DEGRADATION KINETICS OF PATULIN BY ASCORBIC ACID AND
DEVELOPMENT OF PREDICTIVE MODEL USING RESPONSE SURFACE
METHODOLOGY

A Thesis in
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by
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ABSTRACT

The mycotoxin patulin (PAT) is a secondary metabolite produced by several mold species. *Penicillium expansum*, the primary cause of post-harvest decay in stored apples, is the most important source of PAT contamination in apples and processed apple products. Animal studies have demonstrated a variety of acute and chronic effects from ingestion of PAT including carcinogenicity, teratogenicity, and mutagenicity. Based on long term animal and cell studies, FDA has established an action level for PAT in single strength apple juice at 50μg/kg (50 ppb).

The objectives of this study were to:

1) Evaluate the thermal degradation kinetics of patulin (PAT) in the presence of ascorbic acid (AA) and determine factors that affect the rate of the reaction.

2) Establish relationships between factors and the rate constant of the degradation reaction and develop a predictive model.

3) Validate the model and test its predictive power in apple juice (AJ).

4) Identify products formed and elucidate a mechanism for the reaction between patulin and ascorbic acid.

Factors affecting the degradation rate of PAT in the presence of ascorbic acid (AA) were studied using an apple juice model system containing 0.5% malic acid. A mathematical model to describe the kinetics of the reaction was established and a predictive model for the rate constant of the degradation was developed using a Response Surface Methodology (RSM) approach.
Results demonstrated that pH, initial concentration of AA and PAT and temperature significantly (p<0.05) affected the degradation rate of the toxin. Initially a second order kinetic model was used to describe the degradation of PAT and estimate the reaction rate constant. A significant predictive model was developed based on these estimations using an RSM design. Closer examination of the fit of the second order model, for experimental treatments, indicated the need for a more accurate and kinetic model.

Therefore, the data were reanalyzed using the Weibull model to describe the degradation of PAT by AA in both a model system and a commercial apple juice.

A wide range of temperatures was utilized (25-85°C) and the dependency of b and n Weibull parameters on temperature was determined. The reaction rate constant (Weibull parameter b) had an Arrhenius dependence on temperature (Arrhenius plot $R^2 = 0.986$) with an activation energy of $E_a = 29.6$kJ/mol and a z-value of $z = 59.7^\circ C$. The Weibull shape parameter (n) was not temperature dependent. Thus, a simplified Weibull model was applied to the experimental data after setting the Weibull shape parameter n-0.44. The simplified Weibull model produced consistently good fit.

A 3-factor- 3-level Box-Benhken design was then used to determine the effects of the initial levels of PAT, AA, and pH on the rate of the degradation reaction and to develop a model that sufficiently predicts the kinetic parameters at different processing conditions. The developed RSM model, after applying a logarithmic transformation to the response, was highly significant (p<0.001, $R^2 = 0.97$) and the lack of fit was not significant (p>0.05). Four validation experiments were carried out; three using the model
system (malic acid buffer, pH 3.75), and one using a shelf-stable apple juice commercial product. Observed and predicted values were not statistically different (p<0.05), thus, confirming the predictive power of the model.

Exploratory studies were conducted to determine the mechanism for the degradation of PAT in the presence of AA in the model system. HPLC/PDA, LC/ESI/MS and LC/MS/MS were used to monitor the progress of the reaction. Although the results were inconclusive several degradation products of AA were determined and the reduction of PAT levels by AA was evident. The results from our model system studies suggested that PAT could be degrading via a redox mechanism or a Michael-like addition were ascorbate (nucleophile) attacks PAT (electrophile) to form adducts that will eventually lead to the opening of the lactone ring and the detoxification of PAT.
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Chapter 1

Introduction

1.1 Statement of the problem

The mycotoxin patulin (Figure 1.1) is a secondary metabolite produced by several mold genera including *Penicillium*, *Aspergillus* and *Byssoschlamys*. *Penicillium expansum*, is the primary cause of post-harvest decay of apples and the main source of patulin contamination in apples and processed apple products (Paster and others 1995).

Figure 1.1

![Patulin](image)

Figure 1.1: Patulin
Animal studies have demonstrated a variety of acute and chronic effects from ingestion of patulin including carcinogenicity, teratogenicity, and mutagenicity. Only indirect evidence suggests that patulin is harmful to humans, but U.S. and international food regulatory agencies have mandated limits on patulin content in foods. The U.S. Food and Drug Administration (FDA) evaluated dietary exposure of patulin among apple juice drinkers of all ages and concluded that small children are at greatest risk, because they consume higher amounts of apple juice relative to their body weight than other age groups. Based on long term animal and cell studies, FDA set the action level for patulin in single strength apple juice at 50μg/kg (50 ppb) (FDA, 2000). The World Health Organization (WHO) has also recommended a maximum patulin level of 50 ppb based on a toxicological assessment (WHO, 2002) with at least twelve countries regulating patulin at 25-50 ppb (Van Egmond HP.1989).

In response to a series of foodborne disease outbreaks attributed to consumption of apple cider contaminated with human pathogens, FDA mandated that juice processors develop and implement food safety plans based on the principles of Hazard Analysis and Critical Control Point (HACCP) (FDA 2001a; 21 CFR Part 120). Although the juice HACCP regulation was implemented primarily to prevent the occurrence of microbial hazards, processors are also expected to develop control measures to keep patulin levels below 50 ppb.

The FDA Office of Regulatory Affairs considers patulin as an adulterant and may recommend legal action or detention of imports if levels exceed 50 ppb in juice (FDA, 2001b). Failure to comply with the regulations and control patulin levels is considered a violation (FDA, 2002). In order to assure that their products comply with the regulations
juice producers must implement good manufacturing practices, good quality assurance efforts and Hazard Analysis Critical Control Point principles (Proposed by Lopez Garcia and Park 1998 and Park et al., 1999).

1.2 Rational and significance

According to the current Juice HACCP Guidance Document (FDA, 2004), pre-requisite programs such as Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) are critical to the success of a HACCP plan. Nevertheless, controlling patulin levels through pre-requisite programs is difficult to achieve. Research has shown that removal of decayed apples, trimming decayed portions, and wash treatments prior to pressing reduce levels of patulin in apple juice, (Jackson LC. 2003) However, these methods are difficult to monitor and verify under the stringent protocols required for HACCP plans and thus cannot guaranty that the final product will contain patulin levels below the regulatory limit. It has been demonstrated that fungicide resistant populations of *P. expansum* in storage and packinghouses can invade over-ripe fruit apples through wounds and lenticels, and can penetrate through stems during low-oxygen storage. The resulting core rot is extremely difficult to spot during its initial stages, and thus, is not likely to be removed during sorting prior to packing or processing (Rosenberger et al., 1991;1999; Paterson et al., 2000). Moreover, Rosenberg (2003) reported that 20% of bagged, in-store, retailed apples contain visible evidence of decay.
Based on the above here is ample evidence that current industry sanitation practices cannot assure the exclusion of contaminated apples. Therefore, in order to guarantee safe juice products, we must develop and validate effective treatments within HACCP plans. Post processing physical or chemical control measures would be more suitable for the juice industry, because monitoring and verification would be easier to achieve as compared to current culling and trimming methods. However addition of chemical agents in human foods for reducing the level of mycotoxin is not permitted in USA (Park and Troxell 2002). Therefore, only treatments already approved for other purposes are a feasible solution for the juice industry.

1.3 Objectives

There is a need for efficient control strategies in order to address the food safety hazard presented by patulin. To date, the most commonly utilized methods for patulin removal from apple juice are pre-production methods applied during apple harvest, processing and storage such as culling, sorting, trimming and washing. These treatments, although effective, are difficult to control and verify under the stringent protocols required within HACCP systems. More effective control methods must be developed for application during processing or post-processing.

Previous studies indicate that the addition of ascorbic acid can cause reduction of patulin levels in apple juice, but none of these studies adequately considered the effects of temperature, time of storage, and juice composition, and none have proposed a
mechanism for the degradation reaction. There are many reasons to determine the feasibility of using ascorbic acid as a treatment to reduce patulin levels in apple juice. Ascorbic acid is known to increase patulin degradation and decrease toxicity (Alves I. et al. 2000). It has been designated as Generally Recognized As Safe (GRAS) when used in accordance with Good Manufacturing Practices (21CFR 182.3013). Also is relatively inexpensive, is widely recognized by consumers as a beneficial nutrient (vitamin C), and it does not negatively affect the sensory properties of apple juice. Although earlier studies suggest that ascorbic acid may be a feasible solution and preferred over many other forms of treatments, there is a need for further investigation to establish a science-based method for its use.

More work is needed to verify and validate the optimum conditions under which the utilization of ascorbic acid will result in a safe product.

Therefore the objectives of this study were to:

1) Evaluate the thermal degradation kinetics of patulin (PAT) in the presence of ascorbic acid (AA) and determine factors that affect the rate of the reaction.

2) Establish relationships between factors and the rate constant of the degradation reaction and develop a predictive model.

3) Validate the model and test its predictive power in apple juice (AJ).

4) Identify products formed and elucidate a mechanism for the reaction between patulin and ascorbic acid.
2.1 Literature review

2.1.1 History

When PAT was first isolated 1942, it was thought to have a broad spectrum of antibiotic properties (Stott and Bullerman 1975). It was effective against a wide range of fungi (Korzybski and others 1976) and Gram-positive and Gram-negative bacteria (Ciegler and others 1971). The discovery of the compound was claimed by various groups and it has historically been given names such as clavacin (Anslow and others 1943), expansine (Van Luijk 1938), claviformin (Chain and others 1942), clavatin (Bergel and others 1943), gigantic acid (Philpot 1943), and myocin C (DeRosnay and others 1952).

Results originating from uncontrolled studies such as The Army and The Navy trail suggested PAT to have applications in treatment of nasal congestion and the common cold giving hope for a remarkable cure. Not long after, these results were found to be unsupported by one of the first examples of clinical trials (Medical Research
Council 1944; Stansfield and others 1944) which suggested that PAT was very unlikely to be the universal remedy for common cold. Shortly after, various studies suggested PAT to be not only toxic to fungi and bacteria, but also to animals and higher plants (Iyengar and Starky 1953; Norstadt and McCalla 1963, 1968; Berestets’kyi and Synyts’kyi 1973) illustrating an excellent example of the close relationship between anthropogenic benefits and detriments that many compounds exert.

2.1.2 Occurrence and sources of patulin

PAT (4-Hydroxy-4H-furo-[3,2c]pyran-2[6h]one) chemically is an \( \gamma \)-unsaturated, heterocyclic lactone. It is a colorless crystal with a molecular weight of 154, a melting point of 111 \( ^\circ \) C and a maximum absorbance at 276nm. It is soluble in water, ethanol, acetone, ethyl acetate, ethyl ether and chloroform but insoluble in benzene and petroleum ether and it is stable to heat processing at pH < 6.

PAT has been found to be a contaminant in a variety of fruits grains and cheese (Bullerman and Olivigni 1974) but apples and pears are the most frequently contaminated fruits (Mortimer et al. 1985). PAT has also been detected in grapes (Altmayer et al. 1982), blackcurrants (Larsen et al. 1998), cherry juice (Larsen et al. 1998), wheat and barley (Harwing et al. 1977) and corn (Lin et al. 1993). PAT appears to be a safety concern in AJ and AJ products because of its stability at the pH range of AJ.

An FDA survey conducted in 1993 reported that nearly one fifth of AJ samples contained more than the 50 ppb limit (Mycotoxins in agriculture and food safety Kaushal
K. Sinha, Deepak Bhatnagar 2003). Of particular concern are smaller processing operations because poorer quality apples are often used to make cider.

The most likely range of PAT levels is 50-200 ppb but samples with levels in a range of 400-600 ppb have been found (Plant product safety division, FDA Dr. Michael E. Kashtock personal communication, 2006).

Sommer et al., 1974 reported that PAT is produced within the entire temperature growth range *P. expansum* but that the optimum temperature is 25°C. Production of PAT in apples by Penicillium sp. is dependent on the intrinsic properties of the substrate such as water activity, pH, and temperature as well as the strain of the organism (Pasteur et al., 1995). McCallum et al. (2002) reported that PAT production and stability is inversely related to pH and that temperature affects the growth of Penicillium and thus the production of PAT. Jackson et al., 2003 showed that apple cultivar, production and harvesting methods, as well as culling practices affected the amount of PAT in apples and in cider made by infected apples.

### 2.1.3 Toxicity

The health effects from consumption of PAT are based on a wide number of studies. PAT inhibits activities of several enzymes because of its strong affinity for sulphydryl groups (Ashoor and Chu 1973, Arafat et al. 1985, Wouter and Speijers 1996).
It also has been found to be immunosuppressive (Escoula et al. 1988, Bourdial et al. 1990, Paucod et al. 1990, Sharma 1993) and capable of disturbing the mitochondrial plasma membrane.

Acute symptoms of PAT consumption include agitation, dyspnea, pulmonary congestion, edema, ulceration, hyperemia, GI tract distension, intestinal hemorrhage, epithelial cell degeneration, intestinal inflammation, and vomiting and kidney damage (Mckinley et al. 1982). Chronic health risks include neurotoxic, immunotoxic, immunosuppressive, genotoxic, teratogenic, and carcinogenic effects (Mayer and Legator, 1969; Ceigler et al., 1976, Taniwaki et al., 1991; Alves et al., 2000). In a two-generation study conducted on rats, in which males and females were administered 1.5 mg/kg per day orally for 10 or 14 weeks, there was no evidence of teratogenicity (Dailey et al., 1966). Teratogenicity was observed when 12μg of PAT were injected in chick embryos. The major abnormalities observed were malformed feet, ankle, exophthalmal, and exencephaly (Ciegler et al., 1976).

The results from an animal feeding study conducted by Becci et al., 1981 were used to set the “no observed adverse effect level” (NOAEL) for PAT. In this study doses of 0.0, 0.1, 0.5, and 1.5 mg/kg bw were administered to male and female rats three days per week by gastric intubation. PAT at highest dose level caused a significant increase in the mortality rate in both sexes. The cause of death appeared to be increased pulmonary and laryngotracheal inflammation. No adverse effects observed in the group receiving 0.1 mg/kg body weight three times per week. Based on the results of this study, the Food and Drug Administration (FDA 2002) established the NOAEL for PAT at 0.3 mg/kg body weight per week.
2.1.4 Treatments to reduce patulin levels in foods

2.1.4.1 Thermal treatments

PAT is generally stable at high temperatures (Kryger 2001). Wheeler et al., (1987) and Kadakal et al., (2002) reported that high-temperature short-time processing will reduce but not completely destroy the toxin. Scott and Somers (1968) reported that PAT in AJ was stable at 80°C for 10–20 min and there was little decrease after 3 weeks storage at 22°C. Lovett et al. (1973) showed that PAT was resistant to thermal destruction at a pH range of 3.5 – 5.5 when heated up to 125°C. Brackett and Marth (1979) reported PAT half-life values of 55 and 2.6 days, respectively in juice held at 25 °C at pH 6.0 and 8.0 which illustrates the increased stability of PAT at low pH values. Kubacki (1986) reported a 20% PAT reduction in AJ heated 120 °C for 30 min although 30 min at 80 °C had no effect.

2.1.4.2 Physical treatments

Physical treatments are discussed in detail in a comprehensive review by Moake et al (2005). Sorting and hand trimming PAT-containing apple tissues may decrease mycotoxin levels in juice by as much as 90% (Lovett et al., 1975). Nearly complete removal of PAT by filtration through charcoal has also been reported (Sands et al., 1976; Bissessur et al., 2000).
Methods such as centrifugation and filtration and absorption with activated carbon in a “static” or “flow through” system have been evaluated (Huebner and others 2000; Gokmen and others 2001). Clarification and filtration or ultrafiltration of juice results in a reduction of PAT levels as much as 39% (Acar et al., 1998). Gelatin, bentonite, activated charcoal, and adsorbent resins are similarly capable of reducing PAT levels (Gokmen, et al., 2001). Adoption of these types of methods by processors is not practicable due to the fact that the use of activated carbon for industrial scale juice purification will dramatically increase the processing cost. It will be both time consuming and expensive (Leggot et al., 2001). Furthermore it creates excess wastes which has to be dealt with ecologically (Artik et al., 2001) and has negative effects on the overall quality of the juice (color, Brix, pH and fumaric acid content).

Washing apples with water before pressing reduces PAT levels in cider by 10 to 100%, depending on apple cultivar, initial PAT levels, and the type of wash solution used (Jackson, 2003). Sydenham et al., (1997) showed that juice processed from apples that had been stored in the open (“deck storage”) for up 33 days contained significant amounts of PAT (55 to 405 ng/g) even if the fruit had been washed and cull.

It has also been determined that PAT is degraded by high hydrostatic pressure (Brůna et al. 1997). The content of PAT in AJ decreased by 42, 53 and 62% after 1 h treatment at 300, 500 and 800 MPa, respectively, at 20°C.

While effectiveness of these physical methods varies widely and substantial loss of quality may take place, these are the only methods currently recommended by FDA in the Juice HACCP Hazards and Controls Guidance for controlling PAT levels (FDA, 2004).
2.1.4.3 Biological treatments

PAT levels are substantially reduced during fermentation processes. Harwig et al. (1973) reported that PAT in AJ was completely degraded after 2 weeks of fermentation by Saccharomyces spp. Stinson et al. (1978) similarly reported that PAT was reduced by over 99% after fermenting for 2 weeks compared to only a 10% reduction in unfermented juice over the same period. A study by Ough and Corison (1980) reported that PAT rapidly degrades during AJ fermentation to undetectable levels.

While these biological treatments are effective, they are limited to products that can be fermented. Another limitation is that yeasts that are used for the fermentation are sensitive to PAT; Sekiguchi et al. (1983) showed that in concentrations greater than 200 mg/ml, fermentative detoxification by yeasts is completely inhibited.

2.1.4.4 Chemical Treatments

Chemical decontamination of juice may be more suitable for industry due to its consistency of effectiveness and lower cost labor. Chemical treatments can be divided into two groups; those that destroy P. expansum spores on apples and other surfaces, and those that react with the PAT molecule to form non-toxic products. The following review will only discuss chemical treatments that reduce the levels and the toxicity of PAT.

Sulfur containing reducing agents are capable of reducing PAT levels in food. Pohland and Allen (1970) have shown that PAT is unstable in the presence of sulfur.
dioxide. Ough and Corison reported that 100 ppm of sulfur dioxide reduced PAT levels immediately by 50%. Aytar and Acar (1994) reported that by adding 100 mg of sulfur dioxide/kg AJ PAT levels were reduced by 42%. Another study (Burroughs 1977) showed that 200ppm of sulfur dioxide caused a 12% reduction after 24 hours in juice. Valletrisco et al. (1989) reported the disappearance of 100 ppm PAT in buffer within 4-5 days after addition of 1200 ppm cysteine or 800 ppm glutathione. Sulfhydryl groups of cysteine or glutathione react with PAT (Valletrisco et al. 1989) to produce PAT adducts with no toxic effects (Lindroth et al. 1990). Finally a study by Steiner et al.1999, found that PAT degradation by sulfur dioxide is reversible or irreversible depending on the pH of the reaction.

