Thermal and Chemical Inactivation of Ricin and Shiga toxins in Orange Juice

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Dedication

This thesis is dedicated to my advisors for their wonderful guidance and my family for their unconditional love and support.

Abstract

The potential use of ricin and Shiga toxins (Stxs) as bioterror weapons in the food supply is a major concern for homeland security. Denaturation effects of thermal and chemical treatments are expected to reduce the toxicity of ricin and Shiga toxins in water solutions, but their effectiveness and stabilities in food matrices are largely unknown.

The objective of this project was the identification of heat and chemical treatments capable of inactivating ricin and Shiga toxins in orange juice so that large quantities can be safely disposed in the event of an intentional attack.

Diluted ricin was mixed with orange juice for inactivation studies. Thermal stability was determined in capillary tubes using a water bath at high temperatures typical of pasteurization. For chemical inactivation, sodium hypochlorite (NaOCl), sodium hydroxide (NaOH) and peracetic acid (PA) were added alone or in combination to samples with or without thermal treatment. The ricin concentration in samples was determined using an ELISA. The Arrhenius model was used to evaluate temperature dependence.

Enterohemorrhagic *Escherichia coli* strains were used to produce Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). Shiga toxins were added into phosphate buffered saline (PBS) or orange juice to study the inactivation effects. The same inactivation method was also used for heat treatment of Stxs. The concentration of Stxs was determined by an ELISA and a cytotoxicity assay was conducted to confirm the inactivation. Kinetics studies were done to evaluate inactivation parameters.

Heat inactivation of ricin followed first-order kinetics. The half-life ($t_{1/2}$) of ricin at 72, 80, 85 and 90°C were 72.6, 9.0, 2.0 and 0.5 min, respectively. The Z value was 8.8°C indicating high temperature sensitivity. When the concentration of each chemical was increased to a sufficient amount, the detection limit of the

ELISA kit was reached when measuring ricin inactivated within 5 s at room temperature. A significant synergism between NaOCl and NaOH and considerable efficacy with treatment with PA alone were observed.

The heat inactivation of Stxs in PBS and orange juice also followed first-order reaction kinetics. Both Shiga toxins in PBS and orange juice would reach the concentration that was not detectable with ELISA within 30 s at 90°C and 120 s at 85°C. The Z values for Stx1 and Stx2 were 6.7 and 7.2°C in PBS as well as 8.7 and 6.9°C in orange juice, respectively.

This study delivered the first series of time/temperature/concentration conditions that would serve as the basis for recommendations for treating orange juice subjected to intentional adulteration with ricin or Shiga toxins in an orange juice plant with typical pasteurization equipment so it can be safely disposed into the environment.

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1 Introduction

1.1 Bioterrorism and food safety

After the events of September 11, 2001, terrorism became a major concern for homeland security. The goal of terrorists is to attack specific targets resulting in consequences such as chaos, death and economic loss. The food industry which includes the food supply chain starting from producers and a large number of transportation, processing and distribution facilities is vulnerable due to weaknesses related to security, monitoring and tracking. Since all societies are dependent on a stable food supply, the potential for the food system to be a target of terrorists is a top priority issue. Food terrorism was defined by the World Health Organization (WHO) as “an act or threat of deliberate contamination of food for human consumption with chemical, biological or radionuclear agents for the purpose of causing injury or death to civilian populations and/or disrupting social, economic, or political stability” (73).

There are many instances in history where the food supply has been sabotaged deliberately. Some of these attacks have included industrial chemicals as well as biological agents. Among those agents, biological toxins such as ricin and abrin, if used in a terrorist act, would have relatively severe consequences.

1.2 Toxins used as bioterrorism agents in foods

Biological materials and chemicals that cause acute effects on health are the most likely candidates for intentional food contamination. Biological toxins such as ricin, T-2 toxin and Shiga toxins may be used as bioterrorism weapons to attack the food system. Symptoms of intoxication with any of these toxins

develop rather quickly and can be life threatening. In addition to their relatively high toxicity, some of these toxins are fairly easy to produce.

The current food industry, especially the liquid food (milk /juice) production and processing systems are particularly susceptible to deliberate sabotage (35, 72). The toxins could be purposely released at different points in the production chain, such as a holding tank at a farm, a tanker truck transporting milk, or a raw milk silo at the processing facility (72). The centralized storage and processing followed by rapid distribution and consumption of liquid foods would result in several thousands of poisoned individuals in a deliberate toxin attack. Intentional contamination of food may also cause enormous economic loss and trade disruption, by targeting a product, a manufacturer, an industry or a country (72).

1.3 Detection of toxins in foods

In order to detect toxins such as ricin or Shiga toxins, sensitive, accurate and robust assays that can provide critical information are crucial to the prevention and defense against food contamination. There are many types of detection methods and the most common methods used to detect such protein based toxins include cell culture assay, immunochemical assay and mass spectrometry. Several studies (26, 31) have shown generally, that most of the detection methods can't discriminate between inactive and active toxins and only several detection methods are able to differentiate inactive and active toxins.

