Design and Analysis of an Accelerated Stability Test for an In-Vitro Diagnostic Product with Lot-to-Lot Variation

By

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Abstract

This work presents a research project performed in Abbott's Diagnostics Division in Puerto Rico with the purpose of providing an empirical rather than a simulation based method for applying Arrhenius kinetics theory to a set of data gathered from an accelerated stability study of three lots for a quantitative immunoassay reagent kit (i.e. an in-vitro diagnostic product). Four models where developed to relate *product content*, *time* and *temperature*, and they where compared in terms of their ability to fit well the accelerated stability data, the significance of the model parameters considered, and their reliability for extrapolation purposes. These models included the Arrhenius classical approach, a polynomial function, and two nonlinear modifications of the Arrhenius life-temperature equation for a zero and first order kinetic reaction. The Arrhenius nonlinear model for a zero-order reaction was selected as the most appropriate to estimate degradation by means of an accelerated stability test since it provided relevant statistics and compliance with the underlying assumptions of the Arrhenius Life-Temperature relationship. Finally, a validation procedure was developed for the most appropriate model selected, which is based on prediction intervals to contain *m* individual future observations. This procedure could be used routinely to compare degradation patterns of future lots at elevated temperatures and conclude if the lots have similar degradation patterns as the previous good lots from where the prediction intervals where developed.

Resumen

El siguiente trabajo presenta un proyecto de investigación realizado en la división de Diagnóstico de Abbott Puerto Rico, con el propósito de proveer un método empírico, contrario a uno simulado, para aplicar la teoría cinética de Arrhenius a un grupo de datos obtenidos mediante un estudio de estabilidad acelerada en tres lotes de reagente de un ensayo inmunológico cuantitativo (un producto de diagnóstico in-vitro). Cuatro modelos fueron desarrollados para relacionar *contenido del producto*, *tiempo* y *temperatura*, y todos ellos fueron comparados en términos de su capacidad para ajustar los datos, la relevancia de los parámetros del modelo considerado, y la habilidad para realizar extrapolaciones. Entre los modelos evaluados se encontraba el concepto clásico de Arrhenius, una función de polinomio, y dos modificaciones no-lineales de la ecuación de tiempo-temperatura de Arrhenius para reacciones cinéticas de cero y primer orden. El modelo no-lineal de Arrhenius para una reacción de cero orden fue seleccionado como el más apropiado para estimar degradación del producto basándose en un estudio acelerado de estabilidad, ya que provee análisis estadísticos relevantes y es capaz de cumplir con todas las presunciones que involucran la relación de vida y temperatura de Arrhenius. Finalmente se desarrolló un procedimiento de validación para el modelo seleccionado, el cual está basado en intervalos de predicción para contener *m* futuras observaciones individuales. Este procedimiento de validación puede ser utilizado rutinariamente con el propósito de evaluar los patrones de degradación de lotes futuros a temperaturas elevadas y concluir si estos lotes tienen patrones de degradación similares a previos lotes buenos con los cuales se determinaron los intervalos de predicción.

Dedication

To the spiritual force that has giving me the opportunity to live very special moments in my life and which has provided me with the necessary strength, calmness and hope to surpass difficult times in life. Without your love there is no true purpose for living.

To my parents Lillian and Efrain for always giving me the unconditional love and support in every decision of my life, and for providing me with the necessary education and moral values that guided me to be a better man. In special to my mom, who made many sacrifices in her life for me to have one with lots of opportunities. Your spirit will always be in my heart.

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1 Introduction

1.1 Justification

Today's manufacturing businesses regulated by the United States Food and Drug Administration (FDA) require the development of highly reliable products that satisfy patient needs without affecting its treatment. All these requirements need to be fulfilled while maintaining desire levels of productivity in the manufacturing processes and improving the overall quality of the product. For market products regulated by the FDA, a shelf-life (or expiration date period) must be indicated on the label of the product. The shelf-life is defined as the time interval that certain characteristics of the product are within the approved specifications. To establish this expiration date period (specifications), typically, a stability study is performed to characterize the degradation of the product [1]. Even after the products label shelf-life is approved by the FDA, every manufacturing site requires a product stability monitoring procedure (stability protocol) to ensure that products in the market can still meet the approved specifications.

Two types of stability test procedures are most widely used in the practice: real-time stability test and accelerated stability test. In a real-time stability procedure, products are stored in normal storage conditions (or normal use conditions) and a specific characteristic (e.g. concentration) is monitored to evaluate product behavior (degradation, if any) until its expiration date [2]. The majority of diagnostic and medical devices products are monitored up to one month extra of the expiration date indicated by the manufacturer. In an accelerated stability test, the product is put into different elevated temperature levels at which degradation will occur more rapidly than at the normal conditions. Therefore, a detectable amount of degradation is induced at a short period of time. Then, a statistical model is fitted using the data collected at high temperatures to predict product behavior at the normal conditions. The Arrhenius equation is usually used in this type of test model since it provides a mechanism to relate degradation rate and temperature [3].

This project will focus on the development and characterization of an accelerated stability test for an In-Vitro Diagnostic product manufactured in Abbott Diagnostic International Ltd. (ADI) in Barceloneta PR, which is an FDA-regulated manufacturing

plant. This intervention pursues the improvement of the current stability procedure by providing an accelerated degradation model, which can predict product content with high precision and accuracy in less time than the current procedure. This will also improve customer service because learning about any possible issue of product degradation faster will benefit the company's response time for product and process improvement before the issue is observed in the market by the customer.

1.2 Company Description

Abbott Laboratories was founded in 1888 by Dr. Wallace Calvin Abbott, a Chicago physician. Abbott Laboratories is a broad-based health care company that discovers, develops, manufactures and markets products and services that extends the range of care systems from prevention and diagnosis, to treatment and cure [5]. Abbott Diagnostic International Ltd. (ADI) in Barceloneta Puerto Rico, serves as an operation plant for Abbott Diagnostic Division (ADD). Its operations started in august of 1984 with six products and 37 employees. Currently, ADI manufactures 337 products with approximately 500 employees. The division of medical products group for diagnostics offers a range of innovative instruments and diagnostic test products to serve clinical laboratory customers worldwide. The immunoassay and clinical chemistry branch includes systems an assays to measure a variety of analyte found in the blood, and to diagnose and monitor diseases and therapies [5]. ADI is responsible for the manufacture and distribution of reagents that serve in these systems. The portfolio of products include reagents for monitoring therapeutic drugs, abuse drugs and toxicology, products for monitoring reproductive hormones, anemia, thyroid, cancer and hepatitis, as well as cardiovascular markers. The stability monitoring procedures currently applied for all of these products are real-time stability tests. Therefore, the need for alternate methods like accelerated stability tests has arise, so that information of product reliability could be obtained more rapidly.

1.3 Objectives

This engineering project pursues the following objectives:

1. Design an accelerated stability test for an In-Vitro diagnostic product.

2. Develop a statistical model capable or relating degradation rate as a function of time and temperature to assess product stability.
3. Verify goodness of fit of the data obtained by the accelerated test to the Arrhenius Life-Temperature Law.
4. Develop a procedure by means of the lot-to lot variability characterized in the accelerated test, which could to be used routinely to compare degradation patterns of future lots at elevated temperatures.

2 Literature Review

2.1 Organization

The literature review has been divided in the following areas:

1. *In-Vitro Diagnostic Products and Immunoassay*
2. *Accelerated Testing.*
3. *Methods of Acceleration.*
4. *Acceleration Models.*
5. *Product Stability Monitoring.*

2.2 In-Vitro Diagnostic Products and Immunoassay

A series of basic concepts and definitions are presented as an overview of the type of product and instrument technology used in the experiments to complete this engineering project.

2.2.1 In-Vitro Diagnostic

In-vitro is a latin word which means "within the glass". It is referred to the method of performing an experiment in a tube or a controlled environment outside a living organism. An In-Vitro Diagnostic Product is defined as any medical device which is a reagent, reagent product, calibrator, control material, kit, instrument, apparatus, equipment or system, that is used alone or in combination for the examination of specimens, including blood and tissue donations. This type of product is not ingested, injected or inoculated in human beings [6]. They are solely used to provide information about a sample collected from human body:

- concerning a physiological or pathological state, or
- concerning a congenital abnormality, or
- to determine the safety and compatibility of a tissue receiver, or
- to monitor therapeutic measures of a condition.

These types of products are used as a system with the purpose of performing a test that could measure some kind of response signal for a specific substance found in a

biological source (e.g. an analyte in blood). These of tests are known as *immunoassays* and are related to the biomedical science branch of diagnostic immunology.

2.2.2 Immunoassay

An immunoassay is a biochemical test that measures the level of a substance in a biological liquid, typically serum or urine. This is done using the reaction of an antibody or antibodies to its antigen. An antibody-antigen binding is also known as an immuno-complex. "Immuno" refers to an immune response that causes the body to generate antibodies, and "assay" refers to a test. Thus, an immunoassay is a test that utilizes immunocomplexing when antibodies and antigens are brought together [7]. The antibody is a protein that is produced by the body to protect itself in response to an "invading" (foreign) substance. The antigen is the substance that the body is trying to "fight off" (eliminate or reduce) by mounting an immune response [8].

The presence of antigen and antibodies can both be measured. For instance, when detecting cancer, some immunoassays test for the presence of antibodies rather than the cancer molecules (antigen). Therefore, if the antibodies are present, this means that invading cancer cells are also present [9]. Other immunoassays test for antigens directly, rather than looking for the antibodies. For example, in a test to measure the concentration of a therapeutic drug, the drug is the antigen that binds to the antibody [7].

Antibodies possess high specificity and affinity for a specific antigen. It is the specific binding of an antibody to an antigen that allows the detection of analytes by a variety of immunoassay methods. An analyte is anything measured by a laboratory test. In immunoassay testing, the analyte may be either an antibody or an antigen. Detecting the quantity of antibody or antigen can be achieved by a variety of methods, which are described in the next section [10].

2.2.3 Immunoassay Detection technology

In the world of immunoassay procedures there are several types of technologies that can be used for analyte detection. These types of technologies included in the AxSYM instrument system developed by Abbott and used in this project are the following:

- Microparticle Enzyme Immunoassay (MEIA)
- Fluorescent Polarization Immunoassay (FPIA), and

- Radiative Energy Attenuation (REA)

For the purpose of this project, only the MEIA technology is explained since this is the technology that is applied in the testing of the product selected for experimentation.

2.2.4 MEIA Reaction Principles

Microparticle Enzyme Immunoassay (MEIA) technology uses a solution of suspended sumicron sized latex microparticles to measure analytes. The particles are coated with a capture molecule specific for the analyte being measured. The effective surface area of microparticles increases assay kinetics and decreases assay incubation time. This permits MEIA assays to be completed in less time than other immunoassays. The components (reactants) necessary for MEIA assays [11] are the following:

- Microparticles coated with a capture molecule (antigen, antibody or viral particle)
- Fluorescent Enzyme Substrate, which is composed of a 4-Methyl Umbelliferone Phosphate (MUP) in solution that is available for a reaction with the enzyme on the antibody.
- Antibody-Enzyme Conjugate, which is an Alkaline Phosphatase enzyme conjugated to the antibody

Figure 1: Components of the MEIA Procedure [1]

[1] Taken from reference [11]

A typical MEIA reaction [11] would occur in the following steps:

1) Sample and microparticles are combined and incubated at reaction temperature. During the incubation period, analytes bind to the microparticle creating an immune complex.
2) The reaction mixture created is aspirated from the incubation well and dispensed into a matrix cell. The immune complex binds irreversible to the glass fiber of the matrix cell. A matrix cell wash removes unbound material. The immune complex is retained by the glass fibers while the excess reaction mixture flows rapidly through the large pores of the matrix.
3) The Alkaline Phosphatase conjugate is added in the matrix cell to complete the antibody-analyte-conjugate "sandwhich". The matrix is washed again.
4) The 4-Methyl Umbelliferone Phosphate (MUP) is then added to the glass fiber matrix. The Alkaline Phosphatase conjugate catalyzes the hydrolysis of the MUP to a 4-Methyl Umbelliferone (MU).
5) Finally, the MEIA optics of the AxSYM instrument measures the rate at which MUP is converted to MU by detecting fluorescent light intensity signals. Up to 15 fluorescent intensity readings are taken immediately after the MUP is added to the glass fiber matrix. A plot of the Intensity (Y-axis) versus Time (X-axis) is created and a linear regression model is fitted as illustrated in Figure 2. The slope of the line (the rate at which MUP is converted to MU on the matrix cell surface) is calculated and the rate value is used to determine the concentration of the analyte in the sample through the use of a calibration curve.

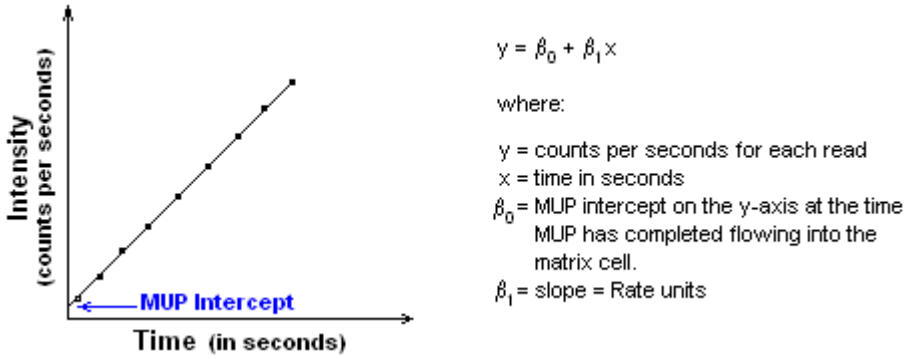


Figure 2: MEIA Rate Determination

A summary of how the components work in combination with the sample to produce a signal and corresponding test result is shown in Figure 3. Notice how the glass fiber matrix serves to anchor the complexes.

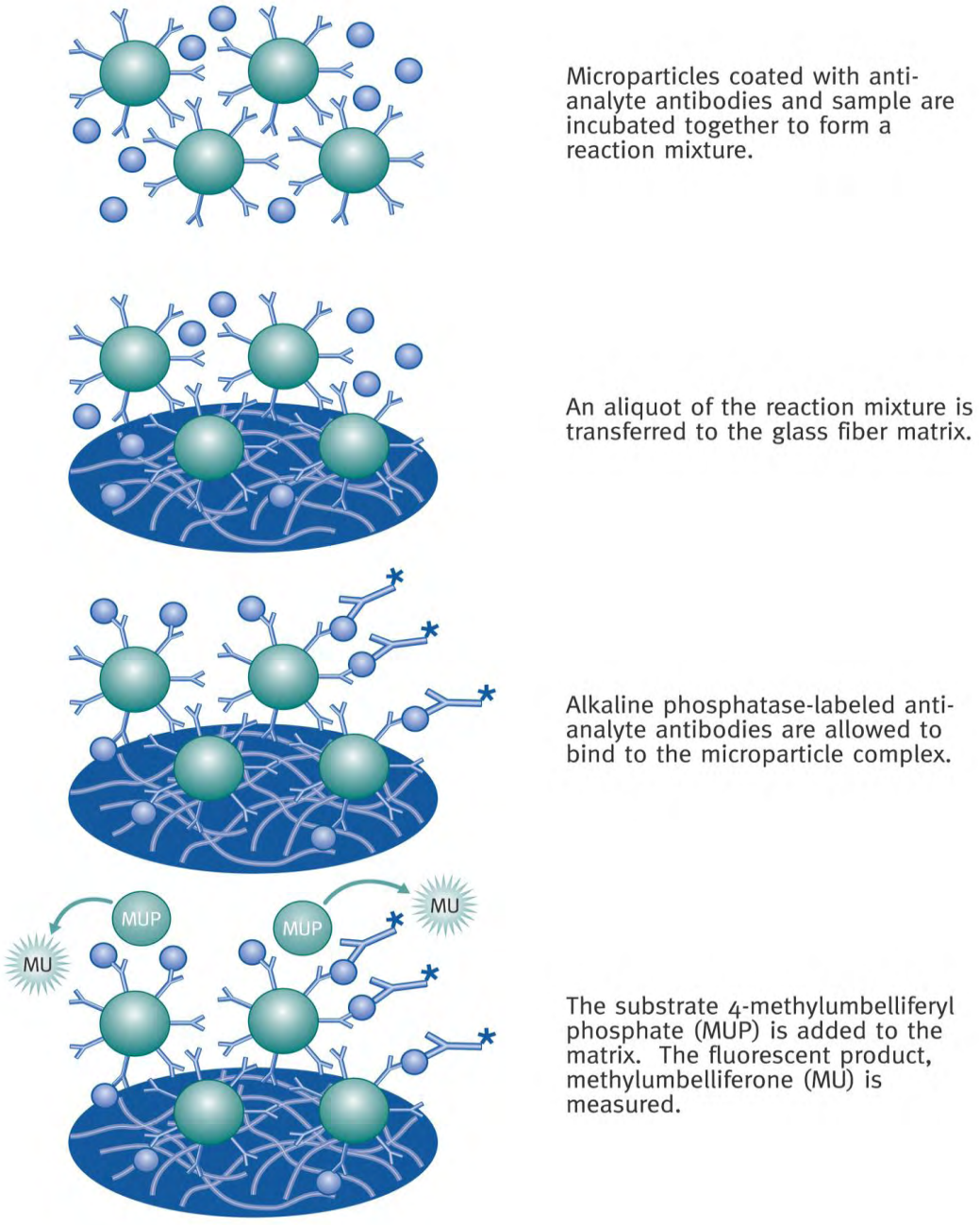


Figure 3: Process of the MEIA Method [2]

[2] Taken from reference [11]

2.3 Accelerated Test

Accelerated tests (AT's) have been used in the manufacturing industries to monitor and achieve highly reliable products. These types of tests have demonstrated to be very useful to identify design and manufacturing deficiencies that have resulted in product and process improvements. These improvements have provided a more robust and reliable product. The main reason for performing an accelerated test is to obtain performance data of devices or components of a product faster, by exposing the product at higher stress levels of one or more accelerating variable (e.g. temperature, use-rate). This procedure will shorten the life of the product or speed up the degradation of its performance. The resulting data is then extrapolated to obtain information of products life or performance at a future time (t), and at normal storage or usage condition [12]. This type of testing saves time and money not only in the early stages of product design or component certification, but also in the investigation procedure when a product demonstrates to have performance issues (e.g. degradation is below specifications before shelf-life) and it's already in the market. Accelerated tests have been used with food, drugs, chemicals, as well as with pharmaceutical and biological components. Accelerated variables include temperature, humidity, load, oxygen, and many others. As Meeker [13] states: "AT's have become increasingly important because of rapidly changing technologies, more complicated products with more components, higher customer expectations for better reliability, and the need for rapid product development".

In general, accelerated tests are divided in two classes of reliability experiments: Accelerated Life Tests (ALT's) and Accelerated Degradation Tests (ADT's). The main common characteristic between these types of AT's is that they usually extrapolate outside the range of the data obtained by the acceleration conditions, to determine an estimate at the use conditions. The following definitions establish the main differences between these two types of AT's [12].

- Accelerated Life Test (ALT) – In this type of test, the failure and/or censoring times of a device subject to an elevated stress is recorded. Then, this data is used to estimate the failure time distribution at the stress conditions, and extrapolation is performed to estimate the failure time distribution at the use conditions. A disadvantage of this type of test is that if a highly reliable device

is being tested, the ALT will probably provide little information to the person performing the experiment since very few or no failures will occur at the design stress levels and the duration for failure may be quite long [14].

- Accelerated Degradation Test (ADT) – In this type of test, degradation in the performance of a device subject to an elevated stress is recorded. This data is then used to obtain specific information of the physical mechanism that causes the degradation on the performance of the characteristic investigated and to make suggestions on the behavior of the device at low levels of stress (use or storage conditions). In contrast to the ALT, for a highly reliable product, ADT's can provide information on performance degradation for the characteristic investigated long before a failure actually occurs. This type of data may be considered very valuable since it uncovers information on the failure modes of the device used in the test [15]. The following four major inputs are required when designing and Accelerated Degradation Test.

    1. Determine the accelerated variable to use in the test.
    2. Select the stress levels to be used for the accelerated variable.
    3. Determine the proportions of devices to be put in each stress level.
    4. Select the time period (intervals) to measure the devices.

    All of these input decisions can be justified by using previous publications that incorporate test characteristics similar to the test environment to be used or by consulting scientists or engineers that have very good knowledge on the physical, and chemical behavior of the devices to be used in the test.

It is very important to clarify that many of the fundamental physical model assumptions, concepts and procedures are equal for the ALT's and the ADT's. The particular thing that differs is the type of model fitted to the data and the methods of analysis used [16].

2.4 Methods for Test Acceleration

In general there are three types of methods for accelerating a test. All of these methods have a common objective, which is to perform a test at stress or accelerated condition for then to extrapolate outside the range of the available data. These types of acceleration test methods are summarized in the following sections.

2.4.1 Use–Rate Increase

In this type of method, the use-rate of the product is increased to accelerate product failure or to cause wear or degradation, which could provide useful information of product performance. As indicated by Meeker and Escobar [13], there is a basic assumption that is fundamental for this type of acceleration method, which is that the useful life of the test units must be adequately modeled by the cycles of operation and cycling rate, and they should not affect the cycles-to-failure distribution. This is obtained when the test is performed using cycles that simulate the actual use and when the frequency of these cycles is low enough to permit the units to return to a steady state after each stress cycle.

2.4.2 Aging–Rate Increase

In this type of acceleration method, the aging rate of the product is augmented by increasing the level of the experimental variables (e.g. temperature or humidity). These types of modifications accelerate certain failure modes such as chemical degradation, which can reduce product performance or can even develop system failures [12]. For example, exposing an in-vitro diagnostic product like a reagent kit to increase levels of temperatures causes the chemical structure of the antibody component to weaken and degradation of the drug concentration to increase. As it has been illustrated in so many publications that assess the study of reliability ADT's, increasing the temperature is the most common method used to accelerate the chemical degradation process and to obtain degradation data more rapidly. The Arrhenius relationship has been widely used to model product life as a function of temperature. The Arrhenius Law is used for simple chemical reaction rates and its relation is used to describe many products that fail as a result of degradation due to chemical reactions or metal diffusion [12].

2.4.2.1 Arrhenius Life-Temperature Relationship

The Arrhenius equation is a simple but exceptionally accurate expression that explains the dependence of the rate constant (*k*) of a chemical reaction on the temperature. The equation was provided with a physical justification and interpretation in 1889 by the Swedish chemist Svante Arrhenius. According to the Arrhenius Law, the rate constant (*k*) of a chemical reaction is given by the following expression:

$$k = A \cdot e^{\left(\frac{-E_a}{R \cdot T}\right)} \tag{1}$$

where k is the reaction (degradation) rate or rate constant.

E_a is the activation energy of the reaction.

R can be defined as the gas or the Boltzmann constant (depending the units used).

T is the absolute *Kelvin* temperature. It equals ^{o}C + 273.16

A is the Arrhenius constant. This factor is characteristic of the product. failure mechanism and test conditions.

In this equation, the activation energy is defined as the minimum energy necessary for a specific chemical reaction to occur. When the activation energy is given in molecular units instead of molar units (e.g. joules per molecule instead of joules per mol), the Boltzmann constant is used instead of the gas constant. It can be seen from the Arrhenius equation that either increasing the temperature or decreasing the activation energy, the result is an increase in the reaction rate [17]. They are three necessary requirements for a reaction to occur, which are known as the "collision model" requirements:

1) *the molecules must collide to react*
2) *there must be enough energy (energy of activation) for the molecules to react*
3) *the molecules must be orientated with respect to each other correctly*

The higher the temperature, the more likely the reaction overcomes the energy of activation. The constant *A* is the frequency factor for the reaction and it expresses the probability that the molecules contain a favorable orientation and will be able to

proceed in a collision [18]. The units of this constant are the same as the units of the rate constant (k) and will vary depending the order of the reaction. Based on the statements established, for a reaction to take place and overcome the activating energy; the temperature, orientation, and energy of the molecules must be considerable. Therefore, the Arrhenius equation manages to relate all these things [17].

Given the small temperatures ranges in which kinetic studies are performed, it is rational to approximate the activation energy as being independent of the temperature. Taking the natural logarithm to the Arrhenius expression in equation (1) the following equation is obtained:

$$\ln k = \ln A - \frac{E_a}{R \cdot T} \tag{2}$$

Therefore, when a reaction has a rate constant that obeys the Arrhenius equation, a plot of ln(k) versus T^{-1} gives straight line whose intercept and slope can be used to determine the Arrhenius constant (A) and the activation energy (E_a).

Since equation (1) is dependent of temperature, it can also be written as follows:

$$k(T) = A \cdot e^{\left(\frac{-E_a}{R} \cdot \frac{1}{T}\right)} \tag{3}$$

From equation (3), an accelerated factor (AF) is calculated as the ratio of the degradation rate at an elevated temperature $k(T_e)$, to the degradation rate at the storage temperature $k(T_s)$. The following expression is obtained after taking the ratio of the reaction rates:

$$AF = \frac{k(T_e)}{k(T_s)} = A \cdot e^{\left[\frac{-E_a}{R}\left(\frac{1}{T_s} - \frac{1}{T_e}\right)\right]} \tag{4}$$

where T_e and T_s correspond to the elevated and storage Kelvin temperatures, respectively. As observed in equation (3), the ratio gives an Accelerating Factor that only depends on two temperature levels and the activation energy [14,19].

2.4.2.2 Chemical Kinetic Reaction and Orders

It is evident from studies of chemicals kinetics that different chemical correspond to different order of reactions [20]. If a reaction of two components is driven as illustrated below:

$$A + B \rightarrow C$$

then, the reaction rate is given by the following differential equation between concentration and time

$$\frac{dC(t)}{dt} = -k_{r_A + r_B}[A]^{r_A}[B]^{r_B} \tag{5}$$

In this equation, *C(t)* is the content of the product being studied at time *t*, [*A*] and [*B*] represents the concentrations of components *A* and *B*, and *k* is the rate constant as previously described in Section 2.4.2.1

In the studies of accelerating testing, it is often common to see the use of one of the following three reaction orders to describe the chemical effect been studied (i.e. zero, first and second reaction). The main difference between each order is mainly the number of reactants been considered. Therefore, a zero-order reaction is one in which the reaction rate does not depend on the concentrations or activities of the reactants. In contrast, in a first and second order reaction rate, the reaction rate is dependent of one or two reactants, respectively. Each of these reaction orders is mathematically determined following the procedure presented subsequently [20].

For a *zero-order reaction*, the differential equation is given by

$$\frac{dC(t)}{dt} = -k_0 \tag{6}$$

Integrating both sides of the equation (6) we obtain

$$\int \frac{dC(t)}{dt} = -\int k_0 \, dt \tag{7}$$

Solving these integrals gives the following expression

$$C = C_0 - k_0 t \tag{8}$$

where C_0 and C are the product contents at time zero and time *t*, respectively. It can be observed in equation (8) that a product following a zero-order reaction will

degrade at a constant rate over time independently of the concentrations at time zero (t_0) and time t.

In contrast, the amount of degradation is proportional to the concentration at time t for a *first-order reaction*. The corresponding differential equation for a first-order reaction is given by

$$\frac{dC(t)}{dt} = -k_1 C(t) \tag{9}$$

Integrating both sides as done previously, we obtain

$$\int \frac{dC(t)}{C(t)} dt = -\int k_1 \, dt \tag{10}$$

Solving these integrals gives the following expression

$$\ln C = C - k_1 t \tag{11}$$

Therefore if the initial concentration at time zero is represented by $\ln C_0$, then equation (11) can be re-written as follows:

$$\ln C = \ln C_0 - k_1 t \tag{12}$$

Many literature books [12, 20] use the following exponential format of equation (12) to describe a first-order reaction.

$$C = C_0 \cdot e^{(-k_1 t)} \tag{13}$$

In a *second-order relation*, the reaction occurs at a constant rate that is proportional to the square of the concentrations. The differential equation for this reaction is given by

$$\frac{dC(t)}{dt} = -k_2 C^2(t) \tag{14}$$

Integrating as previously, we obtain

$$\int \frac{dC(t)}{C^2(t)} dt = -\int k_2 \, dt \tag{15}$$

Solving equation (15) yields the following expression

$$\frac{1}{C} = \frac{1}{C_0} + k_2 t \tag{16}$$

In the overall, the subscript numbers used for the rate constant k in all the equations previously presented were used to distinguish the order applicable to each reaction

but this is actually not needed since each reaction order has a unique expression. Therefore, for simplification purposes, equations (8), (13) and (16) will be re-written as follows:

Zero order: $$C = C_0 - kt \quad (17)$$

First order: $$C = C_0 \cdot e^{(-kt)} \quad (18)$$

Second order: $$\frac{1}{C} - \frac{1}{C_0} = kt \quad (19)$$

where k is the rate constant as previously described in Section 2.4.2.1; C_0 and C are the product contents at time zero and time t, respectively. The first-order reaction is probably the most common model used in the pharmaceutical industry to describe the degradation of a product. The zero-order is used in some occasions but is uncommon to see the use of the second order reaction.

The loss of biological function is the result of a chemical or biochemical reaction, which should be described by one of the above equations. In most cases, the loss of catalytic or other functional activity follows a true first-order process. This means that it is a single molecular incident or "strike" that results in loss of activity, or that there is a constant probability per unit time that any intact molecule will become inactivated.

It is easy to determine whether the activity loss with time at a particular temperature in time is first order. Fitting the experimental data obtained at the specific temperature to a first order exponential equation does this verification. Visual observation of the graphic fit and inspection of parameters such as standard errors and chi-square will show if the data is first-order. First order exponentials, in which the rate constant has units or reciprocal time, have the additional useful property that a semi-logarithmic transformation of the data provides a linear plot. That is, if log(y) or ln(y) is plotted against time, a straight line is obtained as illustrated in Figure 5. This property is not shared by second or higher order exponentials [4,12].

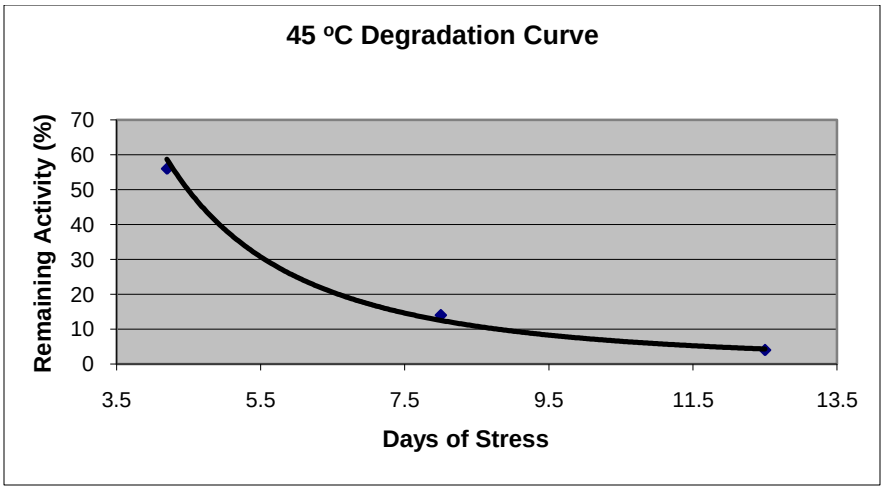


Figure 4: Example of a First-order Degradation Curve

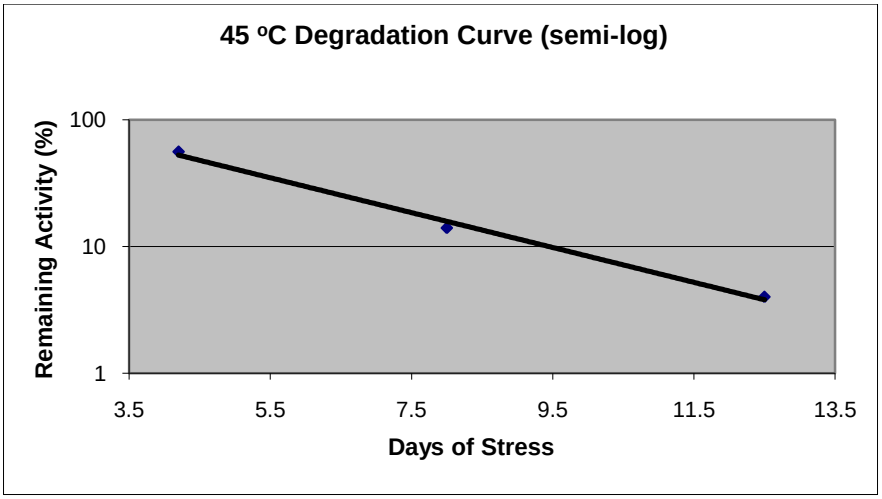


Figure 5: Example of Linear Plot by Semi-log Transformation

If the activity loss with time proves to be first-order reaction, then the Arrhenius equation may be used to analyze the experimental data obtained at different temperatures. As indicated before, this equation relates the activation energy for a particular reaction (i.e. the energy difference between the reactant and the transition state-activated complex) to the temperature and to the first-order rate constant (k). It can be seen from equation (2) that a reaction obeying the Arrhenius equation will yield a linear plot of $\ln(k)$ versus T^{-1}, and the slope of this line represents the term $-E_a/R$. Therefore, the Arrhenius plot provides a basis for determining the activation energy of the chemical reaction since R is a constant already known. In this plot, the natural logarithm of the first-order rate constants

determined at different temperatures is related to the reciprocal of the absolute temperature. The resulting linear plot can be extrapolated to determine the value of the rate constant (*k*) at the temperature of interest (e.g. 281 Kelvin). Therefore, from the rate constant determined by extrapolation, the shelf-life of a product can be determined by a simple calculation once a suitable lower limit of acceptability has been defined (e.g. 80% of staring activity). Refer to Figure 6 for an illustration of this plot [21].

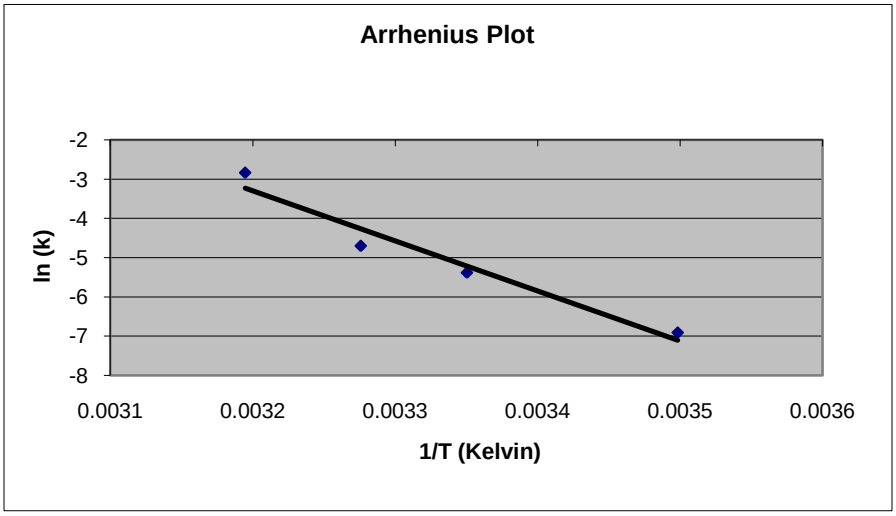


Figure 6: Example of an Arrhenius Plot with 4 different temperatures

2.4.3 Stress Level Increase

In this type of acceleration method, the stress level in which the units operate is increased until failure occurs. In this type of method, failure occurs only when the applied stress goes over the strength level. Therefore, an increase frequency of unit failures is observed as the stress levels increases. Stress loading is a common method used in accelerated tests for investigating physical properties of a material. Types of stress loading include constant, cyclic, step, progressive and random. The most common type of stress loading accelerated method applied is the constant stress since such testing is simple to performed and can be verified empirical for some materials and products [14].

2.5 Acceleration Models

As indicated previously, when performing an accelerated test for some specific variable that will be accelerated, some type of model can be fitted to the data obtained with the purpose of describing the effect that this variable has on the performance on the response variable. Since the final objective is to extrapolate at the lower levels of the accelerated variable, it may be justifiable to use a physical or an empirical acceleration model to perform this extrapolation. These types of models are defined below.

2.5.1 Physical Acceleration Model

Physical acceleration models are based on the physical and chemical aspects of the product being tested. They describe the failure mechanisms of the product by using the range of data obtained from an accelerated test, for then, to extrapolate at use conditions. Since the relationship between the accelerating variables and the failure modes may be complicated to determined, previous feedback from scientists and engineers may be needed to apply this type of model. The main reason to obtain information from these professionals is because they should have very good knowledge of the physical and chemical mechanisms that can affect the performance of the product and which variables may be accelerated to obtain the necessary data to perform the extrapolation. As indicated by Escobar and Meeker [13]: "…failure may result from a complicated chemical process with many steps, but there may be one rate-limiting (or dominant) step and a good understanding of this part of the process that may provide a model that is adequate for extrapolation".

2.5.2 Empirical Acceleration Model

Empirical methods of analysis are also referred to as nonparametric methods or distribution-free methods. The objective when using this type of method is to derive a function or distribution that fits the data when there is very little knowledge of the physical and chemical mechanisms of the product that may cause degradation or failure [12]. Therefore, this type of model is used only when it may be impossible to apply a model based on the physical and chemical aspects that describe the failure mechanisms. It is important to clarify that in some cases this type of model may fit very well the data obtained by the accelerated test but it may result in a model that provides terrible

extrapolation estimates. This is one of the mayor deficiencies for empirical acceleration models. Sensitivity analysis is used in the majority of times to assess the effect of uncertain inputs like inputs related to model assumption. Evans [22] establishes that since accelerated tests may be the only method available to perform fast reliability assessments, the justification to use this type of method may be base only on physical or empirical evidence. Sensitivity analysis should be performed when confronting difficulties with accelerated testing.

2.6 Product Stability Monitoring

2.6.1 General Principles

The purpose for stability monitoring is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of certain environmental factors such as temperature, humidity or light, and to establish a retest period or shelf-life, as well as the recommended storage conditions. It is important to clarify the difference between retest period and shelf life. The term retest period is used when the stability study is performed on a *drug substance*, which is a component of the final drug product. A retest period is defined as the period of time during which the drug substance is expected to remain within its specifications, and therefore, can be used in the manufacture of a given drug product, provided that the drug substance has been stored under the defined conditions [1]. The term shelf-life (also referred to as expiration dating period) is used when the stability is performed on the final *drug product*, and it is defined as the time period during which a product characteristic is expected to remain within the specifications established by the manufacturer [1]. The change of this characteristic is usually called degradation. A product is considered to be degrading when the characteristic(s) of interest (e.g. concentration, potency, polarization, etc) decreases as time increases [23]. This engineering project will be focus on the principles for stability monitoring of shelf-life since the device that will be used for test is a final drug product.

2.6.2 Real Time Stability vs. Accelerated Stability

Before discussing the regulations established by the Food and Drug Administration (FDA) for medical devices and the guidelines that this agency has for designing and performing a stability test, it is important to clarify the major difference between real-

time (long term) and accelerated stability tests, since any of these two models can be used. In a real-time test, the product is stored at the normal storage conditions and monitored for a period of time. It is expected to observed degradation as time increase, but in the majority of products manufactured by sites regulated by the FDA, this behavior is not observed until months after the shelf-life (expiration date) indicated in the label of the product. The reason for this is because manufacturers have a tendency to establish expiration periods shorter than the actual time in which true degradation occurs to provide product assurance to the customer. By contrast, in the accelerated test, the product is subjected to elevated stress conditions in order to increase the rate of degradation and to obtain valuable data that will be used to fit a statistical model capable of performing extrapolation at normal storage or used conditions.

Both methods are equal in that the main purpose is to monitor the performance of a product through time. In addition, when considering lot-to-lot variability, both models are equal in that this variability is considered as random, and is attributed to two sources; variability at time zero and variability of degradation rate. The main difference is that for drug shelf-life, real-time stability test is modeled as a function of time while accelerated stability test is modeled as a function of time and temperature [19].

2.6.3 FDA Regulations and Stability Guidelines

The Food and Drug Administration (FDA) is a federal science-based law enforcement agency mandated to protect public health and safety. FDA accomplishes its mission by establishing and enforcing high product standards and other regulatory requirements authorized or mandated by the Federal Food, Drug and Cosmetic Act (FD&C Act), its amendments, and other public health laws.

2.6.3.1 FDA Regulations

Medical devices are classified and regulated according to their degree of risk to the public. The FDA [1] establishes three different regulatory classes to ensure that each device is subject to regulations that are appropriate:

- Class I – General Controls. These are devices that are subject to a set of general regulations that apply to all devices. General controls include the registration of manufacturers, general record keeping requirements, and compliance with Good Manufacturing Practice (GMP) regulations. Class I devices include clinical

chemistry and clinical toxicology test devices, cell and tissue culture products, as well as immunology (diagnostics) and microbiology test devices.

- Class II – Special Controls. These are devices for which general regulations are not enough to guarantee its safety. A class two device may be subject to specific regulations in order to provide assurance of the product's safety. These specific regulations may include requirements for meeting performance standards recognized by the FDA, post-market surveillance, patient registries, and other appropriate requirements. Class II devices include the same classification of tests devices mentioned for Class I.
- Class III – Pre-market Approval. Devices that are life-supporting or life-sustaining, or is important in preventing impairment of human health. For a Class III device, general controls may be insufficient to provide reasonable assurance of its safety and effectiveness. Under Class III regulations, devices such as heart valves, breast implants, and cranial electrotherapy stimulators must be reviewed for safety and effectiveness, and receive FDA pre-approval before they are marketed

FDA further assures the safety and effectiveness of medical devices by regulating their manufacture and regularly inspecting manufacturing sites to assure they comply with these regulations. The work of this agency does not end when a medical device has been approved since it continuously analyzes reports to ensure that products are safe and to watch for dangerous events related to the use of medical devices.

2.6.3.2 FDA Stability Guidelines for a Drug Product

The stability guidelines established by the FDA [1] are mainly focus on the stability data package of new drug substances and products, but leaves sufficient flexibility to consider the variety of different practical situations that may be encountered due to specific scientific considerations of the materials being evaluated. Other alternative approaches can be used when there are scientifically justifiable reasons. A design of a formal stability study for a drug product should be based on knowledge of the behavior and properties of the drug substances that compose the final drug product and the experience gained from the clinical formulation studies. To perform a stability study the following guidance should be consider:

2.6.3.2.1 Selection of Batches

Data from the stability study should be characterized by testing at least three primary batches of the product. The primary batches should be of the same formulation and packaged in the same container as proposed for marketing. The manufacturing process used for the primary batches should simulate the one to be applied to production batches and should provide product of the same quality and meeting the same specification as that intended for marketing. Where possible, batches of the product should be manufactured by using different batches of raw material or bulk components to capture lot-to-lot variability [1].

Lot-to-lot variability at the time a stability study starts (time zero) can affect the expiration date of the product since a lot with lower product content at this time can reach the failure point more rapidly than a lot with higher product content at time zero, even though they may have the same degradation rate. This is controlled by the manufacturing specifications and is also considered in the specifications of the shelf-life.

2.6.3.2.2 Container Closure System

A container closure system is defined as the sum of packaging components that together contain and protect the final drug product. Stability testing should be conducted on the final drug product packaged in the container closure system for market distribution. This can include any secondary packaging components and labeling [1].

2.6.3.2.3 Specification

Specification is defined as a list of tests that evaluate those product attributes of interest by using one or more analytical procedures. Stability studies should include testing of those attributes that are vulnerable to change during storage and will probably affect product quality, safety and/or efficacy. The testing should cover (as appropriate), the physical, chemical, biological, and microbiological attributes. Analytical procedures (e.g. instruments) used for testing should be fully validated and stability indicating [1].

2.6.3.2.4 Testing Frequency

For *real-time* (long term) stability study, frequency of testing should be sufficient to establish the stability behavior of the drug product. This time interval for testing will depend on the shelf life of the product. The frequency of testing at the long term storage condition should normally be every 3 months over the first year, every 6 months over the second year, and annually thereafter through the proposed shelf life. For a 6-month accelerated stability study, a minimum of three time points, including the baseline and final time points is recommended (e.g. 0, 3 and 6 months). If expectation exists that the drug product put into the accelerated test is likely to approach its specification limits, then increased testing should be conducted either by adding samples at the final time point or by including a fourth time point in the study design. For a 12-month study, a minimum of four time points (e.g. 0, 6, 9, 12 months) is recommended. It is important to clarify that these are general guidelines established by the FDA [1], but that other designs can be also applied, if justified.