Strong oxidizing agents such as ozone and hydrogen peroxide are also capable of degrading PAT. The mycotoxin was found to be completely degraded in 15s in aqueous solutions by 10wt% ozone (McKenzie et al 1997)

The use of various organic acid and vitamins was another method examined for PAT detoxification. Research by Brackett and Marth (1979a) showed that when ascorbic acid (Vitamin C) and ascorbate were added at up to 5% (w/v) to PAT-spiked AJ, complete disappearance of PAT could be observed when measured as a decrease in absorbance at 254nm. In pH 3.5 buffer, PAT (5000 ppm) degradation at 25°C increased with increasing ascorbic acid concentration between 150 and 3000 ppm. An 80% loss of PAT was observed in 3000 ppm ascorbic acid after 8 days at 25°C compared to a 20% reduction in control samples containing no added ascorbic acid. Moreover, in AJ containing 5000 ppm ascorbic acid, a nearly 90% reduction in PAT was observed after 21 days at 10°C. Only 10% was lost in control samples. A study conducted by Aytac and
Acar (1994) showed that treating AJ with 500 mg/kg ascorbic acid reduced PAT levels by 50%. Another study conducted by Fremy et al. (1995) however, showed that ascorbic acid resulted in only 5% degradation after 3 hours and 36% degradation after 44 hours. Canas and Aranda (1996) reported that by adding 100 ppm of ascorbic acid to a sample, the initial PAT level (450 ppm) decreased by 50% after 72 hours and by 85% after 150 hours. The storage temperature was not mentioned. Decreases in PAT concentration were lower in AJ samples compared to buffer samples suggesting that AJ contains substances that may protect PAT degradation by ascorbic acid or sulfhydryl compounds. The researchers in this study (Canas and Aranda 1996) claimed that toxicity studies demonstrated a reduction in toxicity after ascorbic acid treatment but data were not provided to support their claim.

It is noticeable that there is controversy between the different studies regarding the effectiveness of the chemical treatments. In many of the studies researchers use crude spectrophotometric techniques that do not provide sufficient information regarding the reaction end products. Only one paper (Canas and Aranda, 1996) proposes a mechanism for the reaction between PAT and ascorbic acid but the authors did not present data supporting their hypothesis. Fliege and Metzler (1999) proposed a mechanism of reaction between PAT and sulfur containing molecules proving that the electrophilic properties of the mycotoxin were responsible for the reaction. In addition to ascorbic acid, other vitamins have been utilized for PAT degradation; the B vitamins, thiamine hydrochloride, pyridoxine hydrochloride and calcium-b-pantothenate yielded significant PAT reductions (Yazici and Velioglu 2002). Some of the studies propose mechanisms for the degradative pathway of PAT.
2.1.4.5 Electromagnetic irradiation treatments

Studies have shown that electromagnetic irradiation is effective for reducing levels of PAT and other mycotoxins. Zegota et al. (1988) confirmed that the treatments of juice with 0.35 kGy ionizing irradiation caused a 50% reduction of PAT without increasing non-enzymatic browning reactions in the juice. PAT has been also demonstrated to be sensitive to UV light (Doyle et al. 1982, Valletrisco et al. 1990).

2.1.5 Methods of analysis of patulin in apple juice

There are many techniques available for detecting and quantifying PAT. Liquid-liquid partition and solid phase extraction are the most commonly used methods of sample preparation prior to analysis. Analytical methods include Thin-Layer Chromatography (TLC), Gas Chromatography (GC) and Liquid Chromatography (LC). GC and LC are usually coupled with either a UV detector or a Mass Spectroscopy (MS) system that provides additional selectivity and increased sensitivity.

2.1.5.1 Thin-layer Chromatography

This technique is simple and quick but it is semi-quantitative and not as accurate as newer chromatographic techniques. It can, however, be a useful first identification step for the presence of PAT in a sample. Prieta J. et al., (1992) developed the thin-layered technique for PAT identification. The mycotoxin is extracted from the sample using ethyl
acetate in a biphasic dialysis system and it is detected by thin-layer chromatography using a 3-methyl-2-benzothiazolinone-hydrazone solution as spray reagent. The limit of detection is 20 μg/L.

### 2.1.5.2 Gas Chromatography

GC-MS is an alternative analytical technique for PAT analysis and identification and can be used for qualitative PAT confirmation in AJ and concentrate without a prior derivatization step. Rychlik and Schieberle (1999) developed a GC/MS method with a lower detection limit of 12 ppt and lower quantification limit of 35 ppt when the mass spectrometer is operated in the high resolution mode. Rupp and Turnipseed (2000) developed the official AOAC method with a low detection limit of 30 ppb.

### 2.2.5.3 Liquid Chromatography

The official method proposed by the Association of Analytical Chemistry (AOAC) for PAT detection in AJ utilizes liquid-liquid extraction with ethyl acetate and high performance liquid chromatography for the detection and identification of the mycotoxin. The method has a detection limit of 5μg/L.

The liquid chromatographic systems include reverse-phase analytical LC columns with C-18 stationary phase (4μm particle diameter) and a UV or photo diode array detector set at 276 nm (absorption wave length maximum of PAT). The mobile
phase must be developed so that adequate baseline separation of PAT and DHMF, common in processed AJ, occurs. Another method that has been utilized to confirm the presence of PAT is mass spectroscopy. A combination of solid phase extraction, liquid chromatography and mass spectroscopy technique was developed by Rie Yto et al. (2004). Measurements were conducted with selected ion monitoring mode (SIM) with a detection limit of 2.5pg and quantification limit of 5pg in a sample of 5 μl. Roach JAG et al. (2002) developed a combined technique that utilizes HPLC detection and GC/MS conformation for PAT identification.

A slightly modified method for turbid AJ and apple puree was developed by McDonald et al., (2000). In this method, samples prior to extraction, are treated with pectinase for 2 hours at 40°C and then centrifuged for 5 min at 4500 G.

### 2.1.5.4 Micellar electrokinetic capillary electrophoresis

This technique is advantaged over other methods because smaller sample volumes are required and the amount of solvents used is minimized. Separation of PAT from other compounds is achieved by differential migration of charged particles in the run buffer. A technique developed by Tsao and Zhou (2000) achieved a detection limit of 3.8μg/L
2.1.5.5 Sample preparation

Methods for determination of PAT are based on pre-analytical preparation steps that remove background chemical and allow quantification of the analyte. Liquid-liquid extraction with ethyl acetate (Mac Donals and Illida 1997; MocDonald, Long, Gilbert & Felgueiras 2000) and solid phase extraction (SPE) are the methods used for this purpose.

SPE of PAT was developed by Trucksess and Tang (1999). In this method, a copolymer cartridge is used for extraction of the mycotoxin from AJ and then further analysis of the extract is performed by HPLC. Eisele and Gibson (2003) developed a very efficient syringe-cartridge technique for the extraction of PAT. This method requires less equipment than the one proposed by Trucksess and Tang (1999) and it is more time efficient (<30min combined time).

Dysphasic dialysis is another technique used for extracting PAT from AJ (Domínguez et al 1992, Prieta et al. 1992). In this technique, the extraction is achieved using a semi-permeable membrane that separates two immiscible phases. Separation of compounds is achieved based on polarity and molecular weight. Dysphasic dialysis was first coupled with TLC (Domínguez et al 1992, Prieta et al. 1992) and HPLC (Prieta et al. 1993, 1994). Combined dysphasic dialysis and in situ acylation of PAT was coupled with GC/MS for quantification purposes.
Chapter 3

Kinetics of the degradation of patulin in the presence of ascorbic acid

3.1 Abstract

Factors affecting the degradation rate of PAT in the presence of ascorbic acid in an aqueous juice model system were studied. PAT degradation in the presence of ascorbic acid appeared to follow second order reaction kinetics with respect to PAT, although there were some deviations. Using the second order kinetic model, reaction rates were calculated. Temperature, pH and initial concentration of the reactants (PAT and AA) all caused statistically significant (p<0.05) changes to the reaction rate constants. The reaction rate showed an Arrhenius dependency on temperature ($R^2=0.986$). Based on that the activation energy was of the reaction was $E_a= 59.5$kJ/mol and the z-value=34.2°C.

The effects of pH and initial concentration of PAT and ascorbic acid on the degradation of the mycotoxin in a model AJ system were studied using response surface methodology (RSM). A 3-factor-3-level Box-Behnken experimental design was used and reaction rate constants were calculated based on the second order kinetic model. Results demonstrated that pH and initial concentration of AA and PAT significantly (p<0.05)
affected the rate of PAT degradation. After applying a logarithmic transformation the lack of fit for the predictive model was not significant (p>0.05) and it explained 98% of the variability for the rate constant. Three validation experiments were carried out utilizing a model system and a shelf-stable AJ commercial product and each experiment was triplicated. Observed and predicted values were not statistically different indicating the effectiveness of the predictive model developed using RSM. After examining the fit of the second order model for all the experimental, as well as, validation treatments it became evident, especially when AJ was utilized, that although its fit could be considered adequate in most cases, the introduction of error to our predictions was significant. This confirmed the complexity of the degradation mechanism and the need for a more accurate and robust kinetic model.

3.2 Introduction

Although many studies verify the reduction of PAT levels when ascorbic acid is added, none of them have adequately investigated the effect of temperature on the rate of the reaction. Some studies use crude techniques for PAT identification, impractical buffer systems or temperature ranges, and others utilize extraction methods with questionable recovery rates. Although ascorbic acid was shown to cause degradation of PAT, the results from previous studies were conflicting and inconclusive with regards to the efficiency of ascorbic acid addition as a control method. Moreover, none of the studies considered the effects of pH and the initial concentration of the reactants.
To date there is a scarcity of information regarding the degradation kinetics of PAT in the presence of ascorbic acid. The absence of effective critical control points and an understanding on how processing and intrinsic parameters influence the levels of PAT when ascorbic acid is present makes its utilization as an effective treatment difficult. In most cases process optimization is empirical and unpredictable.

Chemical kinetics or reaction kinetics is the study of the rate of chemical reactions. By definition it is the investigation of the influence that different conditions have on the reaction rate. Determination of the reaction rate can also provide useful information about the mechanism by which the reaction progresses and can also be a practical tool for process optimization.

Many factors affect the rate of a reaction but one of the first to be considered is temperature because usually it has a major effect on the reaction rate. When the amount of heat introduced into a system is increased, the thermal and vibrational energy of the molecules is also increased. This results in an increased probability of collisions and a higher reaction rate. Investigating the effect of temperature on the reaction rate and establishing a model to describe it enables us to develop critical control points that could be implemented into HACCP plans, and provide juice processors with a viable solution for PAT control.

The concentration of reactants is also an important factor. Based on the collision theory, molecules must collide in order to react. Thus, the higher the concentration of reactants, the higher the frequency of collision and consequently the higher rate of the reaction. To our knowledge there are no studies adequately considering the effect of the
initial concentration of PAT and ascorbic acid on the reaction rate and on the overall degradation of the toxin.

The nature of the reactants also affects the reaction rate, because the type of the bonds formed will influence the time it takes to form products. The physical state (solid, liquid, or gas) of a reactant is also an important factor influencing the rate of the reaction.

The chemical environment and factors such as pH can also influence the rate of a reaction. The presence of certain chemical species may hinder or facilitate the reaction and pH may influence the physical state of the reactants. Several studies investigated the effect of pH on the stability of PAT, but none of them examined the effects of pH on the reaction of PAT with ascorbic acid. Elucidating the effect of pH is important because it can affect the state and chemical properties of both reactants and consequently influence the rate and the overall degradation of PAT.

In this study we assumed that four factors influence the degradation of PAT in the presence of ascorbic acid: temperature (T), pH of the system (pH) and initial concentrations of reactants, namely PAT (PAT) and ascorbic acid (AA).

Thus, our objectives were to: a) find a kinetic model that sufficiently describes the degradation of PAT in the presence of AA; b) estimate the kinetic parameters of the reaction; c) conduct preliminary experiments to determine if T, pH and initial levels of reactants significantly affect the degradation rate of PAT; d) employ Response Surface Methodology to establish relationships between rate constant and significant factors (T, pH, AA, PAT) and develop a predictive model using RSM e) validate the predictive model using a model system and shelf-stable AJ commercial product.
3.3 Materials and methods

3.3.1 Model system

A model system containing 0.5% malic acid (Sigma-Aldrich, Saint Louis, MO) was used as a reaction medium. The selection of the concentration of malic acid was based on typical levels found in AJ (Mattick L.R. and Moyer J.C., 1983). The pH of the solution was adjusted by adding NaOH solution and was measured using an Accumet AR25 dual channel pH/ion meter (Fisher scientific, Fair Lawn, NJ), which was calibrated weekly.

3.3.2 Sample preparation

PAT (purity ≥98%, Sigma-Aldrich, Saint Louis, MO) was used to prepare internal/external standards and reaction solutions. AOAC procedures (AOAC, 2000) were followed for preparation of PAT solutions. A stock solution was made by adding five milligrams of PAT into 25-mL volumetric flask and bringing to volume with HPLC grade ethyl acetate. One milliliter of the stock solution was transferred to a test tube and dried under a gentle stream of nitrogen. Four milliliters of distilled water acidified to pH 4 with acetic acid was added to produce a 50,000 ppb solution. The solution was vigorously vortexed for 30 sec and kept at 4°C until use.
Dilutions for standards and reaction solutions were prepared and PAT concentrations were confirmed spectrophotometrically, based on the maximum absorption (A) of PAT at 276 nm using the following equation:

\[
\mu g \text{ PAT/mL} = \frac{(A \times MW \times 1000 \times CF)}{\varepsilon} 
\]  

(Eq. 3.1)

Where, \(MW= \) molecular weight of PAT = 154 g/mol, \(CF = \) correction factor, (0.95 to 1.05), and \(\varepsilon = \) extinction coefficient or molar absorptivity = 14,600 mol\(^{-1}\) L cm\(^{-1}\)

Experiments were conducted by holding treatment and control solutions in a controlled temperature water bath. The model system and the AJ samples were spiked with appropriate amounts of PAT and AA to achieve the desired final concentrations at a total volume of 50.0 ml. Each solution was prepared and sampled as follows: Prior to addition of PAT and AA, malic acid was added to 250-ml Erlenmeyer flasks and immersed in the water bath for 30 min to allow the temperature of the solution to equilibrate with that of the water bath. The water level of the bath was at least 1.5 inches above the level of the flask to ensure uniform temperature distribution. Once equilibration was achieved, the flask was removed from the water bath, an appropriate volume of PAT or PAT and AA solution was added, and the mixture was mechanically stirred for 20 sec. One-ml sample was immediately taken from the flask for HPLC analysis. The flask was immediately returned to the water bath after sampling.

Samples were taken and PAT levels were determined by HPLC every two hours during successive eight hour days. Experiments were triplicated for each temperature.
The reactions were followed until PAT was no longer detectable or until no further degradation of PAT was observed.

3.3.3 High Performance Liquid Chromatography (HPLC) analysis

The HPLC system used for all experiments consisted of a Waters 600 controller and HPLC pump delivery system, a Waters 717 plus auto-sampler, and a Waters 996 photodiode array UV detector (Waters Corp. 2002 Milford, MA). Waters Empower Pro software was used to analyze the data.

A column with a new stationary phase (Primesep® D, SIELC, Prospect Heights, IL) that enabled direct injection of samples was used to separate and quantify PAT in the model system. The column was comprised of two types of functional groups: 1) an anion exchange group; and 2) a long alkyl chain chemically bonded to silica support. Strong interactions between the two phases facilitated retention and separation of both ionizable and non-charged compounds. By changing the type and amount of acid and organic modifier in the mobile phase, controlled retention was possible that allowed separation of both PAT and AA. A two-solvent gradient elution technique was developed. Solvent A consisted of 0.1% formic acid (A.C.S. reagent, Sigma-Aldrich Saint Louis, MO) in HPLC grade water adjusted to pH 1.85 with hydrochloric acid (Riedel-de Haen, Germany). Solvent B consisted of HPLC grade Acetonitrile (Fisher scientific Fair Lawn, NJ). Gradient conditions are shown in Table 3.1. Minimum detection levels ranged from 10 to
25 ppb. Lower detection levels were possible in some experiments due to upgrading of the photodiode array detector.

Table 3.1

<table>
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<th>Time (min.)</th>
<th>Flow (ml/min)</th>
<th>%A</th>
<th>%B</th>
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The analytical procedure for PAT quantification resulted in satisfactory peak resolution for the reactants. Chromatograms of AA, PAT, as well as a chromatogram of PAT and AA mixture and are shown in Figure 3.2. The retention time for AA and PAT was 2.6 and 4.46 min, respectively. The maximum absorbency for AA and PAT were 241.9 and 276.0 nm, respectively.
For analysis of AJ samples, a reverse phase Allure C18 column (4.6m X 150mm, 5μm particle size, Restek, Bellefonte, PA) was used. This ultra-aqueous column allowed for adequate wetability of the non-polar stationary phase with the more polar mobile phase, thus preventing peak fronting and resulting in better peak resolution. A two-solvent isocratic elution mode was used. Solvent A (90%) consisted of HPLC grade water adjusted to pH 3.00 with ammonium formate. Solvent B (10%) consisted of HPLC grade acetonitrile.
To prepare juice samples for analysis, a modification of the Solid Phase Extraction (SPE) method developed by Eisele et al. (2003) was used. Waters HLB Oasis® SPE cartridges (Waters Inc.) were activated by successively passing 2 ml of distilled water, 2 ml of methanol, and 2 ml of distilled water at a flow rate of 2-3 drops/sec to avoid cracking the solid phase. Because sample concentration was not necessary, 1 ml of juice was passed through the cartridge at a flow rate of approximately 1 drop/sec. Then 1 ml of 1.5% of sodium bicarbonate was passed through the cartridge followed immediately with 1 ml of 1% acetic acid at a flow rate of approximately 2 drops/sec. Any residual water was removed by placing the cartridge under vacuum for 3 minutes. PAT was then eluted from the solid phase into a test tube using 2 ml of ethyl acetate. The solvent was evaporated under a stream of nitrogen until dry and followed by immediate addition of 1 ml of 0.1% acetic acid. The solution was mixed vigorously and the previously described HPLC method was used to determine PAT content. The recovery of the extraction method based on 10 extractions was 92.2 ± 1.9 % (mean ± S.D.).

3.3.4 Experimental design and data analysis

3.3.4.1 Determination of significant factors

In order to investigate the effect of temperature on reaction rates, malic acid buffer was spiked with 6.4 \times 10^{-6} \text{ M} (1000 \text{ ppb}) \text{ PAT} and 1.25 \times 10^{-3} \text{ M} (221 \text{ ppm})
ascorbic acid. Control solutions without AA were prepared identically. Reaction solutions were held at 25, 35, 45, 55, 65, and 85°C. The significance of initial level PAT, AA, and pH on reaction rate was determined by varying each independent variable at two levels while holding all others constant.