Kalb and Barr (2009) reported a mass spectrometric detection method for ricin and its activity in food samples, with enhanced selectivity and specificity for ricin. It combines three analytical methods: (1) immunoaffinity capture of the ricin protein; (2) examination of the activity of the ricin protein using a DNA

substrate that mimics the toxin's natural RNA target; and (3) analysis of the tryptic digestion fragments of the toxin itself. Babu and others (2008) studied flow cytometry in an apoptosis assay to determine the stability of Shiga toxin 1 and confirmed that this approach is able to provide rapid and high throughput when detecting the activity of Shiga toxin 1 (4). Commercial products such as enzyme linked immunosorbent assays (ELISA) from Tetracore Inc. or Meridian Bioscience, and the cytotoxicity lactate dehydrogenase (LDH) detection kit from Roche Applied Science can be used to detect toxins.

1.4 Inactivation of toxins in foods

There is very limited information about the stability of toxins in foods. Previous research indicated that ricin could be detoxified by thermal treatment during the processing of castor bean. Ricin in flaked castor bean was detoxified at 100-102°C when held for 12 to 15 min (25). Heating castor bean meal for 1 h in steam (~121°C) at 15 psi also inactivated ricin (30). However, the thermal inactivation of ricin added to foods has been largely ignored. Only very recently, researchers at the FDA reported that ricin retained half of its toxicity after heating for 2 min at 90°C in liquid infant formula (29).

Shiga toxins (Stx) and Shiga-like toxins (Stx1 and Stx2) are a relatively large group of cytotoxins produced by certain serotypes of *Shigella* and Shiga-toxin producing *Escherichia coli* (STEC). These toxins are robust, stable, and relatively easy to manufacture and have been the subject of intense research for the last 25 years. Kittell and others (1991) reported the inactivation of verotoxin 1 (Stx1) produced by *E. coli* O157:H7. Stx1 toxicity was stable at 70°C for 60 min, but was reduced by 90% at 80°C for 15 min and 99% at 80°C for 30 min. Verocytoxicity was completely lost at 80°C for 60 min and at 85°C for at least 5 min. Both temperature and pH played an important role on the loss of toxicity. Stability of Shiga-like toxin 1 in fruit punch was studied (4). The

inactivation was achieved with the combination of acidic pH and higher storage temperature (20°C). However, no detailed data were presented about the heat resistance of the toxin.

It should be noted that most of the above data were not produced in food matrices. Due to the complexity of food composition, the inactivation conditions cannot be extrapolated from one system to other food matrices. Thermal susceptibility of toxins can be affected by pH, initial concentration, and food constituents of the specific product. One goal of the Agents group of the National Center for Food Protection and Defense (NCFPD) is to come up with rapid (<30 min) assays for toxins as well as to determine for liquid foods like milk and juice, parameters for heat and chemical inactivation, so if a silo (5,000 to 60,000 gallons) was sabotaged, the processor could effectively reduce the level of toxin such that the liquid food could be disposed according to Environmental Protection Agency (EPA) requirements.

1.5 Research needs

It is important to understand how to inactivate toxins and characterize the thermal and chemical stability of biological toxins, particularly in typical food environments and processing conditions to assess the potential impact of food processes on toxin activity and to identify effective toxin inactivation protocols. It is also critical to develop effective intervention methods that could be used in the event of a deliberate terrorists attack to mitigate the public health impact and serve in decontamination and disposal responses.

1.6 Hypotheses

The null hypotheses for this research are as follows:

1) Heat treatment at high temperature short time (HTST) pasteurization temperatures for processing of orange juice does not inactivate ricin and Shiga toxins.

2) Combinations of heat treatment and chemical compounds treatment do not cause the inactivation of ricin and Shiga toxins in orange juice.

1.7 Objectives

To address these hypotheses, the objectives of this research are as follows:

1) Characterize the thermal stability of ricin and Shiga toxins at HTST pasteurization temperatures in orange juice.

2). Determine the detoxifying time/temperature/concentration conditions of heat combined with sanitizing compounds against the same toxins in orange juice.

2 Literature review

2.1 Ricin overview

2.1.1 Castor bean and castor oil

Castor beans are the seeds of the castor oil plant, *Ricinus communis*, a flowering plant. Ricin is present in the seed coat. Approximately 50% of the weight of the castor seeds is castor oil. It is valued for a number of industrial and medicinal applications such as lubricants, plastics, wax, and transparent soaps in industry or as a laxative, purgative and cathartic in medicinal applications (3). After the oil is extracted from the seeds, the remaining part is called seed cake which could be used as fertilizer or animal feed after careful boiling or heating to inactivate any remaining toxins including ricin. Ricin naturally protects the castor plant from insect pests. The production of castor beans exceeds one billion pounds annually worldwide for these applications and the waste mash contains 5-10% of ricin (75). The readily availability of ricin from the seed cake makes it a potential concern as an easily obtainable biological weapon.