2.6.3.2.5 Storage Conditions

In general, a drug product should be evaluated under storage conditions that test its thermal stability, and if applicable, its sensitivity to moisture. The FDA established guidelines [1] for six different product storage environments that can be followed for stability testing. When performing a stability study, the user has the freedom of using the type of environment that most likely applies to the kind of product put into test. The six drug storage environments covered by FDA guidelines are listed below:

a. General Case
b. Drug products packaged in impermeable containers
c. Drug products packaged in semi-permeable containers
d. Drug products intended for storage in a refrigerator
e. Drug products intended for storage in a freezer
f. Drug products intended for storage below –20°C

Based on the type of product (e.g. in-vitro reagent) that will be used in this engineering project, the accelerated stability test proposed will focus on cases (b)

and (d) of the guidelines, since the product is packaged in impermeable containers and recommended to be stored in a refrigerator. For products packaged in impermeable containers that provide a permanent barrier to passage of moisture or solvent, the guidelines establish that sensitivity to moisture or potential solvent loss is not a concern. Therefore, stability studies for product stored in impermeable containers can be conducted under any controlled or ambient humidity condition.

2.6.3.2.6 Evaluation

As indicated before, the purpose of the stability study based on testing a minimum of three batches of the product is to establish a shelf-life applicable to all of the future batches manufactured and packaged under similar conditions. The degree of variability of the individual batches used in the stability study affects the confidence that a future production batch will remain within specifications through the shelf life proposed.

The FDA [1] indicates that an approach for analyzing data of quantitative attribute that is expected to change with time is to determine the time at which the 95 percent confidence limit (for the mean curve) intersects the acceptance criterion. The nature of the degradation relationship is going to determine whether the data should be transformed for linear regression analysis. The relation can be presented by a linear, quadratic or cubic function on an arithmetic or logarithmic scale. The guidelines also establish that statistical methods should be used to test the goodness of fit on all batches and combined batches (where appropriate) to the assumed degradation line or curve.

For extrapolation purposes the FDA comments that limited extrapolation of data from the real-time study beyond the observed range can be done to extend the shelf-life at the approval time of the product, if this can be justified. The justification has to be based on what is known of the degradation mechanisms, the results obtained by testing at accelerated conditions, the goodness of fit of any mathematical model and/or existence of stability supporting data.

2.6.4 Principles of Design for an Accelerated Stability Test

As indicated by Kirkwood [3, 4], there are three principal reasons why accelerated degradations tests for biological products (e.g. In-Vitro Reagent kits) need to be planned carefully. These reasons are:

1) The experimenter has to ensure that the size of the study is adequate to have results with acceptable statistical precision, without the need of wasting time by repeating experimental runs and increasing the cost of the experiment by wasting expensive material.
2) A second consideration is that in most of the cases, a biological product is improbable to degrade very fast even at moderate temperatures (e.g. 20°C). Therefore, the measurable loss of activity may be small in comparison with the experimental error. If the error is random, this results in low statistical precision of the degradation rate that is being estimated, but if the error is systematic; then, this could cause serious bias in the estimates. The study design should aim to eliminate any cause of systematic error, as well as to minimize pure random error.
3) A satisfactory design should have tests of the two major assumptions used in accelerated degradation tests. These assumptions are that
    (i) degradation follows a first order kinetic reaction,
    (ii) degradation rates obey the Arrhenius equation.

    Violation of either of these assumptions can put into question the validity of the accelerated degradation test results.

Using the assumption that the reaction for degradation follows a first order process, the results from the accelerated test are used to calculate the relative degradation rate at each elevated temperature. These degradation rates are then used to fit the Arrhenius equation by the statistical technique of maximum likelihood.

2.6.4.1 Degradation Model

The degradation of particular product, *D*, as a function of time for a first order reaction is given by

$$D = \alpha \cdot e^{(-k \cdot t)} \tag{20}$$

In this relation, α is the product performance at time zero, *k* is the degradation rate and *t* is the time ($t > 0$). In general, the observed sample degradation (Y_{ij}) of lot *i* at

time j is the actual degradation of the lot plus a measurement error (ε_{ij}), which is given as

$$Y_{ij} = D_{ij} + \varepsilon_{ij} = \alpha_i \cdot e^{\left(-k_i \cdot t_{ij}\right)} + \varepsilon_{ij} \tag{21}$$

Lots of the same product should perform within the manufacturing specifications and lot-to-lot variability is assumed random for a process under control, therefore α_i is assumed to random and independent for most applications. The degradation rate (k_i), depends on the characteristics of the lot, therefore this parameter is also consider random and independent for most applications. The time (t) is fixed, and does not need to be the same for all lots. The experimental error (ε_{ij}) is also random, independent and distributed as $\varepsilon_{ij} \sim NOR\ (0,\sigma_\varepsilon)$. In the majority of the problems where degradation models have been applied it can be observed that the models obtained use a bivariate normal distribution for the intercept and the degradation rate parameters [19].

An accelerated model can be obtained by inserting the accelerating factor (AF) equation that was previously presented in equation (4), since this equation relates temperature and time by the means of the Arrhenius equation. The degradation as a function of time and temperatures is given by

$$D = \alpha \cdot \mathrm{e}^{\left(-k \cdot AF \cdot t\right)} \tag{22}$$

Therefore, the observed sample degradation (Y) of lot i at time j and temperature k is

$$Y_{ijk} = D_{ijk} + \varepsilon_{ijk} \tag{23}$$

As it can be observed, equation (22) is similar to equation (20), with the exception of the added acceleration factor (AF). The acceleration factor equation adds the activation energy (E_a) in equation (22) as another parameter to be determined. It is important to clarify that E_a is considered a fixed effect since it is a material property that does not depend on temperature and that is considered constant from lot-to- lot. Therefore, AF is also fixed [13, 19].

2.6.5 Parameter Estimation

For estimating the parameters of an accelerated degradation model, the maximum likelihood method (ML) can be used. Evaluation of the likelihood functions requires computation of integrals of multi-dimensions and maximization with respect to the

parameters of the accelerated model. In general, this procedure require numerical approximation of *n* integrals of dimension *k*, where *n* is the number of sample paths and *k* is the number of random parameters in each path [13, 19]. A simplified maximum likelihood representation for the accelerated model can be expressed as follows:

$$L(\boldsymbol{\mu}_{\boldsymbol{\beta}}\Sigma_{\boldsymbol{\beta}}, \sigma_{\varepsilon} \mid \mathbf{Y}) \tag{24}$$

In this expression, $\boldsymbol{\mu}_{\boldsymbol{\beta}}$, is the mean vector and $\Sigma_{\boldsymbol{\beta}}$ is the covariance matrix of the $\beta_1, \ldots \beta_k$ parameters, and **Y** is the vector of the observations. Therefore, the parameters to be estimated are μ_α, μ_R, σ^2_α, σ^2_R, E_a and σ^2_ε. The approach developed by Pinheiro and Bates [24] can be used to determine the ML estimates of these parameters. The computational program of Pinheiro and Bates has been already implemented in several software statistic applications.

Another procedure for estimating the parameters of an accelerated model is the use of least squares procedures. The procedure can be applied when using linear or nonlinear models. The main difference between linear and non-linear models will be briefly discussed in the following sub-sections but the explanation will be focused on nonlinear least squares since this project is focused on determining nonlinear models to estimate degradation by means of the Arrhenius Life-Temperature relationship.

2.6.5.1 Linear and Non-linear Regression models

Linear regression models include first order relationships, polynomial and other more complex models. They are called linear regression models since they are linear in the unknown parameters (β_j). In general, a linear regression model is expressed as follows:

$$y = \mathbf{x}'\boldsymbol{\beta} + \varepsilon = f(\mathbf{x}, \boldsymbol{\beta}) + \varepsilon \tag{25}$$

where **x** is an *n* x *p* matrix of the observations, $\boldsymbol{\beta}$ is a *p* x 1 vector of the regression coefficients and ε is a *n* x 1 vector of uncorrelated random error [25].

In contrast, any model that is not linear in the unknown parameters is called a nonlinear regression model. The symbol θ is used to represent a parameter in a nonlinear model to highlight the difference between the linear and nonlinear parameter. In general, a linear regression model is expressed as follows:

$$y = f(\mathbf{x}, \boldsymbol{\theta}) + \varepsilon \tag{26}$$

where $\boldsymbol{\theta}$ is a p x 1 vector of unknown parameters and ε is also an uncorrelated random error term with $E(\varepsilon) = 0$ and $\text{Var}(\varepsilon) = \sigma^2$. In nonlinear regression it is also assumed that errors are normally distributed like in linear regression [26]. The main difference between linear and nonlinear regression is that in nonlinear regression the derivatives of the expected function $f(\mathbf{x}, \boldsymbol{\theta})$ are function of the unknown parameters θ_j.

2.6.5.2 Non-linear least squares

The standard procedure for estimating parameters in nonlinear regression is least squares. For the general expected function $f(\mathbf{x}, \boldsymbol{\theta})$, the estimator $\hat{\theta}$ is chosen to be the value of θ that minimizes the following residual sum of square function:

$$RSS(\theta) = \sum_{i=1}^{n} [y_i - f(\mathbf{x}_i, \theta)]^2 \tag{27}$$

Finding least squares estimates generally requires an iterative function minimization routine. Convergence of an algorithm to the least squares estimate may be sensitive to the selection of the starting values and to the parameterization of the model. Many algorithms require the calculation of the first and possibly second derivatives of the expected function $f(\mathbf{x}, \boldsymbol{\theta})$ with respect to each of the parameters in the model. Computer programs that do not require formulas for derivatives will often approximate them numerically [27].

2.6.5.2.1 Inferential Statements and Diagnostic Methods

Inferential statements for nonlinear regression depend heavily on normality assumptions and are accurate only for very large samples. In smaller samples, the accuracy of results will vary greatly from problem to problem and can also depend on the selection of parameterization. Standard errors produced using small samples of data can understate or overstate the precision of an estimate. Procedures for studying assumptions like normality, constant variance, etc, are yet not well developed for nonlinear models. However, graphical methods like a normal probability plot of residuals can help in the study of these kinds of assumptions [27].

3 Methodology

The following steps summarize the methodology applied in this engineering project to design and to analyze the accelerated stability test for an In-Vitro diagnostic product manufactured in Abbott's Diagnostic plant in Barceloneta Puerto Rico.

3.1 Selection of Product for Accelerated Stability Testing

Because this project needed to be completed in a time frame that provided beneficial results to Abbott, a product with the smallest shelf life (expiration date) was selected. The product selected for testing is an immunoassay regent kit (an in-vitro product) that has an expiration date of six months. It is important to clarify that most expiration dates established by the manufacturer provide some customer assurance, but there is actual knowledge of Abbott scientists that if the product is stored at the recommended conditions, it can still be within the acceptance specifications for more than the shelf life granted for use.

3.2 Selection of Batches

To capture the contribution of lot-to-lot variability in the results of the accelerated model and following the guidelines established by the FDA [1], samples from three (3) different batches (or lots) of the product were put into test. The FDA stability guideline indicates that a single lot does not permit assessment of lot-to-lot variability and that two lots provide an unreliable estimate. Therefore, to provide a more precise estimate of the product degradation, it is preferred to use as many lots as possible to perform stability testing. However, there are some practical considerations for the pharmaceutical industry like costs, resources, and product capacity that may prevent to perform stability testing for more lots.

In addition to satisfying the minimum requirement of 3 lots, the samples used in this project came from batches manufactured with different lots of raw material or bulk components to comply with an optional requirement that is established in the FDA guidelines. The accelerated stability study was conducted with samples of the product packaged in the same container closure system that is used for market distribution. This complies with the second FDA guideline requirement previously discussed in section 2.6.3.2.1.

3.3 Selection of the Accelerated Variable

Temperature will be the accelerated variable used in test. The selection of this variable was based on previous knowledge of Abbott scientists of the physical and chemical aspects of the product failure modes. In general, a reagent kit (the product used) is composed of three components that in combination are used to diagnose a condition or a disease. These components are (1) a solution with latex microparticles that are coated with antibody to bind the specific analyte being measured, (2) a solution of antibody-enzime conjugate, which binds to the microparticle and (3) a specimen diluent solution that reacts with the enzyme on the antibody. Increasing the temperature will most affect the second component (the antibody-enzime conjugate) by breaking its molecular structure and deteriorating the antibody agents, which causes degradation in product content. On this report, only general information about the product components will be given to protect proprietary information of the company.

3.4 Selection of Stress Levels

For the purpose of testing at accelerated conditions, four temperatures (45, 37, 30 and 17 °C) were selected based on feedback given by the scientist. As indicated by Nelson [12], the temperatures selected need to be sufficiently high to provide a more accurate estimate of the parameter for the effect of temperature, but at the same time, it is also necessary to consider that the temperatures needed to provide the necessary quantity of degradation data (test points) to performed an appropriate model fit. In this experiment, a temperature range of 30 to 45°C is expected to provide the necessary degradation data, based on the scientific knowledge of the physical and chemical properties of the product. In addition, the product was also exposed to a room temperature of 17°C to have data near the product storage temperature (2 to 8°C). Having data near the storage condition minimizes the amount of extrapolation required; therefore, the extrapolation estimates are expected to be more accurate.

3.5 Proportion of Devices Exposed to Stress Levels

For each lot, twelve units of product were exposed to each of the high temperatures (30, 37 and 45 °C) and 10 units were exposed to the temperature of 17°C. Fewer units were run at the lowest temperature for limitations of time to complete the project since

units at low temperature degrade more slowly, therefore, they have to be run for a longer period of time to observe desirable degradation patterns. The number of units to be tested was determined based on previous knowledge of the failure mechanisms of the product, the cost of each unit, and by considering previous publication on accelerated degradation test planning. As justified by Kirkwood using Monte Carlo simulation studies on accelerated degradation test for biological standards [4], a minimum of 10 units (for each stress temperature) put on accelerated test is reasonable to have a test that provides estimates with good precision. The cost of product was also considered in the number of units to be tested for the reason that a total of 138 units at a cost of $37.27 each (the total cost of testing material only is $5,143.26) were used and destroyed once tested. Units were destroyed once tested since a biological product that has been exposed to a high temperature in an incubator and then is taken out of the incubator to perform the test in normal room temperature can develop microbial contamination if is continually exposed to frequent cycles of heating and cooling during the complete time range of the accelerated stability study. Therefore, to eliminate the possibility of adding another variable to the experiment, the product was discarded once used for testing.

3.6 Determination of Testing Frequency

The following table illustrates the testing time intervals that were used in the accelerated test.

Table 1: Testing Scheme for the Accelerated Stability Test

Testing Device	Temperature Stress Levels	Number of Units per Lot located at Stress Levels	Testing frequency
Reagent kits from Lot #1, Lot #2 and Lot #3	45°C	12 units	Every 2 days
	37°C	12 units	Every 6 days
	30°C	12 units	Every 10 days
	17°C	10 units	Every 30 days

The testing frequency in Table 1 was established based on recommendations from Abbott scientists and the literature review on previous Monte Carlo simulation studies on accelerated degradation test for biological standards [3, 4]. In addition, units exposed to the lowest stress temperature (17°C) were tested beyond the indicated shelf-life of the product (180 days) to verify if the products continues to degrade slowly without failure until it converges to a constant value. This would indicate that the actual shelf-life of the

product could be extended to a value inside the time range covered by the accelerated stability study, where the product demonstrated to provide acceptable results.

3.7 Model Building and Estimation of Parameters

As the complete data was obtained from the accelerated stability study, it was used to develop several mathematical models that would best fit all the data gathered. The models used included the Arrhenius classical approach, which involves a two-step sequential procedure of linear regressions, as well as several nonlinear models. The reason of using nonlinear models instead of the classical approach is because we were interested in finding a one-step procedure that could relate *product content*, *time* and *temperature* in only one equation by means of a nonlinear mixed-effects approach. Two softwares (S-Plus & Table Cure 3-D) were initially selected for the development of nonlinear models but after comparing software efficiency in the estimation of models parameters, Table Curve 3-D was the software selected because it was faster than S-Plus. These approaches will be discussed in detail in Chapter 4.

3.8 Statistical Analyses and Test of Underlying Assumptions

Statistical tests are performed to assess the significance of the estimated parameters in the model. An *F* test is applied to validate the significance of the regression coefficients at each level of the signal factor. The procedure is used to test the following hypothesis:

$$H_0 : \beta_1 = \beta_2 = ... = \beta_k = 0$$
$$H_1 : \beta_j \neq 0 \quad \text{for at least one } j$$

The statistical test is

$$F_0 = \frac{MSR}{MSE} \tag{28}$$

In this expression, MSE is the mean squares due to the residuals and MSR is the mean squares due to the regression. H_0 is rejected if $F_0 > F_{\alpha,p-1,n-p}$. Rejection of the null hypothesis indicates that at least one of the regressor variables contribute significantly in the model.

Once it is determined that at least one of the regressors is important to the model, then the coefficients of the regression are examined to identified which ones contribute to

the model. This verification is important since the coefficients of the regression are associated with a factor or the combination of factors in the experiment. The statistic *t* test is used to perform this verification. This statistics is given by:

$$t_0 = \frac{\hat{\beta}_j}{\sqrt{MSE \cdot C_{jj}}} \tag{29}$$

where C_{jj} is the diagonal element of $(\mathbf{X'X})^{-1}$ corresponding to $\hat{\beta}_j$. The null hypothesis is rejected if $|t_0| > t_{\alpha/2,n-k-1}$

Other important statistics used to assess the overall adequacy of the model are the coefficient of determination (R^2) and the adjusted R^2. The coefficient of determination is a measure of the total variability of the data explained by the model. This statistic is represented by the following expression:

$$R^2 = \frac{SSR}{SST} = 1 - \frac{SSE}{SST} \tag{30}$$

Since there could be misleading conclusions when using R^2 for the reason that this statistic always increases as more regressor variables are included in the model, then, the adjusted R^2 it is preferred. This statistic is defined as follows:

$$R^2_{Adj} = 1 - \frac{SSE/(n-p)}{SST/(n-1)} \tag{31}$$

It is important to clarify that exact statistical test based on the *t* and *F* distributions are always obtainable in linear regression when the errors are normally and independently distributed. Nevertheless, this is not case for nonlinear regression, even when the errors are normally and independently distributed, for the reason that nonlinear least squares (or maximum likelihood) estimates of the models do not necessary provide unbiased-ness and minimum variances like in linear regression models. Adequacy of statistical estimates in nonlinear regression depends on **large-sample** or **asymptotic** results [24]. The key asymptotic results can be summarized as follows; when the sample size of the experimental results is large, the expected value of $\hat{\theta}$ is approximately equal to the true vector of parameters θ. The covariance matrix of $\hat{\theta}$ is approximately $\sigma^2(\mathbf{Z'Z})^{-1}$, where $\mathbf{Z}$ is the matrix of partial derivates evaluated at the final iteration least square estimate $\hat{\theta}$. Therefore, statistical inference for nonlinear regression when the sample size is large is

similar to inferences for linear regression. In our study, we have **1,233** observations to estimate the vector parameters $\hat{\theta}$, consequently it is reasonable to assume that large-sample results can be applied.

In addition to the previous statistical tests discussed, other tests are performed to verify the assumptions of the Arrhenius theory since there is no absolute guarantee that degradation follows a first order kinetic reaction and that degradation rates obey the Arrhenius equation. The first assumption is verified by testing for linearity in the ln(*k*) versus T^{-1} plot that was done in the model developed with the Arrhenius classical approach. The second assumption is verified by testing the goodness of fit of the data to the Arrhenius model.

3.9 Validation of Model Selected

Once a statistical model to estimate degradation by an accelerated stability test was selected, it was validated using the following procedure.

3.9.1 Identifying a lot without stability issues:

Prediction intervals were determined for some specific times and temperatures considered in the accelerated stability test, after selecting the model that most adequate fitted the data from the three lots used for stability testing based on relevant statistics and compliance with underlying assumptions previously discussed. The types of statistical intervals determined are prediction intervals to contain **all** of *m* individual future observations. These intervals are different from the usual intervals given by statistical softwares, which contain the mean of future observations.

Additional accelerated stability test points were obtained to validate if the statistical model developed with the accelerated stability data, could be used to discriminate a good lot. The lot used to gather additional accelerated stability test points was a new approved lot from market different from the three previous lots used to create the model. Reagent kits of this new lot were exposed to the same temperatures used with the previous three lots (45, 37, 30 and 17 °C) to accelerate degradation and test at some of the time intervals that were considered for the previous lots. If all the replicates of the new lot are within the prediction intervals

calculated from the most adequate model that fitted the data from the initial three lots, then it can be concluded that this new lot has similar degradation patterns (at the four elevated temperatures) as the previous three good lots used in the developmental phase of this study. Thus, it is expected that it will degrade similarly at the storage temperature until the expiration date.

3.9.2 Identifying a lot with stability issues:

Using the same approach presented in Section 3.9.1, it was also desirable to verify if the prediction intervals developed with the model that best fitted the accelerated stability data of the initial three lots, were going to be capable of discriminating a lot with a bad stability performance. Therefore, reagent kits of a bad performance lot were also exposed to the same stress temperatures experimented with the three good lots of the initial accelerated study, and also tested at some time intervals previously considered. Assessing the prediction intervals of the temperatures and time intervals considered, it was expected to observe some replicates out of the prediction intervals calculated from the most adequate model that fitted the data from the initial three lots. This would demonstrate that the lot does not exhibit the same degradation patterns at elevated temperatures that were characterized with the previous three good lots used in the developmental phase of the accelerated stability study. Therefore, this lot would be expected to behave unstable and with high probability of being out of the specification limits much earlier than its expiration date.

4 Accelerated Stability Models

4.1 Introduction

The shelf-life of a product is usually determined based on primary stability data that it is obtained from long-term stability studies under approved stability protocols with ambient storage conditions. However, these types of studies usually take too much time to complete since products under ambient storage conditions degrade very slowly and it make take more than one year to observe significant degradation. Accelerated stability tests have been recently applied to provide stress conditions that could increase the rate of chemical or physical degradation of the product so that the kinetic parameters of the reaction rate could be estimated, and then to estimate the shelf life under normal storage condition of the product by extrapolation.

The use of accelerated models to monitor the stability of a product and to determine its shelf life has been widely applied and documented in the manufacture of electronics and pharmaceutical products, but it has not been applied and documented for biological products in the diagnostic business, specifically for an in-vitro diagnostic product like a reagent kit. Taking this need in consideration, this chapter will focus in the development of mathematical models that could be used to fit the data obtained from the accelerated stability test that was designed and presented in Chapter 3 for an in-vitro diagnostic reagent kit.

Four (4) models were developed and evaluated based on statistical analyzes and test of underlying assumptions previously discussed in Chapters 2 and 3. The first model built was the Arrhenius Classical Approach model, which is based on a two-step sequential procedure of linear regressions. After the Arrhenius Classical Approach was applied, it was decided to find a way of fitting all the data (1,233 values) by relating *product content*, *time* and *temperature* in only one equation by means of nonlinear models which include a polynomial function model as well as nonlinear approach of the Arrhenius equations for a zero and first order degradation. These models were compared on advantages and disadvantages to facilitate the decision of selecting the most adequate model to be used for validation procedures.

4.2 Arrhenius Classical Approach Model

The Arrhenius equation is often used in pharmaceutical industries when performing accelerated stability test procedures since it provides a mechanism to relate degradation reaction rates of the product and temperature for estimating product shelf-life. The first model assessed to fit the data from the accelerated stability study was the Arrhenius model with the classical approach. This approach consists on performing two sequential steps of linear regressions. In the first step, it is required to select a proper order (zero, first or second) for the degradation reactions that best describes the behavior of the data by relating the product content (*rate values*) versus *time* at several elevated temperatures. Then the rate constant (k) at all elevated temperatures is determined from the equations of the degradation order fitted. In the second step, the mean rate constants (k) are used to apply linear regression using the modified Arrhenius equation previously presented in Equation 2, which relates ln k versus T^{-1}. This regression is then used to predict (extrapolate) the rate constant k at the usage condition of the product.

4.2.1 Results for the Arrhenius Classical Approach

A first order reaction was used to relate the product content (rate values) versus time for the four elevated temperatures considered in the accelerated stability study performed. Three response variables (control low, medium and high) of product content were used to monitor the degradation of the reagent kits for the three lots used in the experiment. The reason to use these three response variables is that they are the actual components that are evaluated against customer specifications when a reagent kit is used inside an AxSYM laboratory instrument to determine the rate and concentration of the analyte[3] in a patient sample through the use of a calibration curve as described in Section 2.2.4. If control levels are within specifications, then it is concluded that the calibration curve is good to monitor patient samples. Figures 7, 8 and 9 illustrate the execution of the first step of the Arrhenius classical approach for the three response variables considered. As it can be observed in these figures, the application of a first order reaction is well fitted for the highest temperatures but fitting adequacy is lost as the temperature is near the normal or storage condition of the product (8°C or 281°K).

[3] An analyte is anything measured by a laboratory test. It can be either an antibody or an antigen.

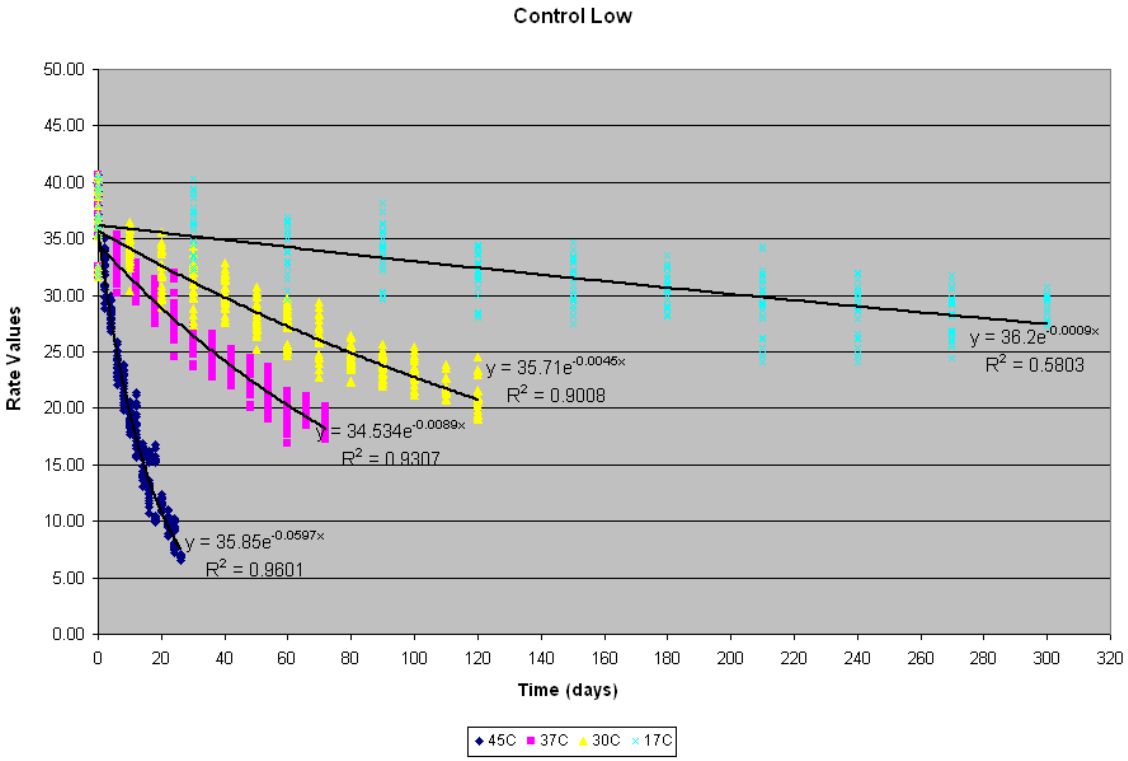


Figure 7: First Order Degradation Curves for Control Low

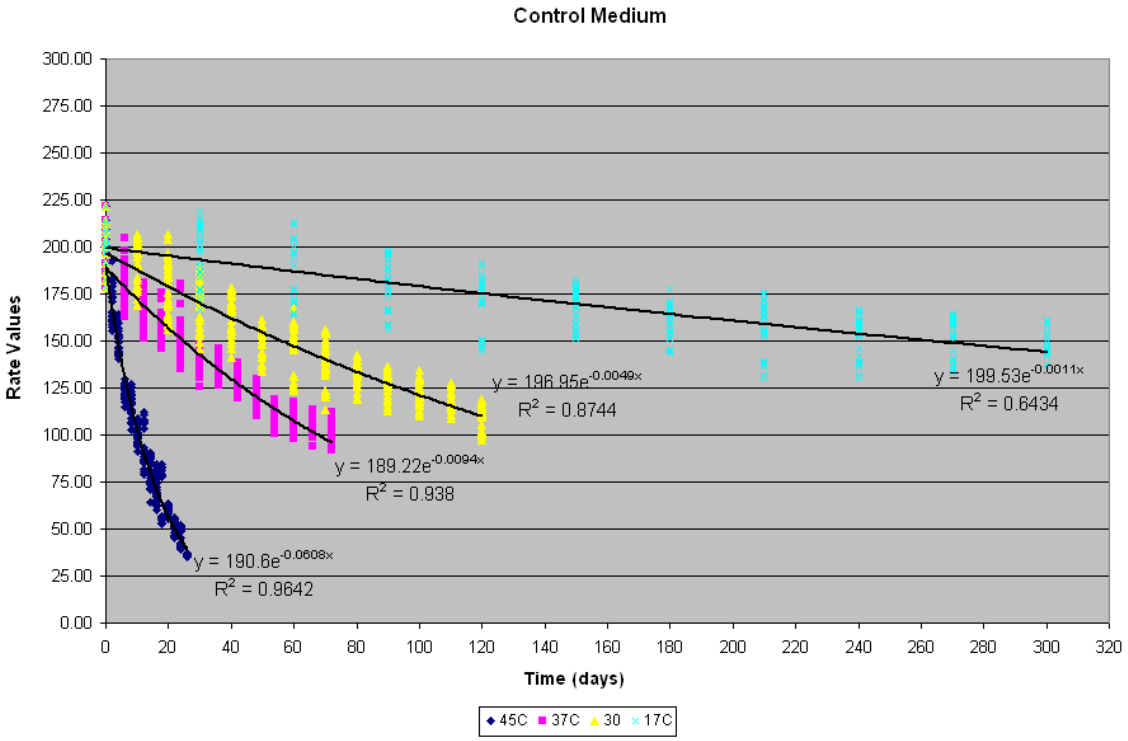


Figure 8: First Order Degradation Curves for Control Medium

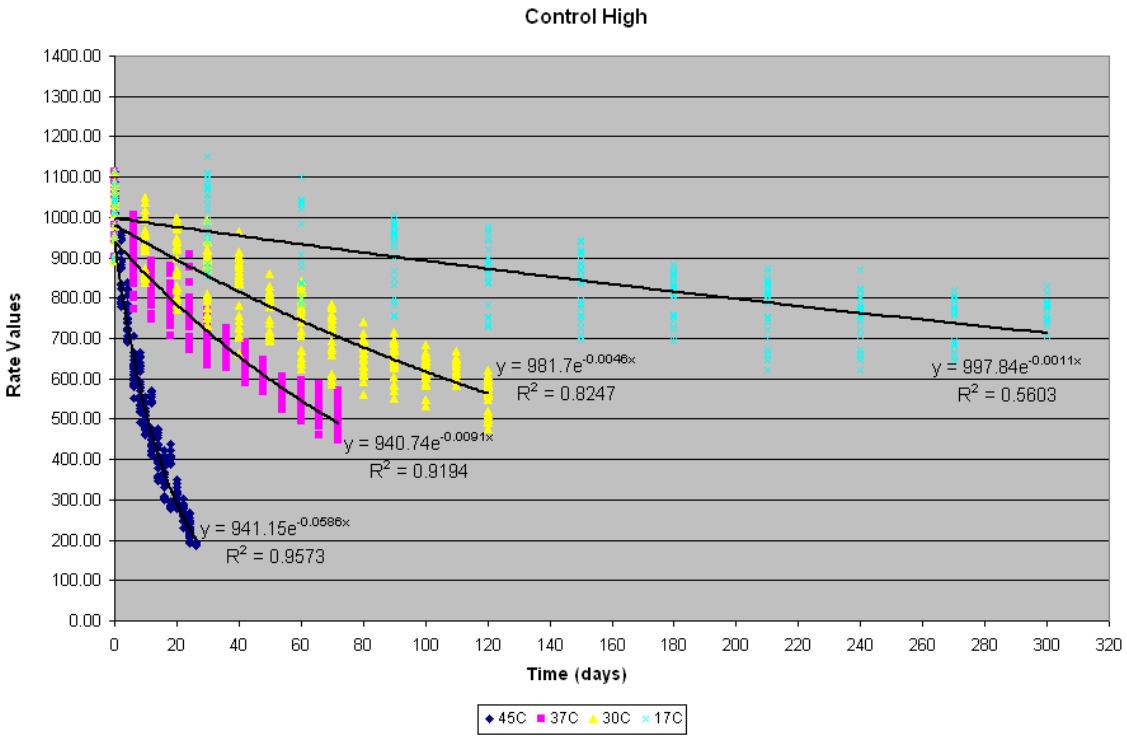


Figure 9: First Order Degradation Curves for Control High

The second step of the Arrhenius classical approach was then applied using the mean rate constants obtained in Figures 7 to 9. The following tables contain the parameters used to apply linear regression using the modified Arrhenius equation previously presented in equation (4), which relates ln k versus T^{-1}.

Table 2: Control Low Parameters for the Arrhenius Plot

Control Low				
T (°C)	**Mean rate constant (*k*)**	**ln *k***	**T (°K)**	**1/T (°K)**
45	0.0597	-2.8184233	318	0.0031
37	0.0089	-4.721704	310	0.0032
30	0.0045	-5.4036779	303	0.0033
17	0.0009	-7.0131158	290	0.0034

Table 3: Control Medium Parameters for the Arrhenius Plot

Control Medium				
T (°C)	**Mean rate constant (*k*)**	**ln *k***	**T (°K)**	**1/T (°K)**
45	0.0608	-2.8001655	318	0.0031
37	0.0094	-4.6670456	310	0.0032
30	0.0049	-5.3185201	303	0.0033
17	0.0011	-6.8124451	290	0.0034

Table 4: Control High Parameters for the Arrhenius Plot

Control High				
T (°C)	**Mean rate constant (*k*)**	**ln *k***	**T (°K)**	**1/T (°K)**
45	0.0586	-2.8370206	318	0.0031
37	0.0091	-4.6994809	310	0.0032
30	0.0046	-5.381699	303	0.0033
17	0.0011	-6.8124451	290	0.0034

Figures 10 to 12 illustrate the Arrhenius Plots that are obtained for each of the response variables (Control Low, Medium and High) considered in the study as a result of fitting a linear model to describe the relationship between ln *k* and 1/T.

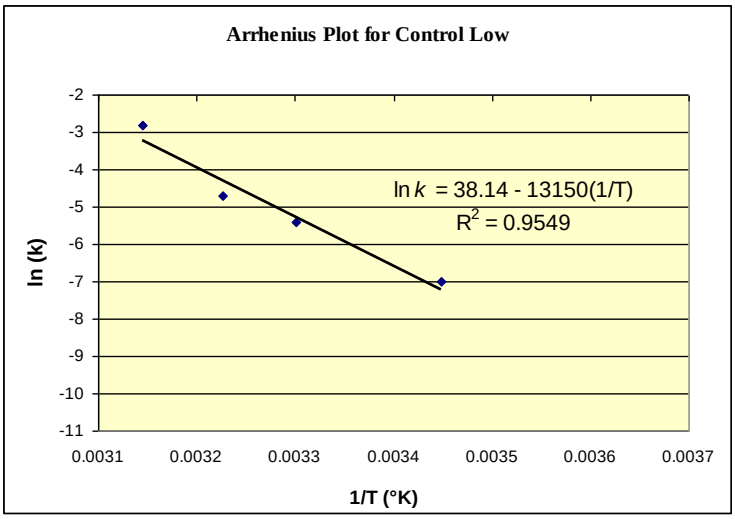


Figure 10: Classical Arrhenius Plot for Control Low

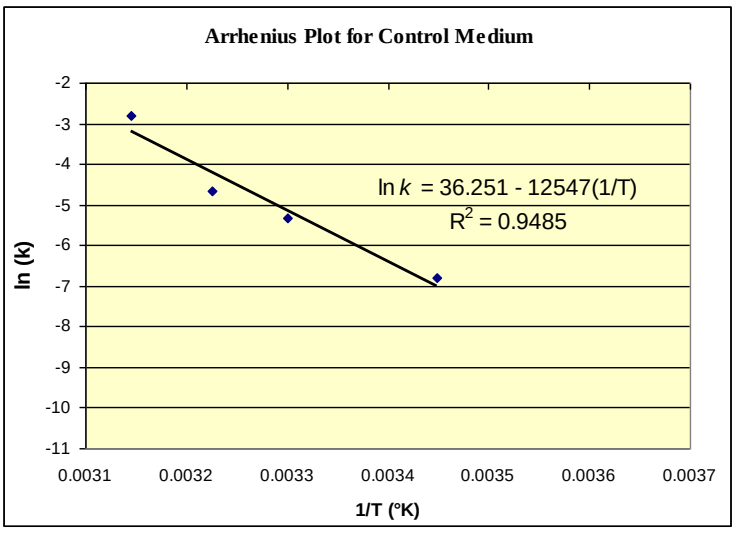


Figure 11: Classical Arrhenius Plot for Control Medium

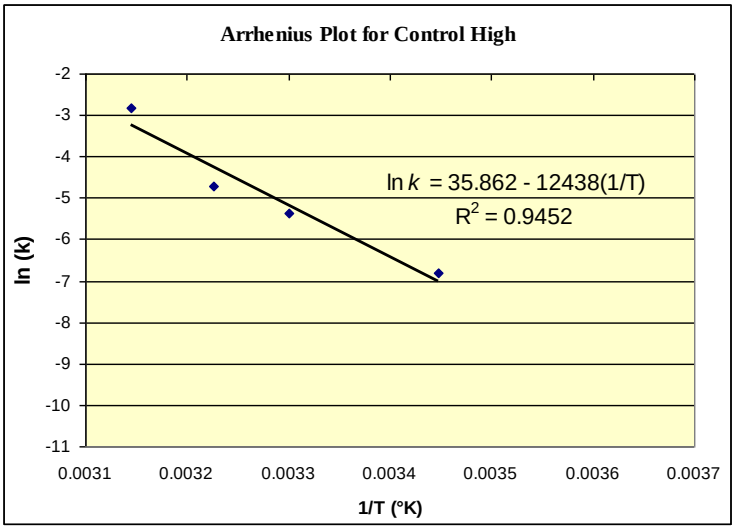


Figure 12: Classical Arrhenius Plot for Control High

Based on these plots, it is established that the equations of the fitted models for each of the response variables are the following:

$$\textit{Control Low}: \quad \ln k = 38.14 - 13150\,(1/T) \tag{32}$$

$$\textit{Control Medium}: \quad \ln k = 36.251 - 12547\,(1/T) \tag{33}$$

$$\textit{Control High}: \quad \ln k = 35.862 - 12438\,(1/T) \tag{34}$$

It can be noticed that the Arrhenius constant A and the activation energy of the reaction E_a can be easily determined from the equations that were fitted. In these equations, the intercept represents the value $\mathbf{ln}\ \boldsymbol{A}$ and the slope of the regressor variable $1/T$, represents the relation $\boldsymbol{E_a/R}$ of the Arrhenius equation. Therefore, using the gas constant (R) value of 0.00199 (kcal mol^{-1} K^{-1}), then, we can determine the activation energy of the reaction (E_a). Table 5 provides a summary of the Arrhenius equation parameters that were obtained for each of the response variables (control levels) considered in the study.

Table 5: Arrhenius Equation Parameters for the Classical Approach

Control Level	$\ln A$	A	E_a/R	E_a
Low	38.140	3.6627E+16	13150	26.168
Medium	36.251	5.5412E+15	12547	24.968
High	35.862	3.7538E+15	12438	24.752

In addition, once the Arrhenius Plot has been developed, the equation that describes this plot can be then used to predict (extrapolate) the mean rate constant (k) at the storage condition (8°C or 281°K), and consequently the rate value of each control level at

expiration day. This prediction is determined by using the same equation of reaction order previously used when applying the Arrhenius classical approach. In this case, Equation 18 was used since a first-order reaction was selected to describe the degradation rate of each control level used in the study. Table 6 contains the predicted rate value of each control level at the expiration date of the product.

Table 6: Prediction of Controls Rate Values using the Arrhenius Classical Approach

Control Level	Mean rate value at t=0	Predicted mean rate constant (k) at 281°K	Expiration Date (days)	Predicted rate value at Exp. Date	95% Prediction Interval
Low	36.75	0.00017	180	35.62	16.31 - 36.71
Medium	201.38	0.00022	180	193.39	64.80 - 201.09
High	1007.60	0.00022	180	967.72	447.05 - 1006.39

Predictions in Table 6 appeared to be a little suspicious since it is previously known by Abbott scientists that the product content loss, measured as a percent difference of rate values between t=180 and t=0, must be between 10 and 15 percent for the specific product that was used in the accelerated stability test. In contrast, the predicted results obtained with the Arrhenius classical approach appear to indicate that the product had a lag of degradation in 180 days since after calculating a percent difference of rate values between t=180 and t=0, only a 3.08%, 3.97% and 3.96% difference is obtained for control low, medium and high, respectively. The 15% limit was establish as a mode of control to assure the product to be within the rate specifications of each control level during the time is in the customers hands since it is known that the product can degrade a little more than this value and maintain its functionality. Table 7 contains the stability specifications in rate values of the product as measured by each control level.

Table 7: Stability Specifications in Rate Values for each Control Level

Control Level	Specifications
Low	26.52 – 40.33
Medium	140.85 – 219.17
High	692.44 – 1150.94

Is important to highlight that the predictions that were done at the storage condition (8°C or 281°K) can be considered of less value since the 95% prediction intervals obtained are significantly wide, and at the same time, they are unsymmetrical to the mean predicted value. This is one of the big problems that the Arrhenius classical approach has, which has been documented in the literature. In addition, when comparing the results of the prediction intervals with the specifications in Table 7, it can be observed

that the lower limits of the prediction intervals are outside of the product specifications. Therefore, this is something that is not appropriate for extrapolation purposes since it indicates that Arrhenius model with the classical approach is capable of predicting rate values outside the specification limits, even when it was previously known that the lots used in the study behave inside the product specification until their expiration date. This is a major disadvantage provided by the model.

4.2.2 Statistical Analyzes and Test of Underlying Assumptions

As indicated previously, statistical tests are performed to provide significance of the estimated parameters in the model. The following figures contain the output of the statistical tests done in for the Arrhenius classical approach models that were fitted to the accelerated stability data of each control level. These statistical test outputs were obtained using STATGRAPHICS Plus software, version 4.1.

```
Regression Analysis - Linear model: Y = a + b*X
------------------------------------------------------------------------------
Dependent variable: ln_k
Independent variable: 1/T
------------------------------------------------------------------------------
                             Standard          T
Parameter       Estimate         Error       Statistic        P-Value
------------------------------------------------------------------------------
Intercept        38.1396        6.63223        5.75064         0.0289
Slope           -13150.0        2020.99       -6.50669         0.0228
------------------------------------------------------------------------------


                          Analysis of Variance
------------------------------------------------------------------------------
Source              Sum of Squares     Df  Mean Square    F-Ratio      P-Value
------------------------------------------------------------------------------
Model                     8.64353      1      8.64353      42.34       0.0228
Residual                  0.40832      2      0.20416
------------------------------------------------------------------------------
Total (Corr.)             9.05185      3

Correlation Coefficient = -0.977185
R-squared = 95.4891 percent
R-squared Adj. = 93.2336 percent
Standard Error of Est. = 0.451841
```

Figure 13: Statistical Analysis for the Classical Arrhenius Model fitted to Control Low Data

Regression Analysis - Linear model: Y = a + b*X

Dependent variable: ln_k
Independent variable: 1/T

Parameter	Estimate	Standard Error	T Statistic	P-Value
Intercept	36.251	6.78538	5.34252	0.0333
Slope	-12546.8	2067.66	-6.06811	0.0261

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	7.86878	1	7.86878	36.82	0.0261
Residual	0.427396	2	0.213698		
Total (Corr.)	8.29618	3			

Correlation Coefficient = -0.973901
R-squared = 94.8483 percent
R-squared Adj. = 92.2724 percent
Standard Error of Est. = 0.462275

Figure 14: Statistical Analysis for the Classical Arrhenius Model fitted to Control Medium Data

Regression Analysis - Linear model: Y = a + b*X

Dependent variable: ln_k
Independent variable: 1/T

Parameter	Estimate	Standard Error	T Statistic	P-Value
Intercept	35.8615	6.94862	5.16096	0.0356
Slope	-12438.1	2117.4	-5.87425	0.0278

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	7.7331	1	7.7331	34.51	0.0278
Residual	0.448208	2	0.224104		
Total (Corr.)	8.18131	3			

Correlation Coefficient = -0.972222
R-squared = 94.5216 percent
R-squared Adj. = 91.7823 percent
Standard Error of Est. = 0.473396

Figure 15: Statistical Analysis for the Classical Arrhenius Model fitted to Control High Data

As it can be observed in Figures 13 to 15, the statistical analyses of the fitted linear regressions indicate that all models provide statistically significant relationships between ln *k* and 1/T at the 95% confidence level since the *p-value* in the ANOVA table is less than 0.05. In addition, when verifying significance of the regression coefficients verified in order to identify which ones are making a genuine contribution to the model, it is concluded that all coefficients are significant to the model since the results of the statistic *t* test indicate that *p-values* are less than 0.05.