Table 3.2 indicates the levels for each of the variables. The range of PAT levels was chosen based on a worst case scenario. The most likely range of PAT is 50-250 ppb but samples with 400-600 ppb have been observed (Plant product safety division, FDA Dr. Michael E. Kashtock personal communication, 2006). Levels for AA were chosen based on the Daily Value (DV) for Vitamin C published in the Code of Federal Regulations (21CFR101.9) which is at or near values for Dietary Reference Intakes (DRI) and Recommended Dietary Allowances (RDA) determined by the Institute of Medicine (2000). RDA values for AA are presented in Table 3.3. The pH range was selected to include the average variation found in AJ (Mattick L.R. and Moyer J.C., 1983, Eisele T.A. amd Drake S.R., 2004).

In order to estimate the reaction rate constant for the degradation of PAT in the presence of AA, a zero, first and second order kinetic model was fitted to the data obtained from the previously described experiments. From that the model with the best fit was determined and used to estimate the kinetic parameters of the reaction.
Table 3.2

Table 3.2: Low and high initial levels for PAT, AA, and pH utilized to investigate the importance of each factor.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAT</td>
<td>$1.6 \times 10^6$M</td>
<td>$6.4 \times 10^6$M</td>
</tr>
<tr>
<td>AA</td>
<td>$1.25 \times 10^3$M</td>
<td>$3.76 \times 10^3$M</td>
</tr>
<tr>
<td>pH</td>
<td>3.0</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Table 3.3

Table 3.3: Recommended Dietary Allowances for AA by life stage and gender group determined by the Institute of Medicine.

<table>
<thead>
<tr>
<th>Life stage</th>
<th>Age</th>
<th>Males (mg/day)</th>
<th>Females (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants</td>
<td>0-6 months</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Infants</td>
<td>7-12 months</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Children</td>
<td>1-3 years</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Children</td>
<td>4-8 years</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Children</td>
<td>9-13 years</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>Adolescent</td>
<td>14-18 years</td>
<td>75</td>
<td>65</td>
</tr>
<tr>
<td>Adults</td>
<td>19 years and older</td>
<td>90</td>
<td>75</td>
</tr>
<tr>
<td>Smokers</td>
<td>19 years and older</td>
<td>125</td>
<td>110</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>18 years and younger</td>
<td>-</td>
<td>80</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>19 years and older</td>
<td>-</td>
<td>85</td>
</tr>
<tr>
<td>Breastfeeding</td>
<td>18 years and younger</td>
<td>-</td>
<td>115</td>
</tr>
<tr>
<td>Breastfeeding</td>
<td>19 years and older</td>
<td>-</td>
<td>120</td>
</tr>
</tbody>
</table>

3.3.4.2 Response Surface Methodology (RSM) design and treatments

Response surface methodology was utilized. RSM explores the relationships between several factors and a depended variable or response (in the present study, the
rate constant of the reaction). In other words, RSM approximates the true function that describes the relationship between depended and independent variables with the appropriate empirical model. RSM models may include just main effects (linear model), or complemented with interactions, quadratic and cubic terms to account for curvature. RSM designs have many desirable properties such as adequate distribution of information across the experimental region (rotatability), good lack of fit detection, internal estimation of errors and they require the minimum number of treatment combinations.

Box-Behnken statistical design was used to investigate and evaluate main effects, interaction effects, and quadratic effects of the initial level of PAT, AA, and pH on the rate constants for the reaction of PAT with AA. The 3-factor-3-level design used (Figure 3.1) was suitable for exploring quadratic response surfaces and constructing second-order polynomial models. This 3-dimensional design, shown in Figure 3.1, is given by a set of points at the midpoint of each edge of a cube and a center point replicate. Replicates of the central point allow for error estimation. The relationship between the dependent and independent variables was further elucidated using response surface plots and contour plots.
Assuming that a function \( (f) \) exists that links the factors \( (x_i) \) with the response \( y \), we can identify the relationship between factors and response. Nonetheless, this function is usually either unknown or too complex, so a non-linear quadratic model was considered to approximate the true function. The nonlinear quadratic model is given the following equation:

\[
y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2
\]  

Figure 3.2: Three dimensional Box-Behnken design
Where \( y \) is the measured response, \( b_0 \) is the intercept, \( b_1 \) to \( b_{33} \) are the regression coefficients and \( x_1, x_2, x_3 \) are the independent variables. This Box-Behnken design is also rotatable and encloses many of the desirable properties of the RSM. The independent variables selected and their associated levels are shown in Table 3.4.

Table 3.4

<table>
<thead>
<tr>
<th>Factor</th>
<th>Label</th>
<th>Low (-1)</th>
<th>Center (0)</th>
<th>High (+1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAT</td>
<td>PAT</td>
<td>( 1.60 \times 10^{-6} )</td>
<td>( 4.00 \times 10^{-6} )</td>
<td>( 6.40 \times 10^{-6} )</td>
</tr>
<tr>
<td>AA</td>
<td>AA</td>
<td>( 1.25 \times 10^{-3} )</td>
<td>( 2.51 \times 10^{-3} )</td>
<td>( 3.76 \times 10^{-3} )</td>
</tr>
<tr>
<td>pH</td>
<td>pH</td>
<td>( 3.00 )</td>
<td>( 3.75 )</td>
<td>( 4.50 )</td>
</tr>
</tbody>
</table>

The minimum and maximum levels were determined as previously described. The center point represents the midpoint as required by the experimental design. Thus, the experimental ranges for each factor include values of commercial interest, while taking into consideration the limitations of the statistical design. The geometry of the design suggests a sphere within the experimental space due to the “missing corners” in the cubic space. Thus, we can expect poor predictability at the extreme levels for each factor. Based on the number of factors and levels, the generated runs for the design were determined (Table 3.5).
3.3.4.3 RSM Validation experiments

For the validation of the model three experiments were performed utilizing the model system and one experiment using the self-stable AJ with no added AA. Each experiment was triplicated. Shelf-stable, made-from-concentrate AJ (White House apple juice, National Fruit Company, Winchester, VA) was used for validation experiments to assess the effectiveness of the model to predict PAT degradation in AJ. The label indicated that no AA was added and analysis of the juice with an AA titration kit (Model, Hach) confirmed the absence of vitamin C. Experimental conditions are presented in Table 3.6.

Table 3.5: Experimental treatments for the 3-factor-3-level Box-Behnken design using coded values -1, 0, 1 which are low, central and high level of each factor respectively.

<table>
<thead>
<tr>
<th>Run</th>
<th>PAT(M)</th>
<th>AA(M)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>-1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>-1</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>11</td>
<td>-1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3.6

Table 3.6: Employed levels for initial PAT concentration, initial AA concentration, and pH for the validation experiments utilizing the model system and apple juice. AJ: apple juice, MS: Model system. Samples were kept at 45 °C.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Reaction medium</th>
<th>PAT (M)</th>
<th>AA (M)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MS</td>
<td>1.9 X 10^{-6}</td>
<td>3.26 X 10^{-3}</td>
<td>4.30</td>
</tr>
<tr>
<td>2</td>
<td>MS</td>
<td>4.0 X 10^{-6}</td>
<td>2.51 X 10^{-3}</td>
<td>3.75</td>
</tr>
<tr>
<td>3</td>
<td>MS</td>
<td>3.8 X 10^{-6}</td>
<td>3.26 X 10^{-3}</td>
<td>3.40</td>
</tr>
<tr>
<td>4</td>
<td>AJ</td>
<td>2.8 X 10^{-6}</td>
<td>2.51 X 10^{-3}</td>
<td>3.70</td>
</tr>
</tbody>
</table>

3.3.4.4 Statistical analysis

The data obtained from the preliminary experiments were analyzed using Minitab (Minitab, Inc., State College, PA USA). t-test was conducted to assess the significance of the treatment and the factors on the response. Significance was set at the 5% probability level.

SAS and MODEE software were used for the analysis of the RSM design and generation of response surface contour plots. The overall effects of pH and initial concentration of both reactants were determined, and the predictive model was generated. Linear, quadratic and interaction effects were considered. The adequacy of the model was tested by the lack of fit test, and the regression coefficient $R^2$. The lack of fit test helps to assess the correctness of the model, meaning how well the function fits the experimental data. The coefficient of determination $R^2$ describes the percentages of variability explained by the predicted model and thus is an indication of the adequacy of the model.
3.4 Results and Discussion

3.4.1. Effectiveness of the treatment

The addition of AA resulted in PAT reduction and the statistical analysis showed that there was a significant difference between the treatment and the control solutions (p<0.05) at the entire temperature range utilized for this project. The effectiveness of the treatment opposed to the control is apparent in Figure 3.3 as well as in Table 1 in the Appendix. The reduction of PAT is significantly greater in the treatment solutions. A positive correlation appeared to exist between temperature and the percentage reduction of PAT in both, treatment and control samples. When AA was present, and T increased from 25 to 85°C, the overall reduction of PAT increased from 37.27 to >91.34%. The time to achieve the overall degradation was decreased meaning, that the rate of the reaction increased.
3.4.2 Kinetic studies

The plot of the concentration of PAT over time (Figure 3.4) suggested that the thermal degradation of PAT (control) appeared to follow zero order kinetics. This means that the reaction rate is independent of concentration (Concentration vs Time generated a straight line). However, the degradation of PAT in the presence of AA seemed to follow more complex kinetics.
To study the degradation kinetics of PAT in the presence of AA, and to find a model that describes PAT degradation, characteristic kinetic plots for zero, first and second order reactions were constructed using data obtained at 45°C (the midpoint of our T range). The characteristic kinetic plots for a specific reaction order generate straight lines and from that, the slope and then the rate constant of the reaction can be calculated.

Initially the concentration of PAT over time was plotted (Figure 3.5) to examine if the reaction can be described by zero order kinetics, however it became clear from the graph, that there was an obvious pattern in the data and the zero order kinetic model did not adequately describe the degradation of PAT. The $R^2$ generated from the regression was 0.835.

Figure 3.4: Plot of the concentration of PAT over time in treatment (AA $C_0 = 221$ ppm or $1.25 \times 10^{-3}$ M) and control samples (no AA). The initial concentration of PAT was $C_0 = 1000$ ppb or $6.4 \times 10^{-6}$ M) and the pH was set at 3.75. Both samples were kept at 25°C.
Given that the zero order kinetic model did not sufficiently describe the data, the characteristic kinetic plot for a first and second order reaction were constructed by plotting the natural logarithm of the concentration of PAT over time (Figure 3.6) and the reciprocal of the concentration over time (Figure 3.7), respectively. The results indicated that the fit of the first order model was better given the higher $R^2 = 0.938$ but there was still a noticeable pattern in the data that the model could not account for. The second order kinetic model seemed to have the best fit ($R^2 = 0.986$).
Figure 3.6: Regression plot of the natural logarithm of the concentration of PAT over time ($R^2=0.938$). The model system was spiked with PAT (Co=6.4x10$^{-6}$ M) and AA (Co=1.25x10$^{-3}$ M), pH was set at 3.75 and samples were kept at 45°C. The data are the average of three replications.
The second order kinetic plot (1/C vs Time) resulted in the highest $R^2$ and appeared to have good linearity. Therefore it was applied to the data obtained from the entire temperature range. Good regression coefficients were obtained ($R^2 = 0.855-0.99$), but when normal probability plots of residuals and residual plots were examined a deviation from linearity was observed. An example of that deviation is apparent in Figure 3.8 were the normal probability plot of residuals for the reaction of PAT with AA at 45°C (midpoint of T range) is shown. Residuals can be thought as the variation that cannot be explained by the fitted model. Thus, the residuals are a form of error and the same
general assumptions apply to the residuals that apply for errors (normally distributed and (approximately) independently distributed with a mean of zero and constant variance)

Figure 3.8

Figure 3.8: Normal probability plot of residuals for the fit of the second order kinetic model. Data or the reaction of PAT \((C_0 = 1000\text{ppb or } 6.4\times10^{-6}\text{M})\) and AA \((C_0 = 221\text{ppm or } 1.25\times10^{-3}\text{M})\) at 45°C.

The deviation from normality for the residuals indicated that there was a pattern in our data which the kinetic model could not account for and that the reaction follows more complex kinetics. Nonetheless, it was thought that the second order model would describe the degradation of PAT sufficiently. The introduction of error in our kinetic parameter estimation was also believed not to be great, based on residual plots, and that
the error will be balanced by the benefits of employing a simple mathematical model to describe the kinetics of the degradation reaction of PAT.

Using the second order kinetics model the rate constant of the degradation of PAT in the presence of AA was calculated from the integrated rate law (Eq.3.3). The rate constant is equal to the slope of the kinetic plot between 1/C vs Time.

Eq. (3.3)

\[ [A] = \frac{[A_0]}{1 + k t [A_0]} \Rightarrow \frac{1}{[A]} = \frac{1}{[A_0]} + k t \]  

(Eq. 3.3)

3.4.3 Effect of temperature

Rate constants were calculated for every temperature level. From the results, presented in Table 3.7, it became clear that higher temperature resulted in higher reaction rates.

In order to determine if the change in temperature was a significant factor, affecting the rate of the degradation of PAT, ANOVA statistics was performed. From the results it was concluded that temperature changes significantly affected the reaction rate (p< 0.05). The effect of temperature is shown graphically in Figure 3.9.
Table 3.7

Table 3.7: Rate constants and regression coefficients obtained from second order plots for the reaction of PAT ($C_0=1000$ppb or $6.4 \times 10^{-6}$M) with AA ($C_0=221$ppm or $1.25 \times 10^{-3}$M) at 25, 35, 45, 55, 65, 85°C. Standard deviations are based on three replications.

<table>
<thead>
<tr>
<th>$T$ °C</th>
<th>$k \pm SD$ (L mole$^{-1}$ sec$^{-1}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.15±0.02</td>
<td>0.86</td>
</tr>
<tr>
<td>35</td>
<td>0.27±0.02</td>
<td>0.94</td>
</tr>
<tr>
<td>45</td>
<td>0.70±0.03</td>
<td>0.99</td>
</tr>
<tr>
<td>55</td>
<td>0.96±0.05</td>
<td>0.97</td>
</tr>
<tr>
<td>65</td>
<td>2.04±0.25</td>
<td>0.97</td>
</tr>
<tr>
<td>85</td>
<td>9.30±0.41</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Figure 3.9

Figure 3.9: Second order reaction kinetic plot of the reaction of PAT ($C_0=1000$ppb or $6.4 \times 10^{-6}$M) with AA ($C_0=221$ppm or $1.25 \times 10^{-3}$M) at 25(black), 35(red), 45(green), 55(blue), 65(pink), 85°C (yellow).
An Arrhenius behavior was observed indicating the dependence of the rate constant $k$ of a reaction on the absolute $T$ (K) according to Equation 3.4

\begin{equation}
    k = Ae^{\frac{-E_a}{RT}}
\end{equation}

(where $k$ is the rate constant, $A$ is the pre-exponential factor, $E_a$ is the activation energy of the reaction, $T$ is the absolute temperature and $R$ the gas constant. In its original form the pre-exponential factor $A$ and the activation energy $E_a$ are considered to be $T$ independent. Taking the natural logarithm of the above equation it yields Equation 3.5.

\begin{equation}
    \ln k = -\frac{E_a}{R} \frac{1}{T} + \ln A
\end{equation}

When a reaction has a rate that obeys the Arrhenius equation, a plot of $\ln(k)$ versus $1/T$ generates a straight line. From the Arrhenius plot (Figure 3.10) it became apparent that the rate constant for the reaction of PAT and AA had Arrhenius dependency on $T$. ($R^2 = 0.986$). The activation energy of the degradation of PAT in the presence of AA was 59.5kJ/mol. Activation energy, is defined, as the energy needed for one mole of reactants to react.

The pre exponential factor ($A$) was equal to $3.77*10^9$. $A$ is an empirical estimation of the collision frequency factor $Z$ which represents the average number of collisions between reacting molecules per unit of time.
Given the activation energy of the reaction, the z-value was calculated. The z-value indicates the temperature change in centigrade or Kelvin for the rate of the reaction to change by a factor of 10 and its relationship with activation energy is described in Eq. 2.5. The z-value of a reaction is also useful for comparing processes and how sensitive they are to temperature change.

Eq.(4.6)

\[ z = \ln(10) \cdot \frac{E_a}{R} \cdot \frac{T_1}{T_2} \]  

(Eq. 3.6)

Where \( T_1 \) and \( T_2 \) 298.15 and 358.15 K, respectively, which is the T range for which the activation energy was evaluated. The calculated z-value for the degradation of PAT in the presence of AA is 34.2 °C.
3.4.4 Effect of pH

Preliminary experiments demonstrated that pH had a significant effect on the reaction rate. At higher pH levels the rate constant of the reaction was significantly higher. The results are presented in Table 3. A t-test was also performed to compare the different treatment conditions confirming the significance of pH (p<0.05).
The reaction mechanism between PAT and AA is not known but based on the reactants chemical behavior and previous studies it can hypothesized that AA attacks PAT via a redox reaction or Michael like addition that results in adduct formation.

It has been demonstrated by Filege and Metzier (2000a, 2000b) that PAT has electrophilic properties and nucleophiles like sulfhydryl compounds can attack PAT via Michael addition reaction to form adducts. Ascorbate can act as a nucleophile and it can be hypothesized that attack on PAT by AA proceeds by a mechanism analogous to the one proposed by Filege and Metzier. AA attacks first at the C-6 of PAT followed by attack at C-4 or C-2 of PAT to form an AA- PAT complex that, upon further molecular rearrangement, leads to an opening of the lactone ring and thus detoxification (Appendix, Figure 1). It has been established that compounds containing a lactone ring are responsible for a wide range of cytotoxic, mutagenic, and carcinogenic properties (Dickens and Jones, 1961,1965) and chemical reactions that cleave the lactone ring decrease their toxicity as evidenced by studies with aflatoxin (Lee et al., 1981) and zearalenone (Hideaki et al., 2002; Takahashi-Ando, et al., 2002). The first pKa of AA is

---

Table 3.8: Rate constants (k) for the degradation of PAT at low and high levels of pH. Levels of PAT and AA were fixed at 6.4x10⁻⁶ M, 1.25x10⁻³ M respectively. T was held at 45°C. The generated rate constants were found to be significantly different. Rate constants are presented as the mean value ± standard deviation calculated from three replications.

<table>
<thead>
<tr>
<th>pH level</th>
<th>k± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.57±0.03</td>
</tr>
<tr>
<td>4.5</td>
<td>1.38±0.04</td>
</tr>
</tbody>
</table>
4.17 increased level of pH will result in higher concentration of ascorbate and in higher degradation reaction rate constant.

Even if the degradation of PAT involves redox reactions, pH levels above the first pKa of AA will also expedite the generation of free radicals from AA and thus, the degradation reaction. Transition metals are present in buffer solutions and can mediate the formation of ascorbate free radical (AFR) from ascorbate as it is shown in Figure 3.11.

**Figure 3.11**

![Formation of ascorbate free radical (AFR) from ascorbate in the presence of transition metals.](image)

The AFR is a resonance-stabilized tricarbonyl species and it is formed from the one-electron oxidation of ascorbate. The reduction potential of the Ascorbate/AFR is $E^{\text{or}} = +282$ mV which is relatively low. This means that practically every oxidizing radical or agent present in the system could lead to the one-electron oxidation of ascorbate which leads to the formation of AFR

J. Van Der Zee et al. (1998) demonstrated that as the concentration of ascorbate increased, generation of AFR increased, and in the presence of dehydroascorbic acid (DHAA) and ascorbate, AFR can be formed by a different pathway shown in Figure 3.12.
Increased pH thus, results in higher levels of ascorbate, which contributes to both hypothesized reaction pathways resulting in higher reaction rates.