2.1.2 Structure and mechanism of action of ricin

The structure and mechanism of action of ricin have been extensively characterized (11, 17, 19). Ricin is a globular protein (66 kDa) which is comprised of two chains, A and B, linked by a disulfide bond. The A chain (32 kDa), a *N*-glycosidase, is able to irreversibly inactivate eukaryotic ribosomes through release of a single adenine residue from the 28S ribosomal RNA loop contained within the 60S subunit and therefore inhibits protein synthesis (3). The B chain (34 kDa), a lectin, is able to bind to galactose-containing

glycoproteins and glycolipids expressed on the surface of cells and then facilitate the entry of ricin into the cytosol. Ricin is first taken up and contained in an endosome, and then it could be digested in lysosomes. Most likely, it could pass through the endomembrane compartments and the A chain is removed into the cytosol. Then the A chain enzymatically cleaves a specific adenine residue and as a result, protein synthesis is inhibited and the cells die eventually, as shown in Figure 2-1.

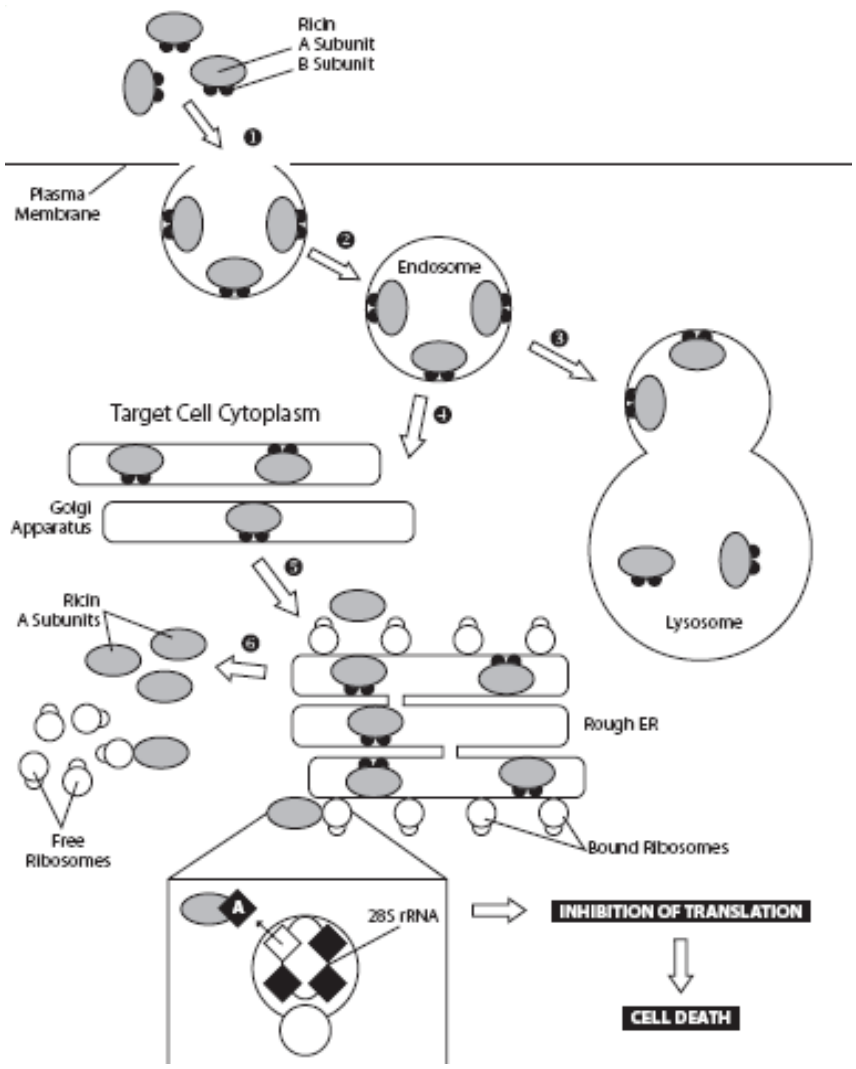


Figure 2-1. Illustration that depicts the mode of the toxic action of ricin toxin. Reprinted with permission from Pommerville, 2003 (54).

The ricin A chain inhibits protein synthesis only when it is internalized into the cytosol with the help of the ricin B chain. If these two subunits are separate, the ricin A chain is not able to penetrate the membrane and would not inhibit protein synthesis (28). However, when they are linked together or even the two separate subunits are mixed together, they can reassociate covalently to form toxic ricin (15).

2.1.3 Use of ricin as a bioterror agent

In recent years, ricin was involved in a number of cases as an agent of terror. As early as 1978, the Bulgarian dissident Georgi Markov was assassinated in Great Britain by an embedded pellet containing ricin in the tip of an umbrella used to puncture Georgi in the foot releasing the pellet into his blood stream. In 2003 and 2004, a letter containing ricin and sealed in a “ricin-contaminated” envelope addressed to the White House was discovered and intercepted in a South Carolina mail sorting facility (7). In 2008 and 2009, ricin was found in a hotel room in Las Vegas and a suburban home in Washington.