Another important factor used to assess the overall adequacy of the model is the adjusted R^2. As it can be observed in Figures 13 to 15, the R^2_{Adj} statistic indicates that the variability in the response variable (ln *k*) is very well explained by the models fitted since the lowest value obtained for the three models was a R^2_{Adj} value equal to 91.78%. Model adequacy is also demonstrated by the examination of the results for the correlation coefficient (*r*) since all values obtained are very close to a value of 1.0, which indicates that there is a very strong relationship between the variables used in the models.

In addition to the previous statistical tests discussed, this project pursued a verification of compliance of the assumptions established by the Arrhenius Life-Temperature Law by using the data obtained from the accelerated stability test (refer to Section 3.8). This verification will guarantee that degradation follows a first order kinetic reaction and that degradation rates obey the Arrhenius equation. The first assumption is verified by testing for linearity in the ln (*k*) versus T^{-1} plot that was done for the Arrhenius model with classical approach. Compliance with this assumption is clearly demonstrated by examining the correlation coefficient (*r*) values of the Arrhenius plots, since all results of the *r* statistic are very close to 1.0, which indicate that there is a strong linear relationship between $\ln(k)$ and T^{-1}. The second assumption is verified by testing the goodness of fit of the data to the Arrhenius equation. Compliance to this assumption is provided by the examination of results for the R^2_{Adj} statistic since as previously mentioned, all models demonstrated to fit well the accelerated data. Therefore, it is concluded that all models fitted by the Arrhenius classical approach demonstrated to be in compliance with both assumptions of the Arrhenius theory.

4.2.3 Advantages and Disadvantages of the Arrhenius Classical Approach Model

With the purpose of illustrating the relevance and limitations of the regression models developed to fit the accelerated stability data, all models presented in chapter will be assessed in terms of their relative merits and disadvantages. In the case of the Arrhenius model with the classical approach, the advantage that the methodology has is that this approach has been used many times in the past. Therefore, a lot of literature and case studies can be found, which can significantly help the user to understand the chemical kinetics theory in which the procedure is based and the principal requirements that surrounds the application of the Arrhenius equation. In addition, this approach tends to be more attractive to people with little knowledge on mathematical methods since it is applied using simple linear regressions, which can be performed using a scientific calculator or an Excel spreadsheet.

In contrast, for the more skilled mathematical person, the Arrhenius classical approach has several disadvantages that make the procedure not so attractive. These drawbacks are summarized in the following bullets:

- Since this approach uses the mean rate constants (k) of each elevated temperature used in the study, the prediction of the final model is also a mean rate estimate of the shelf-life. Therefore, the total variability contributed by the individual observations of the accelerated study is totally lost in the final model.
  - If someone would like to use this model for evaluating m future observations, then, prediction intervals would not be possible to be determined since the model was developed to evaluate the mean rate constant (k) and not individual observations as they are naturally obtained from the study.
- The methodology cannot use the data in the natural form at it is gathered to relate product degradation, time and temperature in only one equation and to provide a direct estimation of the shelf-life with relevant statistics.
  - The two-step procedure of the classical approach was feasible in the past when there was absence of computer programs capable of solving complex mathematical problems with large data sets in minimal time.

- The approach provides wide and unsymmetrical statistical intervals as illustrated in Table 6 since the fitting procedure distorts the error structure of the data and degrees of freedom are severely reduced in the final model.

4.3 Polynomial Function Model

Based on the disadvantages of the Arrhenius classical approach mentioned before, it was decided to find a way of fitting the complete set of data (1,233 values) obtained from the accelerated stability study by relating *product content* (rate values of each control level), *time* and *temperature* in only one equation. Therefore, the mathematical expression of the model developed would be as follows:

$$Z = f(X,Y) + \varepsilon \tag{35}$$

where

X = independent variable related to time (in days)

Y = independent variable related to temperature (in Kelvin units)

Z = dependent variable related to the rate values on each control level

ε = experimental error

Three-dimensional scatter plots of the XYZ accelerated stability data to be fitted are presented in Figures 16 to 18.

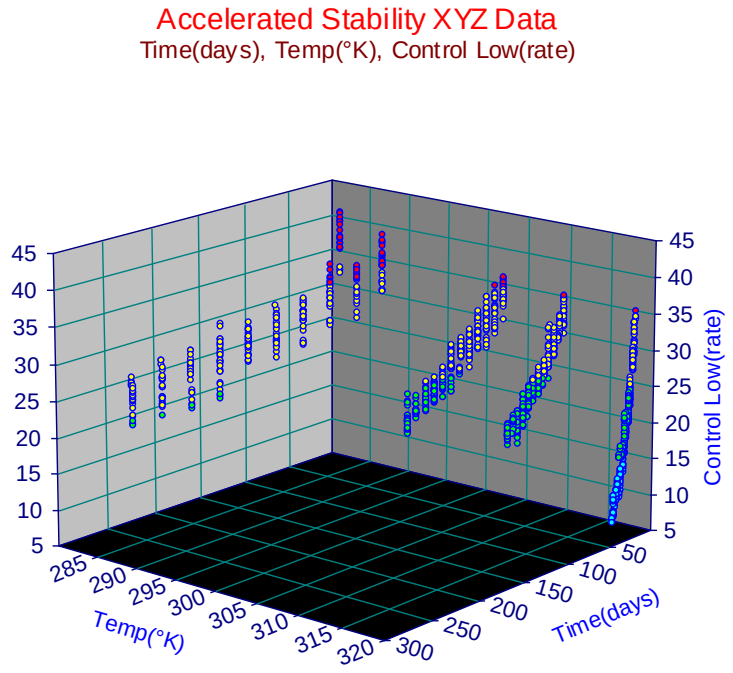


Figure 16: Accelerated Stability Data Scatter Plot for Control Low

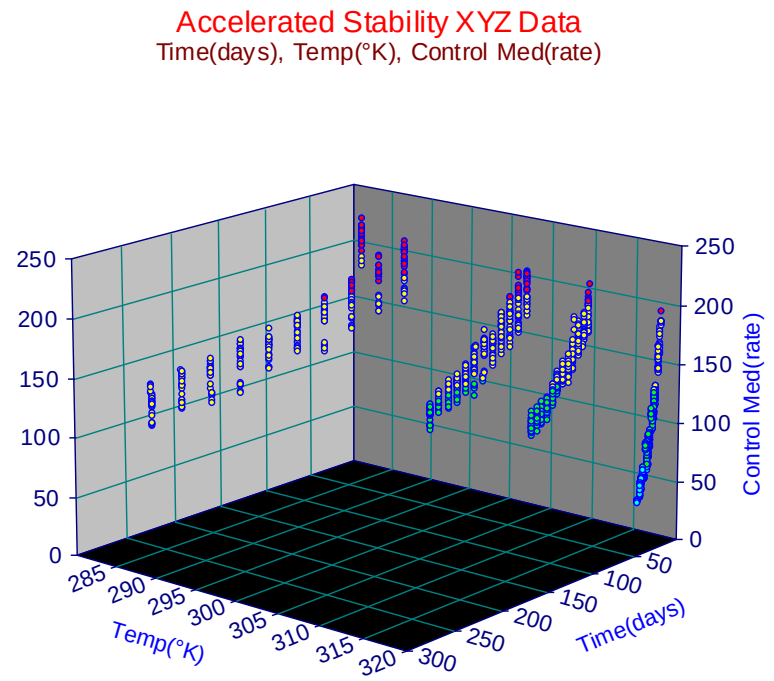


Figure 17: Accelerated Stability Data Scatter Plot for Control Medium

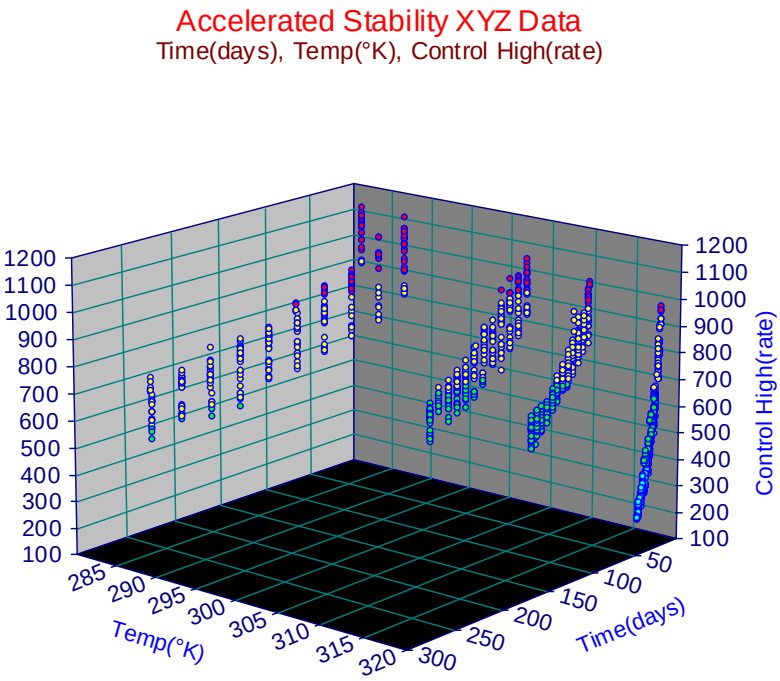


Figure 18: Accelerated Stability Data Scatter Plot for Control High

Table Curve 3D was the software selected to provide a one step approach that would relate all variables considered in the accelerated stability study since it provides thousands of built-in equations to describe empirical data, including a wide array of linear and nonlinear models, as well as user defined functions that can be manually entered by the user. Using the software, 349 built-in equations were fitted for the stability data of each control level but only equations that provided R^2 values higher than 90% were reported. Appendix 2 contains the list of equations fitted and reported by the software for each control level. Since the goal was to provide a model that would best fit all the data gathered in the study, the *adjusted* R^2 was the first statistical criteria used to sort and select the best built-in equation provided by the software. Then, the lowest fitted Standard Error was also used as second criteria for selection.

4.3.1 Results for the Polynomial Function Model

From the list of equations provided by Table Curve 3D, a polynomial function was selected as the best model that fitted the data based since it provided the highest R^2_{Adj} value and smallest fitted Standard Error. Figures 19 to 21 illustrate the surface plots that were obtained for each of the response variables (Control Low, Medium and High) considered in the study as a result of fitting a polynomial function model to describe the relationship between *product content* (rate values of each control level), *time* and *temperature* in a single equation. Based on these surface plots, it is established that the equations of the polynomial models fitted to each response variables are the following:

- **For Control Low** (36)

$$Z = 25471081 - 58549.927\ln X - 13358880\ln Y + 48.2539(\ln X)^2 + 2335395(\ln Y)^2 + 20573.586\ln X\ln Y - 0.0011025523(\ln X)^3 - 136087.45(\ln Y)^3 - 1807.111\ln x(\ln Y)^2 - 8.6929107(\ln X)^2\ln Y$$

- **For Control Medium** (37)

$$Z = 129253300 - 247129.25\ln X - 67828997\ln Y + 123.68613(\ln X)^2 + 11864697(\ln Y)^2 + 87054.674\ln X\ln Y - 0.0086169269(\ln X)^3 - 691774.94(\ln Y)^3 - 7665.3904\ln x(\ln Y)^2 - 22.985864(\ln X)^2\ln Y$$

- **For Control High** (38)

$$Z = 6327724400 - 1304090\ln X - 3.32010200\ln Y + 459.60233(\ln X)^2 + 58066122(\ln Y)^2 + 459273.56\ln X\ln Y - 0.045605305(\ln X)^3 - 3385019.5(\ln Y)^3 - 40430.758\ln x(\ln Y)^2 - 87.07018(\ln X)^2\ln Y$$

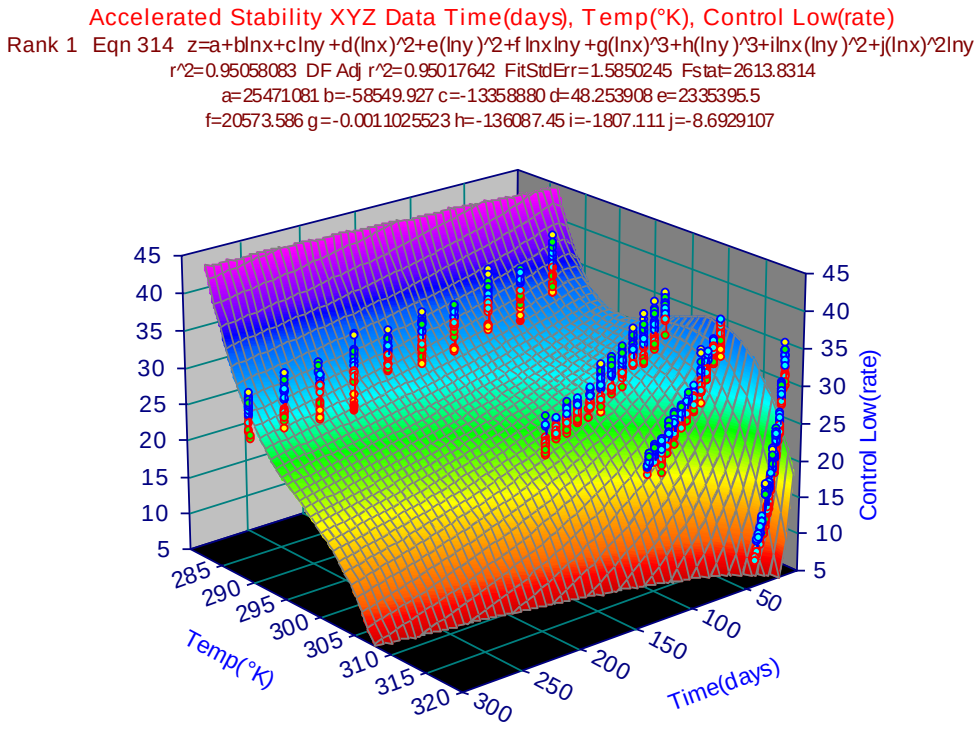


Figure 19: Surface Plot of Polynomial Function Model for Control Low

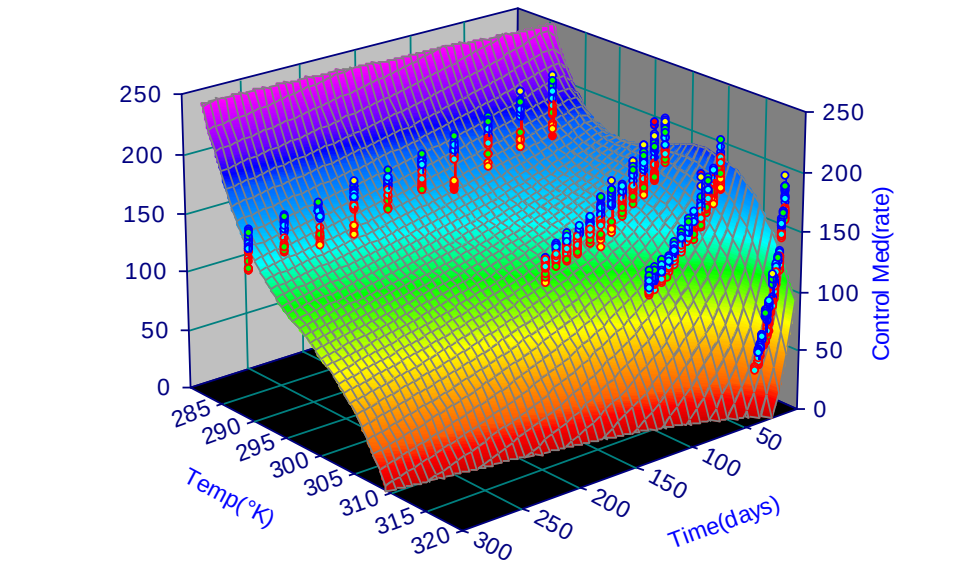


Figure 20: Surface Plot of Polynomial Function Model for Control Medium

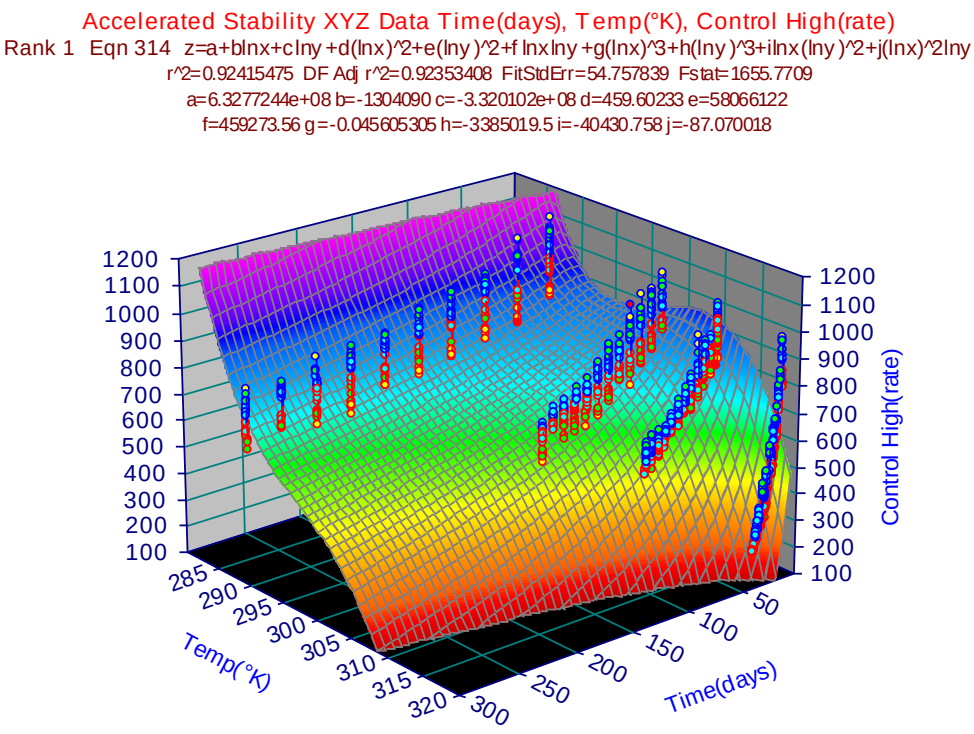


Figure 21: Surface Plot of Polynomial Function Model for Control High

The equations that describe these plots can be then used to predict (extrapolate) the rate value of each control level at the expiration date and at the storage temperature (8°C or 281°K) of the product. Table 8 contains the results of the predicted rate value of each control level at the expiration date.

Table 8: Prediction of Controls Rate Values using the Polynomial Function Model

Control Level	Mean rate value at t=0	Storage Temp. (°K)	Expiration Date (days)	Predicted rate value at Exp. Date	95% Prediction Interval
Low	36.75	281	180	61.44	57.62 – 65.26
Medium	201.38	281	180	327.20	304.63 – 349.77
High	1007.60	281	180	1588.64	1456.71 – 1720.57

Examining the results obtained from the extrapolation it is observed that polynomial function model does not provide reliable prediction results even though the adjusted R^2 statistics indicates that each model fits very well the accelerated stability data. Results should demonstrate that the product suffers some kind of degradation from the date the accelerated stability study started (t_0) until the expiration date (t_{exp}) of the product. Therefore, the product content (rate values) of each control level at time t_{exp} should be

lower than the product content at t_0. Based on this, we can establish that the actual predicted results provided by the polynomial function model at lower temperatures are incongruent with the chemical-physical behavior that is expected. The reason for this incongruence behavior will be further discussed in details in Section 4.3.3

4.3.2 Statistical Analyzes for the Polynomial Function Model

Statistical tests were used similarly as it was applied for the Arrhenius classical approach model to verify statistical significance of the polynomial function model that was fitted to the accelerated stability data. The following figures contain the output of the statistical tests done for each response variable. These statistical test outputs were obtained from the output menu of Table Curve 3D software, version 4.0

Regression Analysis

Rank 1 $z = a+blnx+clny+d(lnx)^2+e(lny)^2+flnxlny+g(lnx)^3+h(lny)^3+ilnx(lny)^2+j(lnx)^2lny$

x = time y = temp

Parameter	Value	Std Error	t-value	95.00% Confidence Limits		P>|t|
a	2.54711e+07	888258.3818	28.67530666	2.37284e+07	2.72138e+07	0.00000
b	-58549.9268	5488.478244	-10.6677888	-69317.8029	-47782.0507	0.00000
c	-1.3359e+07	463795.0459	-28.8034123	-1.4269e+07	-1.2449e+07	0.00000
d	48.25390833	15.17418012	3.180001024	18.48359955	78.0242171	0.00151
e	2.3354e+06	80723.4915	28.930804	2.17702e+06	2.49377e+06	0.00000
f	20573.58645	1903.621696	10.80760242	16838.8604	24308.31249	0.00000
g	-0.00110255	0.000360836	-3.05554563	-0.00181048	-0.00039463	0.00230
h	-136087.448	4683.395605	-29.0574317	-145275.828	-126899.068	0.00000
i	-1807.11098	165.0566668	-10.9484277	-2130.93658	-1483.28538	0.00000
j	-8.69291068	2.650236212	-3.28005128	-13.8924239	-3.49339744	0.00107

Procedure
GaussElim

r^2 Coef Det	DF Adj r^2	Fit Std Err
0.9505808325	0.9501764203	1.5850245261

Analysis of Variance

Source	Sum of Squares	DF	Mean Square	F Statistic	P>F
Regr	59100.623	9	6566.7359	2613.83	0.00000
Error	3072.5463	1223	2.5123027		
Total	62173.169	1232			
Lack Fit	191.14261	38	5.0300688	2.06866	0.00017
Pure Err	2881.4036	1185	2.4315643		

Description: Accelerated Stability XYZ Data: Time(days), Temp(°K), Control Low(rate)

Figure 22: Statistical Analysis for the Polynomial Model fitted to Control Low Data

Regression Analysis

Rank 1 $z = a+blnx+clny+d(lnx)^2+e(lny)^2+flnxlny+g(lnx)^3+h(lny)^3+ilnx(lny)^2+j(lnx)^2lny$

x = time y = temp

Parameter	*Value*	*Std Error*	*t-value*	*95.00% Confidence Limits*		*P>|t|*
a	1.29253e+08	5.25013e+06	24.61904658	1.18953e+08	1.39554e+08	0.00000
b	-247129.25	32440.16481	-7.61800229	-310773.79	-183484.709	0.00000
c	-6.7829e+07	2.7413e+06	-24.7433323	-7.3207e+07	-6.2451e+07	0.00000
d	123.6861281	89.68841309	1.37906474	-52.2740707	299.6463269	0.16813
e	1.18647e+07	477123.7585	24.86712619	1.09286e+07	1.28008e+07	0.00000
f	87054.67407	11251.53436	7.737138	64980.22593	109129.1222	0.00000
g	-0.00861693	0.002132758	-4.04027451	-0.0128012	-0.00443266	0.00006
h	-691774.937	27681.64845	-24.9903808	-746083.718	-637466.156	0.00000
i	-7665.39039	975.5828912	-7.85724151	-9579.39192	-5751.38887	0.00000
j	-22.9858639	15.6644694	-1.4673886	-53.7180739	7.746346162	0.14253

Procedure
GaussElim

r^2 Coef Det	*DF Adj r^2*	*Fit Std Err*
0.9452677795	0.9448198889	9.3684359427

Analysis of Variance

Source	*Sum of Squares*	*DF*	*Mean Square*	*F Statistic*	*P>F*
Regr	1853840.8	9	205982.31	2346.91	0.00000
Error	107339.77	1223	87.767592		
Total	1961180.5	1232			
Lack Fit	6086.6309	38	160.1745	1.87458	0.00113
Pure Err	101253.13	1185	85.445683		

Description: Accelerated Stability XYZ Data: Time(days), Temp(°K), Control Med(rate)

Figure 23: Statistical Analysis for the Polynomial Model fitted to Control Medium Data

Regression Analysis

Rank 1 $z = a+blnx+clny+d(lnx)^2+e(lny)^2+flnxlny+g(lnx)^3+h(lny)^3+ilnx(lny)^2+j(lnx)^2lny$

x = time y = temp

Parameter	*Value*	*Std Error*	*t-value*	*95.00% Confidence Limits*		*P>|t|*
a	6.32772e+08	3.06867e+07	20.62044023	5.72568e+08	6.92977e+08	0.00000
b	-1.3041e+06	189610.4447	-6.87773313	-1.6761e+06	-932092.247	0.00000
c	-3.3201e+08	1.60227e+07	-20.7212053	-3.6345e+08	-3.0058e+08	0.00000
d	459.6023327	524.2223643	0.876731639	-568.872453	1488.077119	0.38080
e	5.80661e+07	2.78875e+06	20.82152693	5.25948e+07	6.35374e+07	0.00000
f	459273.555	65764.41417	6.983618128	330249.9833	588297.1267	0.00000
g	-0.04560531	0.012465817	-3.65842896	-0.07006206	-0.02114855	0.00026
h	-3.385e+06	161797.2567	-20.9213653	-3.7025e+06	-3.0676e+06	0.00000
i	-40430.7584	5702.212272	-7.0903636	-51617.9605	-29243.5562	0.00000
j	-87.0700183	91.55770407	-0.95098516	-266.69759	92.55755295	0.34180

Procedure
GaussElim

r^2 Coef Det	*DF Adj r^2*	*Fit Std Err*
0.9241547484	0.9235340835	54.757838502

Analysis of Variance

Source	*Sum of Squares*	*DF*	*Mean Square*	*F Statistic*	*P>F*
Regr	44682283	9	4964698.1	1655.77	0.00000
Error	3667068.7	1223	2998.4209		
Total	48349352	1232			
Lack Fit	174660.54	38	4596.33	1.55957	0.01712
Pure Err	3492408.2	1185	2947.1799		

Description: Accelerated Stability XYZ Data: Time(days), Temp(°K), Control High(rate)

Figure 24: Statistical Analysis for the Polynomial Model Fitted to Control High Data

As it can be observed in Figures 22 to 24, the statistical analyses of the fitted linear regressions indicate that all models appear to provide statistically significant relationships between *product content* (rate values of each control level), *time* and *temperature* at the 90% confidence level since the *p-value* in the ANOVA table for the regression model is less than 0.10. In addition, when verifying significance of the regression coefficients to identified which ones are making a genuine contribution to the model, it is concluded that all coefficients are significant for the model fitted to control low data, since the results of the statistic *t* test indicate that *p-values* are less than 0.10. This is not the case for the polynomial models fitted to control medium and control high data since it is observed in Figures 23 and 24, that parameters (d) and (j) have *p-values* higher than 0.10. These regression coefficients are related to the square natural logarithm of the time $(\ln X)^2$. In

this case, we could end up eliminating these parameters from the model but this is not a subject of interest for the objectives of this project since it is known that the test of individual regression coefficients is really a partial or marginal test because the regression coefficient ($\hat{\beta}_j$) depends on all the other regressor variables x_i $(i \neq j)$ that are in the model. Thus, this is a test of the contribution of x_j given the other regressor in the model.

Other ways to assess the overall adequacy of the model is the coefficient of determination (R^2) and the adjusted R^2, which is denoted as (R^2_{Adj}). The equations that describe these statistics were previously defined in Section 3.8. Most model builder statisticians preferred to use the R^2_{Adj} statistic for polynomial models since, in general, the R^2 statistic always increases when a regressor is added to the model, regardless of the value of the contribution of the variable. In contrasts, the R^2_{Adj} statistic will only increase when adding a variable to the model, if the variable added reduces the residual mean square (*MSE*) of the model. Therefore, the R^2_{Adj} will penalize for adding parameters that are not helpful.

Reviewing Figures 22 to 24, it is observed that the lowest value obtained for the three polynomial models was a R^2_{Adj} value equal to 92.3%, which will indicate that the variability in the response variable (*Z*) is very well explained by the models fitted. It is important o clarify that even though results of statistical analyses indicate that these polynomial models have good capabilities to explain the variability of the response variable as measured by large R^2_{Adj} values, this not necessarily means that they are good regression models for extrapolation of the response variable as previously discussed in Section 4.3.1.

4.3.3 Advantages and Disadvantages of the Polynomial Function Model

Using the same approach used previously with the Arrhenius classical approach in section 4.2.3, the polynomial function model will also be addressed in terms of their relative advantages and disadvantages to illustrate its relevance and limitations to fit the accelerated stability data. In the case of the Polynomial Function Model, it is of interest

to highlight that these regression models demonstrated capability to handle the 1,233 results obtained from the accelerated test and to relate all the variables of interest into equations that provided R^2_{Adj} values higher than 92% and with almost all of the regression coefficients demonstrating to be significant to the model.

In the other hand, the polynomial function model has several disadvantages that strongly affect its capability to provide accurate predictions for all control levels considered in the accelerated stability test as previously mentioned in Section 4.3.1. These disadvantages are summarized as follows:

- Even though the accelerated that the stability study done provided data of the response variables at time zero (t_0), the polynomial model was not able to consider these values since the regressor variables of the model include the *ln*( ) of time and temperature, and the *ln*(0) is not defined.
  - A model considering values at time zero would provide much better extrapolation estimates since it will contain the baseline from which the product content of each lot of the study initiated there degradation patterns and from which it was measured. In addition, it is know from Abbot scientists, that the behavior of the product should provide a lag of degradation behavior at temperatures closer to the storage condition (281 °K). Therefore, a plateau behavior should be observed for the temperatures closer to the storage condition in the surface response plots, but this was not the behavior observed in any of the Figures 19 to 21. In contrast, these figures illustrate significant degradation response at temperatures near the storage condition.
- It is observed in Figures 19 to 21 that most of the observed rate values (the response variable *Z*) at the highest temperature experimented in the accelerated test (45°C or 318°K) are not well fitted by the surface response plots since they are clearly seen far-off from the shape of the curvature obtained for this temperature. This indicates that the Polynomial Function model is not adequate for performing interpolations at the highest temperatures of the experiment.
- Finally, if we investigate the surface plots of the partial derivatives of the response variable with respect to the regressor variables (time and temperature), it is found

that there is no simple interpretation to the behavior of the degradation rate observed in these plots. Figures 25 to 27 illustrate the partial derivative surface plots on the performance of control levels with respect to changes in time $(\partial Z / \partial X)$ and Figures 28 to 30 illustrate the partial derivative surface plots on the performance of control levels with respect to changes in temperature $(\partial Z / \partial Y)$. The vertical axes in these plots correspond to the derivative of the degradation rate for each respective control level (Low. Medium or High) with respect to the regressor variable of interest. A negative partial derivative means that the performance of the product (i.e. as measure by the rate values of each control level), is decreasing since an actual degradation is occurring as time and temperature increases. Therefore, the expected behavior of these plots is to have a surface intercepting the vertical axis ($\partial Z/\partial X$ or $\partial Z/\partial Y$) at a value equal to zero, and then to have a downward orientation of the curve as degradation rate increases.

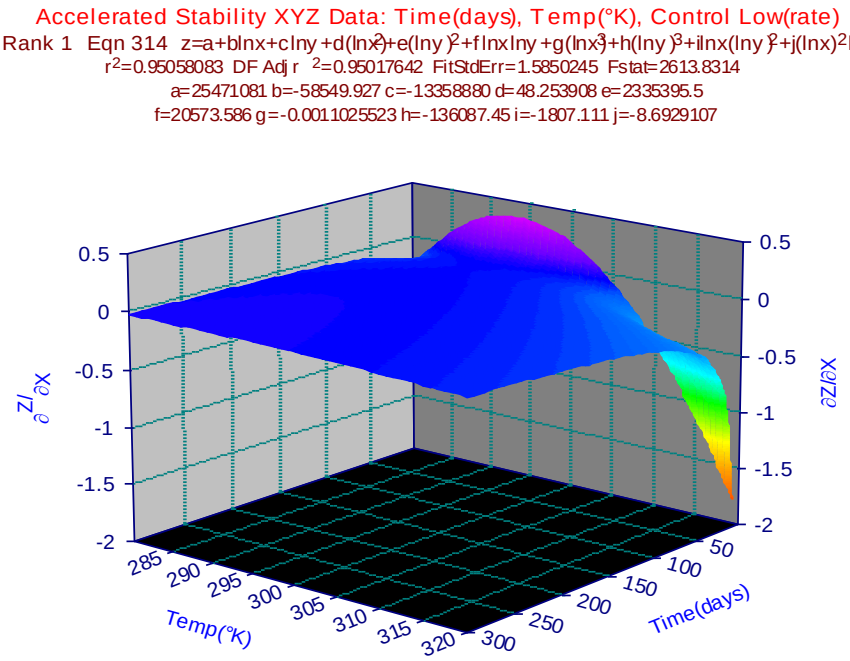


Figure 25: Partial Derivative Plot of Control Low with respect to changes in time for the Polynomial Function Model

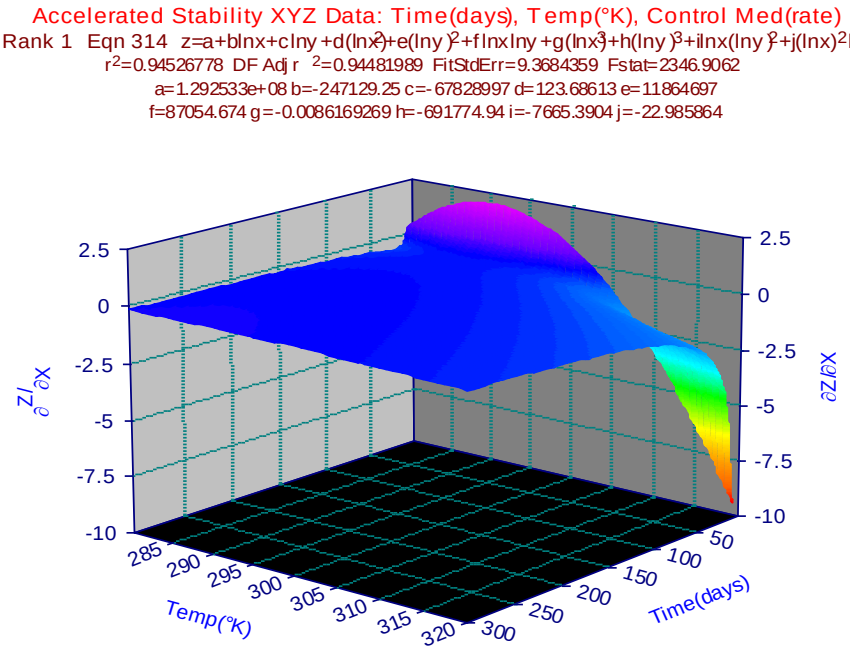


Figure 26: Partial Derivative Plot of Control Medium with respect to changes in time for the Polynomial Function Model

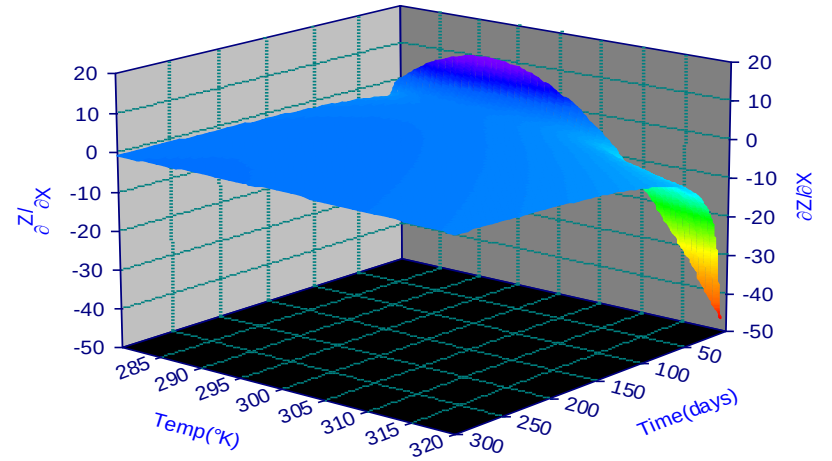


Figure 27: Partial Derivative Plot of Control High with respect to changes in time for the Polynomial Function Model

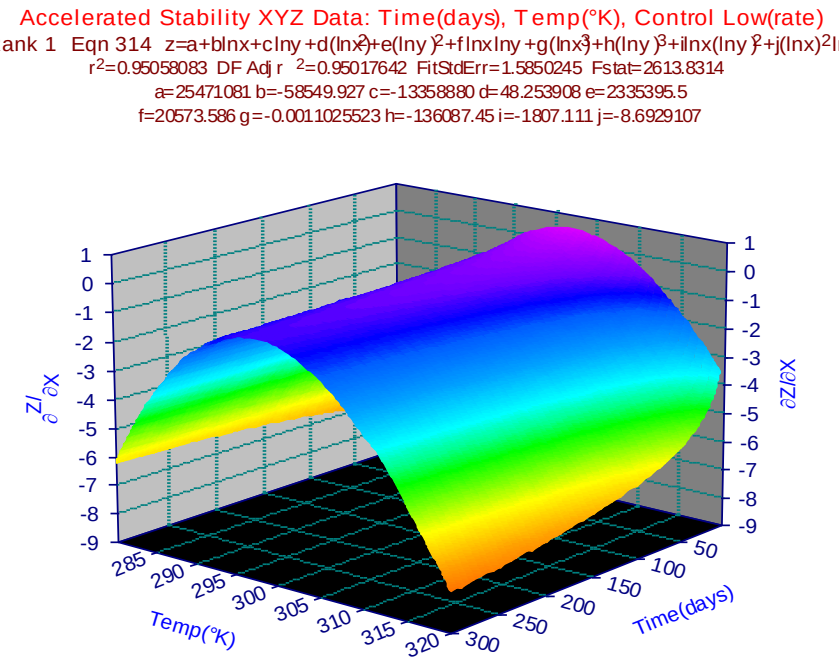


Figure 28: Partial Derivative Plot of Control Low with respect to changes in temperature for the Polynomial Function Model

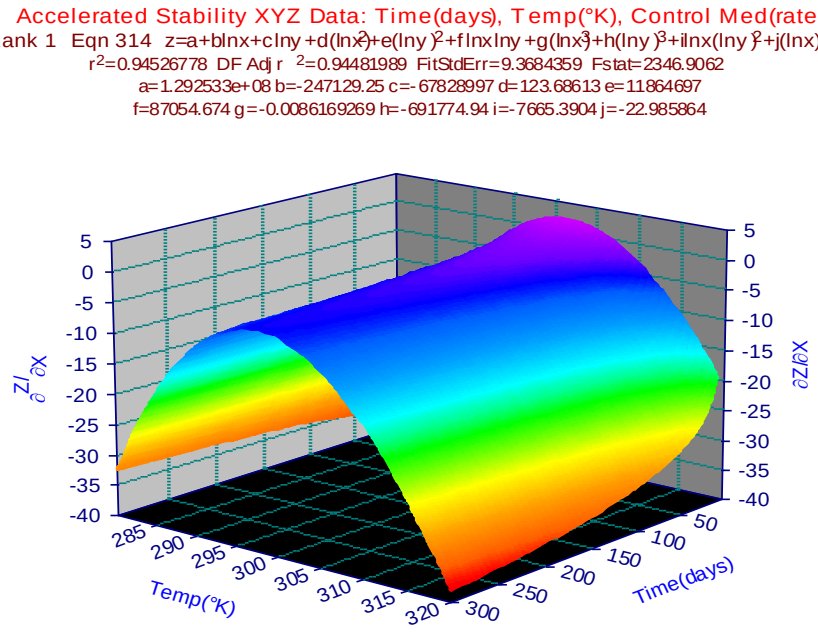


Figure 29: Partial Derivative Plot of Control Medium with respect to changes in temperature for the Polynomial Function Model

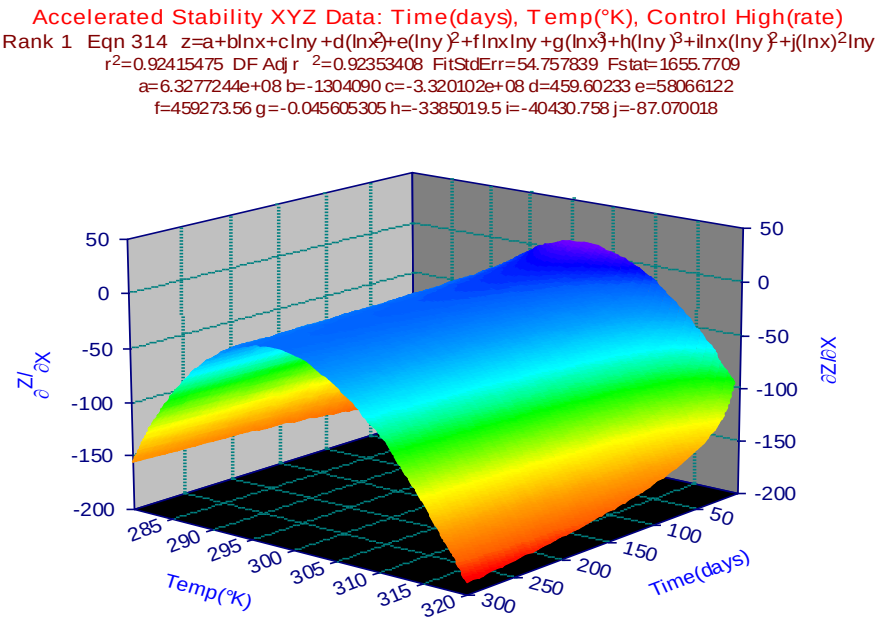


Figure 30: Partial Derivative Plot of Control High with respect to changes in temperature for the Polynomial Function Model

Therefore, if we consider the partial derivative plot for Control Low with respect to changes in temperature (Figure 28) as an example, it is observed that there is no congruence with the expected chemical-physical behavior of the product since at any time value, the surface intercepts the vertical axis near the –6 value, then it increases performance with a positive orientation in the temperature range of 280 to 300 °K, at which it obtains a maximum value, and finally has a degradation of product performance for temperature values greater than 300 °K. The same behavior, but in a different numbering scale, is observed in the partial derivative plots for Control Medium and Control High (Figures 29 and 30). In other words, what these surface plots are saying is that as the product initially receives an incremental stress temperature in any time, it will increase its potency (or product performance) until a temperature near 300°K is achieved. Then, as temperature exceeds the 300°K, the product will start to degrade abruptly until it looses all functionality.

Is important to clarify once again that this is not the expected behavior of the type of product that was used (an immunoassay regent kit) since the product is significantly affected by the exposure of stress temperatures for the reason that one of its components

(the antibody-enzime conjugate) suffers a reaction that breaks its molecular structure and deteriorates the antibody agents, causing degradation in product content. Therefore, these plots should had presented a change in rate values that starts at a value equal to zero and then significantly increases in negative values as it moves further away from the lowest values of temperature and time that were considered in the accelerated test.

This incongruence of the partial derivative plot with the true chemical-physical behavior expected is the main cause why the Polynomial Function Model has significant problems to predict the rate value of each control level at expiration day. Hence, it is preliminary concluded that even though the Polynomial Function Model was able to provide attractive values of the R^2_{Adj} statistic, it also demonstrated to be not appropriate for predicting the stability of the product at the normal conditions of use. Therefore, another statistical model will be developed, that will provide the same attractive results as the Polynomial model in terms of the regression statistical analyses, but in addition, it will provide appropriate prediction estimates that are more congruent with the expected product performance.