### 3.4.5 Effect of initial level of ascorbic acid

Experiments also demonstrated that initial levels of AA had a significant effect on the reaction rate. Higher concentration of AA resulted in higher rate constant (Table 3.9) A t-test showed that the initial concentration of AA significantly affected the reaction rate constant (p<0.05).
Based on the collision theory, when the concentration of the reactants increases the probability of collisions between them also increases and the reaction rate can be expected to increase too. However, even at the low initial level employed in our experiments, was in excess. If AA in the model system reacted only with PAT then, increasing the initial concentration should have had no effect on the rate of the reaction which opposes our results.

This contradiction suggests that AA degrades independently from PAT via two reaction pathways; aerobic and anaerobic. For both treatment samples (low and high initial concentration of AA) AA was initially in excess, so the degradation of PAT during the early stages of the reaction was faster. Later, the degradation of AA started affecting the rate of PAT degradation because AA was no longer in excess. PAT continued to degrade but the rate of that degradation became progressively lower over time. Higher levels of AA resulted in higher degradation rates for PAT because the time needed before the degradation of AA to start affecting the degradation of PAT increased and thus more AA was available to react with PAT.

Table 3.9

<table>
<thead>
<tr>
<th>AA level (M)</th>
<th>Rate Constant ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25x10⁻³</td>
<td>0.71±0.05</td>
</tr>
<tr>
<td>3.76x10⁻³</td>
<td>0.99±0.04</td>
</tr>
</tbody>
</table>

Table 3.9: Rate constants (k) of the degradation of PAT at low and high initial levels of AA. The initial level of PAT was fixed at 6.4*10⁻⁶ M (1000ppb) and the pH was 3.75. The T was held at 45°C. The generated rate constants for the two initial levels of AA are presented as mean value ± standard deviation calculated from three replications.
3.4.6 Effect of initial level of patulin

The initial level of PAT had a negative effect on the reaction rate, meaning, that at higher concentrations of PAT the degradation rate was slower and the overall reduction lower, the results are presented in Table 3.10.

Initially this observation seems to be in opposition to the collision theory. One would expect that increasing the initial concentration will result in higher rates because more molecules are present and the frequency of collision is expected to be higher. The effect of the initial level of PAT can be explained by examining AA and PAT together.

Table 3.10

Table 3.10: Rate constants (k) of the degradation of PAT at low and high initial levels of PAT. AA level was fixed at 1.25*10^{-3} M (221ppm) and pH was 3.75. The T was held at 45°C. The generated rate constants for the two initial levels of PAT were significantly different. Rate constants are presented as mean value ± standard deviation calculated from three replications.

<table>
<thead>
<tr>
<th>PAT level (M)</th>
<th>Rate constant ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6x10^{-6}</td>
<td>1.89±0.10</td>
</tr>
<tr>
<td>6.4x10^{-6}</td>
<td>0.54±0.06</td>
</tr>
</tbody>
</table>

AA reacted with PAT, but as previously mentioned, degraded independently as the reaction progressed. That suggested that initially the reaction progressed rapidly because AA was in excess in the solution. As AA degraded, an increasingly lower amount was available to react with PAT so the reaction rate decreased.

For our experiments two level of PAT were employed and all other parameters were kept fixed. So for both samples, the same levels of AA were available to react with PAT under the same conditions. Thus, regardless the initial level of PAT the same
amount of the mycotoxin degraded before the reduction of AA became appreciable (started affecting the rate), because the starting concentration of AA was the same for both samples and it was in excess.

When the initial concentration of PAT was low most of the toxin present in the sample degraded before the reduction of AA started to affect the rate. On the contrary when the initial concentration of PAT was high the toxin at first degraded fast and at the same time the degradation of AA progressed and as less reactive- towards PAT -species were available the rate of PAT degradation decreased. When low levels of PAT were employed, a higher fraction of the toxin degraded during the same time interval, as compared to when high initial levels of PAT were used. Thus, the overall degradation rate for lower starting levels of PAT was higher.

3.4.7 Response Surface Methodology experiment

The second order kinetic model was fitted to the data and reaction rate constants were calculated as shown in Table 3.11.

Initially the generated model was fitted to the data without applying any transformation to the response but a significant lack of fit was observed (p<0.05). The ANOVA results are presented in Table 3.12 and 3.13.
Table 3.11

Table 3.11: Second order reaction rate constants for the reaction of PAT with AA for the Box-Behnken design experimental treatments. Factor levels for the different treatments are also presented.

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>PAT (mol L⁻¹)</th>
<th>AA (mol L⁻¹)</th>
<th>pH</th>
<th>k (L mol⁻¹ sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.6 X 10⁻⁶</td>
<td>1.25 X 10⁻³</td>
<td>3.75</td>
<td>1.80</td>
</tr>
<tr>
<td>2</td>
<td>1.6 X 10⁻⁶</td>
<td>3.76 X 10⁻³</td>
<td>3.75</td>
<td>13.50</td>
</tr>
<tr>
<td>3</td>
<td>6.4 X 10⁻⁶</td>
<td>1.25 X 10⁻³</td>
<td>3.75</td>
<td>0.69</td>
</tr>
<tr>
<td>4</td>
<td>6.4 X 10⁻⁶</td>
<td>3.76 X 10⁻³</td>
<td>3.75</td>
<td>0.90</td>
</tr>
<tr>
<td>5</td>
<td>4.0 X 10⁻⁶</td>
<td>1.25 X 10⁻³</td>
<td>3.00</td>
<td>0.71</td>
</tr>
<tr>
<td>6</td>
<td>4.0 X 10⁻⁶</td>
<td>1.25 X 10⁻³</td>
<td>4.50</td>
<td>2.70</td>
</tr>
<tr>
<td>7</td>
<td>4.0 X 10⁻⁶</td>
<td>3.76 X 10⁻³</td>
<td>3.00</td>
<td>2.41</td>
</tr>
<tr>
<td>8</td>
<td>4.0 X 10⁻⁶</td>
<td>3.76 X 10⁻³</td>
<td>4.50</td>
<td>10.10</td>
</tr>
<tr>
<td>9</td>
<td>1.6 X 10⁻⁶</td>
<td>2.51 X 10⁻³</td>
<td>3.00</td>
<td>10.30</td>
</tr>
<tr>
<td>10</td>
<td>6.4 X 10⁻⁶</td>
<td>2.51 X 10⁻³</td>
<td>3.00</td>
<td>0.76</td>
</tr>
<tr>
<td>11</td>
<td>1.6 X 10⁻⁶</td>
<td>2.51 X 10⁻³</td>
<td>4.50</td>
<td>22.60</td>
</tr>
<tr>
<td>12</td>
<td>6.4 X 10⁻⁶</td>
<td>2.51 X 10⁻³</td>
<td>4.50</td>
<td>2.00</td>
</tr>
<tr>
<td>13</td>
<td>4.0 X 10⁻⁶</td>
<td>2.51 X 10⁻³</td>
<td>3.75</td>
<td>1.20</td>
</tr>
<tr>
<td>14</td>
<td>4.0 X 10⁻⁶</td>
<td>2.51 X 10⁻³</td>
<td>3.75</td>
<td>1.23</td>
</tr>
<tr>
<td>15</td>
<td>4.0 X 10⁻⁶</td>
<td>2.51 X 10⁻³</td>
<td>3.75</td>
<td>1.06</td>
</tr>
</tbody>
</table>

It became apparent from the ANOVA results that although the predictive model was significant (p<0.05) there was also a significant lack of fit (p<0.05) indicating that the model did not fit the data adequately which could result to biased estimations of the response (over- or under-estimation). PAT and pH were significant at p<0.05 but AA and the quadratic effects of PH and PAT were only significant at p<0.1.
Due to the lack of fit a logarithmic transformation was applied on the response and results are shown in Tables 3.14 and 3.15. After the transformation the model was highly significant at p<0.001 (Table 3.15) with an $R^2$ of 0.98. Linear (PAT, AA and pH) and quadratic effects (PAT*PAT and pH*pH) were also significant at p<0.05 (Table 3.13):

### Table 3.12

<table>
<thead>
<tr>
<th>Source</th>
<th>Master Model</th>
<th>Predictive model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>PAT</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PH</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PAT*PAT</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PAT*AA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PAT*PH</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AA*AA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AA*PH</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PH*PH</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Error</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Lack of fit</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Pure Error</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>3</td>
<td>362.7</td>
<td>120.90</td>
<td>0.005</td>
</tr>
<tr>
<td>Quadratic</td>
<td>3</td>
<td>111.5</td>
<td>37.16</td>
<td>0.056</td>
</tr>
<tr>
<td>Cross Product</td>
<td>3</td>
<td>71.68</td>
<td>23.89</td>
<td>0.118</td>
</tr>
</tbody>
</table>

Table 3.13: ANOVA results for the significance of linear, quadratic and interaction effects of the factors on the reaction rate constant (k).
3.15). Although cross-product were not significant at p<0.05 (Table 3.16) there was one significant interaction between PAT and AA. The lack of fit was also not significant (p>0.05) (Table 3.15). The error term used in the test for the lack of fit was the pure error estimated from the data on the replicated experiments of the three center points of the design (experiment 13-15), thus, the model represents the data sufficiently. Regression coefficients along with their standard errors for the significant factors are presented in Table 3.17. Based on the regression coefficients initial levels of PAT seemed to have the most significant influence on the degradation rate of PAT followed by pH and initial levels of AA.

Table 3.14

<table>
<thead>
<tr>
<th>Source</th>
<th>Master Model</th>
<th>Predictive model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>SS</td>
</tr>
<tr>
<td>PAT</td>
<td>1</td>
<td>9.46</td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>2.95</td>
</tr>
<tr>
<td>PH</td>
<td>1</td>
<td>2.51</td>
</tr>
<tr>
<td>PAT*PAT</td>
<td>1</td>
<td>1.01</td>
</tr>
<tr>
<td>PAT*AA</td>
<td>1</td>
<td>0.77</td>
</tr>
<tr>
<td>PAT*PH</td>
<td>1</td>
<td>0.008</td>
</tr>
<tr>
<td>AA*AA</td>
<td>1</td>
<td>0.0001</td>
</tr>
<tr>
<td>AA*PH</td>
<td>1</td>
<td>0.004</td>
</tr>
<tr>
<td>PH*PH</td>
<td>1</td>
<td>2.33</td>
</tr>
<tr>
<td>Model</td>
<td>9</td>
<td>18.85</td>
</tr>
<tr>
<td>Error</td>
<td>5</td>
<td>0.38</td>
</tr>
<tr>
<td>Lack of fit</td>
<td>3</td>
<td>0.36</td>
</tr>
<tr>
<td>Pure Error</td>
<td>2</td>
<td>0.01</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>19.23</td>
</tr>
</tbody>
</table>
Table 3.15: ANOVA results for the significance of linear, quadratic and interaction effects of the factors on the reaction rate constant (k) after log-transformation applied.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>3</td>
<td>14.92</td>
<td>4.97</td>
<td>0.0002</td>
</tr>
<tr>
<td>Quadratic</td>
<td>3</td>
<td>3.15</td>
<td>1.05</td>
<td>0.0072</td>
</tr>
<tr>
<td>Cross Product</td>
<td>3</td>
<td>0.78</td>
<td>0.26</td>
<td>0.1078</td>
</tr>
</tbody>
</table>

Table 3.16: Regression coefficients of significant factors.

| Term       | Estimate | SE  | t    | Pr > |t| |
|------------|----------|-----|------|------|-------|
| PAT        | -1.087   | 0.078 | -13.96 | 0.0001 |
| AA         | 0.607    | 0.078 | 7.80  | 0.0001 |
| PH         | 0.560    | 0.078 | 7.20  | 0.0001 |
| PAT*PAT    | 0.522    | 0.115 | 4.57  | 0.0020 |
| PAT*AA     | -0.437   | 0.110 | -3.97 | 0.0041 |
| PH*PH      | 0.795    | 0.114 | 6.95  | 0.0001 |

### 3.4.7.1 Main and interaction effects

Based on the predictive model the individual effects of pH and initial concentration of PAT and AA are shown in Figure 3.13. The reaction rate constant for the degradation of PAT decreases as the initial concentration of PAT increases from $1.6 \times 10^{-6}$ M to $5.0 \times 10^{-6}$ M and between that and $6.4 \times 10^{-6}$ M no significant change was observed (all other factors being constant at their middle values pH=3.75, AA= $2.51 \times 10^{-3}$M). The
significant quadratic effect of PAT on the rate was also observed. The reaction rate constant had an almost linear dependency on the initial concentration of AA and increased with the increase of AA from $1.25 \times 10^{-3}$ M to $3.76 \times 10^{-3}$ M. The rate constant of the reaction was increased when pH increased but it is apparent the most significant change on the response happened when pH increased from 3.5 to 4.5. The significance of the quadratic effects of the pH was also observed in Figure 3.11.

**Figure 3.13**

![Main effect plots for rate constant of the reaction with respect of PAT, AA and pH (from left to right). Rate constant is presented on logarithmic scale with 95% confidence interval. Factors not presented on each of the graphs are fixed at central levels.](image)

The interaction between the initial concentration of PAT and AA was significant at $p<0.05$ (Table 3.16) and is shown in Figure 3.14. At low levels of PAT, the rate constant was significantly higher for the high level of AA compared to the low AA level.
As the initial concentration of PAT increased the effect of AA was reduced and that is apparent in Figure 3.12. The difference between the effects of the low and high level of AA was minimized for higher concentrations of PAT which indicated that higher concentrations of AA when PAT levels are high were not as effective as compared to low levels of PAT.

Figure 3.14

![Interaction Plot for pat*AA, resp. Rate Constant](image)

Figure 3.14: Interaction effect between initial concentration of PAT and AA.

3.4.7.2 Response surface plots

Figure 3.15 shows contour plot of the reaction rate constant for the degradation of PAT generated using the predictive model for various treatments combinations within
1.25\times10^{-3} \text{ M} to 3.76 \times10^{-3} \text{ M} AA concentration, 1.6\times10^{-6} \text{ M} to 6.4\times10^{-6} \text{ M} PAT concentration and 3.00 to 4.50 pH of the solution, giving a good comprehensive picture of the system. In Figure 3.15 (a) it can be seen that there was a minimum for the rate constant when pH levels were within 3.1-3.6 and PAT levels within 4.8\times10^{-6}-6.4\times10^{-6} \text{ M} and AA fixed at 1.25\times10^{-3} \text{ M}. So a higher AA concentration will be more effective for the degradation of PAT when the initial level of the toxin is within 4.8\times10^{-6}-6.4\times10^{-6} \text{ M} and the pH of AJ within 3.1-3.6.

From Figure 3.15 (b) it was observed that at every PAT level, the highest rate constant was achieved when pH and AA were at the higher end of the experimental range. At the same time the maximum achievable rate constant decreased as initial levels of PAT increased. It was also observed that at high levels of PAT, when the pH was above 4.1 (or above the first pKa); AA was not a significant factor because the rate constant was influenced only by pH. A minimum of the rate constant was again observed when pH level was between 3.1-3.6, the AA level at low and the level of PAT at the high end.

In Figure 3.15 (c) the low and central level of pH seemed to have almost identical effects further confirming the previous observation that the pH range 3-3.7 will result lower rate constants. The high level of pH on the contrary had a more pronounce effect.

Based on the developed predictive model, pH levels close to or above the first pKa of AA, low levels of PAT, and high levels of AA will result in higher rate constants resulting in a more effective treatment against PAT.
Figure 3.15: Response surface contour plots for the reaction rate constant. a) contour plot of PAT over pH, AA is fixed, b) contour plot of AA over pH, PAT is fixed c) contour plot of PAT over AA, pH is fixed. Co: initial concentration. Fixed factors are at low, central and high level from left to right.
3.4.7.3 Validation of the RSM model

Three different processing conditions for the model system and one for AJ were selected to test the validity of the predictive model. The predicted and observed rate constants for the degradation of PAT are presented in Table 3.17. Preparation of samples was identical to previously described and the observed data were reported as the mean value of three replications and a confidence interval calculated from the standard deviation. Predicted values were reported as the mean value and a confidence interval generated by SAS software.

Table 3.17

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Reaction medium</th>
<th>PAT (M)</th>
<th>AA (M)</th>
<th>pH</th>
<th>Predicted (95% C.I.)</th>
<th>Observed (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MS</td>
<td>1.9 X 10^{-6}</td>
<td>3.26 X 10^{-3}</td>
<td>4.3</td>
<td>18.90 (13.93 - 25.63)</td>
<td>21.08 (16.92 - 25.24)</td>
</tr>
<tr>
<td>2</td>
<td>MS</td>
<td>4.0 X 10^{-6}</td>
<td>2.51 X 10^{-3}</td>
<td>3.75</td>
<td>1.16 (0.91 - 1.49)</td>
<td>1.09 (0.95 - 1.23)</td>
</tr>
<tr>
<td>3</td>
<td>MS</td>
<td>3.8 X 10^{-6}</td>
<td>3.26 X 10^{-3}</td>
<td>3.4</td>
<td>1.73 (1.34 - 2.22)</td>
<td>1.37 (1.19 - 1.55)</td>
</tr>
<tr>
<td>4</td>
<td>AJ</td>
<td>2.8 X 10^{-6}</td>
<td>2.51 X 10^{-3}</td>
<td>3.7</td>
<td>2.06 (1.64 - 2.60)</td>
<td>2.12 (1.60 - 2.64)</td>
</tr>
</tbody>
</table>

T-test were performed and the predicted and observed values were not statistically different (p<0.05). Based on the results the RSM model adequately predicts the degradation rate of PAT in the aqueous model system as well as AJ. Although the RSM
model was successful the fit of the second order kinetic model introduced error in our estimations and that become more pronounced when AJ was used (Figure 3.16) (Residual vs fit plot can be found in the Appendix Figure 2) with an $R^2$ of 0.80.

An $R^2$ of 0.80 is acceptable in most cases, but ignoring an obvious pattern can lead to an over/under-estimation of the real rate of the degradation reaction. In this case, at the initial stages of the reaction the rate constant is underestimated and that can introduce significant error because the majority of the degradation of the toxin occurs then. In addition at the final stages of the reaction the rate constant is overestimated which again can lead to incorrect estimations for the remaining PAT in AJ.
From observed pattern in Figure 3.16 it became noticeable that the data could be described by two distinct regression lines if the data were grouped into two time intervals, and that is depicted in Figure 3.17. It was as well observed that the majority of the toxin reduction occurs during the initial stages of the reaction thus, a question arose of whether the observed degradation of PAT at the final stages of the reaction for treatment samples (added AA) was the result of thermal degradation alone or the added AA contributed to the reduction of PAT.
In order to determine if indeed AA caused the degradation of PAT, and not thermal degradation alone, the data from treatment and control samples were separated into two groups based on time intervals and the second order kinetic model was fitted to all. The estimated rate constants for treatment and control samples (kept at same T) were compared to determine if AA or thermal degradation was the cause of PAT reduction.