Beyond the cases of ricin as a bioterrorism weapon in envelopes and facilities, it has also been detected in food. In 2004, ground-up castor beans which were far less toxic than purified ricin were found in two jars of Gerber banana yogurt dessert sold in a supermarket. The parents of the two families found notes in the jars after they fed their babies warning that the jars were contaminated but the babies were not harmed. The Gerber Products Company stated that the product tampering did not occur at its facilities. Nonetheless, Gerber’s products were removed from all southern California grocery and drug stores. The FDA recommended people to check food packages for any signs of tampering (53). These events had reinforced the concerns regarding the use of ricin for mass urban terror, especially in food systems.

2.1.4 Toxicity and clinical features of ricin

The physical state of ricin determines the route of exposure in humans. Since castor beans are the source of ricin, ricin can be made either in the form of a crude extract, in a purified form as ricin crystals, a powder or dissolved in solvents. The exposure for humans is possible through injection, inhalation or ingestion. The route of exposure determines toxicity and mortality.

A few publications have focused on human exposure to ricin via injection. The LD_{50} (lethal dose) value of crude ricin was reported as 0.2 μg (8 μg/kg) in mice by intraperitoneal injection in 1973 (51). The LD_{50} in mice was reported as approximately 5 to 10 μg/kg body weight through injection (3) while the intravenous LD_{50} in mice was from 55 to 65 ng/kg (75), i.e. the latter being 1000 times more lethal. The symptoms start after ~20 h and the tissue at the injection site could be damaged. Ricin toxic effects on the body can be characterized by laboratory tests which include elevated liver transaminases, amylase, and creatinine kinase, hyperbilirubinemia, myoglobinuria, and renal insufficiency.

Only a few studies have reported toxicity of ricin by human inhalation. The inhalation LD_{50} in mice was reported at 3 to 5 μg/kg (3) or 4 to 6 mg/min/kg (75). The particle size of ricin significantly affects the lethality after ricin inhalation. Smaller particles tend to deposit deeper in the respiratory tract which leads to higher death rate, while larger particles often deposit on the surface and could be swept up by physiological activities in the respiratory tract after inhalation. Symptoms include airway inflammation, rhinitis, and ocular irritation.

No literature has been found indicating human lethality from ingestion of purified ricin. Almost all reported human cases are associated with ingestion of whole or ground castor beans. The physicochemical characteristics of ricin make it easy to be dissolved in food or drink available for ingestion. The median oral lethal dose (LD_{50}) in mice was reported as 30 mg/kg which is ~1,000 times more than the lethal dose found through injection or inhalation. LD_{50} was also reported as

3~5 μg/kg in mice (34, 69, 77), however, we could not obtain the original references. The lethal oral dose in humans was 1 to 20 mg/kg body weight, estimated from consumption of 8 castor beans (6, 75). The minimum number of castor beans associated with an adult human death was 2 and symptoms from mild condition to lethality were mainly reported in the range of one half to 30 castor beans in human (3). Symptoms usually started within 4 to 10 h after ingestion. The symptoms include abdominal pain, vomiting, diarrhea, heartburn, and oropharyngeal pain. A summary of LD_{50}s through different routes of exposure are listed in Table 2-1.

Table 2-1. Summary of LD_{50}s of ricin through injection, inhalation or ingestion.

Route of exposure	Animal (Reference No.)	LD_{50}
Injection	Mouse (3)	Injection (skin) 5-10 μg/kg
	Mouse (75)	Intravenous 55-65 μg/kg
	Mouse (intraperitoneal) (51)	8 μg/kg
Inhalation	Mouse (3)	3-5 μg/kg
Ingestion	Mouse (whole bean) (6, 75)	30 mg/kg
	Mouse (34, 69, 77)	3-5μg/kg
	Human (whole bean) (6, 75)	1-20 mg/kg

Since the estimated oral lethal dose for a 20 kg child with an uncertainty factor of 100 is 4 mg or 1 μg, bioterrorists would need to add ~424 g or only 0.1 g of pure ricin into a 7,000 gal silo of orange juice. The small amount of ricin needed makes it quite easy for the bioterrorists to accomplish this.

2.1.5 Detection of ricin

Detection of ricin and monitoring of decontamination of ricin released to the environment are big concerns and developing suitable, reliable and rapid methods therefore becomes extremely important. There are different technologies for quantifying ricin and the most promising ones include mass spectrometry, cell culture assays, and immunochemical assays. Each assay has its advantages and disadvantages that the researchers should consider when choosing a suitable method according to their needs.

Kalb and Barr (31) reported a mass spectrometric detection method for ricin and its activity in food samples. It combines three analytical methods: immunoaffinity capture, examination of the activity of the ricin protein using a DNA substrate and analysis of the tryptic fragments of the toxin itself. Mass spectrometry is a very sensitive and selective measurement which can detect at very low levels. However, the measurement needs expensive equipment, highly trained people and thus can't be used as a screening method in the field, for example at the point of unloading a tanker truck into a silo at a processing plant.