4.4 Nonlinear Arrhenius Models

Although the Arrhenius classical linear approach has been widely used in the past, the methodology demonstrated to suffer significant drawbacks since it only employs the mean rate constants obtained at elevated temperatures to predict a future value of product content (rate values of each control level) at the shelf-life or expiration date. Therefore, the experimental errors associated with the total number of values (*n*) obtained from the accelerated stability test are not included in the two-step procedure of the Arrhenius classical approach. In addition, several literature reports of people that have used this approach, indicated that the methodology had another problem related with the statistical interval that is obtained for the prediction at the storage or room temperature, since the statistical interval obtained from the fitted linear regression can be so wide that it could make the estimated shelf-life of little or no value.

Because of these intrinsic problems of the Arrhenius classical approach as well as the disadvantages that demonstrated the Polynomial Function Model, it was decided to develop alternate models that could relate *product content*, *time* and *temperature* of the

accelerated stability study in a nonlinear approach. A model is considered non-linear when the partial derivatives of one of the unknown parameters, is a function of one of more of the other unknown parameters. Therefore, a single step matrix solution as applied by linear least squares procedure is not possible. Non-linear model fitting consists of an iterative procedure that begins with an initial set of estimates for the parameters until it converges to the optimization objective.

These models had the advantages that they were based on the applicable reaction orders of the Arrhenius life-temperature relationship, and contrary to the classical approach, they were able to consider the experimental errors associated with the total number of values obtained from the accelerated stability test (n = 1233). Only the zero and first order of reactions were used to relate temperature by the Arrhenius equation in a nonlinear way since the degradation of the product that was used is caused by the interaction of one molecule of interest (the antibody-enzime). Second order reactions are related to pharmaceutical or biological products that have interactions of two or more molecules and the stress factor has a cumulative effect on degradation.

These nonlinear equations were derived by substituting the Arrhenius equation (Eq.1) presented in Section 2.4.2.1 into the equations of a zero and first order reaction (Eq.17 and 18) established in Section 2.4.2.2 of this report. Hence, the corresponding nonlinear Arrhenius equation for a zero-order reaction is given by

$$C = C_0 - A \cdot t \cdot e^{\left(\frac{-E_a}{R \cdot T}\right)} \tag{39}$$

and for a first-order reaction is given by:

$$C = C_0 \cdot e^{\left[-A \cdot t \cdot e^{\left(\frac{-E_a}{R \cdot T}\right)}\right]} \tag{40}$$

In general, the true degradation of product content (C) at any temperature and as a function of time can be expressed as follows:

$$C = f(\mathbf{t}, \boldsymbol{\beta}) \tag{41}$$

where, $\mathbf{t}$ is the vector of observed time points and $\boldsymbol{\beta}$ is the vector of the parameters. Therefore, the observed response as a function of time and temperature for the zero and first reaction orders are respectively:

$$Z = C + \varepsilon = C_0 - A \cdot t \cdot e^{\left(\frac{-E_a}{R \cdot T}\right)} + \varepsilon \tag{42}$$

$$Z = C + \varepsilon = C_0 \cdot e^{\left[-A t \cdot e^{\left(\frac{-E_a}{R \cdot T}\right)}\right]} + \varepsilon \qquad (43)$$

where,

C_0 = is the intercept of the surface (performance at time zero)

A = the Arrhenius constant

t = time (in days)

E_a = the activation energy (kcal mol $^{-1}$)

R = the gas constant, equal to 0.00199 (kcal mol $^{-1}$ K^{-1})

T = temperature in Kelvin (K)

ε = the experimental error

All of these parameters are considered to be fixed except for the experimental error, which are considered to be random, independent, and normally distributed with $\mathrm{E}[\varepsilon] = 0$ and constant variance.

4.4.1 User Defined Functions

For the purpose of fitting the nonlinear Arrhenius equations for zero and first order reactions, we used the "User-Defined Function" (UDF) option that is available in Table Curve 3D software. This option contains all the necessary information to fit the equation, including the function name, the parameter count, the function's formula, and the starting estimates and constraints for each parameter. A special "Adjust" item allows you to graphically adjust the starting estimates to better assure a successfully converged fit. In addition, it enables you to inspect the partial derivatives of the UDF to find instances of multiple constants, insignificant parameters, and to expose conditions would be likely to fail. To enter a UDF in Table Curve 3D, you must perform the following steps:

1) *Enter the Function Name*:

   This is the name that will be used to represent the function in the surface fit graphs.

2) *Enter the Coefficient Count*:

   This is the number of parameters in the UDF model. For example, if you enter 4 as the coefficient count, the UDF must contain parameters #A, #B, #C, #D (or

A0, A1, A2, A3). These are the only letter formats recognized by the program to enter coefficient parameters.

3) *Enter the Mathematical Function or UDF*:

   The program uses a simple ASCII multi-line editor for entering a mathematical function. You may define as many constants as needed. These constants are evaluated once and the numeric result is stored. Variables must be assigned to a F1-F9 (or #F1-#F9) or Z expression because if not, they will be assumed to be a constant. Any expression containing X, Y or any of the function parameters #A-#H (or A0-A7) must be assigned to an F1-F9 expression or to Z. The Z expression must always be last line in the UDF

Taking in consideration the requirements previously mentioned to create UDF models into Table Curve 3D, it was needed to modify the nonlinear Arrhenius equations (Eq.42 and Eq.43) established in Section 4.4 to a format that would be understood by the software. The following equations are re-written formats of the nonlinear Arrhenius equations for zero and first order reactions, respectively:

$$Z = a - b \cdot X \cdot e^{\left(\frac{-c}{Y}\right)} + \varepsilon \tag{44}$$

$$Z = a \cdot e^{\left[-b \cdot X \cdot e^{\left(\frac{-c}{Y}\right)}\right]} + \varepsilon \tag{45}$$

where,

Z = rate values of each respective control level (Low, Medium or High)

a = is the intercept of the surface (performance at time zero)

b = the constant A of the Arrhenius equation

X = time (in days)

Y = temperature in Kelvin (K)

c = the relation E_a /R of the Arrhenius equation

The specific UDF entries that was done in Table Curve 3D for these nonlinear equations will be presented in Sections 4.4.3 and 4.4.4

4.4.2 Parameter Estimation

The parameters for the nonlinear equations (Eq.44 and Eq.45) were estimated using the nonlinear least-squares approach. This approach requires an iterative function minimization routine. Therefore, the Levenburg-Marquardt algorithm provided by Table Curve 3D was used for fitting these nonlinear Arrhenius equations. This algorithm provides a numerical solution to minimize nonlinear functions and its mainly applied in the least-squares curve fitting problems. Therefore, given a set of empirical data pairs of independent and dependent variables (x_i, y_i), the algorithm optimizes the parameters β of the model $f(\mathbf{x}, \beta)$ to minimize the following residual sum of squares function:

$$RSS(\beta) = \sum_{i=1}^{n} [y_i - f(\mathbf{x}_i, \beta)]^2 \quad (46)$$

The rate of convergence of the Levenberg-Marquardt algorithm is among the best of available methods and it is considered more robust than the Gauss-Newton algorithm, since in many cases it finds a solution even if the iteration procedure starts very distantly from the final minimum.

4.4.3 Results for the Arrhenius Zero-Order Nonlinear Model

The third model developed was an Arrhenius zero-order reaction model which related *product content*, *time* and *temperature* of the accelerated stability study in a nonlinear approach as illustrated in Eq.44. The model was included as a nonlinear User Defined Function (UDF) into Table Curve 3D software. Figures 31 to 33 illustrate the function formula with the format required by the software as well as the parameters starting points and constraints used in the UDF for each of the response variable (Control Low, Medium and High) considered in the accelerated study. Parameters *a*, *b* and *c* in Eq.44 are related to important factors of the Arrhenius equation, therefore, their starting points were determined by using the estimates obtained for the Arrhenius models developed with the classical approach (Model 1) as reference values.

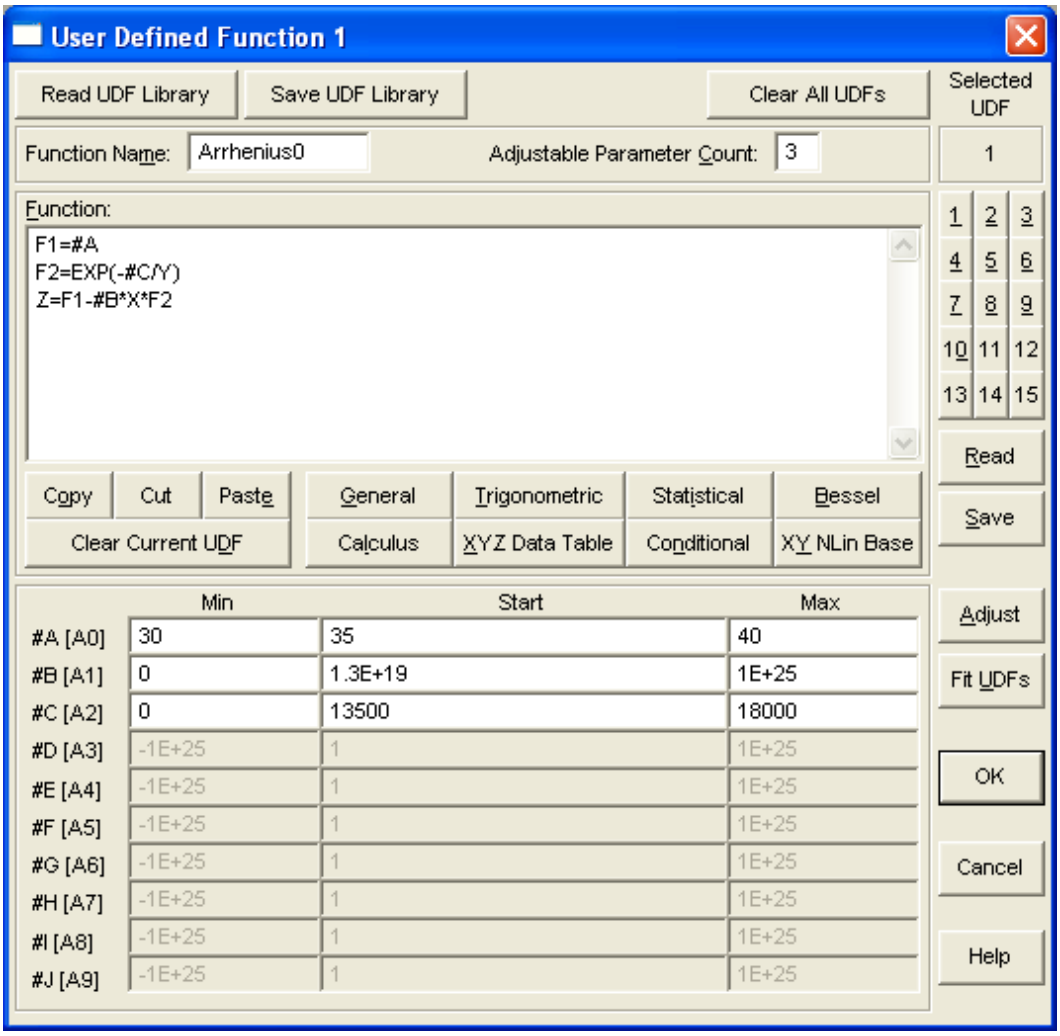


Figure 31: UDF of the Arrhenius Zero-Order Nonlinear Model for Control Low

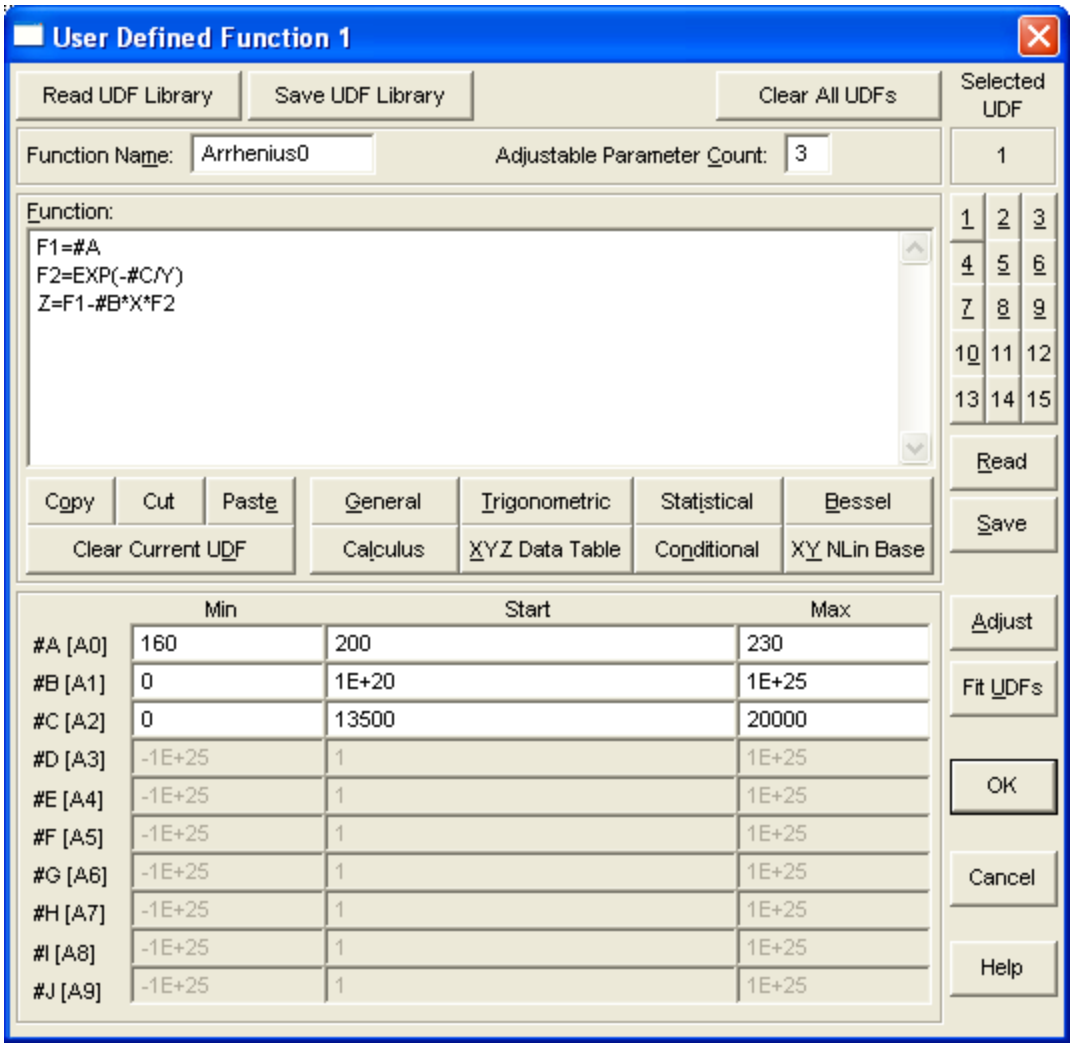


Figure 32: UDF of the Arrhenius Zero-Order Nonlinear Model for Control Medium

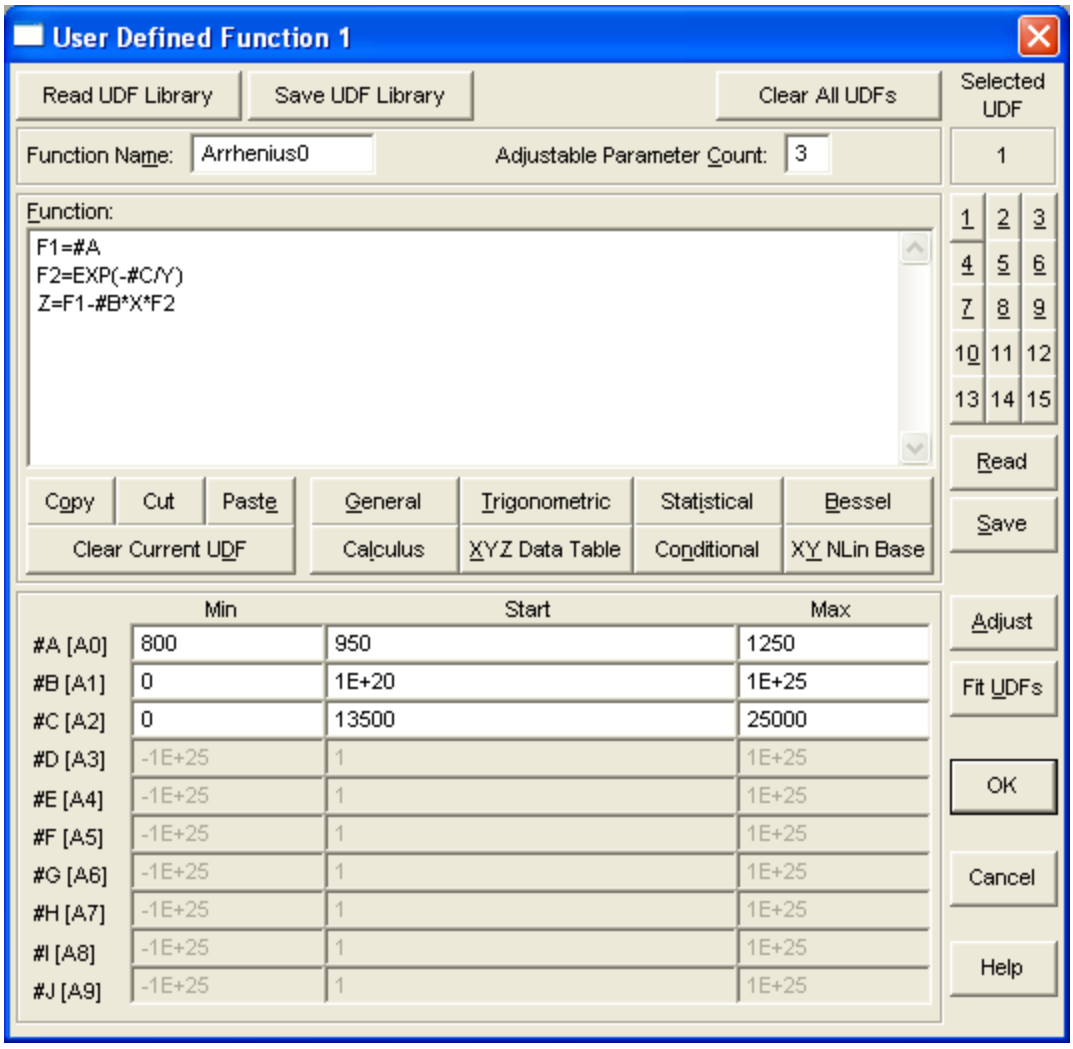


Figure 33: UDF of the Arrhenius Zero-Order Nonlinear Model for Control High

The following equations were obtained as a result of fitting a nonlinear Arrhenius UDF for each of the response variable (Control Low, Medium and High) considered in the accelerated study.

- **For Control Low**

$$Z = 32.90585 - 1.80867 \times 10^{21} \cdot t \cdot e^{\left(\frac{-1557432673}{T}\right)} + \varepsilon \qquad (47)$$

- **For Control Medium**

$$Z = 178.1744 - 6.93593 \times 10^{21} \cdot t \cdot e^{\left(\frac{-1545773317}{T}\right)} + \varepsilon \qquad (48)$$

- **For Control High**

$$Z = 890.94847 - 1.93637 \times 10^{22} \cdot t \cdot e^{\left(\frac{-1528180923}{T}\right)} + \varepsilon \quad (49)$$

Using some algebra modifications, the equations can be re-written as follows:

- **For Control Low**

$$Z = 32.90585 - t \cdot e^{\left(48.94688 - \frac{1557432673}{T}\right)} + \varepsilon \quad (50)$$

- **For Control Medium**

$$Z = 178.1744 - t \cdot e^{\left(50.2910 - \frac{1545773317}{T}\right)} + \varepsilon \quad (51)$$

- **For Control High**

$$Z = 890.94847 - t \cdot e^{\left(51.31769 - \frac{1528180923}{T}\right)} + \varepsilon \quad (52)$$

Figures 34 to 36 illustrate the surface fit plots that correspond to these nonlinear Arrhenius equations for a zero-order reaction.

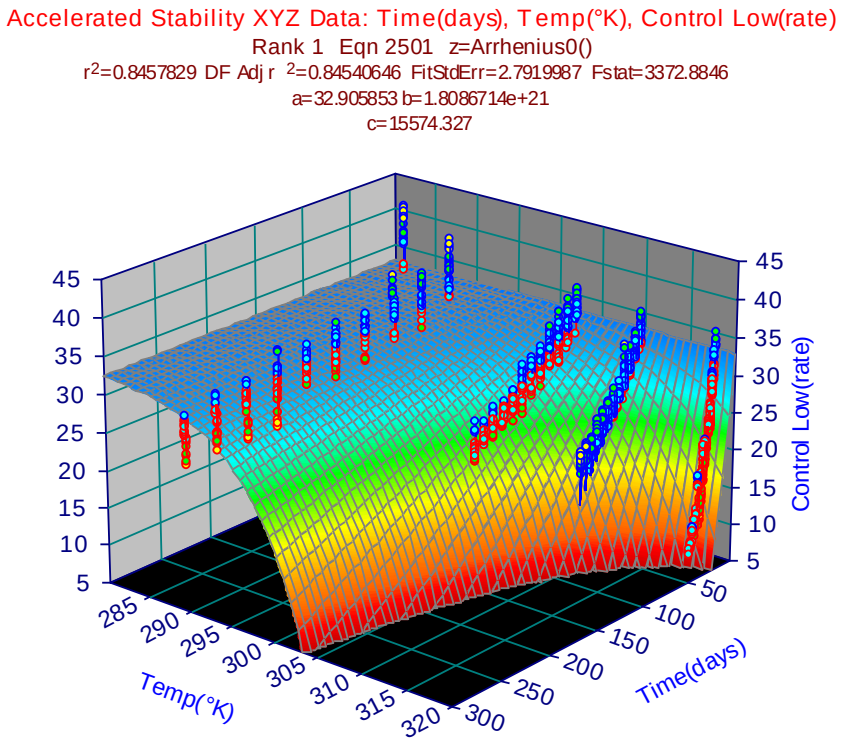


Figure 34: Surface Plot of the Arrhenius Zero-Order Nonlinear Model for Control Low

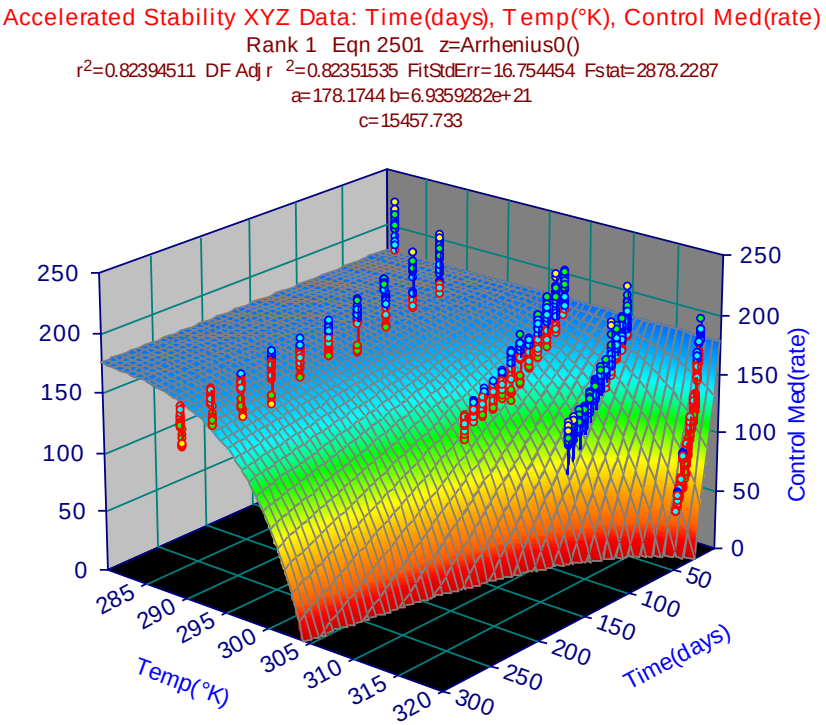


Figure 35: Surface Plot of the Arrhenius Zero-Order Nonlinear Model for Control Medium

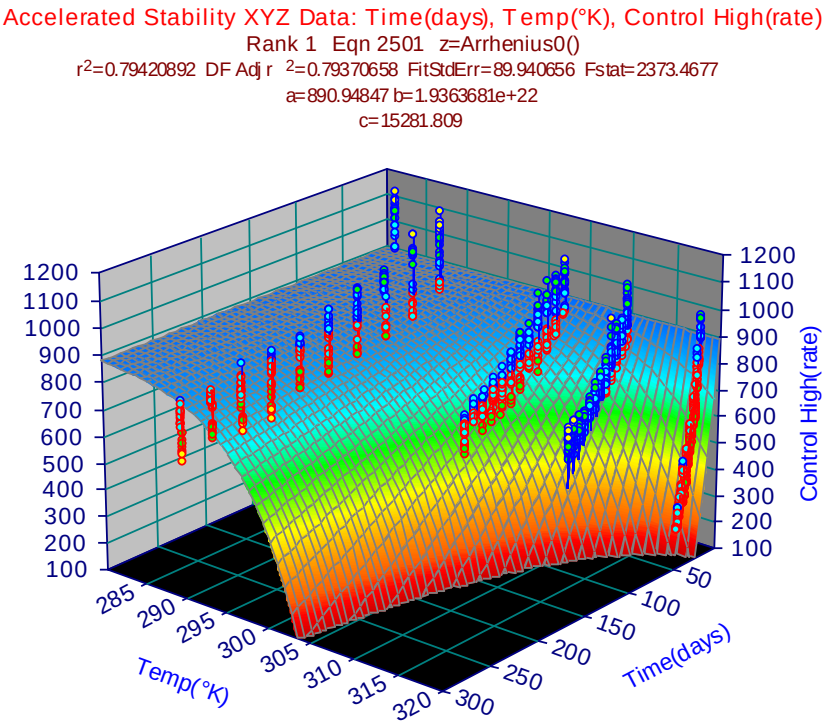


Figure 36: Surface Plot of the Arrhenius Zero-Order Nonlinear Model for Control High

Since the Arrhenius Zero-Order nonlinear model is capable of considering the product content at time zero (t_0) for each of the lots considered in the study, then it is clearly observed that the surface plots for each of the control levels follow the degradation patterns that was expected by Abbott scientists. The product should provide a lag of degradation at the beginning of the study and at temperatures close to the storage condition (281 °K), forming a kind of a plateau at these temperatures. Then, it should start to degrade significantly as temperature and time increases. In our case, this was the exact behavior that was obtained in the surface plots of the accelerated stability data that was fitted for all control levels.

In addition, since these nonlinear models considered values at time zero, it was expected that they would provide much better extrapolation estimates since they were considering the baseline from which the product content of each lot of the study initiated there degradation pattern. Therefore, the equations that described these plots (Eq.50 to Eq.52) were used to predict the rate values of each control level at the expiration date (180 days) and at the storage temperature (8°C or 281°K). Table 9 contains the results of the predicted rate value of each control level at the expiration date.

Table 9: Prediction of Controls Rate Values using the Arrhenius Zero-Order Model

Control Level	Mean rate value at t=0	Storage Temp. (°K)	Expiration Date (days)	Predicted rate value at Exp. Date	95% Prediction Interval
Low	36.75	281	180	32.63	27.15 – 38.11
Medium	201.38	281	180	176.57	143.66 – 209.47
High	1007.60	281	180	882.56	705.91 – 1059.21

Examining the results obtained for the prediction of product content (rate values) at the expiration date we can establish that the Arrhenius Zero-Order Nonlinear Model is congruent with the chemical-physical behavior that is expected since the predicted rate values of each control level is lower than the mean rate value of the product at time zero. If these values are compared in terms of percent difference, it is determined that the product had a product content loss of 11.2%, 12.3%, and 12.4% at the 180 days. These percents of degradation were the results expected by Abbott scientist since at 180 days, a product content loss between 10 and 15% should be observed for the specific product that was used in the accelerate stability test. Table 7 in Section 4.2.1 contains the rate specifications that were established to monitor the stability of the product (a reagent kit)

as measured by each control level. As presented in Table 9, the predicted rate values of each control level are within the specifications. Therefore, these predictions are also in congruence with the behavior of the three lots used to perform the accelerated stability study since these were lots that passed the internal manufacturing specifications for selling them in to the market, and once in the customer hands they were within the stability specifications until they expired.

4.4.3.1 Statistical Analyzes for the Arrhenius Zero-Order Nonlinear Model

Using the statistical tests in a similar way as it was done with the two previous models, it can be establish that the Arrhenius zero-order nonlinear model is capable of providing significant statistical results when relating *product content*, *time* and *temperature* since a *p-value* = 0.00000 was obtained for the regression models of each control level (refer to the ANOVA table in Figures 37 to 39). In addition, when testing the significance of individual regression coefficient, it is found that all parameters of the Arrhenius zero-order nonlinear model are significant to the model since the results of the statistic *t* test indicate that *p-values* are less than 0.10.

Rank 1 z=Arrhenius-Zero Order

Parameter	Value	Std Error	t-value	95.00% Confidence Limits		P>|t|
a	32.90585296	0.140858876	233.6086572	32.6295027	33.18220322	0.00000
b	1.80867e+21	9.20629e+20	1.964604127	2.49454e+18	3.61485e+21	0.04968
c	15574.32673	161.0252993	96.71975028	15258.41208	15890.24139	0.00000

Procedure	Minimization	Iterations
Lev-Marq	Least Squares	100

r^2 Coef Det	DF Adj r^2	Fit Std Err
0.845782901	0.8454064557	2.7919986991

Analysis of Variance

Source	Sum of Squares	DF	Mean Square	F Statistic	P>F
Regr	52585.003	2	26292.502	3372.88	0.00000
Error	9588.1658	1230	7.7952567		
Total	62173.169	1232			
Lack Fit	6706.7621	45	149.03916	61.2935	0.00000
Pure Error	2881.4036	1185	2.4315643		

Description: Accelerated Stability XYZ Data, Time(days), Temp(°K), Control Low(rate)

Figure 37: Statistical Analysis for Arrhenius Zero-Order Nonlinear Model Fitted to Control Low Data

Rank 1 z=Arrhenius-Zero Order

Parameter	Value	Std Error	t-value	95.00% Confidence Limits		P>|t|
a	178.1744037	0.84754122	210.2250598	176.5116172	179.8371901	0.00000
b	6.93593e+21	3.77925e+21	1.835263792	4.7857e+20	1.43504e+22	0.06671
c	15457.73317	172.3491871	89.68845998	15119.60224	15795.8641	0.00000

Procedure	Minimization	Iterations
Lev-Marq	Least Squares	100

r^2 Coef Det	DF Adj r^2	Fit Std Err
0.8239451062	0.8235153546	16.754454066

Analysis of Variance

Source	Sum of Squares	DF	Mean Square	F Statistic	P>F
Regr	1615905.1	2	807952.55	2878.23	0.00000
Error	345275.43	1230	280.71173		
Total	1961180.5	1232			
Lack Fit	244022.3	45	5422.7177	63.4639	0.00000
Pure Error	101253.13	1185	85.445683		

Description: Accelerated Stability XYZ Data, Time(days), Temp(°K), Control Med(rate)

Figure 38: Statistical Analysis for Arrhenius Zero-Order Nonlinear Model Fitted to Control Medium Data

Rank 1 z=Arrhenius-Zero Order

Parameter	Value	Std Error	t-value	95.00% Confidence Limits		P>|t|
a	890.9484679	4.568178378	195.0336424	881.9861838	899.9107521	0.00000
b	1.93637e+22	1.14373e+22	1.693025569	3.0751e+21	4.18025e+22	0.09070
c	15281.80923	186.7879319	81.8136861	14915.351	15648.26745	0.00000

Procedure	Minimization	Iterations
Lev-Marq	Least Squares	100

r^2 Coef Det	DF Adj r^2	Fit Std Err
0.7942089159	0.793706578	89.940655553

Analysis of Variance

Source	Sum of Squares	DF	Mean Square	F Statistic	P>F
Regr	38399486	2	19199743	2373.47	0.00000
Error	9949865.5	1230	8089.3215		
Total	48349352	1232			
Lack Fit	6457457.3	45	143499.05	48.6903	0.00000
Pure Error	3492408.2	1185	2947.1799		

Description: Accelerated Stability XYZ Data, Time(days), Temp(°K), Control High(rate)

Figure 39: Statistical Analysis of Arrhenius Zero-Order Nonlinear Model Fitted to Control High Data

In addition, when reviewing the R^2_{Adj} statistic as other ways the assess the adequacy of the model, it is observed in Figures 37 to 39 that the Arrhenius Zero-Order Models appear to explain well the variability of the response variable Z since the lowest value obtained for all the models fitted was an R^2_{Adj} value equal to 79.37%

4.4.3.2 Advantages and Disadvantages of the Arhenius Zero-Order Nonlinear Model

Addressing the nonlinear approach of the model developed for an Arrehnius zero-order reaction it can preliminary conclude that this model demonstrated to have significant advantages when compared with the other two models that were discussed previously. The following bullets summarize the advantages that presented this model when it was analyzed in detail.

- The model is obtained by a one step nonlinear approach that relates product content, time, and temperature for the treatment of the accelerated stability data that was experimentally gathered.
- The regression equations that were fitted provided good results in the statistical test that were done since a *p-value* of 0.00000 was obtained for the surface fit of all control levels. In addition, all of the individual regression coefficients demonstrated to have a significant contribution in the model since their *p-value* where less than 0.10.
- The model considers data of the response variable at time zero (t_0) and it is also capable of considering the experimental error (variability) associated with the total number of observations obtained from the accelerated stability test (n = 1233). Therefore an improvement was observed in the prediction of the product content at the expiration date, when compared with the Arrhenius classical approach and the Polynomial Function Model.
- Using the predictions performed at the expiration date (180 days) and at the storage temperature (8°C or 281°K), it was observed that the Arhenius zero-order nonlinear model provided much smaller and symmetrical 95% prediction intervals, when compared with the Arrhenius classical approach.

- Finally and most important, is that the partial derivative plots of the response variable with respect to the regressor variables (time and temperature) are aligned with the expected chemical-physical behavior of the type of product that was used since all surface plots intercept the vertical axis ($\partial Z/\partial X$ or $\partial Z/\partial Y$) at a value equal to zero, and then show a continuous downward orientation as time and temperature increases. Refer to Figures 40 to 42 for the partial derivative surface plots on the performance of control levels with respect to changes in time $(\partial Z/\partial X)$ and Figures 43 to 45 for the partial derivative surface plots on the performance of control levels with respect to changes in temperature $(\partial Z/\partial Y)$.

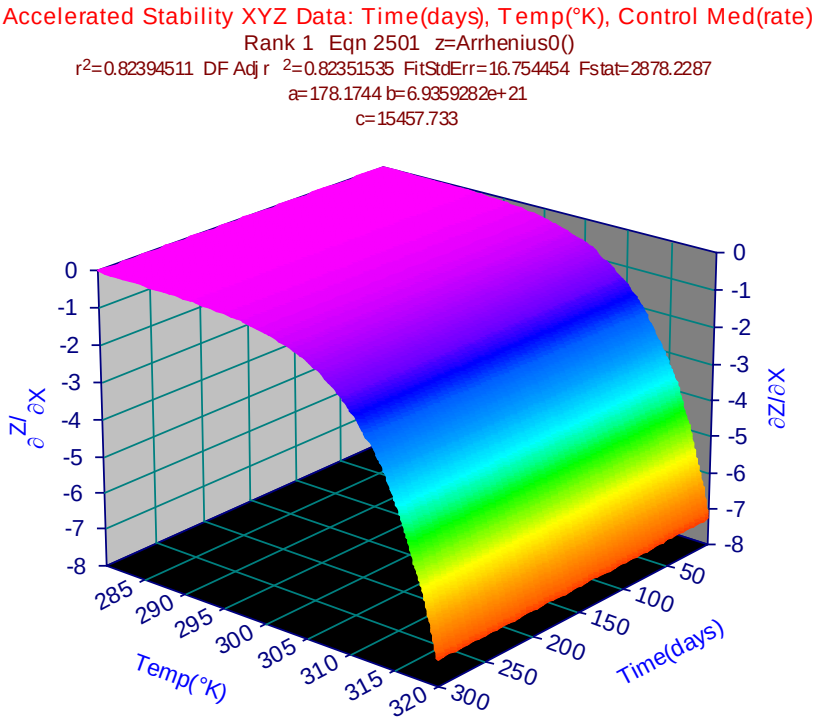


Figure 40: Partial Derivative Plot of Control Low with respect to changes in time for the Arrhenius Zero-Order Nonlinear Model

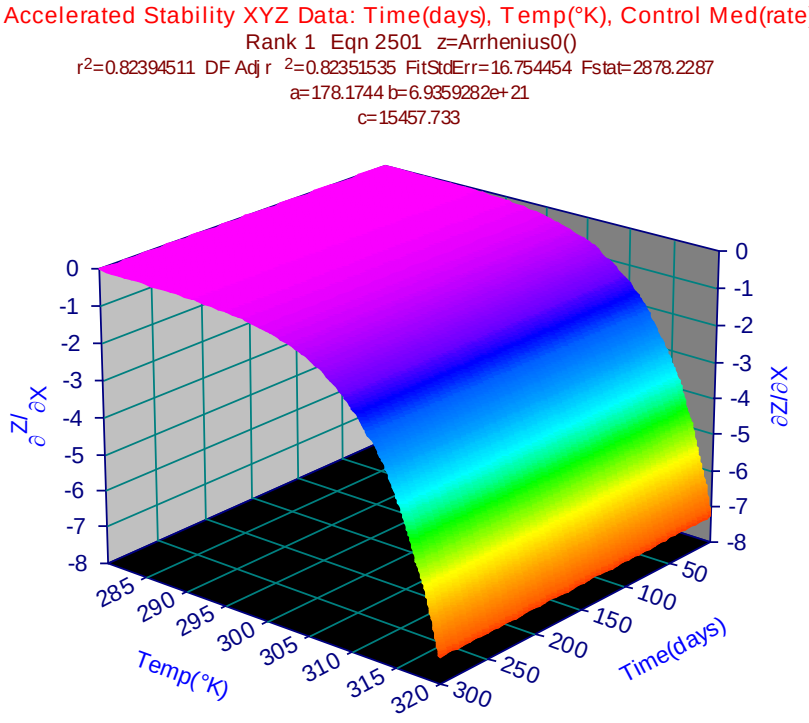


Figure 41: Partial Derivative Plot of Control Medium with respect to changes in time for the Arrhenius Zero-Order Nonlinear Model

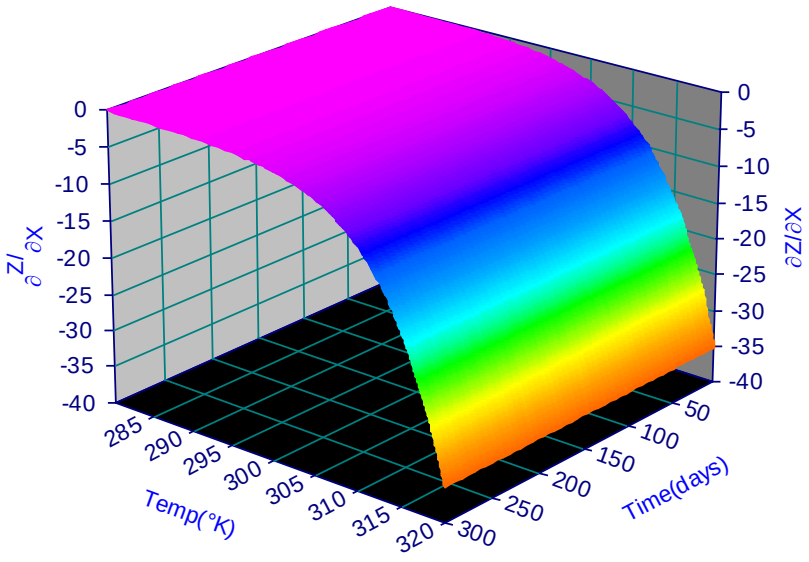


Figure 42: Partial Derivative Plot of Control High with respect to changes in time for the Arrhenius Zero-Order Nonlinear Model

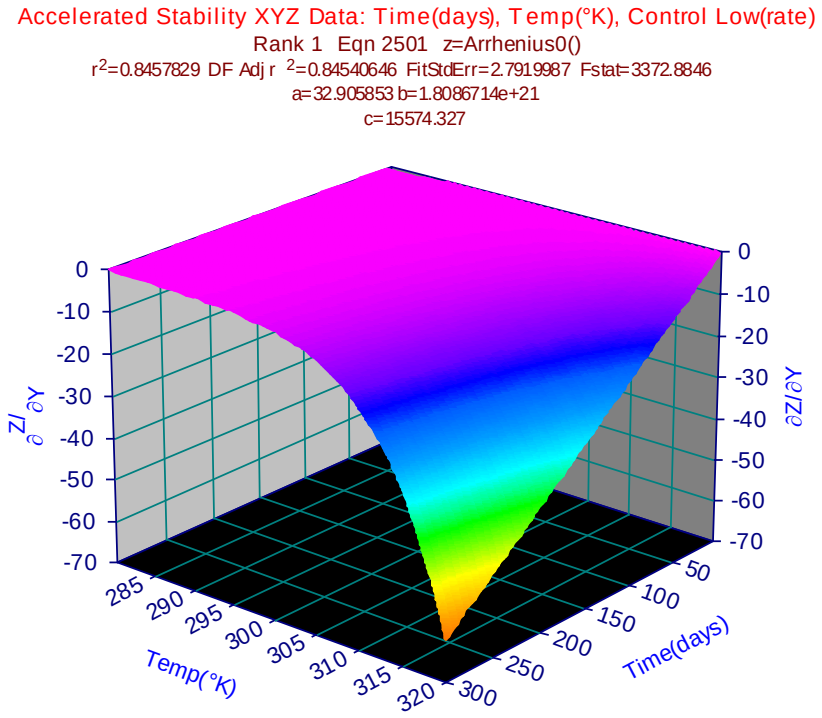


Figure 43: Partial Derivative Plot of Control Low with respect to changes in temperature for the Arrhenius Zero-Order Nonlinear Model

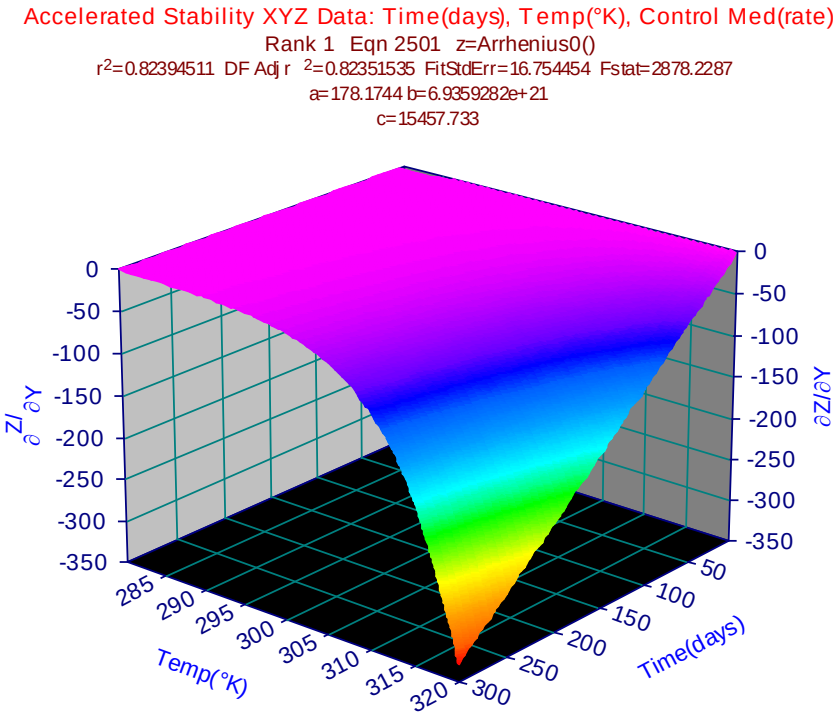


Figure 44: Partial Derivative Plot of Control Medium with respect to changes in temperature for the Arrhenius Zero-Order Nonlinear Model

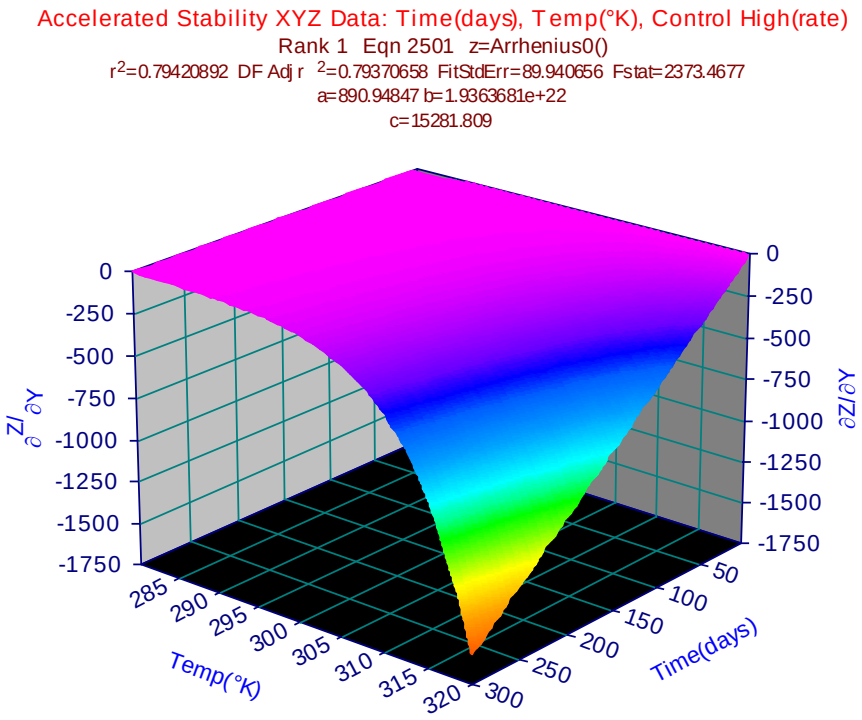


Figure 45: Partial Derivative Plot of Control High with respect to changes in temperature for the Arrhenius Zero-Order Nonlinear Model

4.4.4 Results for the Arrhenius First-Order Nonlinear Model

The fourth and final model developed to relate *product content*, *time* and *temperature* of the accelerated stability test was an Arrhenius first-order nonlinear model. This type of reaction order was considered since it is well known by the literature of the Arrhenius Law that zero and first order reactions can explain chemical reactions that involve only one molecule. The exponential degradation pattern of the first order reaction is one of the most used and presented in the literature. In our specific case, we could apply this type of reaction order to fit a model since the degradation of the product that was used is caused by the interaction of one molecule of interest (the antibody-enzime).