From the results presented in Table 3.18 it was concluded, that although the rate of the reaction was considerably lower at the later part of the reaction compared to the initial one, the presence of AA significantly increased the rate of PAT degradation (t-test
p<0.05). Thus, the degradation of PAT for the later part of the reaction was not the result of thermal degradation alone.

Table 3.18

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (hours)</th>
<th>Treatment k ±S.D.</th>
<th>Control k ±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0-30</td>
<td>0.415±0.020</td>
<td>0.018±0.0010</td>
</tr>
<tr>
<td></td>
<td>30-144</td>
<td>0.095±0.004</td>
<td>0.015±0.0011</td>
</tr>
<tr>
<td>35</td>
<td>0-30</td>
<td>0.589±0.043</td>
<td>0.026±0.0020</td>
</tr>
<tr>
<td></td>
<td>30-144</td>
<td>0.251±0.030</td>
<td>0.022±0.0015</td>
</tr>
<tr>
<td>45</td>
<td>0-30</td>
<td>0.897±0.051</td>
<td>0.043±0.0030</td>
</tr>
<tr>
<td></td>
<td>30-144</td>
<td>0.683±0.040</td>
<td>0.041±0.0028</td>
</tr>
<tr>
<td>55</td>
<td>0-30</td>
<td>1.676±0.097</td>
<td>0.077±0.0034</td>
</tr>
<tr>
<td></td>
<td>30-144</td>
<td>0.934±0.081</td>
<td>0.076±0.0036</td>
</tr>
<tr>
<td>65</td>
<td>0-30</td>
<td>3.037±0.125</td>
<td>0.216±0.0081</td>
</tr>
<tr>
<td></td>
<td>30-120</td>
<td>1.937±0.120</td>
<td>0.205±0.0075</td>
</tr>
<tr>
<td>85</td>
<td>0-48</td>
<td>8.972±0.276</td>
<td>2.556±0.084</td>
</tr>
</tbody>
</table>

The observed curvature in the data could be explained by looking at the complex chemistry of the system. In figure 3.18, a schematic representation of the reactive pathways of AA is shown. Both aerobic and anaerobic degradation of AA takes place in an aqueous acidic solution (Yuan & Chen 1998), but the aerobic pathway is the prevailing one under our experimental conditions.
The results from this chapter show that AA reacts with PAT and also degrades independently; the degradation of PAT can be expected to be faster at the initial stages of the reaction because AA is in excess. As the depletion of AA proceeds, the degradation of PAT slows down and reaches a plateau when AA is completely degraded. This pattern is shown in Figure 3.16.

The more pronounced curvature observed in the AJ experiment could have been a result of the extraction method (increased error due to recovery variations) applied before the analysis in combination with the experimental conditions (factor levels). The experiment utilizing AJ overstated an already existing pattern which could lead to a significant error for the estimated reaction rate constant.

Figure 3.18

Figure 3.18: Hypothesized schematic representation of reactive pathways of AA. AA⁺: ascorbate, AA⁻: free radical ascorbate, DHAA: dehydroascorbic acid, DKG: diketogulonic acid, DP: degradation products
3.5 Conclusions

Addition of AA caused a significant reduction of PAT levels in both model system and AJ. A second order kinetics model was used to describe the degradation of PAT in the presence of AA and to estimate the rate constant of the reaction. A wide range of T was utilized in order to cover storage and thermal processing conditions and it was shown that T had a significant effect on the reaction rate and the overall degradation of the toxin. An Arrhenius dependency was observed and the activation energy of the reaction as well as the z-value was calculated. Initial concentrations of AA and PAT, and pH also significantly affected the rate of the reaction.

The employed RSM design generated a predictive model and established relationships between the rate constant and the significant factors (PAT, pH, AA). The model was significant at p<0.001 and explained 98% of the variability for the rate constant. The lack of fit was not significant (p>0.05) after applying a logarithmic transformation. PAT had the most significant effect on the response followed by pH and AA. The validation experiments confirmed the predictive power of the RSM model because t-test indicated that the predicted rate constants were not significantly different than the observed ones.

Although a predictive model was developed and validated it was thought that a different approach was necessary because the error introduced during the estimation of the kinetic parameter (using the second order kinetic model) was significant. There was a need to find a kinetic model that fitted the experimental data for both AJ model system and AJ and accurately described the degradation reaction.
Chapter 4

Weibull modeling and response surface optimization of patulin degradation in the presence of ascorbic acid

4.1 Abstract

The Weibull model was used to describe the degradation of PAT by added AA in both, model system and AJ. A wide range of T was utilized (25-85°C) and the dependency of b and n parameters on T was studied.

The reaction rate (parameter b) was found to have an Arrhenius dependency on T. The plot of ln(b) vs 1/T generated a straight line ($R^2 0.986$). Activation Energies and the reaction z-value were calculated for PAT degradation by added AA. The shape parameter (n) did not demonstrate a T dependency as parameter estimates for the entire T range were not significantly different.

A Box-Behnken response surface design was utilized to determine the effects of the initial levels of PAT and AA and pH on the rate of the degradation reaction and to develop a model that sufficiently predict the kinetic parameters at different processing conditions. A simplified Weibull model was applied to the experimental data and it produced consistently good fits. The simplified model produced fits comparable to the full model and predicted model generated from the RMS design provided sufficient
predictions of kinetic parameters. The lack of fit for the predictive model after applying a log transformation was not significant (p > 0.05) and the analysis of variance indicated that the model explained 0.973 variability for the rate parameter.

Three validation experiments were carried out utilizing model system and one with AJ, every experiment was triplicated. Observed and predicted values were not found to be statistically different further confirming that the rate constant of the degradation of PAT can be reliably predicted.

The results indicate that the Weibull model was superior to the second order model discussed in chapter 3.

4.2 Introduction

In order to design an effective treatment for the reduction of PAT in AJ by added AA, knowledge of the kinetic parameters of the degradation reaction is necessary. Knowledge of the mechanism of the reaction can facilitate the development of a model that can predict the kinetic parameters at all times but when the mechanist pathway is unknown the development of the kinetic model is hindered.

Although the complexity of the mechanism of the reaction between PAT and AA was known, the second order kinetics in chapter 3 initially was considered to be adequate for describing the degradation of PAT in the presence of AA. After completing all the runs as well as validation experiments using model system and AJ, significant deviations from linearity were observed when the second order kinetic model was fitted on the data. An error was introduced on the estimation of the rate constant of the degradation
reaction. At the initial and final stages of the reaction the rate constant was under- and over-estimated, respectively and a different approach was necessary.

In the past the most common approach, even for highly complex reactions with unknown mechanisms, was the utilization of pseudo –kinetic models like zero, first and second order. The drawback of this approach, because the rate was determined by applying linear regression, was the over- or under-estimation of the rate of the reaction depending on the shape of the curve and the deviation from linearity.

Over the years, in order to overcome the presented problem, many models have been developed and demonstrated to describe degradation kinetics of chemicals, nutrients, enzymes and microorganism. Among those, the Weibull model gained popularity due to its simplicity and flexibility. The Weibull model is useful and applicable in many cases such as modeling the inactivation of microorganisms (Peleg 1998, Van Boekel 2002, Chen and Hoover 2004)) as well as degradation of nutrients, pigments and enzymes (Manso et al. 20000, Corradini and Peleg 2004)

The Weibull model is an empirical failure rate model and has been extensively used in engineering applications (Smith 1991). The loss of any chemical can be viewed as a failure event, where the fraction of intact molecules decreases over time. Thus, regardless of the degradative pathway, the plot of the concentration of PAT over time can be viewed as a survival curve. The Weibul model can be used to describe survival curves and in its simplest form (Eq.4.1), includes a shape parameter (n), and a rate or scale parameter (b), which allows for its application to a number of situations.
Eq. (4.1)

\[ \frac{C}{C_0} = \exp \left( -b(T)t^{n(T)} \right) \]  

(Eq. 4.1)

C and Co are the momentary and initial concentration of PAT respectively, t is time and b(T) and n(T) are the T depended coefficients.

The parameter b is considered to be analogous to the rate constant of the reaction and thus is a characteristic of the reaction. The shape parameter n describes the shape of the distribution curve. The effect of the shape parameter is shown in Figure 4.1. When n>1 there is a downward concavity, when n<1 there is an upward concavity and when n=1 it is linear which corresponds to the familiar first order kinetics model. When n>1 the degradation or failure rate increases with time and when n<1 the degradation rate decreases with time.

Figure 4.1

---

Figure 4.1: Example of the effect of the shape factor on the Weibull function.
The T dependency of the models’ parameters can be described by any empirical model that fits them. The shape factor $n$ in some cases has a weak T dependency and sometimes none at all. Thus, for systems like that the Weibull model can be reduced to Eq.4.2

$$C/Co = \exp(-b(T)t^n)$$

(Eq. 4.2)

Where $n$ is a fixed average or representative value that fit the experimental data. Because the Weibull model has had such a wide range of applicability our objectives were: a) to fit the Weibull model to the experimental data and to determine if it could sufficiently describe the degradation kinetics of PAT in the presence of AA in both model system and AJ; b) define the T dependency of the shape and scale parameter of the model; c) estimate the kinetic parameters of the reaction; d) employ RSM to elucidate the effect of pH, and the initial levels of the reactants on the rate of the reaction as well as develop a mathematical predictive model for the rate constant of the reaction and; e) validate the predictive model.

### 4.3 Materials and methods

The experimental design, the employed factors and their levels, reasoning for choosing the experimental range as well as the experimental treatments, their preparation
and the processing conditions and methods for the validation experiments are described in chapter 2 in Materials and Methods section.

4.3.1 Statistical analysis

The Weibull model was fitted to the experimental data by using non-linear regression procedure and model procedure of SAS (Release 9.1.3 SAS institute Inc, Cary NC) and the model parameters were estimated. The Weibull model was fitted to each experiment to determine the dependency of b and n on T. After establishing a relationship between b and n the Weibull model was fitted to the experimental data from the RMS design and the kinetic parameters were estimated. In order to assess the fit of the Weibull model regression coefficient values ($R^2$), residual plots residuals vs fitted values, and autocorrelation plots of residuals were examined to assess the randomness of the data set thus, assuring that there was no obvious pattern and the model described the data adequately.

Analysis of the RSM design and generation of contour plots was performed with SAS. The overall effects of pH and initial concentration of both reactants on the reaction rate constant were determined and the predictive model was generated. Linear, quadratic and interaction effects were considered.

The adequacy of the predictive model was assessed by using the $R^2$ value, the lack of fit test ($p>0.05$) and residual plots. The lack of fit test helped to examine the correctness of the model, meaning how well the function fitted the experimental data.
The coefficient of determination $R^2$ described the percentages of variability explained by the predicted model and thus was an indication of the adequacy of the model.

4.4 Results and discussion

4.4.1 Effect of temperature

The Weibull model was fitted to the experimental data for the entire T range (25-85°C) in order to establish the dependence of the rate constant ($b$) and the shape parameter ($n$) on T. Using model and non-linear regression procedure by SAS Weibull model produced consistently good fits and parameters $b$ and $n$ were estimated (Table 4.1). Regression coefficients ($R^2$) and Mean Square Error (MSE) values were used to assess the fit of the model (Table 4.1). The smaller the value of MSE the better the model to fit the data and the higher the value of $R^2$ the better is the adequacy of the model to explain the data. Residual and autocorrelation of residuals plots were examined to confirm the randomness of the data and the absence of obvious patterns that would lead to over- or under- estimation of the reaction rate constant.
The shape parameter \( n \) is related to the kinetic mechanisms and may be expected to be independent of \( T \). Plotting the estimated \( n \) parameters over \( T \) (Figure 4.2) showed that there is indeed no dependency as the estimated values were not significantly different. Because of the absence of significant differences in the \( n \) values within the \( T \) range a representative power of \( n \) was chosen and the model was simplified to the following equation:

\[
\text{Eq. (4.3)}
\]

\[
\frac{C}{C_0} = \exp(-b(T)t^{0.44})
\]

The simplified model was fitted to the experimental data from the entire \( T \) range (Figure 4.3) and an Arrhenius plot was constructed after estimating \( b \) (Figure 4.4). The estimated \( b \) values for the entire \( T \) range along with the MSE and \( R^2 \) are presented in Table 4.2. Comparing the MSE and \( R^2 \) values reported in Tables 4.1 and 4.2 it is apparent that the performance of the Weibull model did not change significantly by setting the \( n \) at a value of 0.44 and the fit was still good.

### Table 4.1

<table>
<thead>
<tr>
<th>( T )</th>
<th>( b ) (C.I.)</th>
<th>( n ) (C.I.)</th>
<th>MSE</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.050(0.042-0.058)</td>
<td>0.495 (0.478-0.510)</td>
<td>0.00124</td>
<td>0.930</td>
</tr>
<tr>
<td>35</td>
<td>0.065(0.061-0.069)</td>
<td>0.510 (0.483-0.529)</td>
<td>0.00117</td>
<td>0.946</td>
</tr>
<tr>
<td>45</td>
<td>0.086(0.077-0.092)</td>
<td>0.525 (0.489-0.561)</td>
<td>0.00026</td>
<td>0.978</td>
</tr>
<tr>
<td>55</td>
<td>0.142(0.138-0.151)</td>
<td>0.467 (0.449-0.484)</td>
<td>0.0010</td>
<td>0.970</td>
</tr>
<tr>
<td>65</td>
<td>0.238(0.207-0.269)</td>
<td>0.458 (0.403-0.511)</td>
<td>0.0004</td>
<td>0.982</td>
</tr>
<tr>
<td>85</td>
<td>0.450(0.422-0.487)</td>
<td>0.437 (0.417-0.456)</td>
<td>0.00009</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Table 4.1: Estimated scale (b) and shape (n) parameters within the entire \( T \) range (25-85°C) Mean square errors and regression coefficients of the fitted Weibull model. \( b \) and \( n \) parameters are presented as mean values \( \pm 95\% \) confidence intervals.
Figure 4.2: Interval plot of the shape parameter $n$ over the $T$. Interval bar: 95% C.I. for the mean. Shape parameters for the different $T$ conditions were not statistically different after ANOVA analysis ($p<0.05$).

Table 4.2

Table 4.2: The effect of $T$ on the reaction rate based on $b$ estimates using the simplified Weibull model ($n$ at 0.44). C.I. is the confidence interval calculated from three replicates. MSE and R$^2$ values are also presented in order to evaluate the fit of the Weibull model.

<table>
<thead>
<tr>
<th>$T$ (°C)</th>
<th>$b$ (C.I.)</th>
<th>MSE</th>
<th>R$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.061 (0.056-0.064)</td>
<td>0.0012</td>
<td>0.934</td>
</tr>
<tr>
<td>35</td>
<td>0.075 (0.072-0.078)</td>
<td>0.0014</td>
<td>0.947</td>
</tr>
<tr>
<td>45</td>
<td>0.115 (0.111-0.119)</td>
<td>0.0008</td>
<td>0.972</td>
</tr>
<tr>
<td>55</td>
<td>0.151 (0.147-0.156)</td>
<td>0.0015</td>
<td>0.966</td>
</tr>
<tr>
<td>65</td>
<td>0.207 (0.200-0.215)</td>
<td>0.0007</td>
<td>0.978</td>
</tr>
<tr>
<td>85</td>
<td>0.446 (0.433-0.456)</td>
<td>0.00007</td>
<td>0.999</td>
</tr>
</tbody>
</table>
In Figure 4.3 is shown the fit of the simplified Weibull model on the data from the T experiments. All the plots are the average of three replicates.

Figure 4.3: Weibull model fit for all T experiments. All the plots are the average of three replicates. 25( ), 35( ), 45( ), 55( ), 65( ), 85°C ( ). PAT C: concentration at time t, Co: initial concentration (6.4x10^{-6} M). AA initial concentration for every run was 1.25x10^{-3} M and pH was set at 3.75.

In order to determine if the rate constant of the reaction had an Arrhenius behavior the natural logarithm of b parameter ln(b) was plotted over the inverse of the absolute T (1/T).

Figure 4.4 demonstrated that the rate constant of the reaction between PAT and AA obeyed the Arrhenius equation because the regression plot demonstrated good linearity and fit with an R^2 of 0.987.
From the generated regression equation the activation energy of the reaction as well as the z-value were calculated. The activation energy of the degradation of PAT in the presence of AA was 29.6kJ/mol which is defined, as the energy needed for one mole of reactants to react. The activation energy for the degradation of PAT in the presence of AA was comparable with other degradation reactions, and most notably the degradation of AA (34kJ/mol).
From the activation energy of the reaction the z-value was calculated. The z-value indicates the T change in centigrade or Kelvin for the rate of the reaction to change by a factor of 10 and its relationship with activation energy is described in Eq. 4.3. The z-value of a reaction is also useful for comparing processes and how sensitive they are in T change.

Eq.(4.3)

\[ z = \frac{\ln(10)}{E_a / R} \cdot T1 \cdot T2 \]  

(Eq. 4.3)

Where \( T_1 \) and \( T_2 \), 298.15 and 358.15 K respectively and is the T range for which the activation energy was evaluated. The calculated z-value for the degradation of PAT in the presence of AA is 59.7 °C. After comparing that value, with z-values for microbial inactivation, it became apparent that the rate of the degradation of PAT by AA was not very sensitive to temperature changes. That result was in agreement with previous observations were PAT was found to be heat stable.

The z-value for the rate of the degradation of PAT was also compared with z-values for the degradation of food constituents. It was found to be comparable to those, corresponding to heat stable components. A list of z-values for the inactivation of microorganisms as well as degradation of nutrients and contaminants is presented in Table 4.3.
The reduced Weibull model was also fitted to all RMS design experimental treatments and produced good fits with no apparent patterns. Estimates of \( b \) for every run and their approximate standard error along with \( R^2 \) and MSE values are presented in Table 4.4.

### Table 4.4

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>( b ) (S.E.)</th>
<th>MSE</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.159 (0.0030)</td>
<td>0.0008</td>
<td>0.980</td>
</tr>
<tr>
<td>2</td>
<td>0.276 (0.0045)</td>
<td>0.0095</td>
<td>0.902</td>
</tr>
<tr>
<td>3</td>
<td>0.112 (0.0022)</td>
<td>0.0092</td>
<td>0.970</td>
</tr>
<tr>
<td>4</td>
<td>0.170 (0.0028)</td>
<td>0.0004</td>
<td>0.991</td>
</tr>
<tr>
<td>5</td>
<td>0.096 (0.0030)</td>
<td>0.0002</td>
<td>0.983</td>
</tr>
<tr>
<td>6</td>
<td>0.175 (0.0025)</td>
<td>0.002</td>
<td>0.958</td>
</tr>
<tr>
<td>7</td>
<td>0.192 (0.0090)</td>
<td>0.004</td>
<td>0.960</td>
</tr>
<tr>
<td>8</td>
<td>0.350 (0.0027)</td>
<td>0.0064</td>
<td>0.928</td>
</tr>
<tr>
<td>9</td>
<td>0.195 (0.0100)</td>
<td>0.013</td>
<td>0.890</td>
</tr>
<tr>
<td>10</td>
<td>0.130 (0.0080)</td>
<td>0.0085</td>
<td>0.920</td>
</tr>
<tr>
<td>11</td>
<td>0.450 (0.0200)</td>
<td>0.0028</td>
<td>0.968</td>
</tr>
<tr>
<td>12</td>
<td>0.300 (0.0120)</td>
<td>0.0075</td>
<td>0.942</td>
</tr>
<tr>
<td>13</td>
<td>0.150 (0.0040)</td>
<td>0.0025</td>
<td>0.954</td>
</tr>
<tr>
<td>14</td>
<td>0.145 (0.0030)</td>
<td>0.0018</td>
<td>0.960</td>
</tr>
<tr>
<td>15</td>
<td>0.138 (0.0034)</td>
<td>0.0020</td>
<td>0.967</td>
</tr>
</tbody>
</table>

### Table 4.3

Table 4.3: \( Z \)-values of selected microorganisms, nutrients and food contaminants.