Cell cytotoxicity assays are used to evaluate and differentiate active and inactive toxins. Cole and others (12) investigated the disinfection effect of bleach and monochloramine through measuring the effect on mammalian cell cytotoxicity. The ricin cytotoxicity assay is very sensitive because it measures the enzymatic activity of ricin. However, the measurement of cytotoxicity needs cultivation of mammalian cells with specialized expertise, and any contamination in sample preparation could ruin the whole experiment.

The use of enzyme-linked immunosorbent assay (ELISA) methods for ricin detection has been reported by several researchers. Two recent studies applied ELISA methods to detect residual ricin in a food matrix (24, 29). ELISA is a relatively sensitive and selective measurement for ricin that could be completed within hours. Nonetheless, the cross-reaction and large variance in results are

great limitations of ELISA. Besides, since ELISA protocols are typically based on the immunogenic relationship between antigen (toxin) and antibody, it is not able to reveal the toxic activity of toxins. To address this limitation, Jackson and others (29) correlated the results of ELISA and cell cytotoxicity assays and found a high degree of correspondence, which supported the idea of using ELISA to determine active ricin.

2.1.6 Inactivation studies of ricin

There are few publications on the inactivation of pure ricin but some on the inactivation of whole or flaked castor seeds or castor bean meal. Okorie and Anugwa (50) observed that ricin in castor oil bean seeds prepared for a commercial chicken feed diet couldn't be destroyed by steaming at 80°C for 40 min but activated instead. Steaming for 60 min partially inactivated the ricin and roasting at 140°C for 20 to 30 min was sufficient to destroy ricin. Jenkins (30) reviewed the studies of the inactivation of the toxic components of castor bean meal. That review included autoclaving castor meal at 15 p.s.i. for 30 min, boiling castor cake with saline and heating castor meal at 140°C for 60 to 90 min or heating at 125°C for 15 min. The author concluded that all of those treatments were enough to make animal feed safe to consume by animals. Since 1963, however, very little research on the inactivation of pure ricin or ricin in food matrices had been published.

In one of the recent reports, researchers at the FDA investigated the thermal stability of ricin in different infant formulas (29). This was the first study that determined the half life (time for a toxin to be inactivated by half), activation energy (the threshold or energy barrier that must be overcome or surpassed to permit the transformation of reactants into products) and Z value (change in temperature that accompanies a 10-fold change in D value) parameters of heating ricin in a food matrix. Reconstituted infant formula powders containing

100 µg/mL (27 mg per 8 oz serving) of ricin were heated at 60, 70, 75, 80, 85 and 90°C for up to 5 h in glass test tubes in a block heater. Tubes were removed at different time intervals from the block heater and cooled in an ice bath. Calibrated thermocouples were utilized to monitor the come up time (less than 2 min). However, the come up time wasn't subtracted from the final heating time which would affect the kinetics parameters. The treated samples were then quantified using an ELISA kit and a cytotoxicity assay. Their results showed that ricin inactivation in infant formula at 60 to 90°C could be modeled based on first-order kinetics and the toxicity of ricin decreased with the increasing heating times and temperatures.

According to Jackson and others (29), the half lives of ricin cytotoxic activity in a milk-based infant formula at 60, 70, 75, 80, 85 and 90°C were >100, 9.8, 5.8, 5.1, 3.1 and 1.8 min, respectively, while the values for a soy-based infant formula were >100, 16, 8.7, 6.9, 3.0 and 2.0 min. The activation energy was 92 kJ/mole and Z values for ricin inactivation were 25°C in both formulas. However, when the data was recalculated, the activation energy was 130 and 128 kJ/mole in milk and in soy based infant formulas, respectively, and the Z values were both 18°C, as shown in Figure 2-2. This was convincing evidence indicating that conventional pasteurization processing (63°C for 30 min, 72°C for 15 s, 89°C for 1.0 s and 90°C for 0.5 s) would not be sufficient to ensure complete inactivation of ricin present in infant formulas. Based on the prior calculation, one would need 10 × 1.8 min or a process time of 18 min at 90°C to achieve the needed 10 half lives. For a 4 kg infant, this would need to be inactivated for >150 min at 90°C for both soy and milk based formulas. Very importantly, this study showed that the OD value for the heat treated ricin obtained from the ELISA method could give a good estimation of the amount of residual toxicity of ricin from the cytotoxicity test.