The nonlinear first-order expression presented in Eq.45 was included as a nonlinear User Defined Function (UDF) into Table Curve 3D in the same manner as it was done for the nonlinear zero-order expression. Figures 46 to 48 illustrate the function formula that was included in the software to represent a nonlinear first-order expression as well as the

starting points and constraints that were used for the parameters. The starting points values used in this approach were very similar, and in some cases, equal to the values used with the nonlinear zero-order expression.

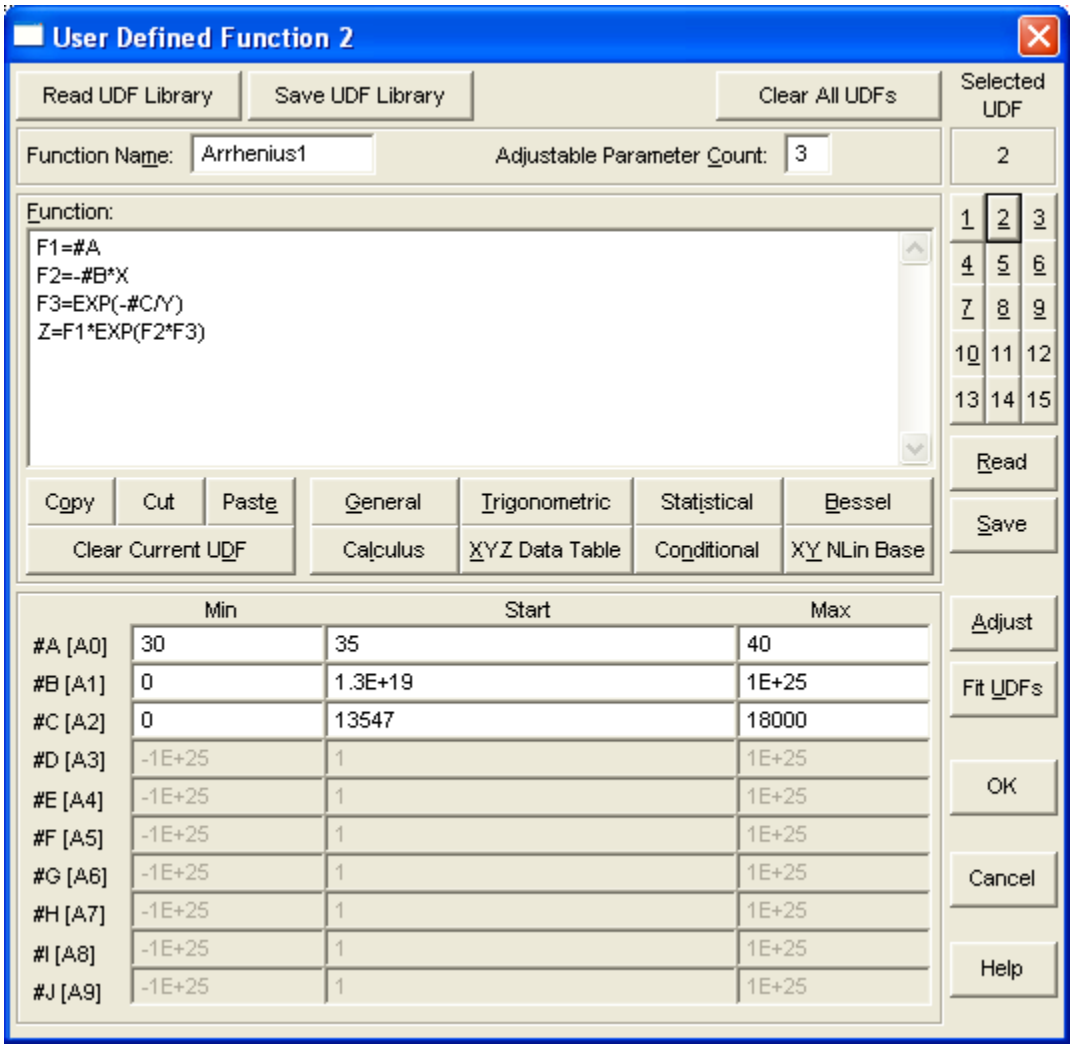


Figure 46: UDF of the Arrhenius First-Order Nonlinear Model for Control Low

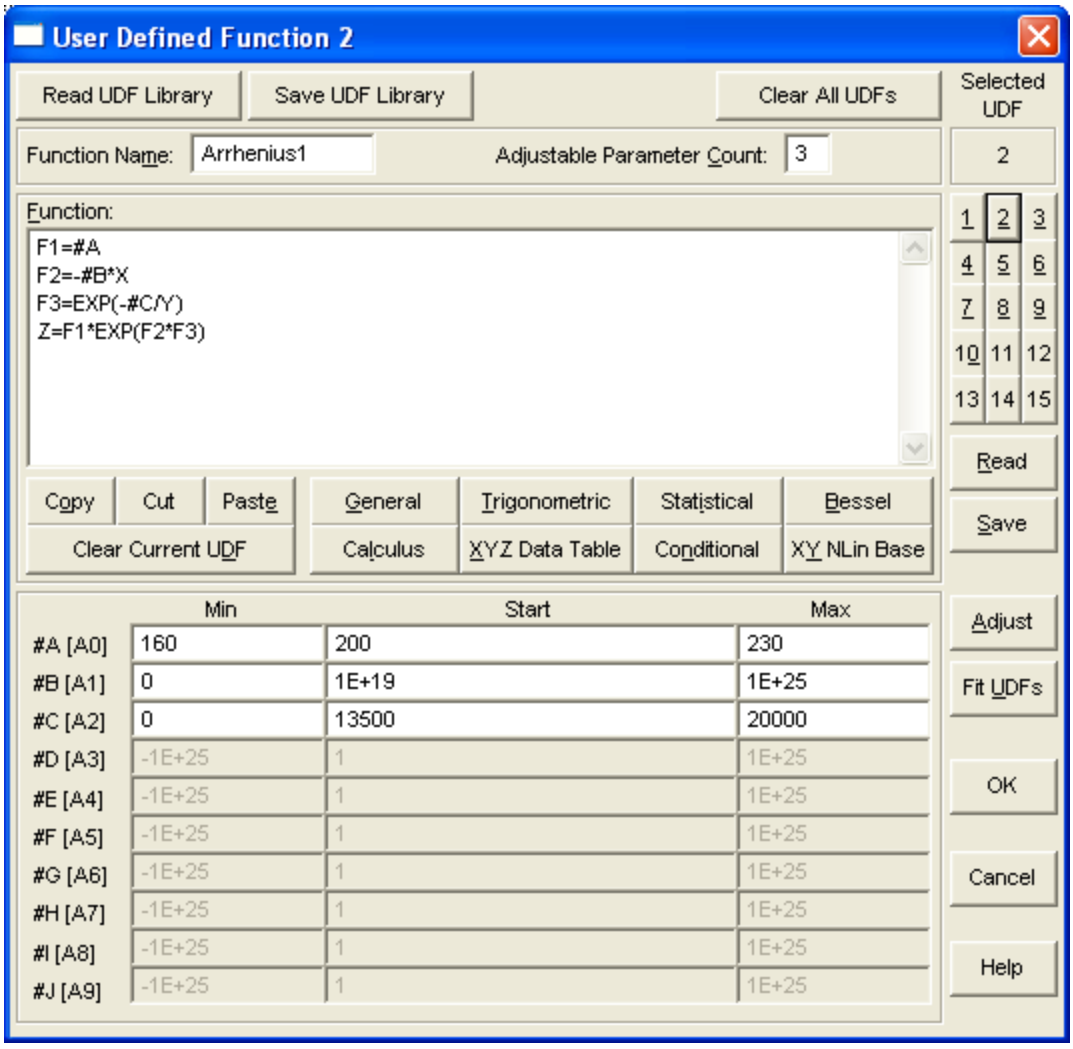


Figure 47: UDF of the Arrhenius First-Order Nonlinear Model for Control Medium

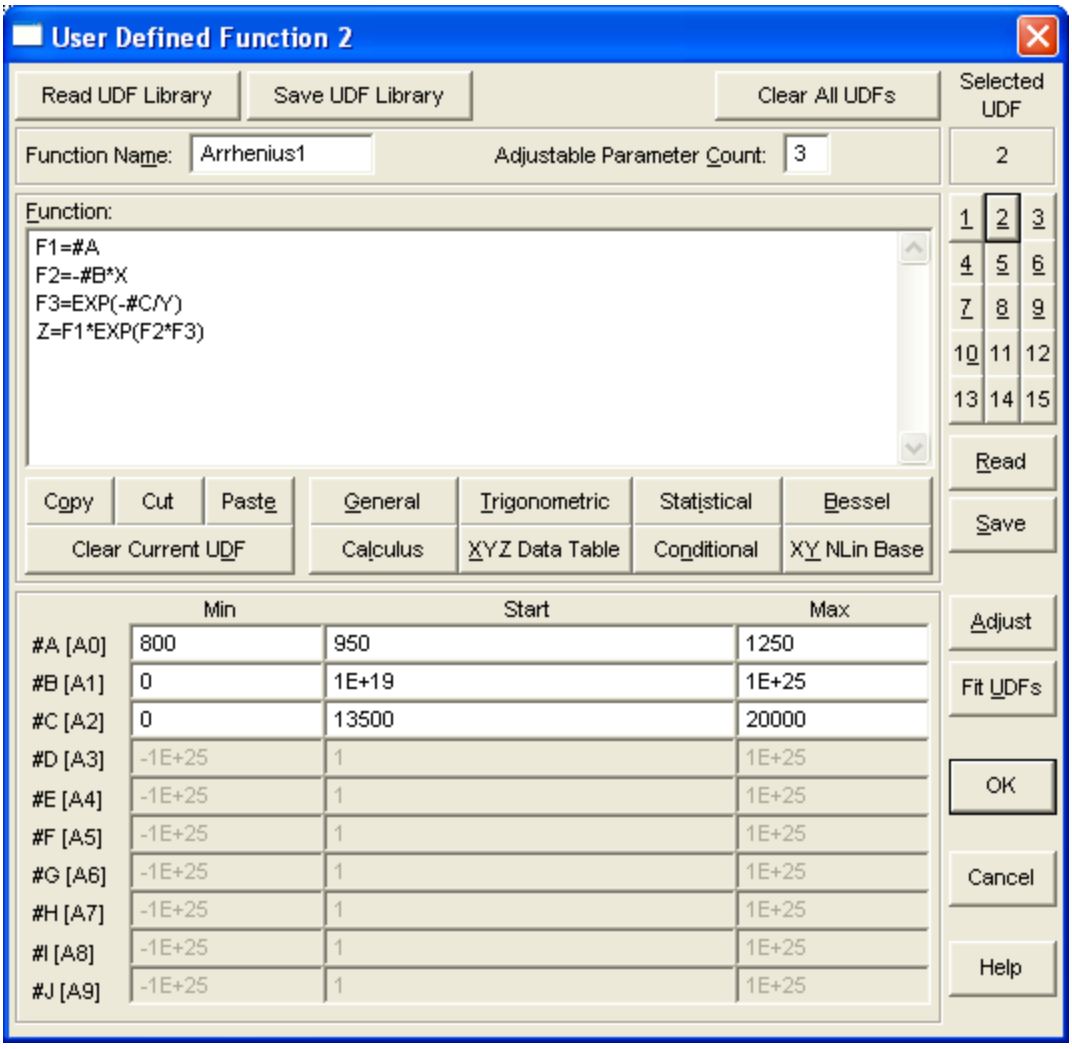


Figure 48: UDF of the Arrhenius First-Order Nonlinear Model for Control High

The following equations were obtained as a result of fitting an Arrhenius first-order nonlinear UDF for each of the response variable (Control Low, Medium and High) considered in the accelerated study.

- **For Control Low**

$$Z = 34.208396 \cdot e^{\left[-9.68489\times10^{19}\cdot t\cdot e^{\left(\frac{-15631.42554}{T}\right)}\right]} + \varepsilon \qquad (53)$$

- **For Control Medium**

$$Z = 185.97304 \cdot e^{\left[-7.42522\times10^{19}\cdot t\cdot e^{\left(\frac{-15533.92866}{T}\right)}\right]} + \varepsilon \qquad (54)$$

- **For Control High**

$$Z = 924.73156 \cdot e^{\left[-9.81331\times10^{19}\cdot t\cdot e^{\left(\frac{-1563254007}{T}\right)}\right]} + \varepsilon \quad (55)$$

Using some algebra modifications, the equations can be re-written as follows:

- **For Control Low**

$$Z = 34.208396 \cdot e^{\left[-t\cdot e^{\left(46.01968 - \frac{15631.42554}{T}\right)}\right]} + \varepsilon \quad (56)$$

- **For Control Medium**

$$Z = 185.97304 \cdot e^{\left[-t\cdot e^{\left(45.7540 - \frac{15533.92866}{T}\right)}\right]} + \varepsilon \quad (57)$$

- **For Control High**

$$Z = 924.73156 \cdot e^{\left[-t\cdot e^{\left(46.03286 - \frac{15632.54007}{T}\right)}\right]} + \varepsilon \quad (58)$$

Verifying the influence of different orders of reaction to the final parameters of the model it is observed in Table 10 that all parameters in the first order equation had a little increase in there final estimates when compared to the parameters of the zero-order reaction. If we verify the 95% confidence intervals of the parameters per control level, it can be concluded that there is no statistical difference between reactions orders for parameter *c* since these intervals overlap between each other. This is not the case for parameters *a* and *b*, since the majority of the confidence intervals do not overlap between each other. Parameter *c* is strictly related to the relation E_a /*R* of the Arrhenius equation, where E_a is the activation energy of the reaction and *R* is the gas constant. Therefore, since *R* is a constant, it can be preliminary concluded that the activation energy needed for degradation to occur in our product is going be practically the same in a zero and first order nonlinear model.

Table 10: Influence of Reaction Orders on Final Parameters of the Nonlinear Models

	Control Low					
	Zero-Order			First-Order		
Parameter	***a***	***b***	***c***	***a***	***b***	***c***
Final Estimate	32.91	1.81×10^{21}	15574.33	34.21	9.68×10^{19}	15631.43
95% Confidence Interval	32.63 – 33.18	2.49×10^{18} – 3.61×10^{21}	15258.4 – 15890.2	33.88 – 34.54	7.2×10^{18} – 2.0×10^{20}	15293.3 – 15969.6
	Control Medium					
	Zero-Order			**First-Order**		
Parameter	***a***	***b***	***c***	***a***	***b***	***c***
Final Estimate	178.17	6.94×10^{21}	15457.73	185.97	7.42×10^{19}	15533.92
95% Confidence Interval	176.51– 179.83	4.78×10^{20} – 1.43×10^{22}	15119.6 – 15795.9	184.00 – 187.94	1.01×10^{19} – 1.59×10^{20}	15176.6 – 15891.2
	Control High					
	Zero-Order			**First-Order**		
Parameter	***a***	***b***	***c***	***a***	***b***	***c***
Final Estimate	890.95	1.94×10^{22}	15281.81	924.73	9.81×10^{19}	15632.5
95% Confidence Interval	881.98 – 899.91	3.07×10^{21} – 4.18×10^{22}	14915.4 – 15648.3	914.29 – 935.17	2.51×10^{19} – 2.21×10^{20}	15237.4 – 16027.7

Figures 49 to 51 illustrate the surface fit plots that correspond to the nonlinear Arrhenius equations previously presented for a first-order reaction.

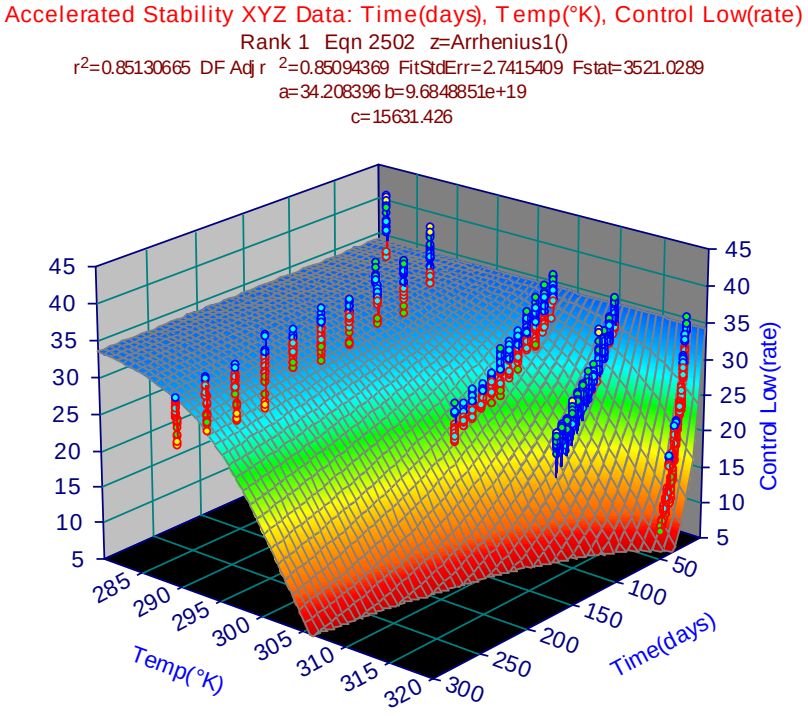


Figure 49: Surface Plot of the Arrhenius First-Order Nonlinear Model for Control Low

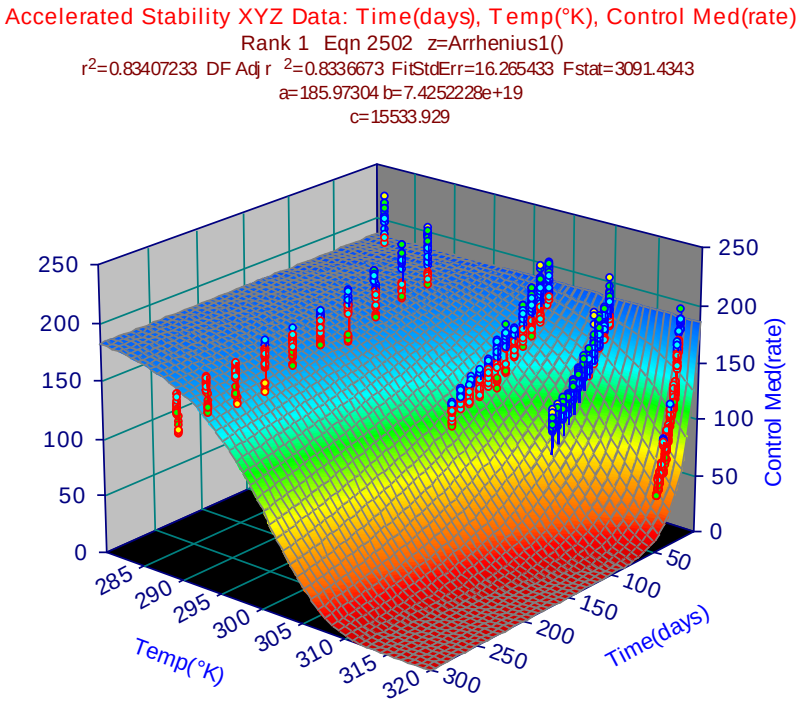


Figure 50: Surface Plot of the Arrhenius First-Order Nonlinear Model for Control Medium

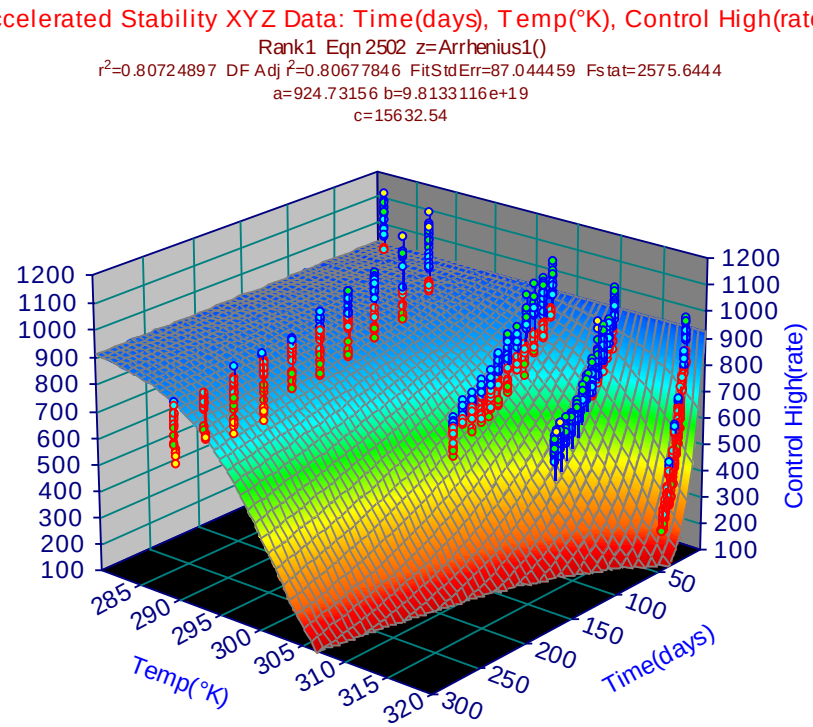


Figure 51: Surface Plot of the Arrhenius First-Order Nonlinear Model for Control High

As with the zero order models, these nonlinear first-order models were capable of considering results of product content at time zero (t_0). Therefore, they also provided the behavior that was expected in there surface plots since a lag of degradation was observed at the beginning of the study and at temperatures close to the storage condition (281 °K), then, a significant continuous degradation was observed as temperature and time increased.

Predictions of the rate values of each control were made at the expiration date (180 days) and at the storage temperature (8°C or 281°K) by using the equations that described these plots. Table 11 illustrates the results obtained for the predicted rate value of each control level at the expiration date.

Table 11: Prediction of Controls Rate Values using the Arrhenius First-Order Model

Control Level	Mean rate value at t=0	Storage Temp. (°K)	Expiration Date (days)	Predicted rate value at Exp. Date	95% Prediction Interval
Low	36.75	281	180	33.80	28.41 – 39.18
Medium	201.38	281	180	183.55	151.59 – 215.51
High	1007.60	281	180	913.52	742.49 – 1084.54

Even though the predictions of the product content (rate values) at the expiration date indicated to be within the stability specifications established in Table 7, these predictions not necessarily represented the expected behavior of the product used for the accelerated stability study since it is previously known that the product content loss of the product, measured as a percent difference of rate values between t=180 and t=0, must be between 10 and 15 percent. In this case, the predictions provided by the Arrhenius first-order nonlinear model indicated a product content loss of 8.0, 8.9 and 9.3 percent for controls low, medium and high, respectively. This incongruence with the expected behavior highlights a possible problem with the partial derivatives plots of the model as it was previously observed with the Polynomial Function Model. The verification of these partial derivatives plots will be performed in Section 4.4.4.2.

4.4.4.1 Statistical Analyzes for the Arrhenius First-Order Nonlinear Model

Significance of statistical test results provided by the Arrhenius first-order nonlinear model were addressed in the same way it was done for the previous three models for the purpose of having a comparative analysis for the selection of the most appropriate model. Verifying the results in Figures 52 to 54 it can be said that all models appeared to be

significant to relate *product content*, *time* and *temperature* since a *p-value* = 0.0000 was obtained for all control levels. In contrast, when testing the significance of individual regression coefficients, it is observed that there is an increase in the *p-values* for parameter *b* of the equation. In specific, this parameter appears to be significant at the 90% confidence level for the models fitted to control low and control medium data, but not in the case of the model fitted to control high data. Since the nonlinear equation for an Arrhenius first-order reaction is an expression that has all the parameters multiplied with each order, then accepting the hypothesis test that the coefficient is equal to zero, would delete the complete expression for the Arrhenius first-order reaction. Therefore, this was not an option for reducing the model.

In addition, even though the models for each control level provided a little increase in the result of the R^2_{Adj} statistic, the predictions obtained with them were incongruent with the expected degradation making the model suspicious and not attractive.

Rank 1 z=Arrhenius-First Order

Parameter	*Value*	*Std Error*	*t-value*	*95.00% Confidence Limits*		*P>|t|*
a	34.20839564	0.167302231	204.4706485	33.88016631	34.53662498	0.00000
b	9.68489e+19	5.30505e+19	1.825596262	7.2307e+18	2.00928e+20	0.06815
c	15631.42554	172.3693414	90.6856487	15293.25507	15969.59601	0.00000

Procedure	*Minimization*	*Iterations*
Lev-Marq	Least Squares	100

r^2 Coef Det	*DF Adj r^2*	*Fit Std Err*
0.8513066489	0.850943687	2.7415408516

Analysis of Variance

Source	*Sum of Squares*	*DF*	*Mean Square*	*F Statistic*	*P>F*
Regr	52928.432	2	26464.216	3521.03	0.00000
Error	9244.7369	1230	7.5160462		
Total	62173.169	1232			
Lack Fit	6363.3332	45	141.40741	58.1549	0.00000
Pure Error	2881.4036	1185	2.4315643		

Description: Accelerated Stability XYZ Data: Time(days), Temp(°K), Control Low(rate)

Figure 52: Statistical Analysis for Arrhenius First-Order Nonlinear Model Fitted to Control Low Data

Rank 1 z=Arrhenius-First Order

Parameter	Value	Std Error	t-value	95.00% Confidence Limits		P>|t|
a	185.9730389	1.004179687	185.1989651	184.0029443	187.9431335	0.00000
b	7.42522e+19	4.29858e+19	1.727367977	1.0081e+19	1.58586e+20	0.08435
c	15533.92866	182.115185	85.29727303	15176.63787	15891.21944	0.00000

Procedure	Minimization	Iterations
Lev-Marq	Least Squares	100

r^2 Coef Det	DF Adj r^2	Fit Std Err
0.8340723304	0.8336672994	16.265433303

Analysis of Variance

Source	Sum of Squares	DF	Mean Square	F Statistic	P>F
Regr	1635766.4	2	817883.21	3091.43	0.00000
Error	325414.11	1230	264.56432		
Total	1961180.5	1232			
Lack Fit	224160.98	45	4981.3551	58.2985	0.00000
Pure Error	101253.13	1185	85.445683		

Description: Accelerated Stability XYZ Data: Time(days), Temp(°K), Control Med(rate)

Figure 53: Statistical Analysis for Arrhenius First-Order Nonlinear Model Fitted to Control Medium Data

Rank 1 z=Arrhenius-First Order

Parameter	Value	Std Error	t-value	95.00% Confidence Limits		P>|t|
a	924.7315551	5.320622313	173.8013903	914.2930553	935.1700549	0.00000
b	9.81331e+19	6.28105e+19	1.562367304	-2.5095e+19	2.21361e+20	0.11846
c	15632.54007	201.4001651	77.61930118	15237.41419	16027.66595	0.00000

Procedure	Minimization	Iterations
Lev-Marq	Least Squares	100

r^2 Coef Det	DF Adj r^2	Fit Std Err
0.8072489672	0.8067784602	87.044458536

Analysis of Variance

Source	Sum of Squares	DF	Mean Square	F Statistic	P>F
Regr	39029964	2	19514982	2575.64	0.00000
Error	9319387.4	1230	7576.7378		
Total	48349352	1232			
Lack Fit	5826979.3	45	129488.43	43.9364	0.00000
Pure Error	3492408.2	1185	2947.1799		

Description: Accelerated Stability XYZ Data: Time(days), Temp(°K), Control High(rate)

Figure 54: Statistical Analysis for Arrhenius First-Order Nonlinear Model Fitted to Control High Data

4.4.4.2 Advantages and Disadvantages of the Arrhenius First-Order Nonlinear Model

After reviewing the results obtained for the Arrhenius first-order nonlinear model and comparing them with the results of the other models previously discussed, we can say that this model shares a lot of the advantages presented by the nonlinear model for a zero-order reaction. These similarities can be summarized in the following bullets:

- The specific Arrhenius kinetic relation is employed in a one-step nonlinear approach that fits the accelerated stability data obtained.
- The regression equations demonstrated to be significant to relate *product content*, *time*, and *temperature* since a *p-value* = 0.00000 was obtained for the surface fit of all control levels.
- It is capable of considering data of the response variable at time zero (t_0) and the experimental error associated with the total number of values obtained from the accelerated stability test (n = 1233).
- In terms of parameters, is much simpler than the Polynomial Function Model and still provide good results for the R^2_{Adj} statistic.
- The 95% prediction intervals of the estimations performed at the expiration date where much smaller and symmetrical, than the intervals provided by the Arrhenius classical approach.

In the other hand, the Arrhenius first-order nonlinear model had several disadvantages that make it less attractive than the nonlinear model for a zero-order reaction. These disadvantages are summarized as follows:

- An increase in the result of the *p-values* was observed when testing significance of the individual regression coefficients at the 90% confidence level. In specific, parameter *b* resulted to be not significant for the model fitted to control high data since a *p-value* higher than 0.10 was obtained. Therefore, if this parameter was eliminated from the equation, then, the Arrhenius approach would no longer applied since we will be eliminating a parameter that is characteristic of the product failure mechanism in the Arrhenius Law for chemical reactions (known as A).

- The model was not able to provide predictions that were aligned with the expected behavior of product content loss at the expiration date since the percent difference of rate values between t=180 and t=0 was not between the range of 10 and 15 percent. Abbott scientists previously characterized this range by using real-time stability monitoring procedures. Therefore, these prediction did not match the degradation rate that was expected for the product been used.
- There is no simple interpretation to the behavior observed in the partial derivatives plots of the response variable with respect to the regressor variables, since the expected behavior of these plots is to have a surface intercepting the vertical $\partial Z/\partial X$ or $\partial Z/\partial Y$ axis at a value equal to zero, and then to have a continuous downward orientation of the curve as degradation rate increase. Therefore, considering once again the partial derivative plot for Control Low with respect to changes in temperature (Figures 58) as an example, it is observed that even though the surface intersects the $\partial Z/\partial Y$ axis at a value equal to zero, the curve has a downward cone shape that do not match the chemical-physical behavior expected. This same strange behavior is also observed in the other partial derivative plots of the model. Refer to Figures 55 to 57 for the partial derivative surface plots on the performance of control levels with respect to changes in time $(\partial Z/\partial X)$ and Figures 58 to 60 for the partial derivative surface plots on the performance of control levels with respect to changes in temperature $(\partial Z/\partial Y)$. This dissimilarities of the partial derivative plots with the true chemical-physical behavior expected by Abbott scientists may probably be the main cause why the nonlinear model for a first-order reaction provided estimates of the shelf-life with less degradation than what it was it obtained with the nonlinear model for a zero-order reaction.

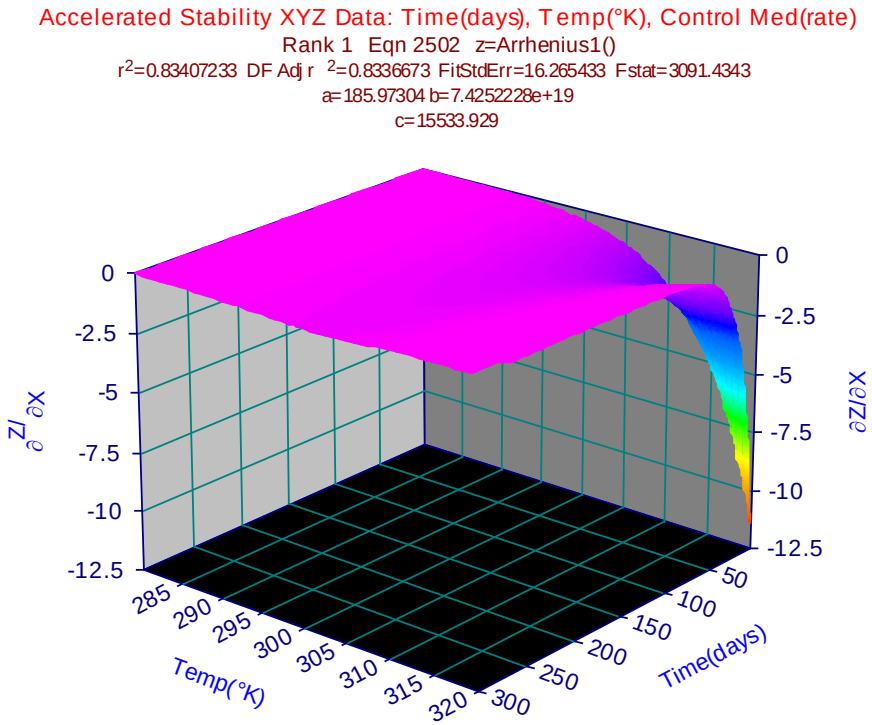


Figure 55: Partial Derivative Plot of Control Low with respect to changes in time for the Arrhenius First-Order Nonlinear Model

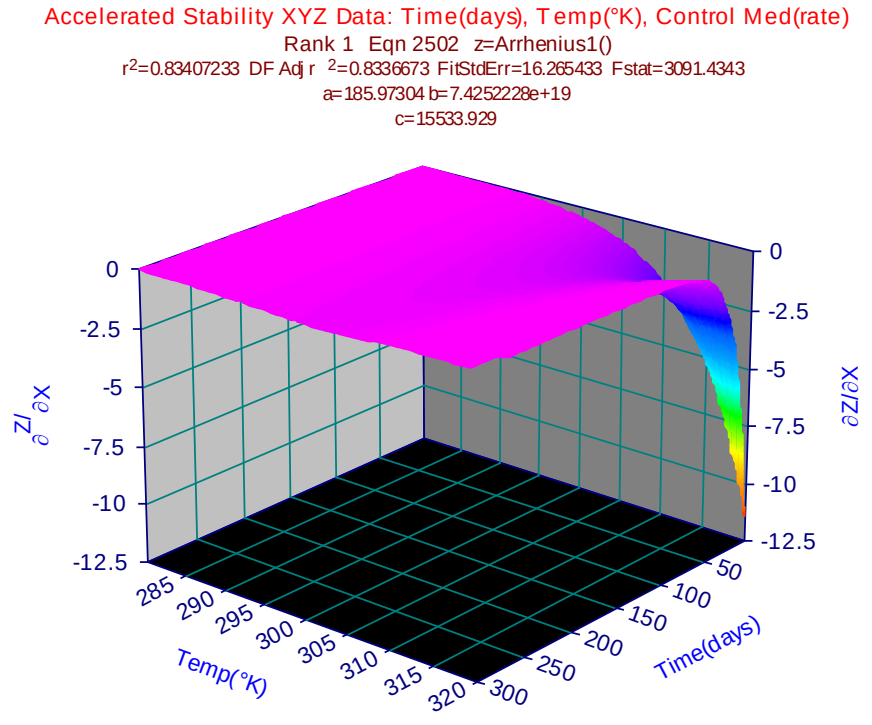


Figure 56: Partial Derivative Plot of Control Medium with respect to changes in time for the Arrhenius First-Order Nonlinear Model

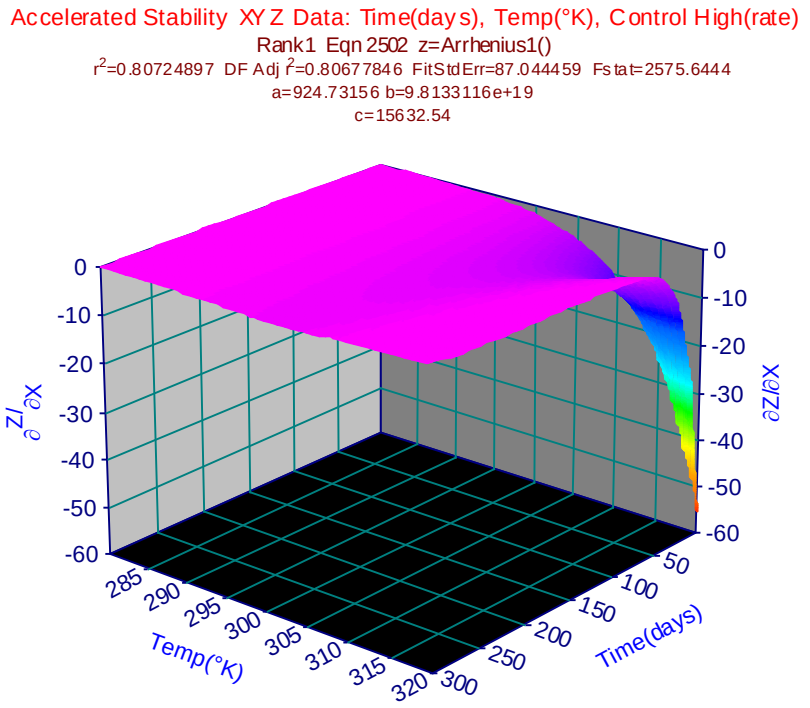


Figure 57: Partial Derivative Plot of Control High with respect to changes in time for the Arrhenius First-Order Nonlinear Model

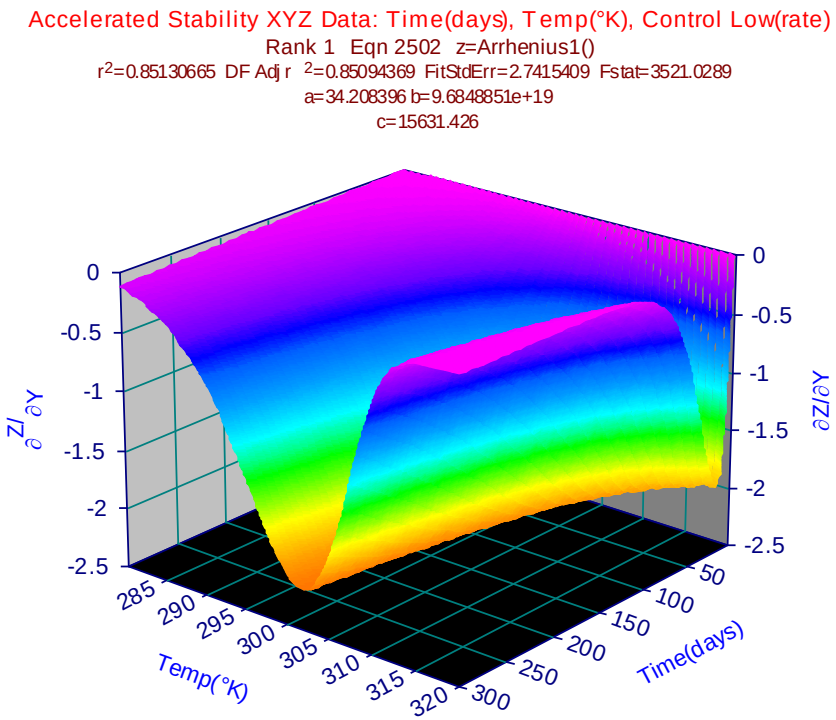


Figure 58: Partial Derivative Plot of Control Low with respect to changes in temperature for the Arrhenius First-Order Nonlinear Model

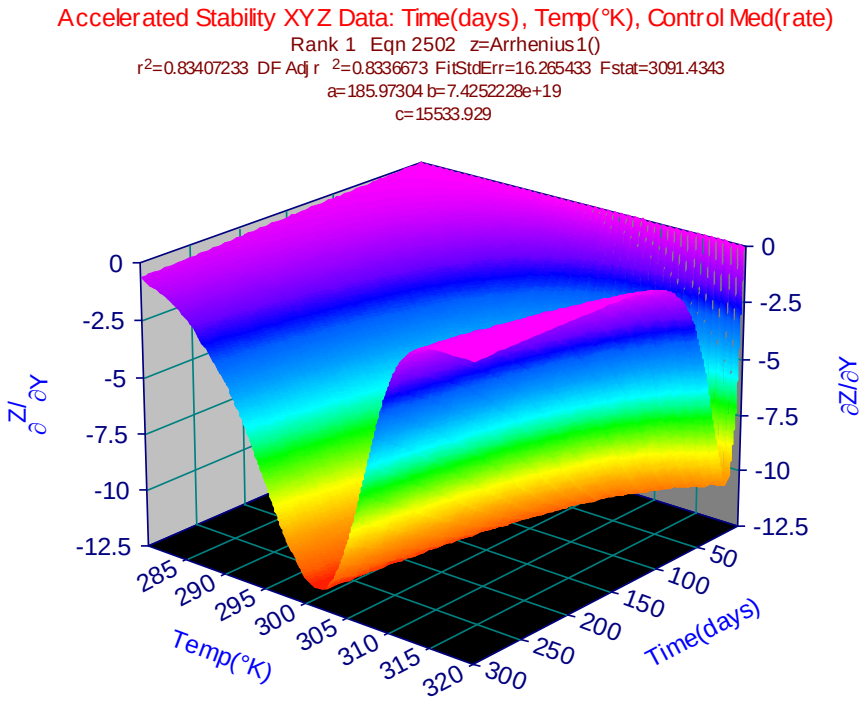


Figure 59: Partial Derivative Plot of Control Medium with respect to changes in temperature for the Arrhenius First-Order Nonlinear Model

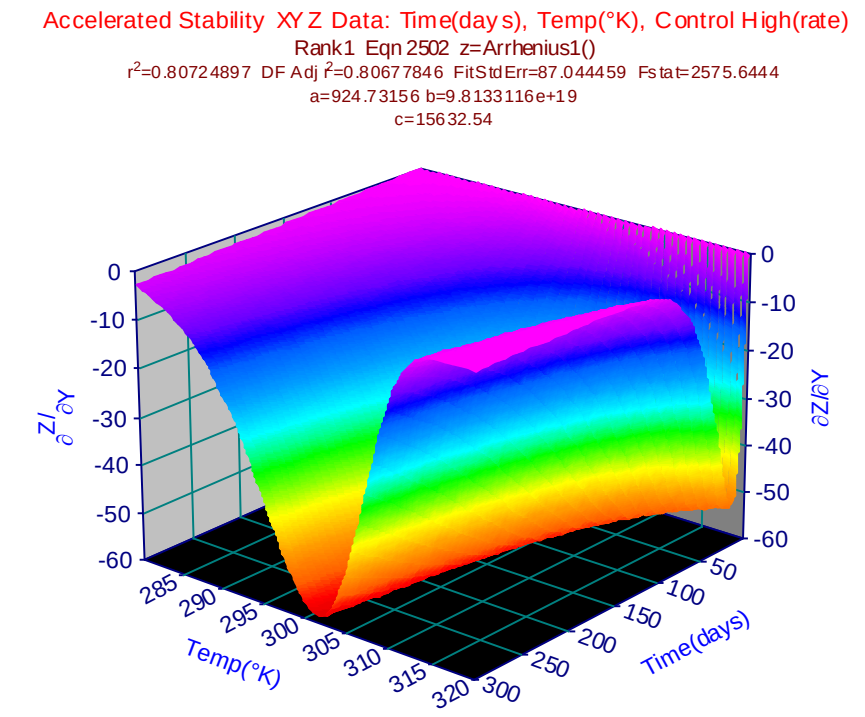


Figure 60: Partial Derivative Plot of Control High with respect to changes in temperature for the Arrhenius First-Order Nonlinear Model

4.5 Model Selection

Based on the relative merits and limitations that were discussed in detail for all the models developed to fit the accelerated stability data it was concluded that the Arrhenius nonlinear model for a zero-order reaction was the most appropriate to estimate degradation by means of an accelerated stability test since it provided relevant statistics and compliance with the underlying assumptions of the Arrhenius Life-Temperature relationship because the degradation of the product that was used, is caused by the interaction of one molecule of interest. Therefore, a zero-order reaction applies to this type of interaction. The major advantages provided by the model were:

- The model provides a well fit of the complete accelerated stability data by relating *product content*, *time* and *temperature* in one equation.
- The regression model as well as all individual regression coefficients demonstrated to be statistically significant at a 90% confidence level.
- The model demonstrated to provide adequate predictions of the product content at the expiration date.
- The behaviors of the partial derivative plots were compatible with the chemical-physical behavior expected by Abbott scientists.