<table>
<thead>
<tr>
<th>Z-value ((^{\circ})C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coxiella burnetti</td>
</tr>
<tr>
<td>C. sporogenes</td>
</tr>
<tr>
<td>Staphylococcal entotoxin A</td>
</tr>
<tr>
<td>Vitamin A</td>
</tr>
<tr>
<td>Vitamin C</td>
</tr>
</tbody>
</table>
4.4.2 Developing the model

The response surface experimental design was statistically analyzed using SAS software. The adequacy of the model was tested by the lack of fit test and the regression coefficient $R^2$. Widely different responses were obtained with the RSM design and are shown in Table 4.5 along with the generated experimental design.

Table 4.5

<table>
<thead>
<tr>
<th>Run</th>
<th>PAT (M)</th>
<th>AA (M)</th>
<th>pH</th>
<th>$b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.6x10^{-6}</td>
<td>1.25x10^{-3}</td>
<td>3.75</td>
<td>0.159</td>
</tr>
<tr>
<td>2</td>
<td>1.6x10^{-6}</td>
<td>3.76x10^{-3}</td>
<td>3.75</td>
<td>0.276</td>
</tr>
<tr>
<td>3</td>
<td>6.4x10^{-6}</td>
<td>1.25x10^{-3}</td>
<td>3.75</td>
<td>0.112</td>
</tr>
<tr>
<td>4</td>
<td>6.4x10^{-6}</td>
<td>3.76x10^{-3}</td>
<td>3.75</td>
<td>0.170</td>
</tr>
<tr>
<td>5</td>
<td>4.0x10^{-6}</td>
<td>1.25x10^{-3}</td>
<td>3.00</td>
<td>0.096</td>
</tr>
<tr>
<td>6</td>
<td>4.0x10^{-6}</td>
<td>1.25x10^{-3}</td>
<td>4.50</td>
<td>0.175</td>
</tr>
<tr>
<td>7</td>
<td>4.0x10^{-6}</td>
<td>3.76x10^{-3}</td>
<td>3.00</td>
<td>0.192</td>
</tr>
<tr>
<td>8</td>
<td>4.0x10^{-6}</td>
<td>3.76x10^{-3}</td>
<td>4.50</td>
<td>0.350</td>
</tr>
<tr>
<td>9</td>
<td>1.6x10^{-6}</td>
<td>2.51x10^{-3}</td>
<td>3.00</td>
<td>0.195</td>
</tr>
<tr>
<td>10</td>
<td>6.4x10^{-6}</td>
<td>2.51x10^{-3}</td>
<td>3.00</td>
<td>0.130</td>
</tr>
<tr>
<td>11</td>
<td>1.6x10^{-6}</td>
<td>2.51x10^{-3}</td>
<td>4.50</td>
<td>0.450</td>
</tr>
<tr>
<td>12</td>
<td>6.4x10^{-6}</td>
<td>2.51x10^{-3}</td>
<td>4.50</td>
<td>0.300</td>
</tr>
<tr>
<td>13</td>
<td>4.0x10^{-6}</td>
<td>2.51x10^{-3}</td>
<td>3.75</td>
<td>0.150</td>
</tr>
<tr>
<td>14</td>
<td>4.0x10^{-6}</td>
<td>2.51x10^{-3}</td>
<td>3.75</td>
<td>0.145</td>
</tr>
<tr>
<td>15</td>
<td>4.0x10^{-6}</td>
<td>2.51x10^{-3}</td>
<td>3.75</td>
<td>0.138</td>
</tr>
</tbody>
</table>

The highest rate constant was 0.450 and generated from experiment 11 were the level of PAT was 1.60x10^{-6} M (low), AA 2.51x10^{-3} M (medium) and that of pH 4.50 (high). Initially the generated predictive model was fitted to the data without applying
any transformation to the response and although it was significant at p<0.001, it had a significant lack of fit (p<0.05). The results from the ANOVA analysis are presented in Table 4.6.

Table 4.6

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>Pr&gt;F</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>9</td>
<td>0.1306</td>
<td>0.0145</td>
<td>0.0070</td>
<td>5</td>
<td>0.126</td>
<td>0.025</td>
<td>0.0001</td>
</tr>
<tr>
<td>PAT</td>
<td>1</td>
<td>0.017</td>
<td>0.017</td>
<td>0.0140</td>
<td>1</td>
<td>0.017</td>
<td>0.017</td>
<td>0.005</td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>0.025</td>
<td>0.025</td>
<td>0.0064</td>
<td>1</td>
<td>0.025</td>
<td>0.025</td>
<td>0.0016</td>
</tr>
<tr>
<td>pH</td>
<td>1</td>
<td>1.055</td>
<td>1.055</td>
<td>0.0011</td>
<td>1</td>
<td>0.055</td>
<td>0.055</td>
<td>0.0001</td>
</tr>
<tr>
<td>PAT*PAT</td>
<td>1</td>
<td>0.009</td>
<td>0.009</td>
<td>0.0390</td>
<td>1</td>
<td>0.010</td>
<td>0.010</td>
<td>0.021</td>
</tr>
<tr>
<td>PAT*AA</td>
<td>1</td>
<td>0.0008</td>
<td>0.0008</td>
<td>0.4380</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAT*pH</td>
<td>1</td>
<td>0.0018</td>
<td>0.0018</td>
<td>0.2790</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA*AA</td>
<td>1</td>
<td>0.0008</td>
<td>0.0008</td>
<td>0.440</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA*pH</td>
<td>1</td>
<td>0.0015</td>
<td>0.0015</td>
<td>0.310</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH*pH</td>
<td>1</td>
<td>0.020</td>
<td>0.020</td>
<td>0.0095</td>
<td>1</td>
<td>0.021</td>
<td>0.021</td>
<td>0.0026</td>
</tr>
<tr>
<td>Error</td>
<td>5</td>
<td>0.0061</td>
<td>0.0012</td>
<td>0.0112</td>
<td>9</td>
<td>0.0112</td>
<td>0.0012</td>
<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td>3</td>
<td>0.0060</td>
<td>0.0020</td>
<td>0.018</td>
<td>7</td>
<td>0.0111</td>
<td>0.0016</td>
<td>0.0224</td>
</tr>
<tr>
<td>Pure Error</td>
<td>2</td>
<td>0.0001</td>
<td>0.0003</td>
<td>0.0000</td>
<td>2</td>
<td>0.0000</td>
<td>0.0000</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>0.1370</td>
<td></td>
<td></td>
<td>14</td>
<td>0.1370</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear</td>
<td>3</td>
<td>0.0966</td>
<td>0.032</td>
<td>0.0017</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quadratic</td>
<td>3</td>
<td>0.0298</td>
<td>0.0099</td>
<td>0.0228</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross</td>
<td>3</td>
<td>0.0042</td>
<td>0.0014</td>
<td>0.4135</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procuts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A logarithmic transformation was applied on the response because the generated model had better fit. The transformation of the data was done to assure that the data were normally distributed and the variance constant because these are assumptions that need to be satisfied for regression analysis. The range of the response in the present study was large (0.096-0.45) which typically leads to non-homogeneous variance.
Variation is usually a constant percentage of the response value, not a constant absolute value. The variance tends to increase with the mean, so as the response value increases, the variance increases too. When a log transformation was applied the Root Mean Square Error (RMSE) converted to percentage error. That resulted in stabilization of variance which became independent from the mean of the response, and also the frequency distribution became more symmetrical. Based on the results after the applied log transformation it was safe to assume that the response followed a lognormal distribution. That can be explained and justified just by looking at the nature of the lognormal distribution and how it relates with chemistry.

Based on the central limit theorem the frequency distributions are generated by a large number of small random effects. For a normal distribution this random effects are additive, but for a log-normal distribution these effects are multiplicative (E. Limbert, W.A. Stahel, M. Abbt 2001). In chemistry, the rate of a reaction depends on the multiplicative effect of the concentrations of the reactants. Equilibrium conditions for a reaction, described by the law of mass action, are also governed by factors that act in a multiplicative way. Thus, it becomes evident that in chemistry, log-normal distributions are favored. When the range of the response is small and the variance constant, normal and log-normal distributions will probably fit well. For cases exhibiting non-constant variance a log-normal will probably fit better.

As shown in Table 4.7 after the transformation, $R^2$ was 0.973. The model was highly significant at $p<0.001$ (Table 4.8) including significant linear and quadratic effects at $p<0.001$ and $p<0.05$, respectively (Table 4.9). The lack of fit was found to be insignificant ($p>0.05$). Residual plot and observed versus predicted values of residuals
plot was also used to assess the fit of the model (Appendix, Figure 3 and 4). The error term used in the test for the lack of fit was the pure error estimated from the data on the replicated experiments of the three center points of the design (experiment 13-15).

Table 4.7

<table>
<thead>
<tr>
<th>Predictive model</th>
<th></th>
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<tbody>
<tr>
<td>R-square</td>
<td>0.973</td>
</tr>
<tr>
<td>Adj.R-square</td>
<td>0.959</td>
</tr>
<tr>
<td>RMSE</td>
<td>0.088</td>
</tr>
<tr>
<td>CV</td>
<td>-5.200</td>
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Table 4.8

<table>
<thead>
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<th>Source</th>
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<th>Predictive model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>SS</td>
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<tr>
<td>Model</td>
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<td>2.570</td>
</tr>
<tr>
<td>PAT</td>
<td>1</td>
<td>0.338</td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>0.693</td>
</tr>
<tr>
<td>pH</td>
<td>1</td>
<td>1.032</td>
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<tr>
<td>PAT*PAT</td>
<td>1</td>
<td>0.180</td>
</tr>
<tr>
<td>PAT*AA</td>
<td>1</td>
<td>0.004</td>
</tr>
<tr>
<td>PAT*pH</td>
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<td>1E-32</td>
</tr>
<tr>
<td>AA*AA</td>
<td>1</td>
<td>0.012</td>
</tr>
<tr>
<td>AA*pH</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>pH*pH</td>
<td>1</td>
<td>0.324</td>
</tr>
<tr>
<td>Error</td>
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<td>0.053</td>
</tr>
<tr>
<td>Lack of fit</td>
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<td>0.049</td>
</tr>
<tr>
<td>Pure Error</td>
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<td>0.003</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>2.623</td>
</tr>
</tbody>
</table>
Table 4.9

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>3</td>
<td>2.064</td>
<td>0.688</td>
<td>64.68</td>
<td>0.0002</td>
</tr>
<tr>
<td>Quadratic</td>
<td>3</td>
<td>0.502</td>
<td>0.167</td>
<td>15.72</td>
<td>0.0055</td>
</tr>
<tr>
<td>Cross Product</td>
<td>3</td>
<td>0.004</td>
<td>0.001</td>
<td>0.141</td>
<td>0.931</td>
</tr>
</tbody>
</table>

4.4.3 Regression coefficients

Regression coefficients for the predictive equation are shown in Table 4.10. Regression coefficients showed that the pH has significant linear and quadratic effects on the rate constant. PAT and AA have significant linear effects with PAT also demonstrating quadratic effects. The results of the canonical analysis are presented in Table 4.11. The eigenvalues and eigenvectors, in the matrix of second-order parameters, are important because they provide information on the significance of the factors and characterize the shape of the response surface. The eigenvectors point in the directions of principle orientation for the surface. The signs and magnitudes of the associated eigenvalues give the shape of the surface in these directions. The larger the absolute value of an eigenvalue, the more distinct is the curvature of the response surface in the associated direction. The coefficients of a specific eigenvector are relatively small except for one, which indicates that the vector points approximately along the axis related with
the factor matching to the large coefficient. Thus, the canonical analysis can be used to in order to establish the sensitivity of the reaction rare constant to variations in that factor.

The results indicate that the stationary point of the response surface is a saddle point which means that the surface at the stationary point that curves up and down in one or more directions. From the absolute values of eigenvalues and eigenvectors, pH seems to have the most significant effects on the rate constant followed by PAT and AA. The Pareto chart of effects (Appendix Figure 4) also confirms the importance of pH.

Table 4.10

| Term     | Estimate | Standard Error | t      | Pr>|t| |
|----------|----------|----------------|--------|------|
| PAT      | -0.206   | 0.031          | -6.62  | 0.0001 |
| AA       | 0.294    | 0.031          | 9.48   | 0.0001 |
| pH       | 0.360    | 0.031          | 11.56  | 0.0001 |
| PAT*PAT  | 0.224    | 0.045          | 4.94   | 0.0008 |
| pH*pH    | 0.300    | 0.045          | 6.60   | 0.0001 |

Table 4.11

<table>
<thead>
<tr>
<th>Eigenvalues</th>
<th>PAT</th>
<th>AA</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.296^a</td>
<td>0</td>
<td>0</td>
<td>1^a</td>
</tr>
<tr>
<td>0.222^b</td>
<td>0.990^b</td>
<td>-0.060</td>
<td>0</td>
</tr>
<tr>
<td>-0.057^c</td>
<td>0.061</td>
<td>0.998^c</td>
<td>0</td>
</tr>
</tbody>
</table>
4.4.4 Main effects

Based on the predictive model the individual effects of pH and initial concentration of PAT and AA are shown in Figure 4.5. The reaction rate constant for the degradation of PAT decreases as the initial concentration of PAT increases from $1.6 \times 10^{-6}$ M to $5.0 \times 10^{-6}$ M and between that and $6.4 \times 10^{-6}$ M no significant change was observed (all other factors being constant at their middle values pH=3.75, AA= $2.51 \times 10^{-3}$M). The significant quadratic effect of PAT on the rate was also observed. On the contrary the reaction rate constant had an almost linear dependency on the initial concentration of AA and increased with the increase of AA from $1.25 \times 10^{-3}$ M to $3.76 \times 10^{-3}$ M. The rate constant of the reaction was increased when pH increased, but it is apparent that the most significant change on the response happened when pH increased from 3.5 to 4.5. The significance of the quadratic effects of the pH was also observed. From the above it becomes clear that when the initial levels of PAT are low and pH is at the higher end of the experimental range a treatment with AA will be more effective as, the rate of patulin degradation will be higher. In addition the higher the added levels of AA the higher the degradation rate.
4.4.5 Response surface plots

Figure 4.6 shows contour plot of the reaction rate constant for the degradation of PAT generated using the predictive model for various treatments combinations within 1.25x10^{-3} M to 3.76 x10^{-3} M AA concentration, 1.6x10^{-6} M to 6.4x10^{-6} M PAT concentration and 3.00 to 4.50 pH of the solution, giving a good comprehensive picture of the system. The absence of interaction effects in the system was apparent as the pattern of the contour plot remained the same at the different levels of the fixed factor.

In Figure 4.5 (a) the response surface contour plots of PAT over AA are presented. As AA levels increased and as PAT decreased, the rate constant of the reaction increased. At the higher levels of pH that increase was more dramatic. The low and
central level of pH seemed to have almost identical effects further confirming the previous observation that the pH range 3-3.6 will result lower rate constants.

In Figure 4.6 (b) the response surface contour plots for pH versus PAT are presented. As pH decreased and PAT levels increased the reaction rate constant decreased finally achieving a minimum when pH levels were within 3.1-3.6 and PAT levels within $4.8 \times 10^{-6}$-$6.4 \times 10^{-6}$M, at every level of AA.

It was also apparent in the same figure that as AA level increased the minimum observed rate constant increased too. Thus, higher AA concentration will be more effective for the degradation of PAT if the initial level of the toxin is within $4.8 \times 10^{-6}$-$6.4 \times 10^{-6}$M and the pH of AJ within 3.1-3.6. The observation of the minimum for the response is in agreement with the hypothesized reaction mechanisms.

The first pKa of AA is 4.11 thus, at the low end of the experimental range for pH only a small fraction of AA was in the form of ascorbate and as pH increased within the range the fraction of ascorbate increased too. Under these conditions the generation of AFR is also favored (as previously described) so as pH increased the concentration of both hypothesized reactive species increased.

From Figure 4.6 (c) it was observed that at every PAT level, the highest rate constant was achieved when pH and AA were at the higher end of the experimental range. There was also as dramatic increase on the rate constant when PAT was at the lower end levels. On the contrary the difference of the central and high level of PAT on the rate was not as pronounced.

Based on the developed predictive model, pH levels close to or above the first pKa of AA, low levels of PAT, and high levels of AA will result in higher rate constants
resulting in a more effective treatment against PAT. Founded on the above observations we could propose that by increasing the pH of the product (blending e.t.c.), close to or higher than 4.11, AA will be a feasible after-processing treatment for PAT since the rate and the overall degradation of the toxin will increase.
Figure 4.6

(a) 

<table>
<thead>
<tr>
<th>pH = 3</th>
<th>pH = 3.76</th>
<th>pH = 4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Contour Plot of PAT over AA, pH is fixed" /></td>
<td><img src="image2" alt="Contour Plot of PAT over pH, AA is fixed" /></td>
<td><img src="image3" alt="Contour Plot of AA over pH, PAT is fixed" /></td>
</tr>
</tbody>
</table>

(b) 

<table>
<thead>
<tr>
<th>AA = 0.00125</th>
<th>AA = 0.002505</th>
<th>AA = 0.00375</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image4" alt="Contour Plot of PAT over AA, pH is fixed" /></td>
<td><img src="image5" alt="Contour Plot of PAT over pH, AA is fixed" /></td>
<td><img src="image6" alt="Contour Plot of AA over pH, PAT is fixed" /></td>
</tr>
</tbody>
</table>

(c) 

<table>
<thead>
<tr>
<th>PAT = 1.5E-6</th>
<th>PAT = 4E-6</th>
<th>PAT = 6.4E-6</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image7" alt="Contour Plot of PAT over AA, pH is fixed" /></td>
<td><img src="image8" alt="Contour Plot of PAT over pH, AA is fixed" /></td>
<td><img src="image9" alt="Contour Plot of AA over pH, PAT is fixed" /></td>
</tr>
</tbody>
</table>

Figure 4.6: Response surface contour plot for the reaction rate constant: a) contour plot of PAT over AA, pH is fixed, b) contour plot of PAT over pH, AA is fixed, c) contour plot of AA over pH, PAT is fixed. Co: initial concentration. Fixed levels are at low, central and high level from left to right.
4.4.6 Validation experiments

In order to assess its ability to describe the degradation of PAT the experimental data obtained from the validation experiments (described in chapter 3) were also fitted with the simplified Weibull model (Eq. 4.4). The processing conditions for the validation experiments both for the model system and the AJ are presented in Table 3.7.