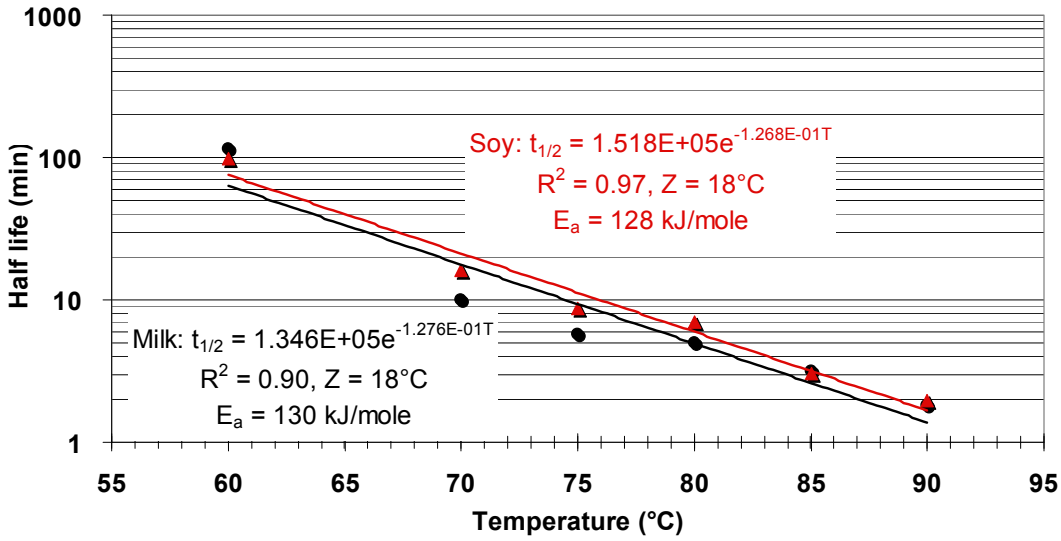


Figure 2-2. Relationship of half life and temperature in the inactivation study of ricin in milk (●) and soy (▲) based infant formula from Jackson and other (2006) FDA.

Xu and others (unpublished) studied the thermal and chemical stability of ricin in milk. A capillary tube method was utilized for thermal inactivation experiments in order to minimize the come up time. The thermal inactivation kinetic parameters of ricin were determined at 72, 80, 85, and 90°C. Several sanitizer chemicals including sodium hypochlorite (NaOCl) from 0.1 to 1% and sodium hydroxide (NaOH) from 0.01 to 0.15 N were evaluated for their efficacy in inactivating ricin in milk at room temperature. Residual ricin in treated samples was detected by an ELISA assay. The half-lives of ricin at 72, 80, 85 and 90°C were 32.1, 3.0, 0.51 and 0.18 min, respectively and the activation energy, Z value and Q_{10} were 305 kJ/mole, 7.8°C, and 18.8, respectively. A significant synergism between NaOCl and NaOH in the inactivation of ricin was observed. There were some larger discrepancies between the latter study results and those reported by Jackson and others (29). Possible reasons included the different characteristics between powder infant formula and milk as well as the

different inactivation methods, especially the come up time.

He and others (26) explored the effect of different food matrices on the biological activity of ricin. In that study, three economically important foods including ground beef, low-fat milk and liquid chicken eggs that had added ricin A chain, pure ricin or crude ricin were subjected to heat and the thermal inactivation was studied. The food (0.1 g) was incubated together with ricin for 5 min at room temperature for absorption followed by heating at 63 or 72°C for 3 min and cooling in an ice bath. Come up time wasn't considered in this study. Residual ricin in the treated samples was analyzed by the CFT (cell free translation) assay. The study revealed that variations of the thermal stability were present in pure and crude ricin in different food matrices. Crude ricin was more sensitive than pure ricin in phosphate buffered saline (PBS) while it was more resistant in ground beef. As seen in Table 2-2, no significant difference between pure ricin and crude ricin in milk was found and the inactivation was dramatically different in eggs at 63 and 72°C between pure and crude ricin. Their results indicated that cooking meats and eggs under the recommended temperature by the FDA could ensure the inactivation of ricin A chain. However, the same temperature was not enough for complete inactivation of the holotoxin in milk.

Table 2-2. Loss of ricin activity after heating for 3 min in different food matrices from He and other (26).

Matrix	Temp. (°C)	Loss of ricin activity (%)		
		Ricin A chain	Pure ricin	Crude ricin
PBS	63	47.34	-1.65	12.65
	72	62.22	4.25	14.80
Beef	63	99.65	17.43	10.92
	72	100.00	30.96	23.78
Milk	63	38.76	-9.90	-9.77
	72	60.21	0.20	-1.40
Egg	63	89.18	5.00	18.00
	72	100.00	42.00	28.00

2.2 Shiga toxins

2.2.1 Shiga toxin classification

Shiga toxin-producing *Esherichia coli* (STEC) and *Shigella dysenteriae* type 1 are two bacteria capable of producing Shiga toxins (Stxs), a class protein of toxin also known as verotoxins, verocytotoxins or Shiga-like toxins. Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) are the two most important Shiga toxins. A single STEC isolate can produce Stx1, Stx2 or both toxins while *Shigella dysenteriae* type 1 can only produce Stx (48).

Many similarities exist among Stxs in various aspects including genetic, structural and functional properties, however significant divergences are also present (47). The difference between Stx1 of STEC and Stx of *Shigella dysenteriae* type 1 is only one amino acid in the A polypeptide and therefore they are usually designated as Stx/Stx1 group versus Stx2 group. Variable

antigenic features are present among the Stx2 group including Stx2, Stx2c, Stx2d and Stx2e while there are no significant differences, in the Stx/Stx1 group. These antigenic features among Stx2s are mainly from the sequence in the B chain and these lead to different toxicity and activity among Stx2 variants in tissue cells or animals. Against Vero cells, the toxicity of Stx/Stx1 and Stx2 are similar while Stx2 is almost 400 times more toxic than Stx/Stx1 to humans.