Therefore, Chapter 5 will present a validation procedure that was developed for the purpose of further evaluating the benefits that this model could provide.

5 Validation

5.1 Introduction

Chapter 5 will focus on the development of a procedure that would validate the statistical model selected in Chapter 4 as the most appropriate model to estimate degradation by means of an accelerated stability test. This validation procedure will use the lot-to-lot variability characterized in the accelerated test to develop prediction intervals that could be used routinely to compare degradation patterns of future lots at elevated temperatures and conclude if these future lots have similar degradation patterns as the previous three good lots that were used to gather the accelerated stability data. Is important to clarify that the three lots used to gather the data were released for sell in the market and demonstrated good performance in the customer hands until their expiration date. Therefore, if any future lot exposed to the same elevated temperatures provide results within the prediction intervals developed with the variability of the previous three good lots, then, it is expected that the future lot will degrade similar at the storage temperature until the shelf-life (expiration date). Section 5.2 will discuss the type of prediction intervals used for the validation procedure and how they were developed. Sections 5.3 and 5.4 provide the results obtained when applying the validation procedure to identify lots with and without stability issues.

5.2 Intervals to Compare Degradation Patterns

The types of statistical intervals used to compare degradation patterns are prediction intervals to contain **all** of *m* individual future observations. These intervals are different from the usual given by statistical softwares, which contain the mean of future observations. As indicated by Hahn and Meeker [28], "these types of prediction intervals are often referred as *simultaneous* prediction intervals, because we are concerned with simultaneously containing *all* of the *m* observations within the calculated interval (with the associated level of confidence)". Therefore, a two sided 100(1-α)% simultaneous prediction interval to contain the values of all of *m* future observation from a previously sample population is given by

$$\bar{x} \pm r_{(1-\alpha;m,n)} \cdot s \tag{59}$$

where:

$\bar{x}$ = sample mean

s = sample standard deviation

$1 - \alpha$ = confidence level

m = number of future observations

n = number of previous observations

These prediction intervals were determined using the degradation data obtained from the accelerated stability test of three lots (n = 1233), which was best fitted by an Arrhenius zero-order nonlinear model. They were determined for the same stress temperatures considered in the accelerated study (45, 37, 30 and 17 °C) and for some specific time points distributed across the time range experimented for each stress temperature. The reason for only using some specific time points of the accelerated study was because it would take more than one year to repeat the complete study for any future lot. Therefore, it was decided to determined prediction intervals for four time points distributed across the time range previously experimented for the temperatures of 45, 37, and 30°C.

Fewer units were tested at the temperature of 17°C since the product degrades more slowly at this temperature level. For this reason, it was decided only to use prediction intervals for the first three time points of this temperature condition (17°C) since they will cover the degradation pattern of 90 days for the future lot. Each of the future lots exposed to the stress temperatures and time points considered in the validation procedure will provide nine replicates of the rate values for each of the response variable (Control Low, Medium and High) considered in the study and tested at these conditions.

The biggest concern with these prediction intervals was the selection of the appropriate confidence level, since it was required to have an interval capable of discriminating between a lot with and without stability issues. Therefore, we did not wanted to have an interval wide enough that it would indicate that a lot is good, even when the lot truly has performance issues, or vice versa; to have an stringent interval that would end up flagging good lots as if they would have stability issues. After gathering the date of the future lots in the validation procedure, it was decided to use predictions intervals at the 90% confidence level since they demonstrated to discriminate a lot with

stability issues better than prediction intervals at the 95% confidence level. This will be discussed in detail in Section 5.4.

The 90 and 95% prediction intervals to contain the rate values of all m = 9 replicates of each control level, were determined using the following expressions:

$$\bar{x} \pm r_{(0.90;\, 9,\, \infty)} \cdot s \tag{60}$$

$$\bar{x} \pm r_{(0.95;\, 9,\, \infty)} \cdot s \tag{61}$$

The sample mean and sample standard deviation in Equations 60 and 61 are obtained from the outputs predicted by Table Curve 3D for each specific time and temperature condition experimented with the previous three good lots, which were fitted by an Arrhenius nonlinear model for a zero-order reaction. The factor $r_{(1-\alpha;\, m,\, n)}$ can be obtained using Table A.13 in Hahn and Meeker's book [28]. Is important to clarify that since n is a large number, in our case (n = 1233), this parameter can be considered infinite (∞) in the factor $r_{(1-\alpha;\, m,\, n)}$. Therefore, using Table A.13, it is obtained that $r_{(0.90;\, 9,\, \infty)} = 2.523.$ and $r_{(0.95;\, 9,\, \infty)} = 2.766.$

Tables 12 and 13 contain the time points for which prediction intervals were calculated at 90 and 95% confidence level to compare degradation patterns at the stress temperatures considered and to discriminate a future lot with or without stability issues. Even though Tables 12 and 13 only contains prediction intervals for the specific time points that were used for discriminating a lot in the validation procedure, Appendixes 3 and 4 illustrates the Excel spreadsheet tables that were developed to determine 90 and 95% prediction intervals to contain m = 9 future observations for all of the combinations of time and temperature conditions used in the accelerated stability study with the initial three good lots. Each of these appendixes contains three tables that are related to each of the response variable (Control Low, Medium and High) considered in the study.

Table 12: 90% Prediction Intervals to Contain all *m*=9 Future Observations

Control level	Temperature Condition			
	45°C	**37°C**	**30°C**	**17°C**
Low	**Day 4** **90% Pred. Interval:** 21.97 – 36.07	**Day 6** **90% Pred. Interval:** 24.21 – 38.31	**Day 10** **90% Pred. Interval:** 24.99 – 39.09	**Day 30** **90% Pred. Interval:** 25.60 – 39.70
	Day 10 **90% Pred. Interval:** 16.14 – 30.24	**Day 18** **90% Pred. Interval:** 20.92 – 35.01	**Day 20** **90% Pred. Interval:** 24.13 – 38.24	**Day 60** **90% Pred. Interval:** 25.34 – 39.44
	Day 12 **90% Pred. Interval:** 14.20 – 28.30	**Day 30** **90% Pred. Interval:** 17.62 – 31.72	**Day 30** **90% Pred. Interval:** 23.28 – 37.38	**Day 90** **90% Pred. Interval:** 25.08 – 39.18
	Day 18 **90% Pred. Interval:** 8.36 – 22.48	**Day 42** **90% Pred. Interval:** 14.33 – 28.43	**Day 40** **90% Pred. Interval:** 22.42 – 36.51	**N/A**
Medium	**Day 4** **90% Pred. Interval:** 114.36 – 198.99	**Day 6** **90% Pred. Interval:** 126.66 – 211.29	**Day 10** **90% Pred. Interval:** 131.01 – 215.64	**Day 30** **90% Pred. Interval:** 134.38 – 219.02
	Day 10 **90% Pred. Interval:** 82.11 – 166.74	**Day 18** **90% Pred. Interval:** 108.28 – 192.87	**Day 20** **90% Pred. Interval:** 126.18 – 210.79	**Day 60** **90% Pred. Interval:** 132.91 – 217.54
	Day 12 **90% Pred. Interval:** 71.35 – 155.99	**Day 30** **90% Pred. Interval:** 89.89 – 174.47	**Day 30** **90% Pred. Interval:** 121.34 – 205.94	**Day 90** **90% Pred. Interval:** 131.43 – 216.06
	Day 18 **90% Pred. Interval:** 39.06 – 123.78	**Day 42** **90% Pred. Interval:** 71.48 – 156.08	**Day 40** **90% Pred. Interval:** 116.50 – 201.09	**N/A**
High	**Day 4** **90% Pred. Interval:** 559.44 – 1013.71	**Day 6** **90% Pred. Interval:** 618.52 – 1072.78	**Day 10** **90% Pred. Interval:** 639.62 – 1093.93	**Day 30** **95% Pred. Interval:** 656.21 – 1110.57
	Day 10 **90% Pred. Interval:** 402.87 – 857.16	**Day 18** **90% Pred. Interval:** 528.02 – 982.09	**Day 20** **90% Pred. Interval:** 615.50 – 1069.71	**Day 60** **95% Pred. Interval:** 648.67 – 1102.98
	Day 12 **90% Pred. Interval:** 350.65 – 805.01	**Day 30** **90% Pred. Interval:** 437.44 – 891.47	**Day 30** **90% Pred. Interval:** 591.36 – 1045.50	**Day 90** **95% Pred. Interval:** 641.13 – 1095.40
	Day 18 **90% Pred. Interval:** 193.87 – 648.66	**Day 42** **90% Pred. Interval:** 346.77 – 800.94	**Day 40** **90% Pred. Interval:** 567.20 – 1021.31	**N/A**

Table 13: 95% Prediction Intervals to Contain all *m*=9 Future Observations

Control level	Temperature Condition			
	45°C	**37°C**	**30°C**	**17°C**
Low	**Day 4** **95% Pred. Interval:** 21.29 – 36.75	**Day 6** **95% Pred. Interval:** 23.53 – 38.99	**Day 10** **95% Pred. Interval:** 24.32 – 39.78	**Day 30** **95% Pred. Interval:** 24.92 – 40.38
	Day 10 **95% Pred. Interval:** 15.46 – 30.92	**Day 18** **95% Pred. Interval:** 20.24 – 35.69	**Day 20** **95% Pred. Interval:** 23.46 – 38.91	**Day 60** **95% Pred. Interval:** 24.66 – 40.12
	Day 12 **95% Pred. Interval:** 13.52 – 28.98	**Day 30** **95% Pred. Interval:** 16.95 – 32.40	**Day 30** **95% Pred. Interval:** 22.60 – 38.05	**Day 90** **95% Pred. Interval:** 24.40 – 39.86
	Day 18 **95% Pred. Interval:** 7.68 – 23.16	**Day 42** **95% Pred. Interval:** 13.65 – 29.11	**Day 40** **95% Pred. Interval:** 21.74 – 37.19	**N/A**
Medium	**Day 4** **95% Pred. Interval:** 110.29 – 203.06	**Day 6** **95% Pred. Interval:** 122.59 – 215.36	**Day 10** **95% Pred. Interval:** 126.94 – 219.72	**Day 30** **95% Pred. Interval:** 130.30 – 223.09
	Day 10 **95% Pred. Interval:** 78.03 – 170.81	**Day 18** **95% Pred. Interval:** 104.21 – 196.94	**Day 20** **95% Pred. Interval:** 122.10 – 214.86	**Day 60** **95% Pred. Interval:** 128.83 – 221.61
	Day 12 **95% Pred. Interval:** 67.28 – 160.07	**Day 30** **95% Pred. Interval:** 85.82 – 178.54	**Day 30** **95% Pred. Interval:** 117.27 – 210.01	**Day 90** **95% Pred. Interval:** 127.36 – 220.13
	Day 18 **95% Pred. Interval:** 34.98 – 127.86	**Day 42** **95% Pred. Interval:** 67.40 – 160.16	**Day 40** **95% Pred. Interval:** 112.42 – 205.16	**N/A**
High	**Day 4** **95% Pred. Interval:** 537.56 – 1035.59	**Day 6** **95% Pred. Interval:** 596.64 – 1094.66	**Day 10** **95% Pred. Interval:** 617.75 – 1115.80	**Day 30** **95% Pred. Interval:** 634.33 – 1132.45
	Day 10 **95% Pred. Interval:** 380.99 – 879.04	**Day 18** **95% Pred. Interval:** 506.15 – 1003.95	**Day 20** **95% Pred. Interval:** 593.62 – 1091.58	**Day 60** **95% Pred. Interval:** 626.79 – 1124.86
	Day 12 **95% Pred. Interval:** 328.77 – 826.89	**Day 30** **95% Pred. Interval:** 415.57 – 913.34	**Day 30** **95% Pred. Interval:** 569.49 – 1067.37	**Day 90** **95% Pred. Interval:** 619.26 – 1117.27
	Day 18 **95% Pred. Interval:** 171.97 – 670.56	**Day 42** **95% Pred. Interval:** 324.90 – 822.81	**Day 40** **95% Pred. Interval:** 545.33 – 1043.18	**N/A**

5.3 Results for a lot without stability issues

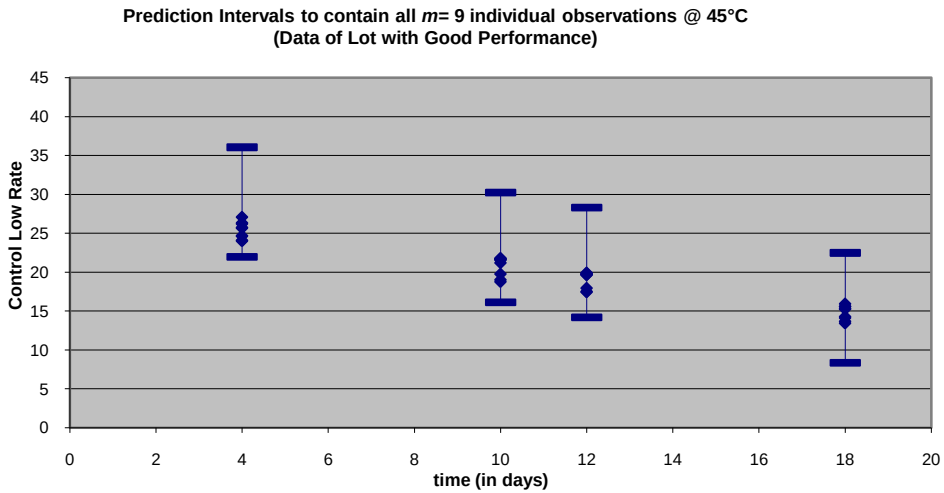
Once completed the data gathering of additional accelerated stability test point of a new reagent lot approved for market, the information was tabulated in an Excel spreadsheet for the purpose of comparing the data against the prediction intervals determined from the most adequate model that fitted the data from the initial three lots. As mentioned previously in Section 5.2, these statistical intervals were determined for all the stress temperatures used with the previous three lots (45, 37, 30 and 17 °C) to accelerate degradation and for some specific time points distributed across the time range experimented for each of these stress temperatures.

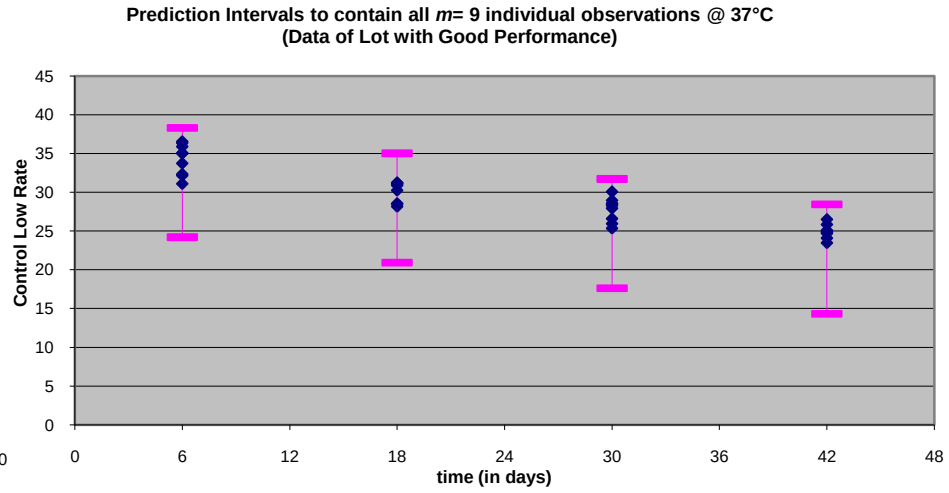
Figures 61 to 63 illustrate the results obtained for each of the response variables considered in the study (Control Low, Medium and High) against 90% prediction intervals for the four stress temperatures considered in the accelerated study. Verifying the results for Control Low in Figure 61 it is observed that all of the $m = 9$ individual observations for each of the specific time points considered in the validation procedure are within the 90% prediction intervals that were determined from the initial three lots. The same results are observed in Figures 62 and 63 for the response variable Control Medium and High.

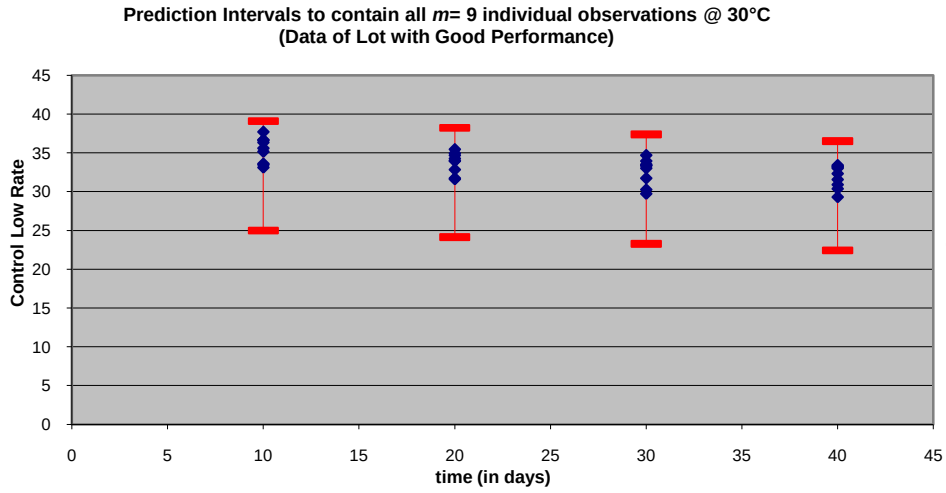
In addition to the prediction intervals determined at the 90% confidence level, we also determined prediction intervals at the 95% confidence level since preliminary when these statistical intervals were calculated, we did not know if the 90% prediction intervals would be to stringent to fail a lot that is truly good, or vice versa, to accept a lot that is truly bad as it will be shown in Section 5.4. Therefore, in the case of assessing a true good lot, if the 90% prediction intervals where capable of this discrimination, then the 95% prediction intervals will also be, as it is demonstrated in Figures 64 to 66 for each of the response variables considered.

Therefore, since all of the replicates of the new lot are within the prediction intervals calculated from the most adequate model that fitted the data from the initial three lots, then it can be concluded that this new lot has similar degradation patterns (at the four elevated temperatures) as the previous three good lots used in the developmental phase of the study. Thus, we are at most 95% confidence that it will degrade similar at the storage temperature until its expiration date.

Figure 61: Control Low Degradation Patterns of Lot with Good Performance against 90% Pred. Intervals at Different Stress Temperatures







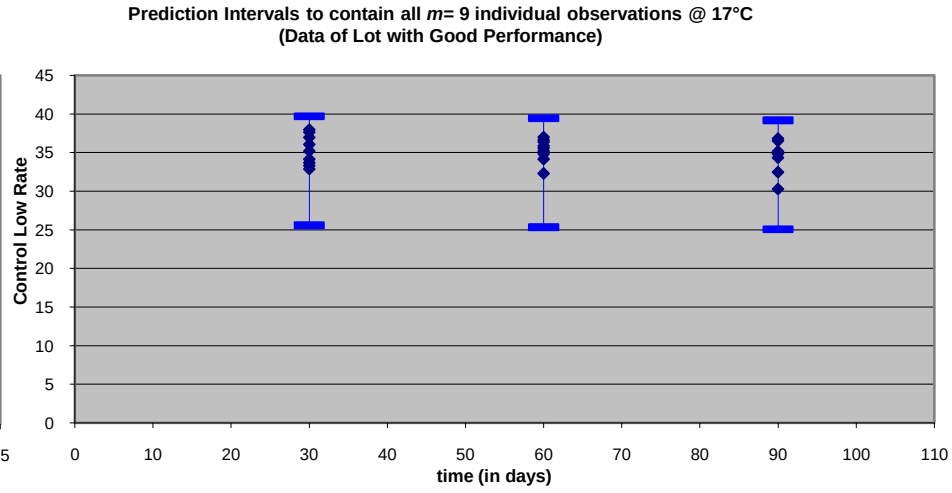


Figure 62: Control Medium Degradation Patterns of Lot with Good Performance against 90% Pred. Intervals at Different Stress Temperatures

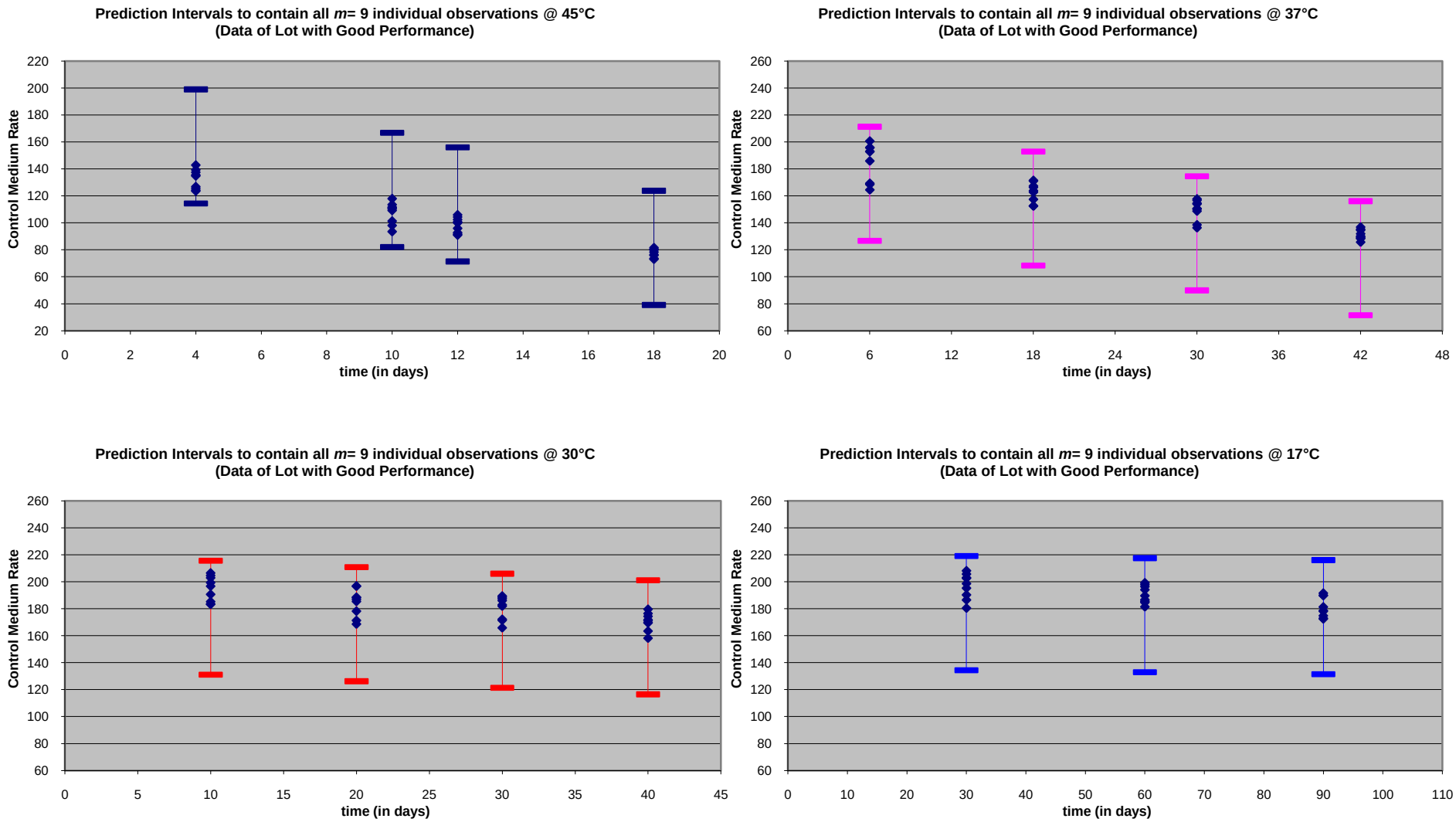
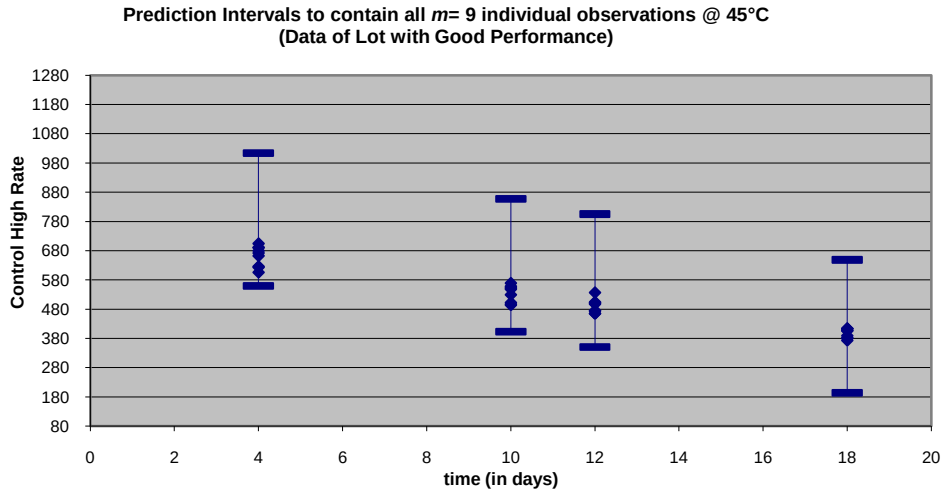
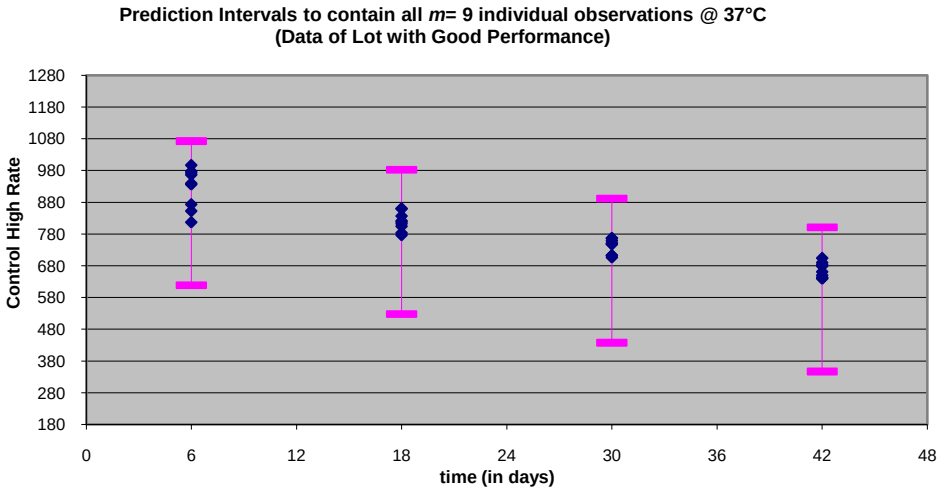
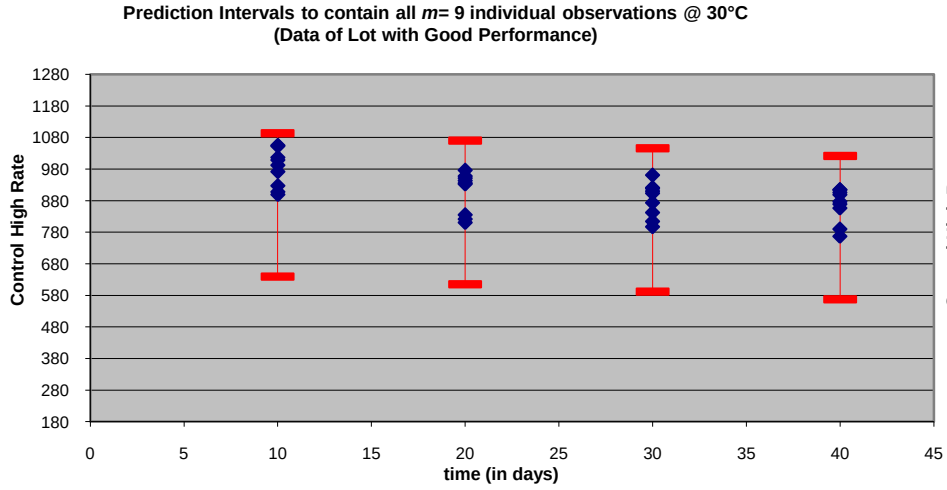


Figure 63: Control High Degradation Patterns of Lot with Good Performance against 90% Pred. Intervals at Different Stress Temperatures







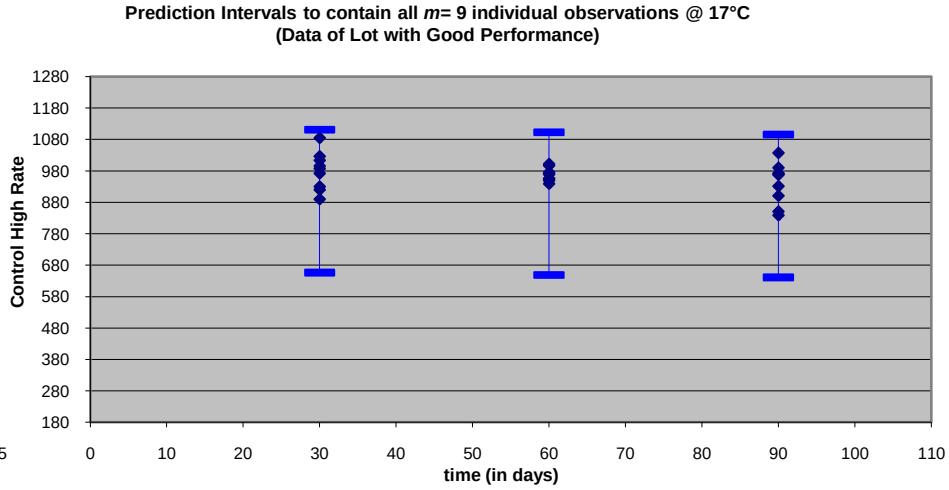
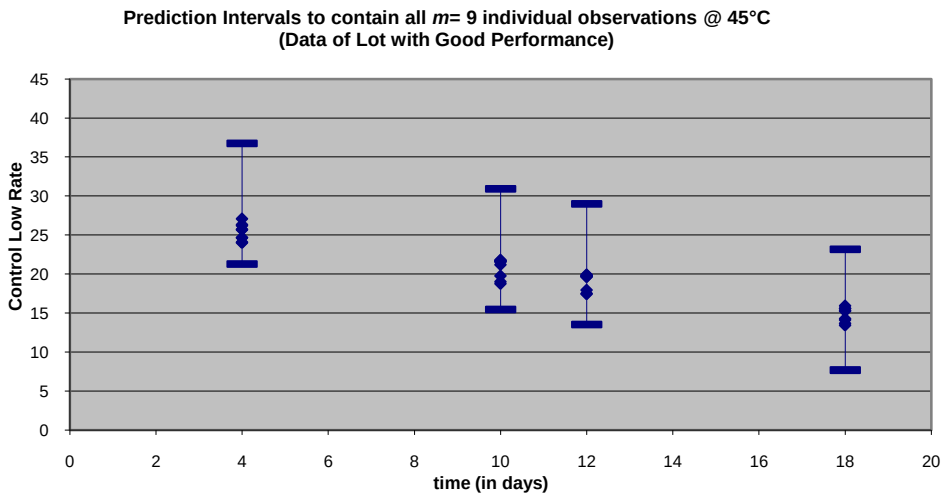
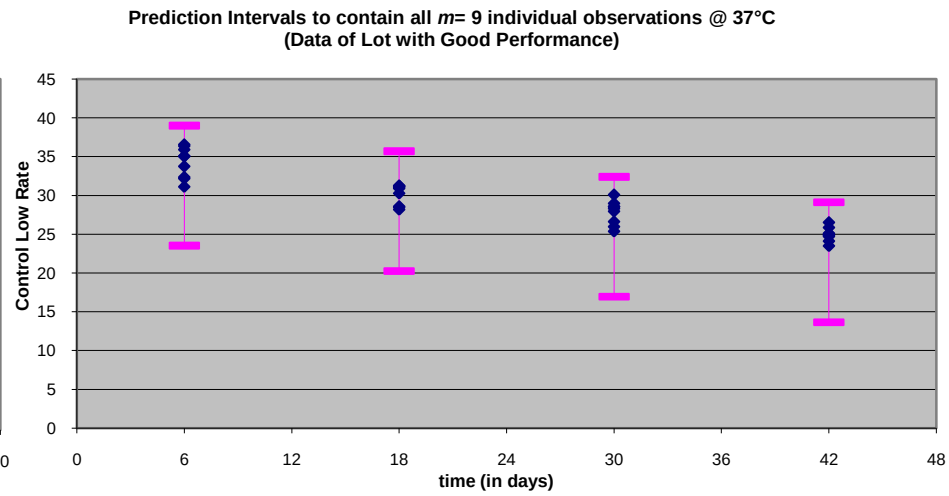
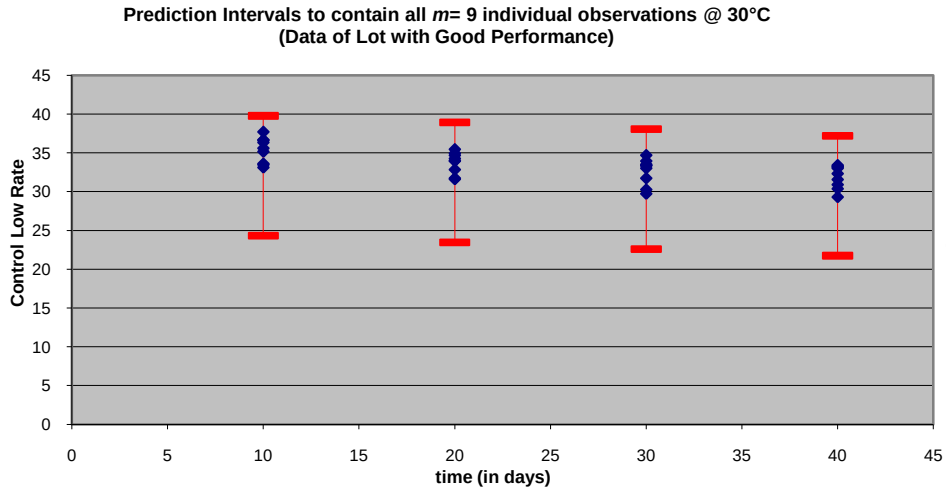


Figure 64: Control Low Degradation Patterns of Lot with Good Performance against 95% Pred. Intervals at Different Stress Temperatures







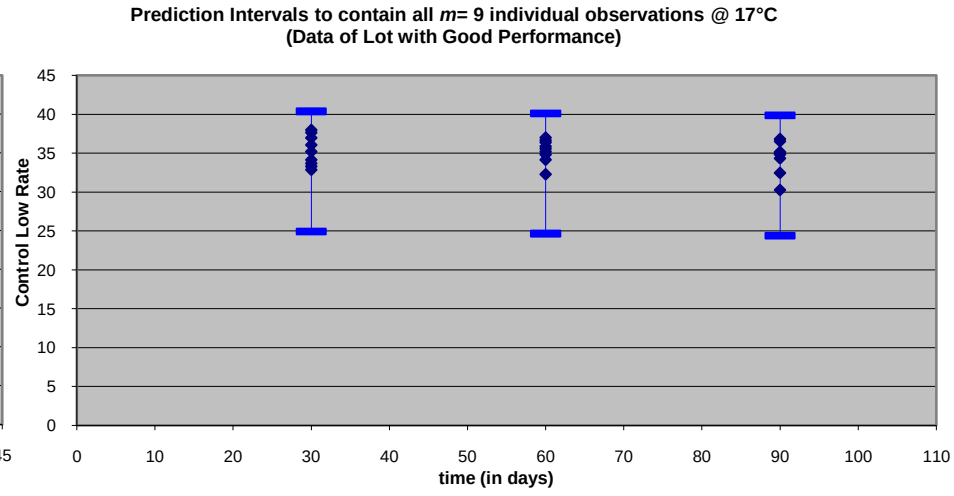
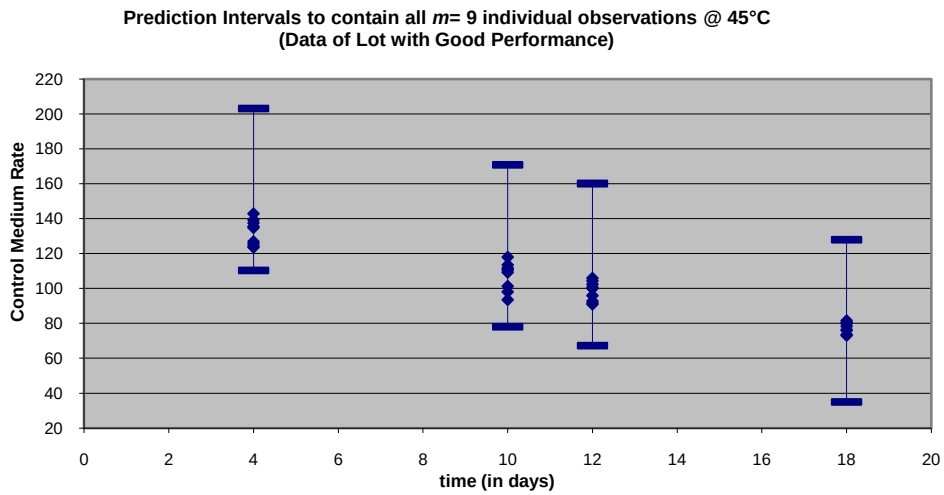
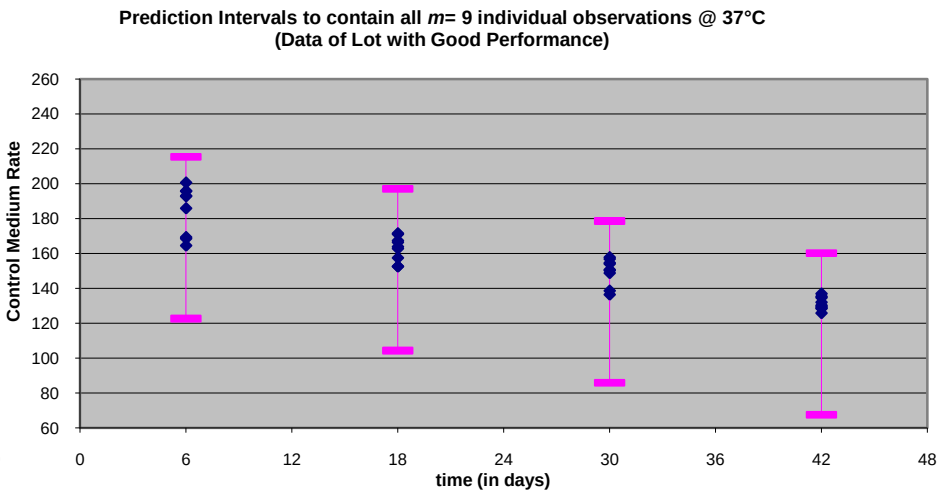
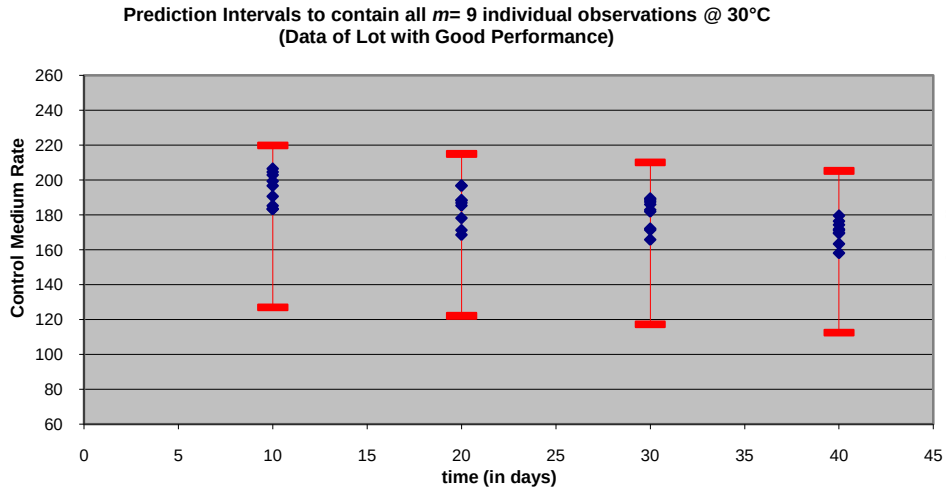


Figure 65: Control Medium Degradation Patterns of Lot with Good Performance against 95% Pred. Intervals at Different Stress Temperatures







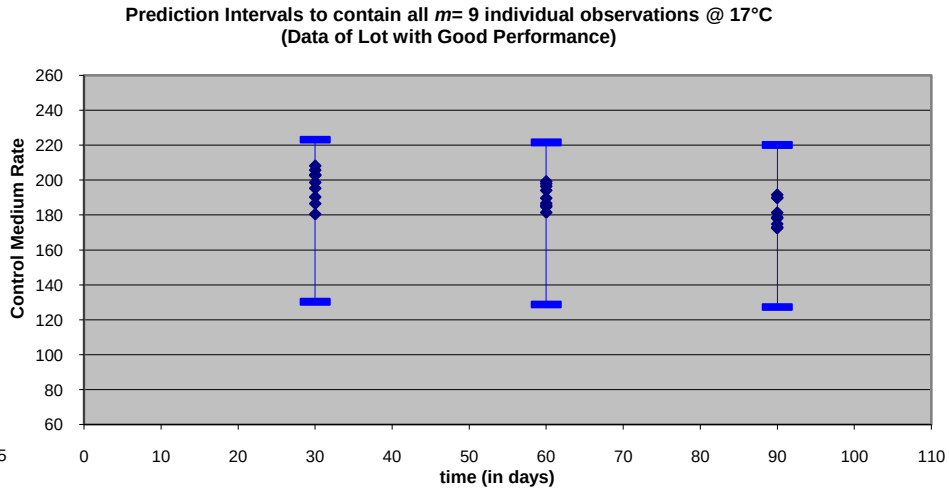
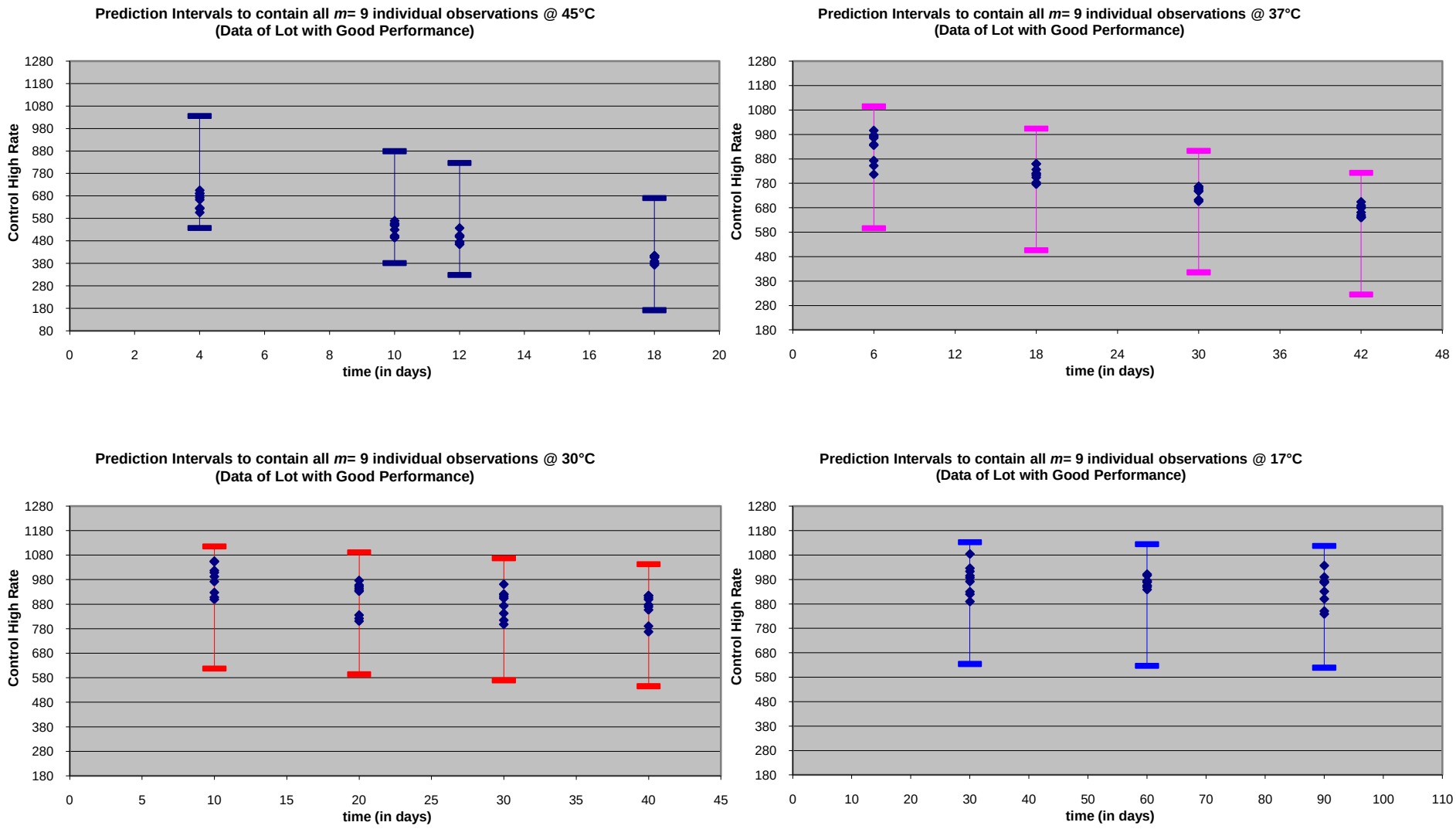


Figure 66: Control High Degradation Patterns of Lot with Good Performance against 95% Pred. Intervals at Different Stress Temperatures



5.4 Results for a lot with stability issues

During the execution of the initial accelerated stability study used to gather the data and develop a most appropriate model, we had the unexpected event that the third lot used in the experiment provided extremely high rate values at t = 30 days, for all of the instruments used to measure the product. This event was only observed at the lowest temperature used in the accelerated study (T = 17°C). Therefore, since this lot was been also monitored at the storage condition (2 to 8°C) in the real time stability program of the company, a failure of the product was also observed at t = 30 days. Is important to clarify that even though we keep monitoring this lot as it was originally scheduled (refer to Table A.1.4 in Appendix 1) for temperatures at 45, 37 and 30 °C, no more results were capable to be gathered at 17°C after t > 30 days, since the instruments used to measure the product started to present error codes that unable them to provide results.