The results for the Weibull model are presented in Table 4.12. Predicted and observed values of b and their 95% confidence interval are reported, along with the MSE and $R^2$ values from the average of replications for each experiment.

Table 4.12:

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Reaction medium</th>
<th>Predicted b (C.I.)</th>
<th>Observed b (C.I.)</th>
<th>MSE</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MS</td>
<td>0.329 (0.297-0.363)</td>
<td>0.365 (0.322-0.409)</td>
<td>0.0014</td>
<td>0.976</td>
</tr>
<tr>
<td>2</td>
<td>MS</td>
<td>0.139 (0.126-0.153)</td>
<td>0.150 (0.140-0.161)</td>
<td>0.0013</td>
<td>0.981</td>
</tr>
<tr>
<td>3</td>
<td>MS</td>
<td>0.159 (0.144-0.176)</td>
<td>0.153 (0.143-0.163)</td>
<td>0.0017</td>
<td>0.993</td>
</tr>
<tr>
<td>4</td>
<td>AJ</td>
<td>0.173 (0.158-0.190)</td>
<td>0.192 (0.166-0.21)</td>
<td>0.0016</td>
<td>0.974</td>
</tr>
</tbody>
</table>

After performing t-test it was concluded that the predicted and the observed values of b were not statistically different ($p<0.05$) for both the aqueous model system and the AJ therefore it was concluded that the reduced Weibull model can efficiently
describe the degradation of PAT in the presence of AA and the developed RSM model can sufficiently predict the value of b (rate constant of the degradation reaction).

Residual as well as autocorrelation residual plots were generated for each fit in order to test the randomness of the data. The plots are presented in the Appendix (Figure 5, 6, 7 and 8) and indicate a random distribution of residuals confirming the good fit of the model. The fits of the Weibull model for the average of the AJ and the model system validation experiments are presented in Figure 4.7 and 4.8 respectively.

**Figure 4.7**

![Graph showing the fit of the Weibull model](image)

*Figure 4.7: Fit of the Weibull model for the validation experiment conducted in AJ.*
Figure 4.8: Fit of the Weibull model for the validation experiments conducted in model system: a) experiment 1, b) experiment 2 and c) experiment 3.
The results obtained from the validation experiments contradict previous studies of Brackett and Marth (1979) and Canas and Aranda (1996) which found that the degradation of PAT by added AA in buffer is faster than that in juice. In the present study, the observed rate constant for the degradation of PAT in AJ was not significantly different from the predicted value of the rate, which was generated from a model developed using data obtained using the model system.

Brackett and Marth found that the addition of ascorbate enhanced the degradation of PAT in both buffer and AJ but the rate of the reaction was significantly higher in buffer. That observation could have been due to the glycine buffer they used. Glycine reacts with many carbonyl containing compounds and it is a reducing agent. When a reducing agent is present the rate of conversion of DHAA back to AA is increased so the degradation of AA is slower and more reactive species are available to react with PAT. Also the storage T for the juice experiment was lower than that for the buffer ones so the results are in no way comparable.

Canas and Aranda reported 54% reduction of PAT immediately after addition of 100ppm of AA in standard solutions but they did not report the composition of the solution which could exert a great effect on the reaction rate and overall degradation. They also reported 25% reduction of PAT in juice after 1 hour of treatment and approximately 50% after 72 hours which taking into account the levels of reactants could be comparable with the results obtained from this study but still they did not report holding T and pH of the juice.
The results from this study seem reasonable, the buffer solution utilized contains the average concentration of malic acid found in AJ and the pH levels utilized were within the commercial range.

Using the model generated from the response surface analysis b can be estimated and from that the remaining concentration of PAT at any given time can be calculated. There is also an established relationship between b and T so the concentration of PAT can be calculated for different storage conditions. Given that the reaction between PAT and AA could be prolonged depending on the storage T and the initial levels of the reactants, it seems that AA could be potentially utilized to reduce the levels of PAT in AJ as a post-production treatment during storage.

Considering a hypothetical scenario, where apple juice is contaminated with 310ppb PAT, has pH of 3.70 (close to the average pH of commercial apple juice) and 600 ppm of AA is added (<3 RDA). The developed predictive model, the Arrhenius equation and the Weibull model were used to estimate the time needed for PAT to degrade below regulatory limits.

Using the predictive model (Eq. 4.4) the rate constant for the degradation of PAT by AA at 45°C was calculated, \( b_{45^\circ C} = 0.245 \text{ h}^{-1} \).

Eq. (4.4)

\[
\ln(b) = 4.12 - 4\times105\text{PAT} + 234.57\text{AA} - 3.526\text{PH} + 3.91\times1010\text{PAT}\times\text{PAT} + 0.534\text{PH}\times\text{PH}
\] (Eq. 4.4)
The Arrhenius equation (Eq. 4.5) was utilized to calculate the reaction rate constant at 25°C $b_{25°C}=0.141 \text{ h}^{-1}$.

Eq. (4.5)

$$\ln(b1/b2)=Ea/R [(1/T2)-(1/T1)] \quad \text{(Eq. 4.5)}$$

And finally the Weibull model (Eq. 4.6) was used to estimate the time needed to reduce levels of PAT below 50ppb which was found to be 15.2 days.

Eq. (4.6)

$$\ln(C/Co)=-b(t)^{0.44} \quad \text{(Eq. 4.6)}$$

The employed level of PAT in the hypothetical scenario is comparable to the ones usually found in AJ, if contamination with PAT occurs (based on a survey contacted by the Plant Product Safety Division of the FDA). The pH is roughly the average value (pH=3.75) of commercial AJ and addition of AA at levels between 2 and 3 RDAs is also common practice for the AJ industry as many commercially available products contain levels of AA within these ranges. Consequently, it became evident that a treatment with AA is feasible approach to address the safety hazard presented by PAT. Given that shelf-stable AJ varies from 1 to 2 years, 15 day storage at room temperature (25°C) before market release is viable as AA provides an economical way of reducing PAT.

There is of course a need to conduct toxicological studies to establish that treatment with AA detoxifies the product. Alves et. al. (2000) showed, using several bioassays (including mammalian cells), that in the presence of AA clastogenicity of PAT is significantly reduced. Nevertheless, testing the final product (treated AJ) and its
toxicity by conducting cell and animal studies will give the level of assurance needed in order for AA to be a feasible commercial treatment. Elucidating the mechanism of the degradation reaction will help to identify individual end-products of the reaction, and eventually determine their effect in biological systems.

4.5 Conclusions

Based on the presented results Weibull model provided a good description of the degradation kinetics of PAT in the presence of AA. The generated fits were consistently good with high \( R^2 \) and low MSE values that indicate good performance of the model.

The \( n \) parameter, after fitting the Weibull model to the data from the entire \( T \) range, was found to be \( T \) independent and the model was simplified by choosing a representative power of \( n \) to the following:

\[
\frac{C}{C_0} = \exp(-bt^{0.44})
\]

The simplified model was used to estimate \( b \) parameters for the entire \( T \) range, as well as the runs from the RSM design, and the validation experiments and it demonstrated good fits giving good \( R^2 \) and MSE values.

The \( b \) parameter (rate constant) was found to have an Arrhenious dependency on \( T \) and from that the activation energy of the reaction and the \( z \)-value was calculated. From the RSM analysis a predictive model for the rate constant was generated, it was highly significant at \( p<0.001 \) (\( R^2=0.973 \)) and it had no significant lack of fit (\( p>0.05 \)).
Linear and quadratic effects were also significant at p<0.05. The relationship between b and the initial concentration of the reactants and pH was established. The most influential factor was pH, followed by PAT and AA. No interactions between the factors were found to be significant.

The RSM model was tested and produced good results for the validation experiments because the predicted values for b were not statistically different from the observed ones (t-test), indicating the efficiency of the model.

The predictive model was validated and the relationship between the rate constant and T was established so it became clear that AA could be a potential treatment for the reduction of PAT in AJ and that the Weibull model along with the RSM model could be used to predict the concentration of PAT at any given time.

Given that the treatment with AA could be prolonged it seemed that it would be a suitable after processing (storage) treatment. Knowing the initial levels of PAT and AA, the storage T and the pH of the juice the rate of the reaction can be calculated and from that the time needed to reduce PAT below regulatory limits.

Owing to higher degradation rates achieved when the initial levels of the toxin are low, it is essential to mention, that AA will be a more effective treatment if used in combination with GAP and GMP; programs already proven to contribute to the reduction of the initial load of PAT producing fungi as well as levels of PAT.
Chapter 5

Investigation of the mechanistic pathway of the reaction between patulin and ascorbic acid and identification of products

5.1 Abstract

The mechanism of the degradation of PAT in the presence of AA in an aqueous AJ model system (malic acid buffer, pH 3.75) was investigated. LC/MS with electrospray ionization (ESI) was used to monitor the progress of the reaction. Although many degradation products of AA were identified and PAT reduction was evident, no adducts or degradation products of PAT were identified. Some preliminary experiments with C-13 labeled PAT and C-13 labeled AA were performed but the results are inconclusive and no suggestions can be made for the mechanism.

The inability to discover degradation products of PAT could have been the result of the low concentrations of PAT employed for the study and/or the use of unsuitable ion generation method or mobile phase and or due to fragmentation of the generated by-products.
5.2 Introduction

As mentioned in previous chapters, although the degradation of PAT by added AA has been demonstrated, no one so far has proposed a mechanism with the experimental background to support it. In this study the effectiveness of AA as a control method for PAT was investigated, factors that affect the degradation of the mycotoxin were identified and a predictive model for the degradation in an aqueous juice like system was developed. In order to verify that the AA can be effectively used to degrade and detoxify PAT there is a need to examine the mechanistic pathway and identify end products of the reaction.

Knowledge of the mechanism of the reaction will help to explain the enhanced destruction of PAT in AJ containing AA and will provide valuable data needed to develop treatments that will minimize exposure of PAT to consumers. Knowing the end products of the reaction will allow toxicity studies, to confirm that the final product is not harmful.

The reaction mechanism between PAT and AA is not established, but based on previous studies it can be hypothesized that AA may attack PAT via a redox reaction, or Michael like addition which results in adduct formation.

It has been demonstrated by Filege and Metzier (2000a, 2000b) that PAT has electrophyllic properties. Nucleophiles, like sulfhydryl compounds, can readily attack PAT via Michael addition reaction and form adducts. Because ascorbate also acts as a nucleophile it can be hypothesized that attack on PAT by AA proceeds by an analogous
mechanism. The hypothesized mechanism is presented in the Appendix (Appendix, Figure 1).

The results on the effect of each factor on the degradation reaction of PAT supported both mechanisms so our objectives were: a) to identify by-products of the reaction that suggest the pathway by which PAT degrades in the presence of AA and; b) identify end-products of the reaction that suggest the detoxification of PAT by cleavage of the lactone ring (Dickens and Jones, 1961, 1965 and Lee et al., 1981).

Identification of degradation products initially attempted using an HPLC coupled with a Photo Diode Array (PDA) detector. Later on a liquid chromatography system coupled with mass spectrometry (LC/MS) was utilized because it is a very powerful tool for quantification and identification and previous studies demonstrated good detection limits for both PAT and AA using ESI sources (Nicola Ciofi et al., 2000, Ito R. et al. 2004).

5.3 Materials and methods

5.3.1 LC/MS Theory

LC/MS is the coupling of liquid chromatography with mass spectroscopy for identification and quantification of compounds. Interfacing these two techniques is not trivial but mass spectrometry provides an excellent tool for identification and as it measures mass to charge ratio (m/z) it can also provide information about chemical
structures. In figure 5.1 a very simplified schematic presentation of a LC/MS system is showed.

Figure 5.1: Schematic presentation of LC/MS system

The sample to be analyzed is introduced into an analytical column, with the help of an HPLC pump, where the different components of the sample are separated based on their polarities. The flow splitter breaks up the flow and directs one fraction to the UV detector and one to the MS for simultaneous detection. The different components entering the MS are introduced into an ion source in liquid phase whereupon are ionized. The ions then pass into the mass analyzer were they are separated based on their mass-to
charge ratio. Next, they reach the ion detector where their presence is recorded and a mass spectrum is produced.

The ionization of the sample can be done in a variety of different ways but in this study, Electrospray Ionization (ESI) was utilized. ESI ionization method is considered a ‘soft’ ionization processes, meaning that predominantly the degradation of the analyte of interest is prevented. The sample is introduced to a capillary that ends in a fine tip. When a high voltage is applied to that tip, solvent molecules become charged and as the charge accumulates, Coulomb repulsive forces increase and, once they overcome the surface tension, droplets of solvent violently disintegrate into smaller droplets. The solvent from the spray droplets then gradually evaporates with the help of nitrogen gas and further disintegration cycles follow until the products of lone ions.

After ionization, the mass analyzer sorts the ions based on their mass to charge ratio. In the present study a quadrupole and a triple-quadrupole were used. Quadrupole analyzer consists of four metal rods (two parallel pairs) and uses an oscillating electrical field to selectively stabilize or destabilize ions passing through a radio frequency field. For given ratio voltages only ions with certain m/z will reach the detector other ions will collide with the rods. The triple quadrupole is a linear series of three quadrupole mass analyzers (Q1, Q2, Q3) Q1 and Q3 act as mass filters and Q2 acts as collision cell. Q2 stimulates collisional dissociation of selected parent ions from Q1. The generated fragments then pass through Q3 where they are fully scanned (Figure 5.2).

This mass analyzer allows study of fragments and gives invaluable structural information. After the sorting of ions, the detector records the change of current that is
produced when ions hit a surface and the signal recorded in the detector during the course of a scan will produce a mass spectrum.

Figure 5.12

Figure 5.2: Schematic presentation of a triple-quadrupole. Q1: quadrupole 1, Q2: quadrupole 2, Q3: quadrupole 3, EM: electromagnetic detector.

5.3.2 HPLC and LC/MS operating conditions

For the identification of degradation products from the reaction between PAT and AA an HPLC system was initially used (instrumentation and operating conditions as described in chapter 2, Materials and methods). In later experiments, a LC/ESI/MS system was used because additional selectivity and increased sensitivity was required. The system consisted of a Shimadzu (Columbia, MD) HPLC system with a SCL-10AVP controller, DGU-14A degasser and a LC-10AVP pump coupled with SPD-10AVP UV/Vis detector and a micromass ZMD MS (Waters Inc.). A primesep D column and the
analytical method previously described (Table 2.1) were used due to their compatibility with the MS analysis. The flow rate of the mobile phase was 1ml/min and the effluent was split postcolumn with a zero dead volume T-splitter (Supelco) with 1 part to the MS and 4 parts to the UV detector.

Typically negative ion mode is employed for PAT identification and quantification, but ion suppression occurred which masked the signal of the parent ion of PAT. The pH of the mobile phase and the presence of formic acid enhance protonation but for negative mode operation deprotonation is desirable the signal was masked. Positive ion mode was therefore used which although produced higher background noise, resulted in detectable signal for the anayltie of interest. The operating conditions that gave the best signal for the parent ion of PAT were as follows: Desolvation gas 410L/hour, cone gas 50L/hour, Cone voltage 25 kV, source block T 100°C and desolvation T 250°C.

5.3.3 Sample preparation

Reaction solutions were prepared using the previously described AJ model system (Chapter 2) spiked with the appropriate concentrations of PAT and AA. The initial concentration of reactants was increased 10fold to enhance detectability (overcome noise and chemical background). The final volume of the solution was 10ml, the concentration of PAT was 3.2x10^{-5} M (10ppm) and the concentration of AA was 0.0205 M (4,420ppm). Labeled $^{13}$C PAT and AA were also used and are presented in Figure 5.3 ($^{3-13}$C PAT Hayashi Pure Chemical Ind. Japan, $^{13}$C AA Sigma-Aldrich Co.). Due to limited
availability of $^{13}$C PAT, reaction volumes containing labeled reactants were decreased at 5ml. AJ model system was utilized and it was spiked with PAT and AA at the same concentrations mentioned above. The preparation of stock and working solutions of PAT and malic acid buffer as well as the reagents are described in chapter 2 (Materials and methods).

![Figure 5.3](image)

Figure 5.3: a) $^{13}$C labeled PAT, b) $^{13}$C labeled AA. * indicates the position of the labeled carbon

Buffer was added into graduated bottles and then placed in a water bath at 45°C and kept for 15min to account for T come up time before reactants were added. The reaction was followed over time and samples were taken at specific time intervals. In order to identify reaction products 4 different reaction solutions were prepared and the combinations between reactants are presented in Table 5.1.
5.4 Results and discussion

5.4.1 Identification of degradation products using HPLC

An HPLC system coupled with a PDA detector was initially utilized for the identification of by-products of the reaction between PAT and AA. The reaction and control samples for AA and PAT were followed over time by injecting samples every 2 hours.

The obtained chromatograms revealed the formation of new peaks (Figures 5.4 a and b) and based on maximum absorbencies (Table 5.2), and retention times the by-products of the reaction appeared to be identical to the degradation products of AA without PAT present and no conclusions were drawn as to how PAT degrades.
At 0 hours peak 1 (AA) and peak 2 (PAT) are visible in the chromatograms obtained from the reaction solution and the control. At 24 and 48 hours peaks 3, 4 and 5 formed and are visible for both solutions. The maximum absorbencies were identical for both the reaction and the AA control solution. It is noticeable from the chromatograms that the intensity of peaks 1 and 2 (AA and PAT respectively) decreased over time and the intensity of peak 1 decreased faster in the reaction solution compared to the AA control solution indicating the higher degradation of AA when PAT is present. Also peaks 3, 4 and 5 emerge and increase faster in the presence of PAT. Based on the maximum absorbencies and their comparison with previous studies (Yuan J.P. and Chen F 1998) most of the peaks were identified as shown in Table 5.2. Peak 3 a had similar absorbance to 2,5-Dimethyl-4-Hydroxy-3(2) Furanone (DMHF) but DMHF is usually observed as a degradation product in the presence of sugars.

Table 5.2

<table>
<thead>
<tr>
<th>PEAK</th>
<th>Max Abs.(nm)</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>241.9</td>
<td>AA</td>
</tr>
<tr>
<td>2</td>
<td>276.0</td>
<td>PAT</td>
</tr>
<tr>
<td>3</td>
<td>286.7</td>
<td>Unknown</td>
</tr>
<tr>
<td>4</td>
<td>277.2</td>
<td>Furfural</td>
</tr>
<tr>
<td>5</td>
<td>293.8</td>
<td>3-hydroxy-2-pyrone</td>
</tr>
</tbody>
</table>
Figure 5.3: a) HPLC-UV chromatograms of the reaction solution at 0, 24 and 48h, HPLC-UV chromatograms of the AA control solution at 0, 24 and 48h. Chromatograms were obtained using a gradient elution (Solvent A: water/formic acid, Solvent B: Acetonitrile). Both reaction and control samples were kept at 45°C and malic acid buffer (pH 3.75) was utilized for their preparation.
5.4.2 Identification of degradation products using LC/ESI/MS

The reaction mechanism between PAT and AA is not established but based on the previous studies it can hypothesized that AA may attack PAT via a redox reaction or Michael like addition which results in adduct formation. A Michael like addition is presented in the Appendix, Figure 1 the detoxification of PAT proceeds with an adduct formation between the mycotoxin and AA which results in the opening of the lactone ring and the detoxification of the toxin. The pH of the model system used for the present study was close to the first pKa of AA thus, a large percentage of that was in the first-deprotonated form (ascorbate).