2.2.2 Structure and mechanism of action of Shiga toxins

Similar to ricin, Shiga toxin also consists of two chains, namely the A and B chains. The A chain (32 kDa) has an A_1 portion of ~28 kDa and an A_2 portion of 4 kDa. The A_1 portion, an *N*-glycosidase, is able to remove a key nucleotide residue from the 28S rRNA of 60S ribosomes and cause inhibition of protein synthesis, similar to the mode of action of ricin (19). The A_2 domain's main function is to connect the A_1 portion to the B chain. The B chain is a pentamer formed by monomers (~7.7 kDa per monomer). It is required to bind to a eukaryotic glycolipid receptor, globotriaosylceramide (Gb_3) (48). Similar to ricin, the A chain can't enter cells by itself and the B chain is required for toxin cell surface recognition and internalization.

There are also differences between the mechanisms of action of Shiga toxins and ricin, illustrated in Figure 2-3 (65). In the case of ricin, the A and B chains are connected by a disulfide bond and in order to have the best enzymatic activity the disulfide bond has to be reduced. In contrast, the disulfide bond is present internally for Stxs and it is required for effective toxicity.

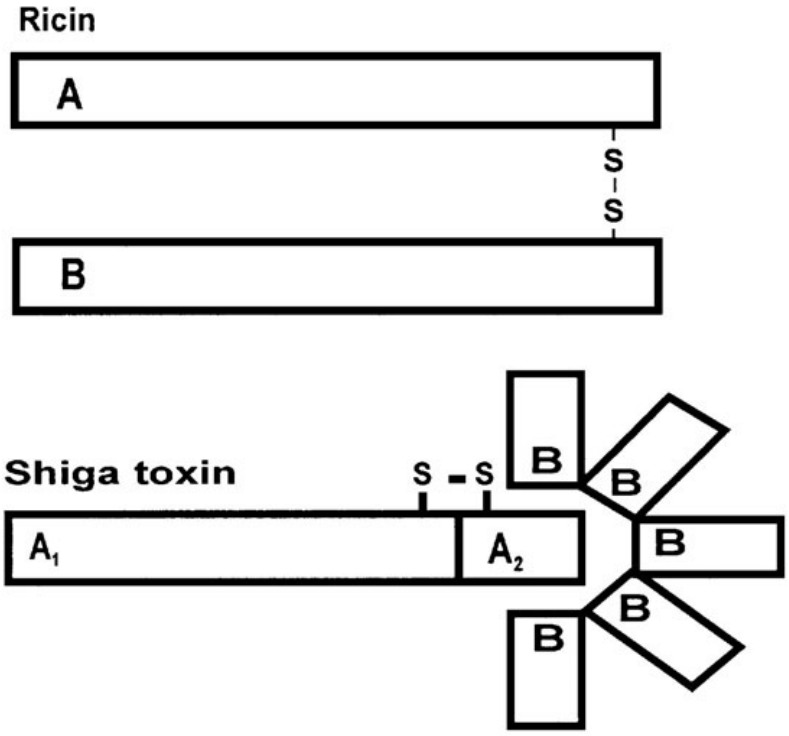


Figure 2-3. Schematic structure of protein toxins and difference between ricin and Shiga toxin (65). Reprinted by permission from Sadvig, 2000.

2.2.3 Use of Shiga toxins as bioterror agents

Since Shiga toxins are protein toxins produced by enterohemorrhagic *Escherichia coli* (Shiga toxin-producing *E.coli*) and *Shigella*, Shiga toxins have always been associated with outbreaks of STEC or *Shigella*. However, there is no documented event of utilization of Shiga toxins as biological weapon despite the fact that they are relatively easy and inexpensive to produce.

2.2.4 Toxicity of Shiga toxins

Several studies have shown different LD_{50} values for Shiga toxins. Melton-

Celsa and O'Brien (48) listed the LD_{50} of Shiga toxin 1 and Shiga toxin 2 as 400 ng and 0.5-2 ng, respectively, for mice intraperitoneally. There are no published reports on the inhalation toxicity of Shiga toxins. The LD_{50} of Shiga toxins in mice was determined by Tesh and others (67) as approximately 5×10^{-5} mg/kg body weight for Shiga toxin 2 and 0.02 mg/kg body weight for Shiga toxin 1, through either intravenous or intraperitoneal injection. These data indicated that Stx2 appears to be much more toxic than Stx1.