Since this project focused on capturing the variability of three good lots and then fitting a statistical model capable of estimating the normal degradation behavior of a future lot, the data of this bad performance lot was not used for the purpose of developing the most appropriate statistical model. The data of this bad performance lot was substituted with another good lot to develop the most appropriate statistical model, but it was used in the validation procedure to verify if the predictions limits determined with the fitted model could discriminate this lot with stability issues.

Using the same approach presented in Section 5.3, we plotted the rate values of this lot against the prediction limits established in Tables 12 and 13. Verifying the results of Control Low against 90% prediction intervals in Figure 67 it is observed that the intervals for the highest temperature of 45°C are not able to discriminate the bad performance lot, but we see that some of the nine replicates begin to fall out the prediction intervals as temperature decreases. Specifically it is observed all of the m = 9 replicates of the lot monitored at T = 17°C and t = 30 days, are outside the prediction limits. Table 14 summarizes the temperature and time conditions for Control Low where results are observed outside the prediction limits. Verifying the results in this table we could establish the question; why we see a lot of observations out of the prediction limits at the lowest temperature and then this observable fact decreases as temperature increases?

Table 14: Results Out of 90% Prediction Limits for Control Low

	Temperature and Time Condition			
	37°C	**30°C**	**30°C**	**17°C**
	Day 30	**Day 10**	**Day 20**	**Day 30**
90% Pred. Interval	(17.62 – 31.72)	(24.99 – 39.09)	(24.13 – 38.24)	(25.60 – 39.70)
Results Out of Limits	31.74	39.76, 39.13	38.38, 38.80	49.42, 52.01, 52.55, 46.22, 46.65, 46.05, 50.81, 50.14, 49.09

The answer to this question is supported by an investigation that was done by Abbott scientists, since the nonconformance report for the lot in issue indicated that one of the raw materials supplied by a vendor and used to manufacture the lot, was contaminated with high molecular weight proteins (cryoglobulins) that were insoluble in the product used for the study at the lower temperatures. Therefore, when the product was measured with Abbott's immunoassay instruments, the cryoglobulins were masked as if there was much more analyte present in the product. This caused high results of rate values for the Control Low response variable, which were significantly observed at the lowest temperature of the study (T = 17°C). In contrast, when verifying the plot of rate values at 45°C, it is observed that all results are within the expected degradation pattern of the prediction intervals, since at this temperature the cryoglobulins are dissolved in the product solution and makes it behave in its normal degradation pattern.

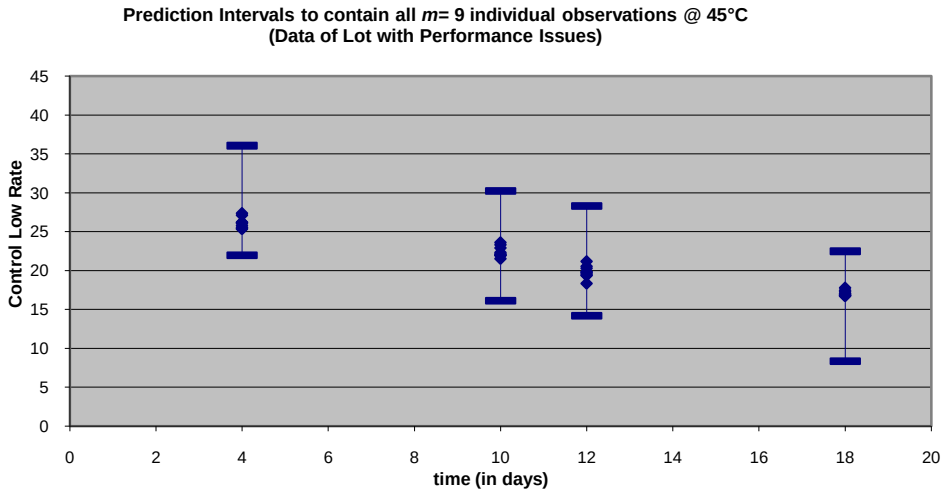
Verifying the plots for Control Medium and Control High in Figures 68 and 69 it is observed that the problem provided by the cryoglobulins is not as noticeable in these response variables in comparison to the behavior seen with the Control Low. Particularly, it is seen for Control Medium in Figure 68 that the lot appears to behave normally at the temperatures of 45, 37 and 30 °C since all of the results are within the 90% prediction intervals, and it is only at the temperature of 17°C that the interval is capable of discriminating the bad performance lot. Lastly, when the results for Control High are verified in Figure 69, it is observed that the rate values in all of the temperature and time conditions monitored are within the 90% prediction limits.

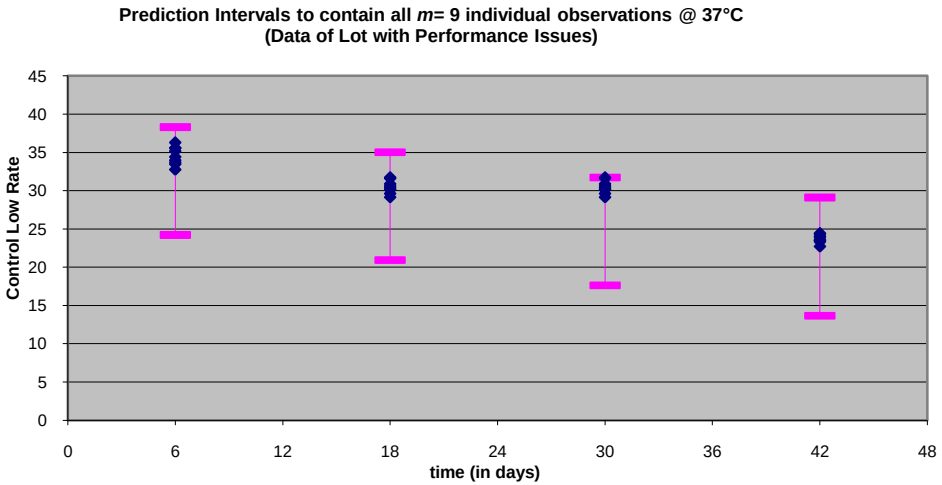
These results provide us with helpful information on what type of response variable is most effective to measure changes of rate signals for the type of product that was used (an immunoassay regent kit) and for demonstrating equivalency in degradation patterns of an experimental lot when compared against previous lots well known to be acceptable.

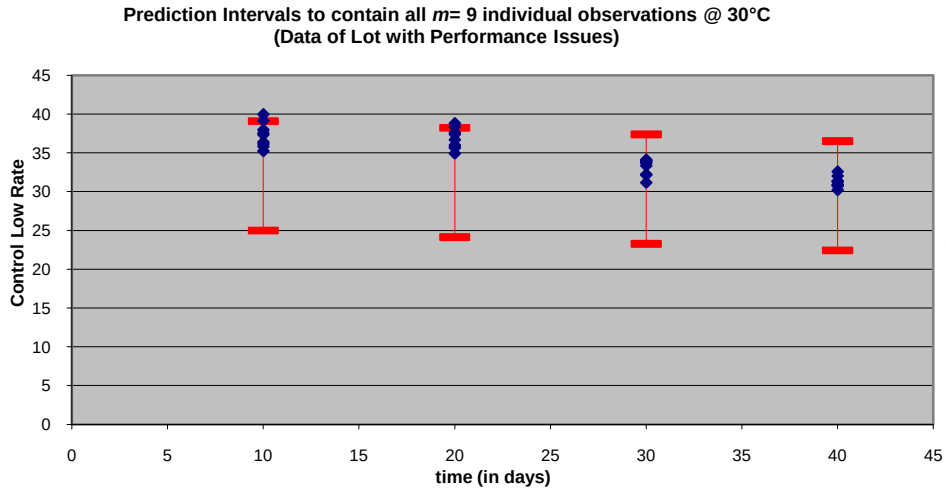
In addition, we could establish the question; why more results are seen out of prediction limits for Control Low than for the other control levels? The answer to this question is based on the purpose that each control level has on the product that was used in this project. In our case, controls are solutions that contain known concentrations of analyte and are used to monitor the accuracy and precision performance of the product (reagent kit) and the analyzer (instrument). The usually are divided in three levels since each of them is supposed to mimic the levels of signal (e.g. rate or concentration) that a patient sample can be measured in the range of values that the product it is used for categorizing the levels of a condition. In addition, the results of one of these control levels will always going to be designed to be near the region where the product is more sensitive to changes in assay signal. In the specific product that we used, the Control Low is the level more sensitive to changes in assay signal and it is the reason why more results were seen out of prediction limits at different temperature conditions.

The final step in this validation procedure was to verify what would be the most appropriate confidence level (90% 95% or other) to use in the prediction intervals to discriminate m=9 individual observations of a future lot with a good or a bad stability performance. Verifying the same results of the lot with bad performance against the prediction intervals determined at the 95% confidence level, it is observed in Figure 70 that results out of limits are only seen for Control Low at the temperature and time conditions of T = 17°C and t = 30 days. In addition, fewer observations are seen out of the limits for Control Medium at this same time and temperature condition, than what it was seen with the 90% prediction intervals (refer to Figures 68 and 71 for this comparison). This clearly indicates that prediction intervals that were determined at the 95% confidence level are less perceptive to discriminate a lot with true stability issues than prediction intervals determined at the 90% confidence level. Therefore, uniting all the facts presented in Section 5.3, as well as in this section, we can conclude that the 90% prediction intervals determined with the lot-to-lot variability characterized in the accelerated test can be used routinely as a validation procedure to compare degradation patterns of future experimental lots at elevated temperatures and conclude if these lots have similar degradation patterns as the previous control lots from where the accelerated stability data was gathered to develop the prediction intervals.

Figure 67: Control Low Degradation Patterns of Lot with Performance Issues against 90% Pred. Intervals at Different Stress Temperatures







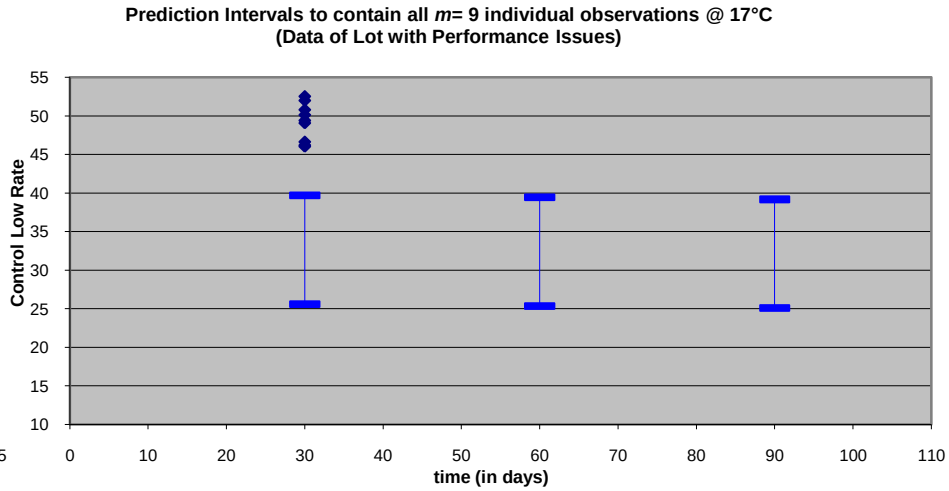


Figure 68: Control Medium Degradation Patterns of Lot with Performance Issues against 90% Pred. Intervals at Different Stress Temperature

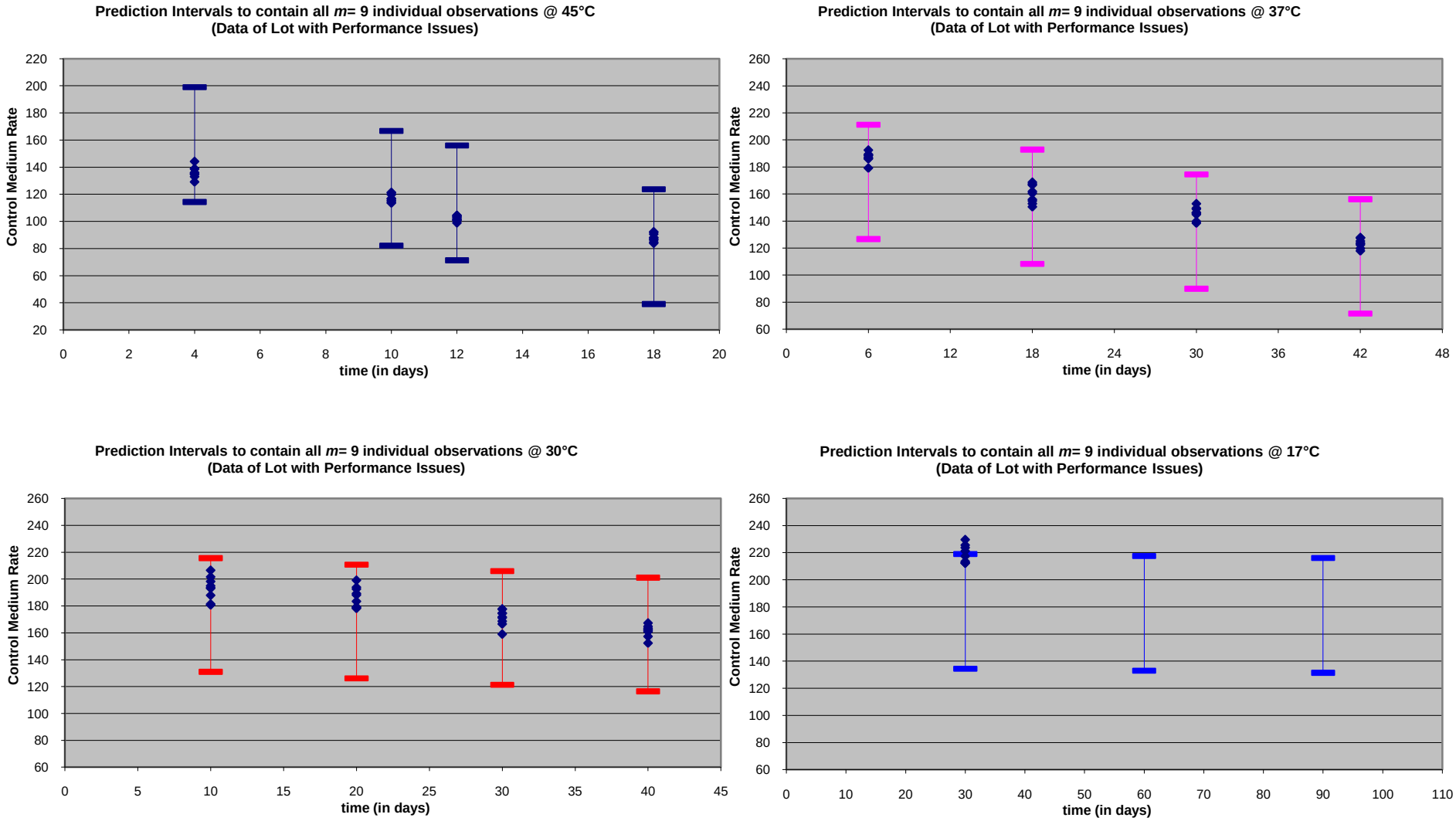


Figure 69: Control High Degradation Patterns of Lot with Performance Issues against 90% Pred. Intervals at Different Stress Temperatures

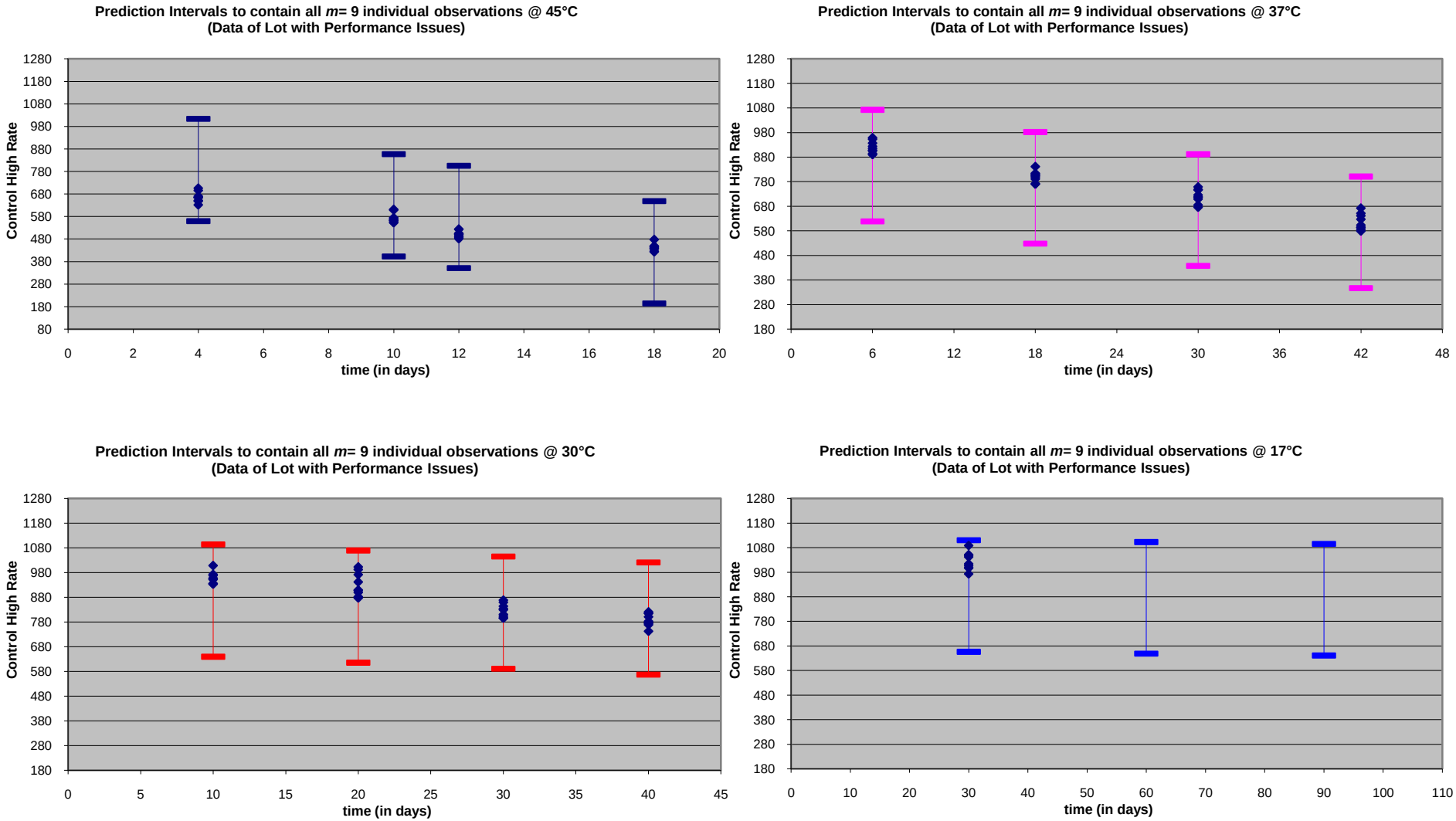
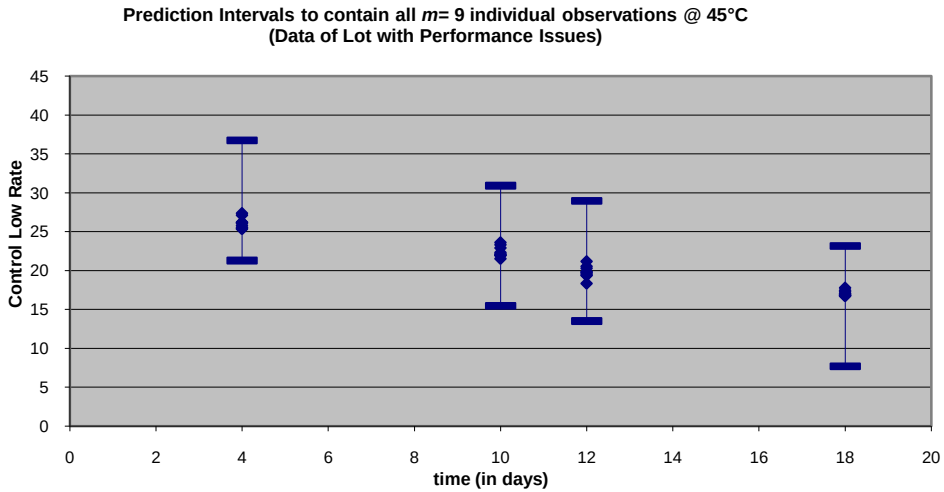
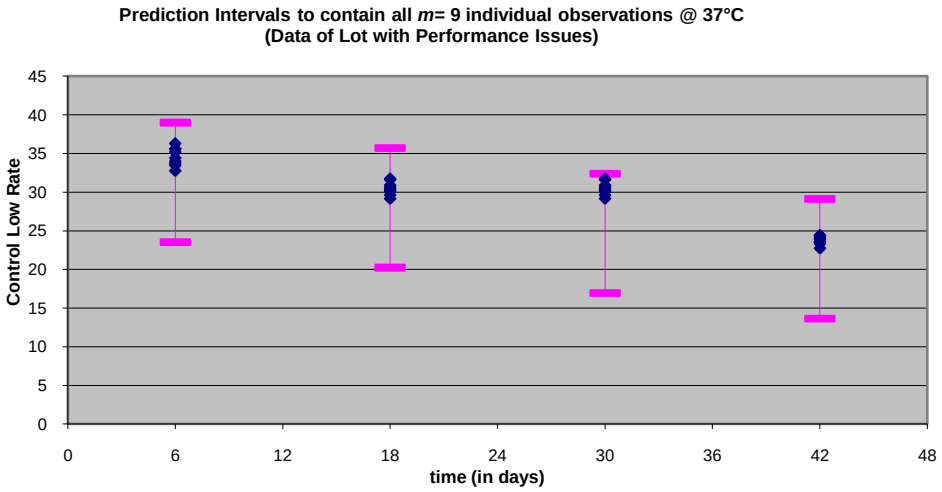
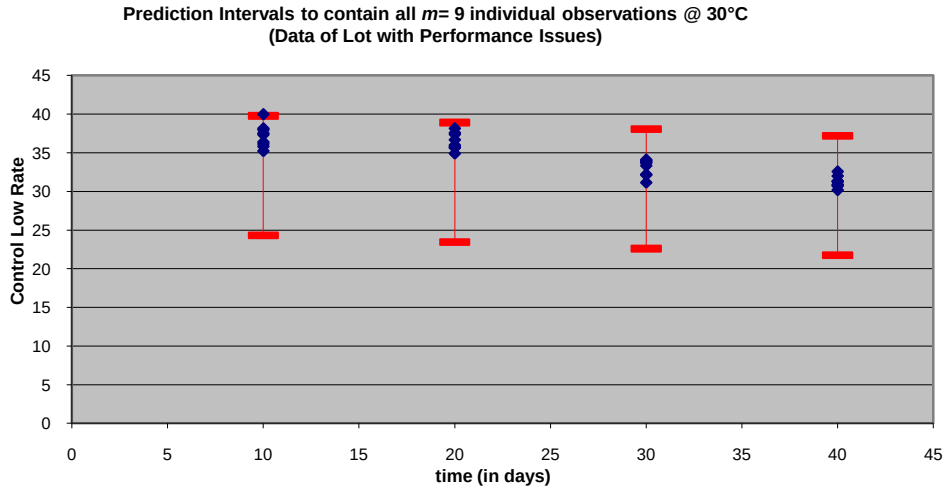


Figure 70: Control Low Degradation Patterns of Lot with Performance Issues against 95% Pred. Intervals at Different Stress Temperatures







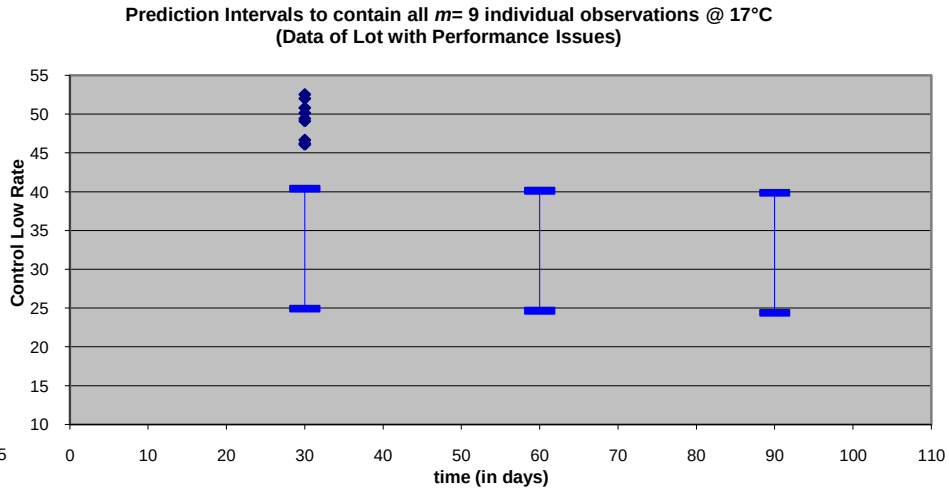


Figure 71: Control Medium Degradation Patterns of Lot with Performance Issues against 95% Pred. Intervals at Different Stress Temperatures

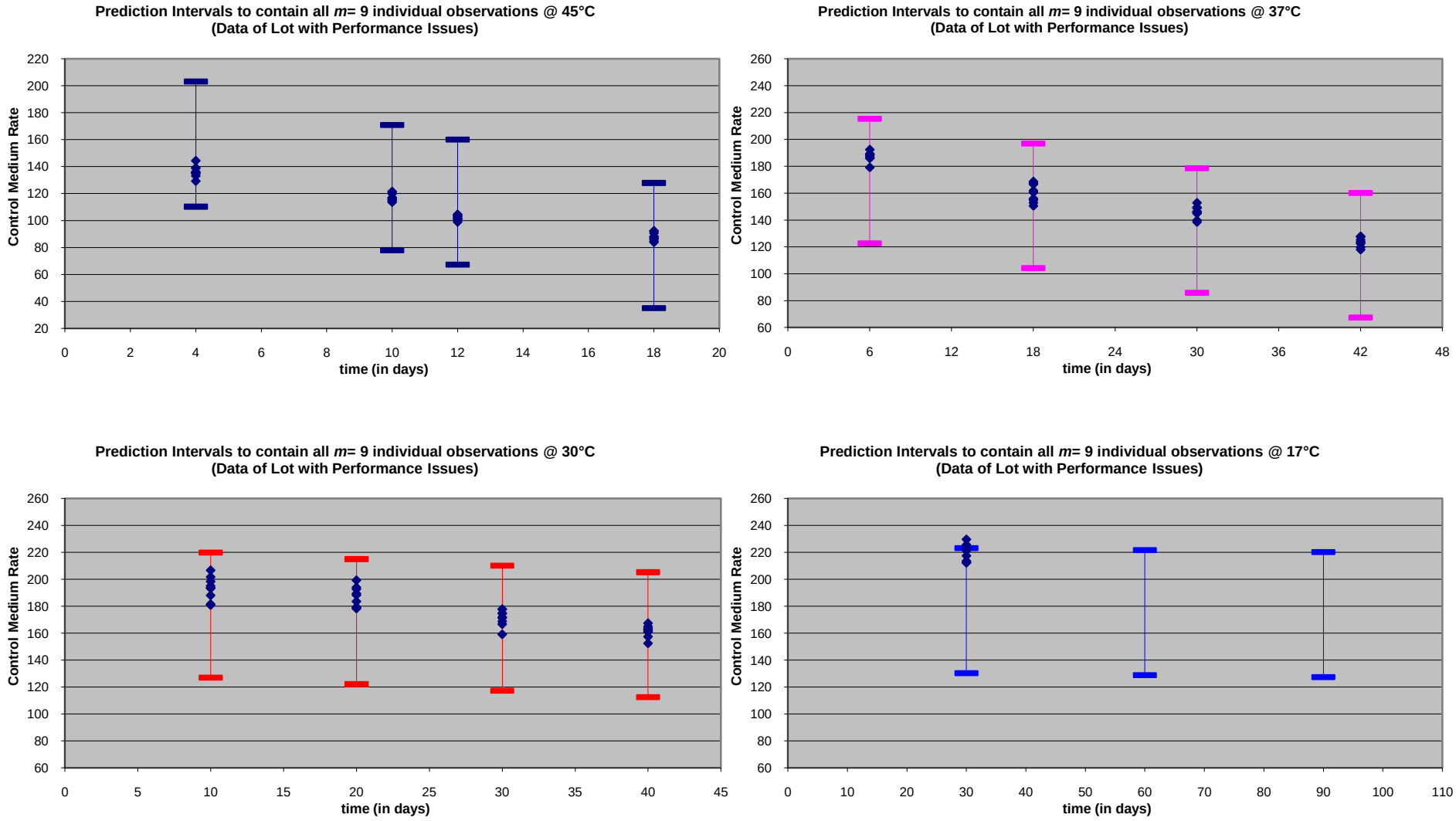
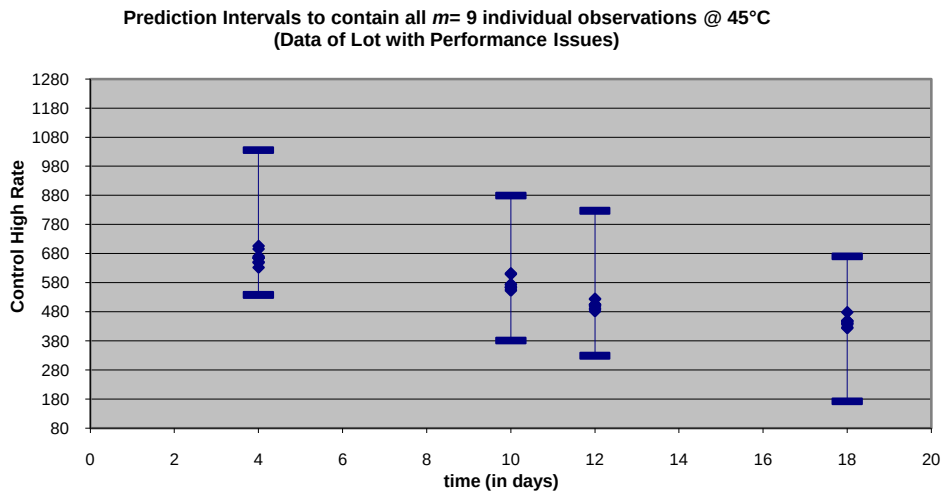
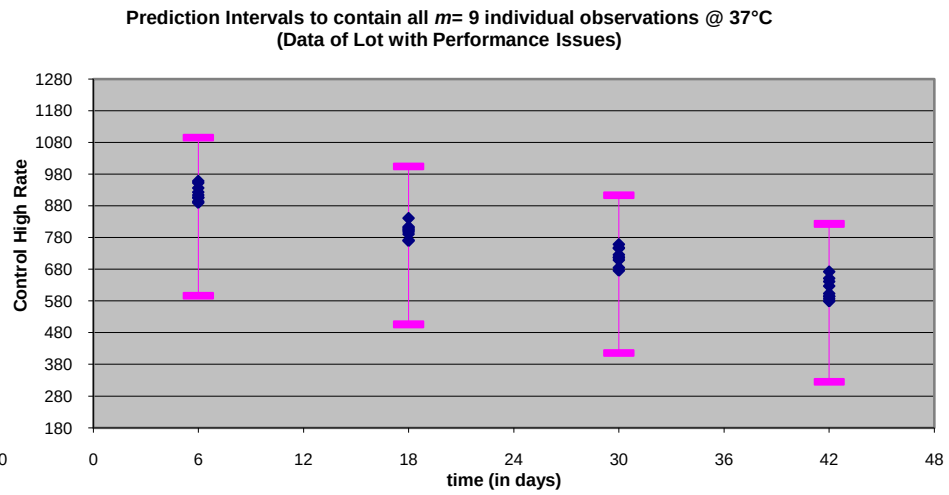
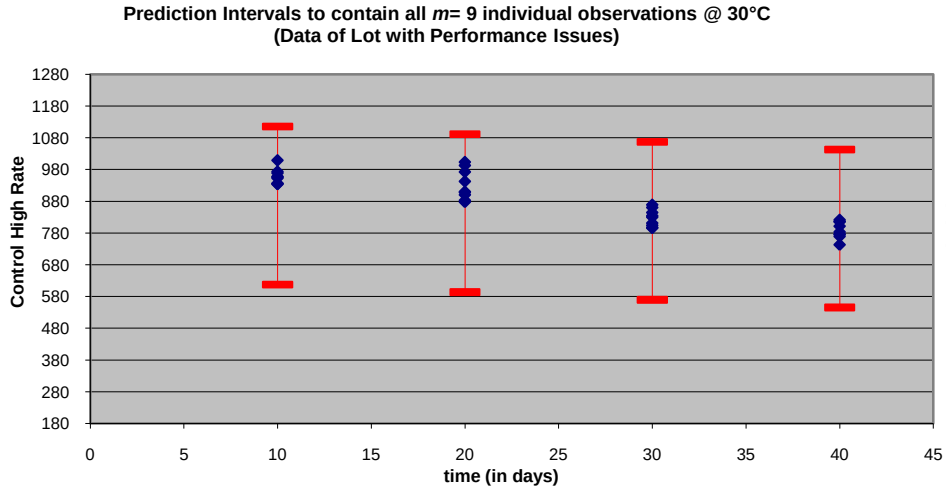
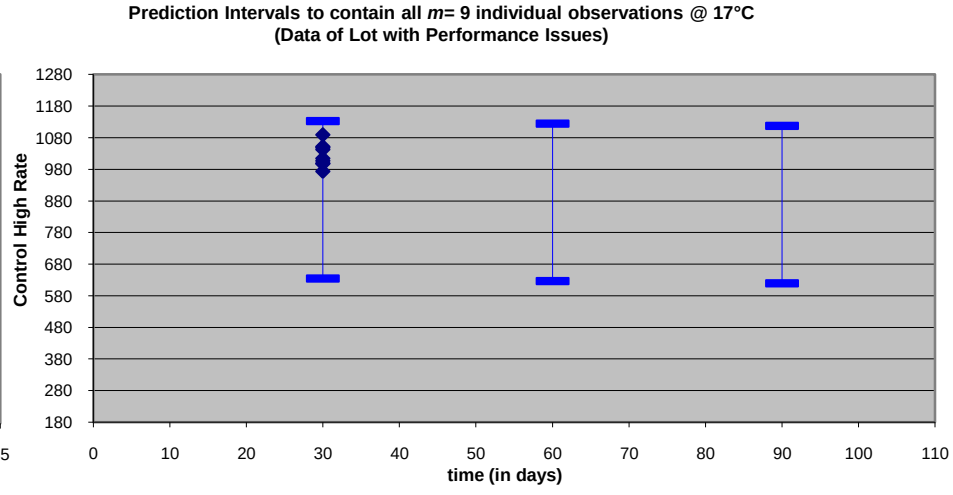


Figure 72: Control High Degradation Patterns of Lot with Performance Issues against 95% Pred. Intervals at Different Stress Temperatures









6 Conclusions and Future Work

6.1 Conclusions

This research project is a significant contribution to the scientific world of in-vitro diagnostics products since it provides an empirical example to design an accelerated stability test for an immunoassay reagent kit and to fit a mathematical model that relates product content, time and temperature in a superior way than it was done in the past with the Arrhenius classical approach. Based on the relative merits and limitations that were discussed in detail for all the models developed to fit the accelerated stability data, it was concluded that the Arrhenius nonlinear model for a zero-order reaction was the most appropriate to estimate product degradation for the product under study since it provided relevant statistics and compliance with the underlying assumptions of the Arrhenius Life-Temperature. In contrast to the two step procedure of the Arrhenius classical approach, this nonlinear model was able to provide a one step approach that accounts all of the variability provided by the accelerates stability data of three lots and provides a direct estimation of product degradation at the storage condition with significant reliability. The major advantages provided by the model can be summarized as follows:

- The model provides a well fit of the complete accelerated stability data by relating *product content, time* and *temperature* in one equation.
- The regression model as well as all individual regression coefficients demonstrated to be statistically significant at a 90% confidence level.
- The model demonstrated to provide adequate predictions of the product content at the expiration date.
- The behaviors of the partial derivative plots ($\partial Z/\partial X$ and $\partial Z/\partial Y$) were compatible with the chemical-physical degradation behavior that was observed for the product under study

Finally, with the lot-to-lot variability characterized in the accelerated test and the best fitted model, we were able to provide a validation procedure that could be used routinely to compare degradation patterns of future lots at elevated temperatures and conclude if these future lots have similar degradation patterns as the previous control lots that had good stability performance until their expiration date and from where the best fitted

model was developed. The validation procedure was based on prediction intervals to contain all of *m* individual observations at specific time and temperature conditions. Its suitability to discriminate a lot with and without stability issues was confirmed by comparing the results of two future lots at elevated temperatures. In our case, the validation procedure demonstrated suitability for use since the 90% prediction intervals determined at the elevated temperatures where very perceptive for identifying a lot with good stability performance as well as for discriminating a lot previously known to have true stability issues. Additional uses of the nonlinear model and the validation procedure developed could include:

1. Predicting the long term stability or expiration dating for new or re-formulated products at the intended storage condition.
2. Showing product behavior equivalency of an experimental and control material that has similar raw materials used for manufacturing or that pertain to the same family of products.
3. Demonstrating product stability performance similarity when a raw material has been supplied by a new vendor.

6.2 Future Work

Even though this research project is a good contribution in the study of the mechanisms of degradation for an in-vitro diagnostic product, there still a lot of work to be done since only a few studies of biological products have been reported in the literature. In our specific case, it would be beneficial to make a future study to determine the minimum time range of data that is needed for each stress temperature condition which could still provide a fitted model capable of provide meaningful predictions of the product content at the storage condition. In addition, it would be interesting to see how the predictions of the model fitted to the accelerated stability data would have improved if we had used the storage condition of the product (2-8°C) as an experimental condition. Further work will also be needed to develop models and estimate parameters for cases in which a significant lot-to-lot variability is present in the data and the average response cannot represent the degradation of individual lots.

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Appendixes

Appendix 1: Testing Schedule of Lots Used in the Accelerated Stability Study

Table A.1. 1: Accelerated Stability Schedule for Lot #1

Test Point No.	Test Date 45ºC	Test Date 37ºC	Test Date 30ºC	Test Date 17ºC
1	01/19/05	01/22/05	01/25/05	01/22/05
2	01/21/05	01/28/05	02/03/05	02/21/05
3	01/23/05	02/03/05	02/13/05	03/23/05
4	01/25/05	02/09/05	02/23/05	04/22/05
5	01/27/05	02/15/05	03/05/05	05/22/05
6	01/29/05	02/21/05	03/15/05	06/21/05
7	01/31/05	02/27/05	03/24/05	07/21/05
8	02/02/05	03/05/05	04/04/05	08/20/05
9	02/04/05	03/11/05	04/14/05	09/19/05
10	02/06/05	03/17/05	04/24/05	10/19/05
11	02/08/05	03/23/05	05/04/05	N/A
12	02/10/05	03/29/05	05/14/05	N/A

Table A.1. 2: Accelerated Stability Schedule for Lot #2

Test Point No.	Test Date 45ºC	Test Date 37ºC	Test Date 30ºC	Test Date 17ºC
1	02/11/05	02/15/05	02/19/05	02/14/05
2	02/13/05	02/21/05	03/01/05	03/16/05
3	02/15/05	02/27/05	03/11/05	04/15/05
4	02/17/05	03/05/05	03/21/05	05/15/05
5	02/19/05	03/11/05	03/31/05	06/14/05
6	02/21/05	03/17/05	04/10/05	07/14/05
7	02/23/05	03/23/05	04/20/05	08/13/05
8	02/25/05	03/29/05	04/30/05	09/12/05
9	02/27/05	04/04/05	05/10/05	10/12/05
10	03/01/05	04/10/05	05/20/05	11/11/05
11	03/03/05	04/16/05	05/30/05	N/A
12	03/05/05	04/22/05	06/09/05	N/A

Table A.1. 3: Accelerated Stability Schedule for Lot #3

Test Point No.	Test Date 45ºC	Test Date 37ºC	Test Date 30ºC	Test Date 17ºC
1	05/18/05	05/22/05	05/26/05	06/15/05
2	05/20/05	05/28/05	06/05/05	07/15/05
3	05/22/05	06/03/05	06/15/05	08/14/05
4	05/24/05	06/09/05	06/25/05	09/13/05
5	05/26/05	06/15/05	07/05/05	10/13/05
6	05/28/05	06/21/05	07/15/05	11/12/05
7	05/30/05	06/27/05	07/25/05	12/12/05
8	06/01/05	07/03/05	08/04/05	01/11/06
9	06/03/05	07/09/05	08/14/05	02/10/06
10	06/05/05	07/15/05	08/24/05	03/12/06
11	06/07/05	07/21/05	09/03/05	N/A
12	06/09/05	07/27/05	09/13/05	N/A

Table A.1.4: Accelerated Stability Schedule for Lot #3A
(Lot with stability issues used for validation purposes)

Test Point No.	Test Date 45ºC	Test Date 37ºC	Test Date 30ºC	Test Date 17ºC
1	03/03/05	03/07/05	03/11/05	03/18/05
2	03/05/05	03/13/05	03/21/05	04/17/05 *
3	03/07/05	03/19/05	03/31/05	05/17/05 *
4	03/09/05	03/24/05	04/10/05	06/16/05 *
5	03/11/05	03/31/05	04/20/05	07/16/05 *
6	03/13/05	04/06/05	04/30/05	08/15/05 *
7	03/15/05	04/12/05	05/10/05	09/14/05 *
8	03/17/05	04/18/05	05/20/05	10/14/05 *
9	03/19/05	04/24/05	05/30/05	11/13/05 *
10	03/21/05	04/30/05	06/09/05	12/13/05 *
11	03/23/05	05/06/05	06/19/05	N/A
12	03/24/05	05/12/05	06/29/05	N/A

* **Note:** No data was obtained for these days since at 3/18/05, the lot demonstrated to have true stability issues at temperatures near the storage condition (2-8 °C)

Table A.1.5: Accelerated Stability Schedule for Lot #4
(Additional lot with good performance used for validation purposes)

Test Point No.	Test Date 8ºC	Test Date 45ºC	Test Date 37ºC	Test Date 30ºC	Test Date 17ºC
0	03/31/06				
1	04/15/06	04/04/06	04/06/06	04/10/06	04/30/06
2	04/30/06	04/10/06	04/18/06	04/20/06	05/30/06
3	05/15/06	04/12/06	04/30/06	04/30/06	06/29/06
4	05/30/06	04/18/06	05/12/06	05/10/06	N/A

Appendix 2: List of Table Curve 3D Built-in Equations Fitted to Control Levels

Appendix 2 presents the list of built-in equations from Table Curve 3D software, which were fitted to the data of each control level. Equations are ranked in decreasing order of the R^2_{Adj} statistic result. Only the equations that provided R^2_{Adj} values greater than 90% were listed.

