgado, González-Caballero, Hunter, Koopal, & Lyklema, 2007; Wilson, Wade, Holman, & Champlin, 2001) depicted in **Figure 25**. This final slipping plane layer is that which determines the aggregative stability of the particle and the system at large and the interface at which interactions with the environment and other compounds occur. A highly-charged ζ-potential corresponds to system stability via electrostatic repulsion; A more neutral ζ-potential indicates a system prone to aggregation and subsequent precipitation or creaming, in the case of oil emulsions (da Silva Malheiros, Patricia et al 2011; Taylor, Gaysinsky, Davidson, Bruce, & Weiss, 2007).

![Figure 25. Rudimentary diagram of a charged particle (grey) with complimentary charged ions (blue) existing in the Stern layer. The slipping plane resides outside this layer and consists of an intermediate layer of complimentary (blue) and oppositely-charged ions (red) which interacts with the medium and other particles’ slipping planes.](image)
The $\zeta$-potential is, in fact, not measured directly. Typically, the electrophoretic velocity of solubilized particles exposed to an electric field is measured and the $\zeta$-potential is calculated by the Henry equation:

$$
u_e = \frac{2 \varepsilon z f(\kappa a)}{3\eta}$$

where $\nu_e =$ the electrophoretic mobility, $\varepsilon =$ the dielectric constant, $z =$ zeta potential, $f(\kappa a) =$ Henry’s function (1.5 for polar solutions), and $\eta =$ viscosity (Delgado et al., 2007; Morfesis, Ana; Schoff, 2007). In this way, electrophoretic mobility can be related to zeta potential of solubilized particles. This technique has not yet been applied to wine for determining system stability during ageing, but theoretically can be used to predict and track aggregation into precipitates during system equilibration/ageing.

6.3 Materials and Methods

6.3.1 Cabernet Sauvignon manufacturing

Cabernet Sauvignon wine was produced by E. & J. Gallo Winery (Modesto, CA) by conventional winemaking methods. The must was sulfited to 60 ppm and enzyme-treated with Rohavin MX pectinase enzyme solution to 0.025 mL/L. Yeast-assimilable nitrogen (YAN) was monitored for two days during initial fermentation and held at 300 ppm. Tartaric acid addition was carried out to obtain a pH of 3.7 and addition of water to aid fermentation (WAF) was done to achieve 24.0 Brix.

Fermentation was carried out with 0.18 g/L dry D254 wine yeast at a temperature of 85F. Following fermentation, the final press target was 0 Brix. Prior to bottling, sulfate treatment was added such that free SO$_2$ measured 35 ppm and acid was added to ensure pH <4.0.
6.3.2 Accelerated ageing design

Fresh Cabernet Sauvignon from the current vintage was provided by E. & J. Gallo Winery. After arrival, wines were gently inverted for 2 minutes and placed in an anaerobic chamber before aliquoting 15 mL into 20 mL Type I borosilicate glass headspace vials and crimp-capped (Fisher Scientific, 168 Third Avenue, Waltham, MA, USA). Vial seals were wrapped with Parafilm M (Fisher Scientific) and vials were then wrapped in aluminum foil and placed into 37°C storage. Downstream analyses for initial wine properties (t=0) were also conducted at this time. Wine samples were pulled and analyzed every successive month for six months.

6.3.3 Suspension turbidity

Turbidity experiments were conducted in triplicate using a Hach 2100Q Turbidimeter (Hach Company, P.O. Box 389, Loveland, CO, USA) which was calibrated identically prior to each monthly analysis. Wine aliquots were opened immediately before each measurement. To normalize the data and to operate the turbidimeter within its working range, wine samples were vortexed for 10 seconds on high speed and 5 mL were taken while swirling. Aliquots were diluted with 10 mL matching ethanolic buffer such that the initial Cabernet Sauvignon samples measured 200 NTUs. Buffer composition was as follows: 12% v/v ethanol in deionized water, 5 g/L tartaric acid (Fisher Scientific), pH adjusted to 3.5 with 5N NaOH, and ionic strength of 70 mM using NaCl. Following dilution, samples were vortexed again for 10 s and measured three times, vortexing between measurements. Each measurement took 5 seconds to complete.
6.3.4 Optical microscopy

Diluted samples were loaded on Corning standard tissue culture dishes (Fisher Scientific) and suspended structures were investigated using a Nikon TS100 (Nikon Instruments Inc., 1300 Walt Whitman Road, Melville, NY, USA) at 100X total magnification with a 10X objective lens. Samples were also imaged in a light box.