Ascorbate can act as a nucleophile and given that PAT has electrophilic properties it was hypothesized that attack on PAT by AA proceeds via Michael addition and a mechanism analogous to that proposed by Filege and Metzier (2000a, 2000b).

The result from previous experiments actually supported both possible mechanisms. The pH of the buffer seemed to have a significant positive effect with a more dramatic increase in the rate of the reaction at pH values above the first pKa (4.17) of AA. Under the described conditions the concentration of the hypothesized reactive species of AA will be higher so the rate of the reaction will increase.

Preliminary experiments with buffer spiked with PAT and dehydroascorbic acid (DHAA) at equivalent levels of those of AA were performed. Because no reducing agents were present in the reaction solutions, conversion of DHAA back to AA did not take place and the generation of ascorbate and ascorbate free radicals was not possible. As it was expected in the absence of the two possible reactive species the reduction of PAT
was not significantly different from that in a control solution (buffer spiked with PAT alone).

Given that it was not possible to identify any degradation products of PAT by using an HPLC coupled with a PDA detector the next step was to utilize a LC/ESI/MS system to detect and identify products which may indicate how PAT degrades and if indeed involves Michael addition or progresses via a redox mechanism. Four different reaction solutions (Table 5.1) were prepared and followed over time. The same reverse phase column was used and the samples were directly injected without any pretreatment.

The MS and the UV/Vis detector were used simultaneously to confirm identification of PAT and AA by maximum absorbance and m/z peak so UV detection at 276nm was performed prior to MS detection. The retention time of AA and PAT were 2.61 and 5.70 minutes respectively and the generated UV chromatogram are shown in Figure 5.4(a). Chromatograms for AA and PAT are also presented in Figures 5.4(b) and 5.4(c).
Using LC-ESI-MS m/z 155 and 158 were observed as the main peaks for PAT and 3-\(^{13}\)C PAT respectively (Figure 5.5) and m/z 177 and 178 for AA and \(^{13}\)C AA (Figure 5.6).
Figure 5.5: Mass spectrum for PAT. The molecular weight of PAT is 154g/mol and 157g/mol for the labeled molecule. The parent ion peak at positive ion mode operation is observable at m/z 155 and 158 respectively. The peaks at m/z 137 and 140 m/z correspond to the dehydrated form of PAT and peaks at m/z 127 and 130 are PAT after loss of carbonyl group. (* indicates $^{13}$C labeled carbon) PAT sample was prepared using our model system (pH 3.75).

It was apparent from Figure 5.6 that the base peak was at m/z 141 and not the peak for the parent ion of AA m/z 177 and that was probably the result of the operating conditions and more specifically the cone voltage. It was observed that cone voltage of 20V and below produce a higher peak for the parent ion of AA. A cone voltage of 25V was utilized because it produced high peak intensity for PAT. The high cone voltage
resulted in increased collisions between the faster moving ions and neutral molecules and that led to fragmentation an intensified peak at m/z 141.

Figure 5.6

![Mass spectrum for AA. The molecular weight of AA is 176g/mol and 177g/mol for the labeled molecule. The parent ion at positive ion mode operation is 177 and 178 m/z respectively. The observed peak at m/z 141 and 142 is AA - 2H2O. The peaks at m/z 199 and 200 are thought to be sodium ascorbate. * indicates 13C carbon.](image)

Although previous experiments indicate that AA may attack PAT via a Michael addition or a redox reaction they do not provide nearly enough evidence to support either mechanism. The reaction was monitored over time for the purpose of detecting m/z peaks
corresponding to the intermediate and final products of the reaction and $^{13}$C-labeled reactants were used along with unlabeled reactants.

Even though PAT was noticeably reduced the determination of its fate was not successful. Degradation products of AA were identified and the results were in agreement with previous publications.

In a peak eluting at 2.30 min (Figure 5.7) m/z peaks attributed to dehydroAA, Diketogulonic acid and 3,4-dihydroxy-5-methyl-2(5H)furanone (DMF) were detected. Dehydroascorhic acid has a molecular weight of 174 g/mol and it is the oxidized form of AA. Diketogulonic acid is produced by the delactonization of DHAA and the addition of a water molecule and has a molecular weight of 192 g/mol so the peak at m/z 193 was credited to it. DMF with a molecular weight of 130 g/mol was also thought to be co-eluted because a peak at 131 m/z was detected. That was in agreement with results by Sawamura M. et al. where DMF was identified as one of the degradation products of DHAA. It was also noticeable that DMF generated the same m/z peak for both reaction solutions ($^{13}$C-labeled and unlabeled AA) because the labeled carbon was lost during the decarboxylation of Diketogulonic acid to L-xylosone.

A signal at m/z 113 was detected in a peak eluting at 5 min in the MS scan (Figure 5.8). There were two degradation products of AA that could generate that peak; 2-furoic acid and 2-hydroxy-3-pyrone. Both are produced from L-xylosone after an intermolecular redox reaction and they have a molecular weight of 112 g/mol. Although the peak could have been attributed to both the maximum absorbance (293.8) suggested that the compound is 2-hydroxy-3-pyrone. This finding was also in agreement with the work of Yuan J. and Chen F. (1998) where they studied the effect of pH on the
degradation of AA. They found that even though 2-furoic acid and 2-hydroxy-3-pyrone were both present at low pH values, the former was at higher levels when pH was within a range of 1-2, and the later was predominant at a pH around 4. Given that the employed pH range in the present study was closer to 4 the peak was credited to 2-hydroxy-3-pyrone. Similar as DHT, 2-hydroxy-3-pyrone generated the same m/z peak for both reaction solutions ($^{13}$C-labeled and unlabeled AA) as the labeled carbon was lost during degradation. Furfural was not detected because it was outside the MS scan range. The MS scan was from 100-1000m/z and based on the molecular weight furfural will give a signal at 97m/z.
Figure 5.7: MS spectra of peak eluting at 2.30 min. Signals that could be attributed to Dehydroascorbic and Diketogulonic acid both labeled and unlabeled were detected. (m/z 175, 176 and 193, 194 respectively). A signal at 131 m/z was also detected and was attributed to 3,4 dihydroxy-5methyl-2(5H)furanone (DMF).
The inability to detect such degradation products of PAT could have been due to a combination of the operating conditions of the LC/ESI/MS and low levels of PAT used for the study. Another possibility is the formation of highly hydrophobic compounds that did not elute from the analytical column. The operation of the ESI probe at positive ion mode although necessary to obtain a signal for the parent ion of PAT resulted in increased noise. The samples were also injected with no pretreatment in order to clean the chemical environment and that resulted in an overall increased background noise.
It was also observed that under the operating conditions, and more specifically the applied cone voltage, AA was inclined to fragmentation and although PAT seemed fairly stable fragmentation products were also obtained. That could suggest that some of the by-products of the reaction underwent fragmentation and that in combination with the high background noise made their identification impossible. In addition time limitations and low availability of labeled reactants resulted to a very small number of runs using the LC/MS/MS thus, the results obtained from that were inconclusive.

The fact that no degradation products of PAT were apparent even when $^{13}$C labeled compound was used supports the hypothesis of the formation of highly hydrophobic degradation products. The development of an analytical method that utilizes higher percentages of acetonitrile and results in satisfactory resolution could solve the problem because highly hydrophobic compounds will elute when the percentage of acetonitrile (non-polar) increases. A different analytical column might also allow adequate separation with a less acidic mobile phase which may solve the ion suppression problem and make the operation of ESI at negative mode feasible.

The employment of Atmospheric pressure photoionization (APPI) which is a ‘softer’ ionization method may decrease the background noise thus, simplifying the identification of the reactions products. APPI is an ionization technique for LC/MS and its operation is based on a high-frequency gas discharge lamp that generates vacuum-ultraviolet photons of 10 and 10.6 eV energy. The generated energy usually exceeds the first ionization potential (IP) of many analytes, because a large number of organic compounds have IPs in the range 7–10 eV. Another advantage of APPI is that the most widely used LC mobile phase solvents have IPs higher than the photon energy
(acetonitrile, 12.2 eV). Hence, the total ion production from mobile phase solvents is low. Takino M. et al (2003) in a comparison study between ESI, APCI (atmospheric chemical ionization) and APPI showed superior characteristics and was proven to be more selective for the analysis of PAT.

Electron paramagnetic resonance (EPR) or electron spin resonance (ESR) Spectroscopy may be a useful tool to determine whether AA degrades PAT via simple adduct formation or by an oxidation-reduction process which results in opening of the lactone ring. EPR is a technique of great specificity and can be used to study compounds that have one or more unpaired electrons (free radicals). AA is readily oxidized by radical and metal ions via two one-electron transfers to yield an ascorbate free radical and then DHAA. Radicals generated from AA can be captured using a standard spin adduct such as 5,5-dimethyl-1-pyrroline-N-oxide (DMPO).

PAT solutions containing added L-AA and the spin adduct can be heated to various T and free radicals that are generated can be captured by the spin adduct. Signal intensity of the spectrum can be utilized to indicate the concentration of the radicals generated. Radicals generated due to AA alone (control) can also be captured and then a comparison of the generated radicals from the reaction mixture and the control solution may determine if PAT reacts with the free radicals generated by the AA or follows a different degradative pathway. The spectra generated can be further analyzed for the radical identification using the standard methods for hydroxyl radicals and superoxide radicals.
5.5 Conclusions

Although PAT noticeably reduced in the presence of AA the detection of degradation products that would indicate the mechanism by which the reaction preceded was not possible. A few degradation products of AA were identified, but no adducts between AA and PAT were detected or any other end-products that would give an indication for the mechanism of the degradation reaction.

The inability to detect such products could have been due to a combination of the operating conditions of the LC/ESI/MS, and low levels of PAT utilized for the study. Another possibility is the formation of highly hydrophobic compounds that did not elute from the analytical column.

Further work is needed to identify the degradation products of PAT and elucidate the mechanism by which the reaction progresses. More extensive experiments with labeled reactants should be carried out. The initial concentration of the reactants should also be increased in order to increase the detectability of PAT and subsequently its degradation products.
Chapter 6

Conclusions and suggested future research

6.1 Conclusions

In the present study addition of AA was found to increase significantly the degradation of PAT and these results were in agreement with previous studies.

Preliminary experiments showed that the initial concentrations of reactants and pH significantly affected the rate of the reaction. It was observed that as pH and initial concentration of AA increased, the degradation rate increased and as the initial concentration of PAT increased the degradation rate decreased. T was also found to significantly affect the reaction rate.

Initially a second order kinetics model was thought to be sufficient for the description of the degradation reaction of PAT by AA but the observed deviations from linearity caused a significant error in the estimation of the rate constant of the reaction. Thus, it was concluded that the second order kinetics model was inadequate for describing the reaction and the Weibull model was used to describe the degradation of PAT in both model system and AJ by added AA.
A T range of 25-85°C was selected because that may be encountered during storage of processing of AJ and the dependency of b (rate constant) and n (shape factor) parameters on T was studied.

The reaction rate constant (parameter b) was found to have an Arrhenius dependency on T and the plot of ln(b) vs 1/T generated a straight line (R² 0.986). Activation Energies and the reaction z-value were calculated for the reaction. The shape parameter (n) did not demonstrate a T dependency; parameter estimates for the entire T range did not significantly differ. A simplified Weibull model was generated by setting n at 0.44 and the model was fitted to the experimental data demonstrating good fits.

A 3-factor 3-level Box-Benken response surface design was utilized to determine the effects of the initial levels of PAT, AA and pH on the rate of the degradation reaction and to develop a mathematical model that can sufficiently predict the kinetic parameters at different processing conditions. The design generated 15 experimental treatments. The Weibull model was fitted to the experimental data obtained from every treatment producing consistently good fits. The shape parameter n did not demonstrate a dependency on any of the factors and the both Weibull model and simplified Weibull model gave good fits with comparable MSE and R².

The predicted model, generated from the RMS design, provided good estimations of kinetic parameters and it was highly significant at p<0.001. The lack of fit for the predictive model after applying a log transformation was not significant (p>0.05) and the analysis of variance indicated that the model explained 0.973 variability for the rate parameter. The residual plots for the model indicated a random distribution which
translates to a good fit. The initial concentration of PAT and AA, the pH as well as the quadratic effects of pH and PAT were found to be significant at $p<0.05$. Based on the canonical analysis and regression coefficients pH was the most influential factor followed by PAT and AA concentration. Additionally, it was shown that the lower initial level of PAT contamination and the higher the pH of apple juice and the added levels of AA, the higher the rate of PAT degradation, indicating that conditions like that are desirable if AA is to be used to reduce levels of PAT.

Three validation experiments were carried out utilizing model system and one with shelf-stable AJ, and each experiment was triplicated. The predicted and the observed values of $b$ were not statistically different ($t$-test $p<0.05$) for both the model system and the shelf-stable juice indicating the adequacy of the developed RSM model in predicting the degradation rate of PAT in the presence of AA at different conditions.

Thus, it was concluded that AA could be an applicable post-processing treatment during storage. Using the model generated from the response surface analysis $b$ can be estimated and from that, the remaining concentration of PAT at any given time can be calculated. The relationship between $b$ and $T$ was also established (Arrhenius) so the remaining concentration of PAT can be calculated for different storage conditions. Given that the reaction between PAT and AA could be prolonged depending on the storage $T$ and the initial levels of the reactants, it seems that AA could be potentially utilized to reduce the levels of PAT in AJ as a post-production treatment during storage. Pre-requisite programs like GAP and GMP could enhance its efficiency of AA as they could help to decrease the initial load of PAT producing fungi and levels of PAT. Increasing the
pH of AJ as well as the added levels of AA will also help to decrease the storage time needed to reduce PAT levels below the regulatory limit.

More experiments should be carried out utilizing different processing condition and multiple AJ samples; differences in composition may affect the rate. Further experimentation will enable the identification of any other factor that may affect the rate and will provide further validation for the model.

The mechanism of the degradation of PAT in the presence of AA in an aqueous AJ model system (malic acid buffer, pH 3.75) was investigated. LC/MS with electrospray ionization (ESI) was used to monitor the progress of the reaction between AA and PAT. Although several degradation products of AA were identified and PAT reduction was evident no adducts or degradation products of PAT were identified. The identified degradation products of AA were: DHAA, Diketogulonic acid and 3,4-dihydroxy-5-methyl-2(5H)furanone (DMF) and 3-hydroxy-2-pyrone.

6.2 Suggested future work

There is a need to further investigate the mechanistic pathway of the degradation reaction to gain a more thorough understanding on how different factors might affect it. In addition identification and isolation of PAT degradation products will allow for
toxicological studies which will further confirm the ability of AA to be used as a treatment to reduce the concentration of the mycotoxin and also result in a safe product.

More experiments utilizing an LC/MS or LC/MS/MS system should be carried out in order to elucidate the mechanism. The utilization of a different analytical column might allow adequate separation with a less acidic mobile phase which may solve the ion suppression problem presented in this study and thus make the operation of ESI at negative mode feasible. The use of a different ionization method like APPI as well as an analytical method that allows the elution of highly hydrophobic compounds may reveal degradation products of PAT and allow conclusions for the mechanistic pathway of the reaction.

Electron paramagnetic resonance (EPR) or electron spin resonance (ESR) Spectroscopy may be a useful tool to determine whether AA degrades PAT via simple adduct formation or by an oxidation-reduction process which results in opening of the lactone ring. EPR is a technique of great specificity and can be used to study compounds that have one or more unpaired electrons (free radicals). AA is readily oxidized by radical and metal ions via two one-electron transfers to yield an ascorbate free radical and then DHAA. Radicals generated from AA can be captured using a standard spin adduct such as 5,5-dimethyl-1-pyrroline-N-oxide (DMPO).

Both microbial and mammalian toxicological tests can be used to determine if the reaction with AA decreases PAT toxicity and whether decreases are reversible. Toxicological changes can be followed by the *Tetrahymena* bioassay and the *Bacillus subtilis* assay. Both assays were proven to be useful for quantifying PAT toxicity and are
easier in comparison with the mammalian lung fibroblasts or other mammalian cell lines for following changes in toxicity during the course of the reaction.

Because the Food and Drug Administration used the 2-year animal study of Becci et al. (1981) to establish a "no observed adverse effect level" (NOAEL) for PAT of 0.3 mg/kg bw per week, similar animal studies will be desirable (gold standard) to confirm loss of toxicity of PAT-containing juice treated with AA.
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Appendix

Table A.1

Table A.1: Reduction (%) of PAT at specific time intervals (average of three replications). At each point the initial level of PAT was 1000 ppb and the pH was 3.75. (AA+): with AA 221 ppm, (AA-): without AA, *: values beyond that time were not significantly different. (p>0.05). N.D.: not detectable.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>25°C</th>
<th>35°C</th>
<th>45°C</th>
<th>55°C</th>
<th>65°C</th>
<th>75°C</th>
<th>85°C</th>
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</thead>
<tbody>
<tr>
<td>AA+</td>
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<td>0.73</td>
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<td>1.32</td>
<td>23.91</td>
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</tr>
<tr>
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<td>35.66</td>
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</tr>
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</tr>
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<td>7.57</td>
<td>68.90*</td>
<td>12.21</td>
<td>72.51*</td>
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Figure A.1: Hypothesized mechanism, ascorbate attacks PAT via Michael addition and results in adduct formation. Eventually the lactone ring opens and PAT is detoxified.
Figure A.2: Residual plot for the second order kinetic model ($1/C$ vs Time) on the data obtained from the apple juice validation experiment. The data demonstrate a clear pattern which means that the linear model does not fit the data.
Figure A.3: Residual plot for the predictive model generated from the RSM analysis. Residuals demonstrate a random distribution indicating no patterns and good fit of the model.

Figure A.4: Observed versus predicted values of residuals plot for the predictive model also indicates a good fit.
Figure A.5: Pareto chart of effects for b parameter (rate constant) of the Weibull model.
Figure A.6: Autocorrelation of residual plots for the apple juice validation experiment (average of three replications) confirms that there is no autocorrelation between the residuals and no existing pattern which means that Weibull model adequately fits the data and describes the kinetics of degradation of PAT in the presence of AA in apple juice.
Figure A.7: Autocorrelation residual plot for validation experiment 1 (buffer) indicates random distribution of residual and thus good fit.
Figure A.8: Autocorrelation of residuals for validation experiment 2 (buffer) indicates random distribution of residual and good fit of the Weibull model.
Figure A.9: Autocorrelation of residuals plot for validation experiment 3 (buffer) indicated random distribution of residuals and good fit of the Weibull model.