Table A.2. 1: Table Curve 3D Built-in Equations Fitted to Control Low Accelerated Stability Data

Rank	R^2	Adj R^2	Fitted Std. Error	F-statistic	Built-in Equation Fitted from Table Curve 3D Software
1	0.950581	0.950176	1.5850245	2613.8314	z=a+blnx+clny+d(lnx)2+e(lny)2+flnxlny+g(lnx)3+h(lny)3+ilnx(lny)2+j(lnx)2lny
2	0.946639	0.946203	1.6470218	2410.7173	z=a+blnx+cy+d(lnx)2+ey2+fylnx+g(lnx)3+hy3+iy2lnx+jy(lnx)2
3	0.946124	0.945683	1.6549586	2386.35	z=a+blnx+c/y+d(lnx)2+e/y2+f(lnx)/y+g(lnx)3+h/y3+i(lnx)/y2+j(lnx)2/y
4	0.945066	0.944389	1.6745518	1496.7177	Chebyshev X,Y Bivariate Polynomial Order 4
5	0.944978	0.9443	1.6758965	1494.1773	Chebyshev X,LnY Bivariate Polynomial Order 4
6	0.94221	0.941498	1.7175318	1418.4468	Sigmoid Series Bivariate Order 4
7	0.940176	0.939538	1.7460568	1597.7702	Fourier Series Bivariate Order 2x2
8	0.934966	0.934541	1.8167798	2515.9175	z=a+LORCUMX(b,c,d)+LORCUMY(e,f,g)+LORCUMX(h,c,d)*LORCUMY(1,f,g)
9	0.925464	0.924977	1.9449864	2172.858	z=a+LORX(b,c,d)+LORY(e,f,g)+LORX(h,c,d)*LORY(1,f,g)
10	0.919082	0.918553	2.0265438	1987.6836	z=a+LDRX(b,c,d)+LDRY(e,f,g)+LDRX(h,c,d)*LDRY(1,f,g)
11	0.913249	0.912682	2.0983137	1842.2705	z=a+LNCUMX(b,c,d)+LNCUMY(e,f,g)+LNCUMX(h,c,d)*LNCUMY(1,f,g)
12	0.912928	0.912359	2.1021901	1834.8376	z=a+SIGX(b,c,d)+SIGY(e,f,g)+SIGX(h,c,d)*SIGY(1,f,g)
13	0.911333	0.910681	2.1222217	1572.5658	z=a+bx+cxlnx+dx0.5lnx+ex0.5+fylny+gy1.5+hy2.5+iy3
14	0.911229	0.910429	2.1252114	1254.3714	Chebyshev X,Y Rational Order 2/3
15	0.91042	0.909834	2.1322585	1778.5533	z=a+EXVCUMX(b,c,d)+EXVCUMY(e,f,g)+EXVCUMX(h,c,d)*EXVCUMY(1,f,g)
16	0.909506	0.90884	2.143979	1537.7211	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gy2+hy2.5+iy3
17	0.909502	0.908836	2.1440332	1537.6356	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gy1.5+hy2.5+iy3
18	0.909498	0.908832	2.1440703	1537.5771	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gylny+hy2.5+iy3
19	0.909497	0.908831	2.1440915	1537.5436	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gy1.5+hy2+iy3
20	0.909497	0.908831	2.1440919	1537.543	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gy+hy2.5+iy3
21	0.909493	0.908827	2.1441292	1537.4842	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gylny+hy2+iy3
22	0.909491	0.908825	2.1441532	1537.4464	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gy+hy2+iy3
23	0.909491	0.908825	2.1441542	1537.4448	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gy1.5+hy2+iy2.5
24	0.909489	0.908823	2.1441857	1537.3951	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gylny+hy1.5+iy3
25	0.908087	0.907259	2.1624868	1207.3237	Chebyshev X,LnY Rational Order 2/3
26	0.906256	0.905644	2.1812446	1691.7934	z=a+bxlnx+cx/lnx+dx0.5+ey+fy1.5+gy2.5+hy3
27	0.906176	0.905563	2.1821794	1690.1943	z=a+LOGNORMX(b,c,d)+GAUSSY(e,f,g)+LOGNORMX(h,c,d)*GAUSSY(1,f,g)
28	0.904783	0.90416	2.1983224	1662.9013	z=a+bx+cx/lnx+dx0.5+eylny+fy1.5+gy2.5+hy3
29	0.904508	0.904041	2.1996944	2324.453	z=a+blnx+cy+d(lnx)2+ey2+fylnx
30	0.904508	0.903883	2.2014961	1657.6061	z=a+LOGISTICX(b,c,d)+LOGISTICY(e,f,g)+LOGISTICX(h,c,d)*LOGISTICY(1,f,g)
31	0.904379	0.903754	2.202975	1655.1463	z=a+bx+cx/lnx+dx0.5+ey+fy1.5+gy2.5+hy3
32	0.904103	0.903476	2.2061572	1649.8706	z=a+bx+cx/lnx+dx0.5+ey+fy1.5+gy2+hy3
33	0.903888	0.90326	2.2086216	1645.8004	z=a+bx+cx/lnx+dx0.5+ey+fy2+gy2.5+hy3
34	0.903601	0.902413	2.2182677	815.49968	Cosine Series Bivariate Order 4
35	0.903472	0.902842	2.2133965	1637.953	z=a+bx+cx/lnx+dx0.5+eylny+fy2+gy2.5+hy3
36	0.902074	0.901595	2.2275514	2260.5797	z=a+blnx+clny+d(lnx)2+e(lny)2+flnxlny
37	0.902031	0.901391	2.2298636	1611.2752	z=a+GCUMX(b,c,d)+GCUMY(e,f,g)+GCUMX(h,c,d)*GCUMY(1,f,g)
38	0.900926	0.900279	2.242399	1591.3598	z=a+bx+cxlnx+dx0.5+ey+fy1.5+gy2.5+hy3

Table A.2. 2: Table Curve 3D Built-in Equations Fitted to Control Med Accelerated Stability Data

Rank	R^2	Adj R^2	Fitted Std. Error	F-statistic	Built-in Equation Fitted from Table Curve 3D Software
1	0.945268	0.94482	9.3684359	2346.9062	z=a+blnx+clny+d(lnx)2+e(lny)2+flnxlny+g(lnx)3+h(lny)3+ilnx(lny)2+j(lnx)2lny
2	0.942016	0.941541	9.6427469	2207.6573	z=a+blnx+cy+d(lnx)2+ey2+fylnx+g(lnx)3+hy3+iy2lnx+jy(lnx)2
3	0.941635	0.941157	9.674359	2192.3668	z=a+blnx+c/y+d(lnx)2+e/y2+f(lnx)/y+g(lnx)3+h/y3+i(lnx)/y2+j(lnx)2/y
4	0.93836	0.9376	9.9624869	1324.4127	Chebyshev X,Y Bivariate Polynomial Order 4
5	0.938277	0.937517	9.9691269	1322.5331	Chebyshev X,LnY Bivariate Polynomial Order 4
6	0.935631	0.934838	10.180608	1264.5811	Sigmoid Series Bivariate Order 4
7	0.933693	0.932986	10.32427	1431.5998	Fourier Series Bivariate Order 2x2
8	0.928517	0.92805	10.69773	2273.1403	z=a+LORCUMX(b,c,d)+LORCUMY(e,f,g)+LORCUMX(h,c,d)*LORCUMY(1,f,g)
9	0.919084	0.918555	11.381742	1987.7287	z=a+LORX(b,c,d)+LORY(e,f,g)+LORX(h,c,d)*LORY(1,f,g)
10	0.914337	0.913777	11.710821	1867.8892	z=a+LDRX(b,c,d)+LDRY(e,f,g)+LDRX(h,c,d)*LDRY(1,f,g)
11	0.914278	0.913647	11.719661	1631.8326	z=a+bx+cxlnx+dx0.5lnx+ex0.5+fylny+gy1.5+hy2.5+iy3
12	0.912375	0.91173	11.849005	1593.0787	z=a+bx+cxlnx+dx0.5lnx+ex0.5+fy+gy1.5+hy2.5+iy3
13	0.912301	0.911655	11.854037	1591.5965	z=a+bx+cxlnx+dx0.5lnx+ex0.5+fy+gylny+hy2.5+iy3
14	0.911985	0.911337	11.875375	1585.3327	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gy2+hy2.5+iy3
15	0.911972	0.911324	11.876221	1585.0851	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gy1.5+hy2.5+iy3
16	0.911964	0.911316	11.876779	1584.922	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gylny+hy2.5+iy3
17	0.911959	0.911311	11.877096	1584.8291	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gy1.5+hy2+iy3
18	0.911959	0.911311	11.877099	1584.8283	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gy+hy2.5+iy3
19	0.911951	0.911303	11.877648	1584.6675	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gylny+hy2+iy3
20	0.911946	0.911298	11.877997	1584.5655	z=a+bx+cxlnx+dx/lnx+ex0.5+f/lnx+gy+hy2+iy3
21	0.910787	0.910204	11.950993	1786.6048	z=a+bxlnx+cx/lnx+dx0.5+ey+fy1.5+gy2.5+hy3
22	0.909523	0.908932	12.035383	1759.1924	z=a+bx+cx/lnx+dx0.5+eylny+fy1.5+gy2.5+hy3
23	0.909123	0.908529	12.061974	1750.674	z=a+bx+cx/lnx+dx0.5+ey+fy1.5+gy2.5+hy3
24	0.908636	0.908039	12.094228	1740.4166	z=a+bx+cx/lnx+dx0.5+ey+fy2+gy2.5+hy3
25	0.908539	0.907942	12.100626	1738.3917	z=a+LNCUMX(b,c,d)+LNCUMY(e,f,g)+LNCUMX(h,c,d)*LNCUMY(1,f,g)
26	0.908377	0.907778	12.111365	1734.9999	z=a+SIGX(b,c,d)+SIGY(e,f,g)+SIGX(h,c,d)*SIGY(1,f,g)
27	0.908223	0.907624	12.121509	1731.8043	z=a+bx+cx/lnx+dx0.5+eylny+fy2+gy2.5+hy3
28	0.90609	0.905476	12.261596	1688.4833	z=a+bx+cxlnx+dx0.5+ey+fy1.5+gy2.5+hy3
29	0.905664	0.905047	12.289365	1680.0715	z=a+EXVCUMX(b,c,d)+EXVCUMY(e,f,g)+EXVCUMX(h,c,d)*EXVCUMY(1,f,g)
30	0.905069	0.904604	12.318021	2339.6305	z=a+blnx+cy+d(lnx)2+ey2+fylnx
31	0.905054	0.904434	12.329011	1668.1601	z=a+bx+cxlnx+dx0.5+ey+fy2+gy2.5+hy3
32	0.903115	0.902482	12.454302	1631.2619	z=a+bx+cxlnx+dx0.5lnx+ex/lnx+fy2+gy2.5+hy3
33	0.903031	0.902397	12.459676	1629.7043	z=a+bx+cxlnx+dx0.5lnx+ex/lnx+fy1.5+gy2.5+hy3
34	0.902977	0.902343	12.463153	1628.6975	z=a+bx+cxlnx+dx0.5lnx+ex/lnx+fylny+gy2.5+hy3
35	0.90284	0.902364	12.461802	2280.3239	z=a+blnx+clny+d(lnx)2+e(lny)2+flnxlny
36	0.902172	0.901533	12.514731	1613.8605	z=a+LOGISTICX(b,c,d)+LOGISTICY(e,f,g)+LOGISTICX(h,c,d)*LOGISTICY(1,f,g)

Table A.2. 3: Table Curve 3D Built-in Equations Fitted to Control High Accelerated Stability Data

Rank	R2	Adj R2	Fitted Std. Error	F-statistic	Built-in Equation Fitted from Table Curve 3D Software
1	0.924155	0.923534	54.757839	1655.7709	z=a+blnx+clny+d(lnx)2+e(lny)2+flnxlny+g(lnx)3+h(lny)3+ilnx(lny)2+j(lnx)2lny
2	0.921008	0.920362	55.882135	1584.4031	z=a+blnx+cy+d(lnx)2+ey2+fylnx+g(lnx)3+hy3+iy2lnx+jy(lnx)2
3	0.92061	0.919961	56.02268	1575.7824	z=a+blnx+c/y+d(lnx)2+e/y2+f(lnx)/y+g(lnx)3+h/y3+i(lnx)/y2+j(lnx)2/y
4	0.916646	0.915619	57.522139	956.74058	Chebyshev X,Y Bivariate Polynomial Order 4
5	0.916553	0.915525	57.554139	955.58027	Chebyshev X,LnY Bivariate Polynomial Order 4
6	0.91378	0.912717	58.502656	922.04712	Sigmoid Series Bivariate Order 4
7	0.912339	0.911404	58.941331	1058.0971	Fourier Series Bivariate Order 2x2
8	0.907578	0.906974	60.396879	1718.4926	z=a+LORCUMX(b,c,d)+LORCUMY(e,f,g)+LORCUMX(h,c,d)*LORCUMY(1,f,g)

Appendix 3: 90% Prediction Intervals to Contain all of *m* Future Observations

Appendix 3 presents Excel spreadsheet tables that were developed to calculate 90% prediction intervals that could contain all of m=9 future observations for each time and temperature conditions considered in the accelerated stability study. Each table is related to a response variable (Control Low, Medium or High) considered in the study. ∞

Table A.3. 1: 90% Prediction Interval to contain all m=9 future observations for Control Low

X = time (day)	Y = Temp (°K)	Z Predicted (rate)	95% Pred Limits for the mean		$r_{(0.90;\,9,\,\infty)}$	Δ = b-a	s	90% Pred Limits for all m=9 observations	
0	281	32.905853	27.421279	38.390427	2.523	10.9691	2.7956	25.8527	39.9590
2	318	30.963002	25.479571	36.446433		10.9669	2.7950	23.9113	38.0147
4	318	29.020151	23.53745	34.502852		10.9654	2.7946	21.9694	36.0709
6	318	27.0773	21.594917	32.559683		10.9648	2.7944	20.0269	34.1277
8	318	25.134449	19.651972	30.616927		10.9650	2.7945	18.0840	32.1849
10	318	23.191598	17.708614	28.674583		10.9660	2.7947	16.1405	30.2427
12	318	21.248747	15.764844	26.732651		10.9678	2.7952	14.1964	28.3011
14	318	19.305896	13.820661	24.791131		10.9705	2.7959	12.2519	26.3599
16	318	17.363045	11.876067	22.850023		10.9740	2.7968	10.3068	24.4193
18	318	15.420194	9.9310619	20.909327		10.9783	2.7979	8.3611	22.4792
20	318	13.477343	7.9856456	18.969041		10.9834	2.7992	6.4150	20.5397
22	318	11.534492	6.0398189	17.029166		10.9893	2.8007	4.4683	18.6007
24	318	9.5916414	4.0935826	15.0897		10.9961	2.8024	2.5211	16.6622
6	310	31.258985	25.776324	36.741646		10.9653	2.7946	24.2083	38.3097
12	310	29.612117	24.130873	35.093361		10.9625	2.7939	22.5632	36.6610
18	310	27.965249	22.484925	33.445573		10.9606	2.7934	20.9175	35.0130
24	310	26.318381	20.83848	31.798281		10.9598	2.7932	19.2712	33.3656
30	310	24.671513	19.191538	30.151487		10.9599	2.7932	17.6242	31.7188
36	310	23.024645	17.544099	28.50519		10.9611	2.7935	15.9766	30.0727
42	310	21.377777	15.896164	26.85939		10.9632	2.7940	14.3284	28.4272
48	310	19.730909	14.247732	25.214086		10.9664	2.7948	12.6795	26.7823
54	310	18.084041	12.598803	23.569278		10.9705	2.7959	11.0300	25.1381
60	310	16.437173	10.949379	21.924966		10.9756	2.7972	9.3798	23.4945
66	310	14.790305	9.2994606	20.281149		10.9817	2.7988	7.7291	21.8516
72	310	13.143437	7.6490477	18.637825		10.9888	2.8006	6.0776	20.2093
10	303	32.045965	26.562838	37.529093		10.9663	2.7948	24.9946	39.0973
20	303	31.186078	25.704054	36.668101		10.9640	2.7943	24.1362	38.2360
30	303	30.32619	24.84493	35.807451		10.9625	2.7939	23.2773	37.3751
40	303	29.466303	23.985463	34.947143		10.9617	2.7937	22.4179	36.5147
50	303	28.606415	23.125654	34.087176		10.9615	2.7936	21.5581	35.6547
60	303	27.746528	22.265504	33.227551		10.9620	2.7937	20.6979	34.7952
70	303	26.88664	21.405012	32.368268		10.9633	2.7941	19.8372	33.9360
80	303	26.026753	20.544178	31.509327		10.9651	2.7945	18.9761	33.0774
90	303	25.166865	19.683003	30.650727		10.9677	2.7952	18.1146	32.2191
100	303	24.306977	18.821486	29.792469		10.9710	2.7960	17.2526	31.3613
110	303	23.44709	17.959628	28.934551		10.9749	2.7970	16.3902	30.5040
120	303	22.587202	17.09743	28.076975		10.9795	2.7982	15.5273	29.6471
30	290	32.648292	27.164439	38.132145		10.9677	2.7952	25.60	39.70
60	290	32.390731	26.907492	37.87397		10.9665	2.7949	25.34	39.44
90	290	32.133169	26.650437	37.615902		10.9655	2.7946	25.08	39.18
120	290	31.875608	26.393276	37.357941		10.9647	2.7944	24.8253	38.9259
150	290	31.618047	26.136007	37.100087		10.9641	2.7943	24.5681	38.6680
180	290	31.360486	25.878631	36.842341		10.9637	2.7942	24.3108	38.4102
210	290	31.102925	25.621148	36.584701		10.9636	2.7941	24.0533	38.1525
240	290	30.845364	25.363558	36.327169		10.9636	2.7941	23.7957	37.8950
270	290	30.587802	25.105861	36.069744		10.9639	2.7942	23.5380	37.6376
300	290	30.330241	24.848056	35.812426		10.9644	2.7943	23.2801	37.3804

Table A.3. 2: 90% Prediction Interval to contain all *m*=9 future observations for Control Medium

X = time (day)	Y = Temp (°K)	Z Predicted (rate)	95% Pred Limits for the mean		$r_{(0.90;\ 9,\ \infty)}$	Δ = b-a	s	90% Pred Limits for all *m*=9 observations	
0	281	178.1744	145.2619	211.08691	2.523	65.8250	16.7759	135.8487	220.5001
2	318	167.42419	134.51858	200.32981		65.8112	16.7724	125.1074	209.7410
4	318	156.674	123.77278	189.57519		65.8024	16.7702	114.3629	198.9851
6	318	145.92378	113.02452	178.82303		65.7985	16.7692	103.6152	188.2324
8	318	135.17357	102.27379	168.07334		65.7996	16.7694	92.8643	177.4829
10	318	124.42336	91.520588	157.32613		65.8055	16.7710	82.1102	166.7365
12	318	113.67315	80.76492	146.58138		65.8165	16.7737	71.3530	155.9933
14	318	102.92294	70.006784	135.83909		65.8323	16.7778	60.5926	145.2533
16	318	92.172729	59.246184	125.09927		65.8531	16.7831	49.8290	134.5164
18	318	81.42252	48.48312	114.36192		65.8788	16.7896	39.0623	123.7828
20	318	70.672311	37.717597	103.62702		65.9094	16.7974	28.2924	113.0522
22	318	59.922101	26.949617	92.894586		65.9450	16.8065	17.5193	102.3249
24	318	49.171892	16.179184	82.1646		65.9854	16.8168	6.7431	91.6007
6	310	168.9753	136.07434	201.87627		65.8019	16.7700	126.6645	211.2861
12	310	159.7762	126.88379	192.6686		65.7848	16.7657	117.4764	202.0760
18	310	150.5771	117.69027	183.46392		65.7737	16.7628	108.2845	192.8697
24	310	141.37799	108.49376	174.26222		65.7685	16.7615	99.0887	183.6673
30	310	132.17889	99.294275	165.06351		65.7692	16.7617	89.8891	174.4687
36	310	122.97979	90.091803	155.86777		65.7760	16.7634	80.6857	165.2739
42	310	113.78069	80.886349	146.67502		65.7887	16.7667	71.4784	156.0830
48	310	104.58158	71.677916	137.48525		65.8073	16.7714	62.2673	146.8959
54	310	95.382481	62.466504	128.29846		65.8320	16.7777	53.0524	137.7126
60	310	86.183378	53.252118	119.11464		65.8625	16.7855	43.8336	128.5332
66	310	76.984276	44.034763	109.93379		65.8990	16.7948	34.6110	119.3575
72	310	67.785173	34.814441	100.7559		65.9415	16.8056	25.3846	110.1857
10	303	173.32931	140.42558	206.23305		65.8075	16.7715	131.0149	215.6437
20	303	168.48422	135.58718	201.38126		65.7941	16.7680	126.1785	210.7900
30	303	163.63913	130.74672	196.53155		65.7848	16.7657	121.3393	205.9390
40	303	158.79404	125.90418	191.6839		65.7797	16.7644	116.4975	201.0906
50	303	153.94895	121.05957	186.83833		65.7788	16.7641	111.6530	196.2449
60	303	149.10386	116.21289	181.99484		65.7820	16.7650	106.8059	191.4018
70	303	144.25877	111.36413	177.15341		65.7893	16.7668	101.9561	186.5615
80	303	139.41368	106.5133	172.31406		65.8008	16.7697	97.1036	181.7237
90	303	134.56859	101.6604	167.47678		65.8164	16.7737	92.2485	176.8887
100	303	129.7235	96.805432	162.64157		65.8361	16.7788	87.3907	172.0563
110	303	124.87841	91.948395	157.80842		65.8600	16.7848	82.5302	167.2266
120	303	120.03332	87.089292	152.97734		65.8880	16.7920	77.6671	162.3995
30	290	176.69791	143.78984	209.60598		65.8161	16.7737	134.3780	219.0179
60	290	175.22141	142.3171	208.12572		65.8086	16.7717	132.9063	217.5365
90	290	173.74492	140.8437	206.64614		65.8024	16.7702	131.4338	216.0561
120	290	172.26842	139.36962	205.16723		65.7976	16.7689	129.9604	214.5765
150	290	170.79193	137.89487	203.68898		65.7941	16.7680	128.4861	213.0977
180	290	169.31543	136.41945	202.21141		65.7920	16.7675	127.0110	211.6198
210	290	167.83894	134.94336	200.73451		65.7912	16.7673	125.5351	210.1428
240	290	166.36244	133.4666	199.25829		65.7917	16.7674	124.0582	208.6667
270	290	164.88595	131.98917	197.78273		65.7936	16.7679	122.5805	207.1914
300	290	163.40945	130.51106	196.30785		65.7968	16.7687	121.1019	205.7170

Table A.3.3: 90% Prediction Interval to contain all *m*=9 future observations for Control High

X = time (day)	Y = Temp (°K)	Z Predicted (rate)	95% Pred Limits for the mean		$r_{(0.90;\,9,\,\infty)}$	Δ = b-a	s	90% Pred Limits for all *m*=9 observations	
0	281	890.94847	714.26693	1067.63	2.523	353.3631	90.0568	663.7351	1118.1619
2	318	838.76167	662.11731	1015.406		353.2887	90.0379	611.5961	1065.9272
4	318	786.57488	609.95449	963.19526		353.2408	90.0257	559.4401	1013.7096
6	318	734.38808	557.77848	910.99769		353.2192	90.0202	507.2672	961.5090
8	318	682.20129	505.58925	858.81332		353.2241	90.0214	455.0773	909.3253
10	318	630.01449	453.38682	806.64216		353.2553	90.0294	402.8704	857.1586
12	318	577.8277	401.1712	754.48419		353.3130	90.0441	350.6465	805.0089
14	318	525.6409	348.94238	702.33942		353.3970	90.0655	298.4057	752.8761
16	318	473.45411	296.70037	650.20784		353.5075	90.0936	246.1479	700.7604
18	318	421.26731	244.4452	598.08942		353.6442	90.1285	193.8731	648.6615
20	318	369.08051	192.17686	545.98417		353.8073	90.1701	141.5815	596.5796
22	318	316.89372	139.89539	493.89205		353.9967	90.2183	89.2729	544.5145
24	318	264.70692	87.600802	441.81305		354.2122	90.2733	36.9475	492.4663
6	310	845.64938	669.03025	1022.2685		353.2383	90.0250	618.5162	1072.7825
12	310	800.3503	623.77758	976.92301		353.1454	90.0014	573.2768	1027.4238
18	310	755.05121	578.50891	931.59352		353.0846	89.9859	528.0169	982.0856
24	310	709.75212	533.22422	886.28003		353.0558	89.9785	482.7363	936.7679
30	310	664.45304	487.92352	840.98256		353.0590	89.9794	437.4351	891.4709
36	310	619.15395	442.6068	795.70111		353.0943	89.9883	392.1134	846.1945
42	310	573.85487	397.27407	750.43566		353.1616	90.0055	346.7710	800.9387
48	310	528.55578	351.92534	705.18622		353.2609	90.0308	301.4081	755.7035
54	310	483.2567	306.56063	659.95277		353.3921	90.0642	256.0246	710.4888
60	310	437.95761	261.17994	614.73528		353.5553	90.1058	210.6206	665.2946
66	310	392.65852	215.7833	569.53374		353.7504	90.1556	165.1960	620.1210
72	310	347.35944	170.37075	524.34813		353.9774	90.2134	119.7510	574.9678
10	303	866.77496	690.14123	1043.4087		353.2675	90.0325	639.6230	1093.9269
20	303	842.60145	666.00424	1019.1987		353.1945	90.0139	615.4965	1069.7064
30	303	818.42794	641.85596	994.99993		353.1440	90.0010	591.3554	1045.5005
40	303	794.25444	617.69637	970.8125		353.1161	89.9939	567.1998	1021.3091
50	303	770.08093	593.52549	946.63637		353.1109	89.9926	543.0297	997.1322
60	303	745.90742	569.34331	922.47153		353.1282	89.9970	518.8450	972.9698
70	303	721.73391	545.14982	898.318		353.1682	90.0072	494.6458	948.8220
80	303	697.5604	520.94504	874.17576		353.2307	90.0231	470.4321	924.6887
90	303	673.3869	496.72898	850.04482		353.3158	90.0448	446.2039	900.5699
100	303	649.21339	472.50163	825.92515		353.4235	90.0722	421.9611	876.4657
110	303	625.03988	448.263	801.81676		353.5538	90.1054	397.7039	852.3759
120	303	600.86637	424.01312	777.71962		353.7065	90.1444	373.4321	828.3006
30	290	883.38757	706.73069	1060.0445		353.3138	90.0443	656.2058	1110.5693
60	290	875.82668	699.19061	1052.4627		353.2721	90.0336	648.6718	1102.9816
90	290	868.26578	691.64669	1044.8849		353.2382	90.0250	641.1327	1095.3989
120	290	860.70488	684.09892	1037.3108		353.2119	90.0183	633.5887	1087.8211
150	290	853.14399	676.54731	1029.7407		353.1934	90.0136	626.0397	1080.2483
180	290	845.58309	668.99186	1022.1743		353.1824	90.0108	618.4858	1072.6803
210	290	838.0222	661.43256	1014.6118		353.1792	90.0100	610.9270	1065.1174
240	290	830.4613	653.86941	1007.0532		353.1838	90.0111	603.3632	1057.5594
270	290	822.90041	646.30242	999.49839		353.1960	90.0142	595.7945	1050.0064
300	290	815.33951	638.73159	991.94743		353.2158	90.0193	588.2208	1042.4582

Appendix 4: 95% Prediction Intervals to Contain all of *m* Future Observations

Appendix 4 presents Excel spreadsheet tables that were developed to calculate 95% prediction intervals that could contain all of *m*=9 future observations for each time and temperature conditions considered in the accelerated stability study. Each table is related to a response variable (Control Low, Medium or High) considered in the study.

Table A.4. 1: 95% Prediction Interval to contain all *m*=9 future observations for Control Low

X = time (day)	Y = Temp (°K)	Z Predicted (rate)	95% Pred Limits for the mean		$r_{(0.95;\,9,\,\infty)}$	Δ = b-a	s	90% Pred Limits for all *m*=9 observations	
0	281	32.905853	27.421279	38.390427	2.766	10.9691	2.7956	25.1733	40.6384
2	318	30.963002	25.479571	36.446433		10.9669	2.7950	23.2321	38.6939
4	318	29.020151	23.53745	34.502852		10.9654	2.7946	21.2903	36.7500
6	318	27.0773	21.594917	32.559683		10.9648	2.7944	19.3479	34.8067
8	318	25.134449	19.651972	30.616927		10.9650	2.7945	17.4049	32.8640
10	318	23.191598	17.708614	28.674583		10.9660	2.7947	15.4613	30.9219
12	318	21.248747	15.764844	26.732651		10.9678	2.7952	13.5172	28.9803
14	318	19.305896	13.820661	24.791131		10.9705	2.7959	11.5725	27.0393
16	318	17.363045	11.876067	22.850023		10.9740	2.7968	9.6271	25.0989
18	318	15.420194	9.9310619	20.909327		10.9783	2.7979	7.6813	23.1591
20	318	13.477343	7.9856456	18.969041		10.9834	2.7992	5.7348	21.2199
22	318	11.534492	6.0398189	17.029166		10.9893	2.8007	3.7877	19.2812
24	318	9.5916414	4.0935826	15.0897		10.9961	2.8024	1.8401	17.3432
6	310	31.258985	25.776324	36.741646		10.9653	2.7946	23.5292	38.9888
12	310	29.612117	24.130873	35.093361		10.9625	2.7939	21.8843	37.3399
18	310	27.965249	22.484925	33.445573		10.9606	2.7934	20.2387	35.6918
24	310	26.318381	20.83848	31.798281		10.9598	2.7932	18.5925	34.0443
30	310	24.671513	19.191538	30.151487		10.9599	2.7932	16.9455	32.3975
36	310	23.024645	17.544099	28.50519		10.9611	2.7935	15.2978	30.7515
42	310	21.377777	15.896164	26.85939		10.9632	2.7940	13.6494	29.1061
48	310	19.730909	14.247732	25.214086		10.9664	2.7948	12.0004	27.4615
54	310	18.084041	12.598803	23.569278		10.9705	2.7959	10.3506	25.8175
60	310	16.437173	10.949379	21.924966		10.9756	2.7972	8.7001	24.1742
66	310	14.790305	9.2994606	20.281149		10.9817	2.7988	7.0490	22.5317
72	310	13.143437	7.6490477	18.637825		10.9888	2.8006	5.3971	20.8898
10	303	32.045965	26.562838	37.529093		10.9663	2.7948	24.3155	39.7764
20	303	31.186078	25.704054	36.668101		10.9640	2.7943	23.4572	38.9150
30	303	30.32619	24.84493	35.807451		10.9625	2.7939	22.5983	38.0540
40	303	29.466303	23.985463	34.947143		10.9617	2.7937	21.7391	37.1936
50	303	28.606415	23.125654	34.087176		10.9615	2.7936	20.8793	36.3336
60	303	27.746528	22.265504	33.227551		10.9620	2.7937	20.0190	35.4740
70	303	26.88664	21.405012	32.368268		10.9633	2.7941	19.1583	34.6150
80	303	26.026753	20.544178	31.509327		10.9651	2.7945	18.2971	33.7564
90	303	25.166865	19.683003	30.650727		10.9677	2.7952	17.4354	32.8984
100	303	24.306977	18.821486	29.792469		10.9710	2.7960	16.5732	32.0408
110	303	23.44709	17.959628	28.934551		10.9749	2.7970	15.7105	31.1837
120	303	22.587202	17.09743	28.076975		10.9795	2.7982	14.8474	30.3270
30	290	32.648292	27.164439	38.132145		10.9677	2.7952	24.9168	40.3798
60	290	32.390731	26.907492	37.87397		10.9665	2.7949	24.6601	40.1214
90	290	32.133169	26.650437	37.615902		10.9655	2.7946	24.4033	39.8631
120	290	31.875608	26.393276	37.357941		10.9647	2.7944	24.1463	39.6050
150	290	31.618047	26.136007	37.100087		10.9641	2.7943	23.8891	39.3470
180	290	31.360486	25.878631	36.842341		10.9637	2.7942	23.6318	39.0892
210	290	31.102925	25.621148	36.584701		10.9636	2.7941	23.3744	38.8315
240	290	30.845364	25.363558	36.327169		10.9636	2.7941	23.1168	38.5740
270	290	30.587802	25.105861	36.069744		10.9639	2.7942	22.8590	38.3166
300	290	30.330241	24.848056	35.812426		10.9644	2.7943	22.6011	38.0594

Table A.4. 2: 95% Prediction Interval to contain all *m*=9 future observations for Control Medium

X = time (day)	Y = Temp (°K)	Z Predicted (rate)	95% Pred Limits for the mean		$r_{(0.95;\,9,\,\infty)}$	Δ = b-a	s	95% Pred Limits for all *m*=9 observations	
0	281	178.1744	145.2619	211.08691	2.766	65.8250	16.7759	131.7722	224.5766
2	318	167.42419	134.51858	200.32981		65.8112	16.7724	121.0317	213.8167
4	318	156.674	123.77278	189.57519		65.8024	16.7702	110.2877	203.0603
6	318	145.92378	113.02452	178.82303		65.7985	16.7692	99.5403	192.3073
8	318	135.17357	102.27379	168.07334		65.7996	16.7694	88.7893	181.5578
10	318	124.42336	91.520588	157.32613		65.8055	16.7710	78.0349	170.8118
12	318	113.67315	80.76492	146.58138		65.8165	16.7737	67.2770	160.0693
14	318	102.92294	70.006784	135.83909		65.8323	16.7778	56.5156	149.3303
16	318	92.172729	59.246184	125.09927		65.8531	16.7831	45.7507	138.5947
18	318	81.42252	48.48312	114.36192		65.8788	16.7896	34.9824	127.8626
20	318	70.672311	37.717597	103.62702		65.9094	16.7974	24.2106	117.1340
22	318	59.922101	26.949617	92.894586		65.9450	16.8065	13.4353	106.4089
24	318	49.171892	16.179184	82.1646		65.9854	16.8168	2.6566	95.6872
6	310	168.9753	136.07434	201.87627		65.8019	16.7700	122.5894	215.3612
12	310	159.7762	126.88379	192.6686		65.7848	16.7657	113.4023	206.1501
18	310	150.5771	117.69027	183.46392		65.7737	16.7628	104.2111	196.9431
24	310	141.37799	108.49376	174.26222		65.7685	16.7615	95.0156	187.7403
30	310	132.17889	99.294275	165.06351		65.7692	16.7617	85.8160	178.5418
36	310	122.97979	90.091803	155.86777		65.7760	16.7634	76.6122	169.3474
42	310	113.78069	80.886349	146.67502		65.7887	16.7667	67.4041	160.1573
48	310	104.58158	71.677916	137.48525		65.8073	16.7714	58.1918	150.9713
54	310	95.382481	62.466504	128.29846		65.8320	16.7777	48.9754	141.7896
60	310	86.183378	53.252118	119.11464		65.8625	16.7855	39.7547	132.6120
66	310	76.984276	44.034763	109.93379		65.8990	16.7948	30.5299	123.4387
72	310	67.785173	34.814441	100.7559		65.9415	16.8056	21.3009	114.2695
10	303	173.32931	140.42558	206.23305		65.8075	16.7715	126.9395	219.7192
20	303	168.48422	135.58718	201.38126		65.7941	16.7680	122.1038	214.8646
30	303	163.63913	130.74672	196.53155		65.7848	16.7657	117.2652	210.0130
40	303	158.79404	125.90418	191.6839		65.7797	16.7644	112.4238	205.1643
50	303	153.94895	121.05957	186.83833		65.7788	16.7641	107.5793	200.3186
60	303	149.10386	116.21289	181.99484		65.7820	16.7650	102.7320	195.4757
70	303	144.25877	111.36413	177.15341		65.7893	16.7668	97.8818	190.6358
80	303	139.41368	106.5133	172.31406		65.8008	16.7697	93.0286	185.7988
90	303	134.56859	101.6604	167.47678		65.8164	16.7737	88.1725	180.9647
100	303	129.7235	96.805432	162.64157		65.8361	16.7788	83.3134	176.1336
110	303	124.87841	91.948395	157.80842		65.8600	16.7848	78.4515	171.3053
120	303	120.03332	87.089292	152.97734		65.8880	16.7920	73.5867	166.4800
30	290	176.69791	143.78984	209.60598		65.8161	16.7737	130.3020	223.0939
60	290	175.22141	142.3171	208.12572		65.8086	16.7717	128.8308	221.6121
90	290	173.74492	140.8437	206.64614		65.8024	16.7702	127.3586	220.1312
120	290	172.26842	139.36962	205.16723		65.7976	16.7689	125.8855	218.6513
150	290	170.79193	137.89487	203.68898		65.7941	16.7680	124.4115	217.1724
180	290	169.31543	136.41945	202.21141		65.7920	16.7675	122.9365	215.6943
210	290	167.83894	134.94336	200.73451		65.7912	16.7673	121.4606	214.2173
240	290	166.36244	133.4666	199.25829		65.7917	16.7674	119.9837	212.7412
270	290	164.88595	131.98917	197.78273		65.7936	16.7679	118.5059	211.2660
300	290	163.40945	130.51106	196.30785		65.7968	16.7687	117.0271	209.7918

Table A.4. 3: 95% Prediction Interval to contain all *m*=9 future observations for Control High

X = time (day)	Y = Temp (°K)	Z Predicted (rate)	95% Pred Limits for the mean		$r_{(0.95;\,9,\,\infty)}$	Δ = b-a	s	95% Pred Limits for all *m*=9 observations	
0	281	890.94847	714.26693	1067.63	2.766	353.3631	90.0568	641.8513	1140.0457
2	318	838.76167	662.11731	1015.406		353.2887	90.0379	589.7169	1087.8064
4	318	786.57488	609.95449	963.19526		353.2408	90.0257	537.5639	1035.5859
6	318	734.38808	557.77848	910.99769		353.2192	90.0202	485.3923	983.3839
8	318	682.20129	505.58925	858.81332		353.2241	90.0214	433.2021	931.2005
10	318	630.01449	453.38682	806.64216		353.2553	90.0294	380.9932	879.0358
12	318	577.8277	401.1712	754.48419		353.3130	90.0441	328.7658	826.8896
14	318	525.6409	348.94238	702.33942		353.3970	90.0655	276.5197	774.7621
16	318	473.45411	296.70037	650.20784		353.5075	90.0936	224.2551	722.6531
18	318	421.26731	244.4452	598.08942		353.6442	90.1285	171.9719	670.5627
20	318	369.08051	192.17686	545.98417		353.8073	90.1701	119.6701	618.4909
22	318	316.89372	139.89539	493.89205		353.9967	90.2183	67.3499	566.4376
24	318	264.70692	87.600802	441.81305		354.2122	90.2733	15.0111	514.4027
6	310	845.64938	669.03025	1022.2685		353.2383	90.0250	596.6402	1094.6586
12	310	800.3503	623.77758	976.92301		353.1454	90.0014	551.4065	1049.2941
18	310	755.05121	578.50891	931.59352		353.0846	89.9859	506.1503	1003.9521
24	310	709.75212	533.22422	886.28003		353.0558	89.9785	460.8715	958.6327
30	310	664.45304	487.92352	840.98256		353.0590	89.9794	415.5702	913.3359
36	310	619.15395	442.6068	795.70111		353.0943	89.9883	370.2462	868.0617
42	310	573.85487	397.27407	750.43566		353.1616	90.0055	324.8997	822.8100
48	310	528.55578	351.92534	705.18622		353.2609	90.0308	279.5306	777.5810
54	310	483.2567	306.56063	659.95277		353.3921	90.0642	234.1390	732.3744
60	310	437.95761	261.17994	614.73528		353.5553	90.1058	188.7249	687.1904
66	310	392.65852	215.7833	569.53374		353.7504	90.1556	143.2882	642.0288
72	310	347.35944	170.37075	524.34813		353.9774	90.2134	97.8292	596.8897
10	303	866.77496	690.14123	1043.4087		353.2675	90.0325	617.7451	1115.8048
20	303	842.60145	666.00424	1019.1987		353.1945	90.0139	593.6231	1091.5798
30	303	818.42794	641.85596	994.99993		353.1440	90.0010	569.4852	1067.3707
40	303	794.25444	617.69637	970.8125		353.1161	89.9939	545.3313	1043.1776
50	303	770.08093	593.52549	946.63637		353.1109	89.9926	521.1615	1019.0004
60	303	745.90742	569.34331	922.47153		353.1282	89.9970	496.9758	994.8391
70	303	721.73391	545.14982	898.318		353.1682	90.0072	472.7741	970.6937
80	303	697.5604	520.94504	874.17576		353.2307	90.0231	448.5565	946.5643
90	303	673.3869	496.72898	850.04482		353.3158	90.0448	424.3230	922.4508
100	303	649.21339	472.50163	825.92515		353.4235	90.0722	400.0736	898.3532
110	303	625.03988	448.263	801.81676		353.5538	90.1054	375.8082	874.2715
120	303	600.86637	424.01312	777.71962		353.7065	90.1444	351.5271	850.2057
30	290	883.38757	706.73069	1060.0445		353.3138	90.0443	634.3251	1132.4501
60	290	875.82668	699.19061	1052.4627		353.2721	90.0336	626.7936	1124.8598
90	290	868.26578	691.64669	1044.8849		353.2382	90.0250	619.2566	1117.2750
120	290	860.70488	684.09892	1037.3108		353.2119	90.0183	611.7143	1109.6955
150	290	853.14399	676.54731	1029.7407		353.1934	90.0136	604.1664	1102.1216
180	290	845.58309	668.99186	1022.1743		353.1824	90.0108	596.6132	1094.5530
210	290	838.0222	661.43256	1014.6118		353.1792	90.0100	589.0546	1086.9898
240	290	830.4613	653.86941	1007.0532		353.1838	90.0111	581.4905	1079.4321
270	290	822.90041	646.30242	999.49839		353.1960	90.0142	573.9210	1071.8798
300	290	815.33951	638.73159	991.94743		353.2158	90.0193	566.3461	1064.3329