6.3.5 Zeta potential measurements

Cabernet Sauvignon samples were analyzed for their zeta potential which may lead to aggregation at values close to neutral charge. Wine aliquots were opened immediately prior to each analysis and diluted as described above. Experiments were conducted on Zetasizer Nano ZS using DTS1070 disposable cuvettes (Malvern Panalytical Ltd, Grovewood Road, Malvern, UK). Refractive index and viscosity parameters were selected to be 1.36 and 1.011 cP respectively. Data were averaged from ~40 readings per sample and measurements were conducted in triplicate.

6.4 Results and Discussion

6.4.1 Suspension turbidity

Wine sample color loss and apparent transformation of solution turbidity over 6 months of accelerated ageing storage was observed. Figure 26 presents aliquots of each sample taken at each month of storage. pH was recorded to verify color change was not related to changes in [H⁺]. A dramatic shift away from red color and apparent browning was observed, as well as an increase in particulate matter size and deposition rate. Additionally, increasing precipitate size was paired with increasing transparency of wine samples, as background text became visible through samples 5 and 6 (months).
Figure 26. Visible turbidity and discoloration of diluted age-accelerated Cabernet Sauvignon. Time (t) is expressed in months. pH was measured to show no change over the course of ageing which could influence precipitate formation.

Turbidity was accurately measured with Hach 2100Q Turbidimeter at each month of sampling following sample agitation. Samples measured at 1 month were used to produce an appropriate dilution that was applied to all subsequent samples such that the original turbidity measured 200 NTUs (see Figure 27). An evolutionary trend was observed in aged wine suspensions over the course of 6 months. Turbidity rapidly increased from the initial time point to 2 months, followed by a gradual increase past 2 and 3 months. A peak turbidity was revealed at 4 months of storage at 37C, which began to decline entering 5 and 6 months. This can be compared directly with apparent turbidity and color change observations where the most significant browning and precipitant size increases occurred between 4 and 5 months, when there exists an inflection point in measured turbidity. Final measured turbidity of 6-month old wine suspensions produced a lower NTU value than wine suspensions measured after 1 month of ageing.
Figure 27. Cabernet Sauvignon aliquot suspension turbidity measured in triplicate over a 6-month period. Turbidity of suspensions peaked between 2-4 months and declined to below the turbidity measured at 1 month at the 6-month analysis point.

6.4.2 Optical microscopy

Aged wine samples were imaged in triplicate. Visualized wine precipitant was captured in a window of 1.78mm diameter as shown in Figure 28. Background slide color is not representative of matrix or sample differences. Rather, image capturing produced unpredictable deviations in color balance and saturation. As early as 0-month (no accelerated storage) wine, small precipitations and crystals were observed, but were scarce and colorless. No notable differences were observed for 1 and 2 months of storage, except for possible crystal growth. At 3 months of accelerated storage, flat and irregularly-shaped precipitates were observed, bearing significant color in the red range. The number, size, and color variation of precipitates increased directly with increasing time such that at 6 months of accelerated storage, images were dominated by slowly-moving large flake-like deposits.
Figure 28. Optical microscopy of age-accelerated Cabernet Sauvignon at a final magnification of 100Xs. Column number denotes sample age in months at 37°C, and each aliquot was sampled and imaged individually in triplicate. Each sample image represents a visible width of 1.78mm.

6.4.3 Zeta potential measurements

Zeta potential was monitored over the same timescale and the sample aliquots that were measured for turbidity were also measured for zeta potential to produce Figure 29. In a similar trend to what was plotted for measured turbidity, there was a rapid neutralization of suspended wine particles from 1-month accelerated wine, with a peak around 3 months. Following peak neutralization, there was another rapid evolution such that measured zeta potential was recovered to near the original values of unaged wine. Particles bearing a more negative charge are less susceptible to further aggregation due to slipping plane charge neutralization. This can be interpreted to mean that following peak zeta potential neutralization and subsequent recovery, aggregation would have become exhausted and further aggregation that would produce additional turbidity would be delayed or nonexistent. This is reflected in Figures 26-27 where turbidity declines.
following peak around the same timeframe as the peak and recovery observed in zeta potential.

Figure 29. Zeta potential of age-accelerated Cabernet Sauvignon was monitored for 6 months at 37C in triplicate. Zeta potential became more neutral between 1 and 3 months, upon which the original negative slipping plane charge was re-established by 4 months and was maintained for the remainder of the accelerated ageing.
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