2.2.5 Production of Shiga toxins

Since Shiga toxins are not commercially available, almost every researcher has to produce Shiga toxins by themselves through different methods. Roberts and others (61) prepared Shiga toxins in a traditional way by growing bacterial cultures in brain heart infusion (BHI) at 37°C for 23 to 26 h followed by centrifugation (8160 × g, 3 min), resuspension of the cell pellets in polymyxin B sulfate solution, incubation for 30 min to release cell bound toxins and filtration through 0.45 μm disc filters. Rocha and Piazza (63) determined the suitability of different broth media for producing Shiga toxin from STEC and concluded that Shiga toxin production could be optimized when bacteria is grown in the presence of antibiotics such as ciprofloxacin. In order to produce a relatively large amount of Shiga toxin for research purpose, Viscardi and others (71) grew *E.coli* O157:H7 in pre-warmed Luria-Bertani (LB) broth at 37°C with antibiotics (5 μg/L ciprofloxacin or 10 μg/L norfloxacin) overnight, centrifuged at 1000 × g for 20 min at 4°C and then filtered using 0.2 μm pore size filter with low protein binding capacity. The toxin was measured with a Premier EHEC kit.

2.2.6 Detection methods of Shiga toxins

In order to detect Shiga toxins, fast, sensitive, accurate and robust assays that can provide critical information are needed. Several studies (26, 31, 58) have shown that, most of the detection methods can't discriminate between inactive and active toxins and only several detection methods are able to differentiate them. There are several types of detection methods such as immunochemical assays, and cell culture assays, with or without the ability to determine the activity of Shiga toxins.

There are a variety of cell-based assays to detect Shiga toxins such as MTT (Methylthiazolyldiphenyl-tetrazolium bromide) assay (66), GFP (green fluorescent protein) (58) assay and LDH (lactate dehydrogenase) assay (61). Most of the cell-based assays for detection of Shiga toxins utilize Vero cells and different reagents to measure the change of Vero cell activity due to the attack of Shiga toxins. Sekino and others (66) utilized the MTT assay to measure the effect of Shiga toxins on Vero cell dehydrogenase activity. The researchers reported the existence of a Stx1-resistant stock of Vero cells and revealed the mechanism of the Stx1 resistance in their Vero cells. A GFP assay was utilized by Rasooly and Do (58) to determine the stability of Shiga toxin 2 in whole milk that was confirmed by the MTT assay. Although these cell-based detection assays are time consuming and labor intensive, they are able to differentiate active toxins and inactive toxins, which is critical in many studies.

One popular cell-based detection assay is the LDH and Vero cell assay. As a confirmatory test, a Vero cell assay is used to determine the virulence potential of STEC (38). However, time consuming and additional microscopic analysis make it inconvenient to be used. An LDH assay (lactate dehydrogenase) could be combined with the Vero cell assay to rapidly determine toxicity. LDH is a stable enzyme that is present in all mammalian cells. When the plasma membrane of Vero cells is damaged or dead in response to the toxin, LDH in

the cell is rapidly released from the cytosol into the cell culture supernatant. The toxin is then determined using a colorimetric assay based on the measurement of LDH. A large number of samples in an LDH assay could be measured simultaneously in a 96-well plate. LDH assays have been utilized frequently and one example is to distinguish Shiga toxin producing *E.coli* from non-Shiga toxin producing strains (61). The LDH and Vero cell assay is capable of determining the virulence factors and therefore is a good detection and confirmation method although it is still time consuming.

Immunoassays are common detection methods, which are commercially available. ELISA formats are among the most popular commercial immunoassays, because they are easy, fast and convenient compared with other methods such as PCR or cell culture assay. Willford and others (74) evaluated three commercially available Shiga toxin ELISA kits including Premier EHEC test (Meridian Bioscience Inc., Cincinnati, OH), the Ridascreen Verotoxin Enzyme Immunoassay (r-Biopharm AG, Darmstadt, Germany), and the ProSpecT Shiga toxin *E.coli* (STEC) Microplate Assay (Remel Inc., Lenexa, KS). The researchers concluded that all the three tests had similar specificities. The first two kits had comparable sensitivity while the one from Remel Inc. was 10-fold less sensitive. In addition, they indicated the lack of ability of all three tests to detect Stx2d and Stx2e variants, which is absolutely a disadvantage of ELISA commercial kits. In addition, although the ELISA kit is available, easy and fast, it was uncertain if the method could differentiate between active and inactive Shiga toxins. Further studies are needed to correlate ELISA results with the cytotoxicity assay for detection of Shiga toxins.

2.2.7 Inactivation studies of Shiga toxins

A number of inactivation studies of Shiga toxins were done in the past years. Most of the media include water, buffer or a food matrix. Different inactivation

conditions were studied involving pH, temperature, time and so on, while different temperature and time points were tested according to researchers' objectives.

Kittell and others (37) studied the effect of heat, pH, and various cations and lipids on the activity of Shiga toxin 1. Forty 1.25 mL samples were thermally inactivated in Tris-buffered saline (50 mM Tris-HCl) in a water bath at 25, 37, 45, 55, 65, 70, 75, 80, or 85°C for 15, 30 or 60 min or at 75, 80, or 85°C for 5 min. The timing started once the samples were immersed. The results showed that Stx1 was stable when treated at 45-70°C for as long as 60 min. The inactivation of Stx1 was time-dependent when heated above 70°C. The toxicity of Stx1 was reduced by 90% at 80°C for 15 min and by 99% at 80°C for 30 min. Heat treatments at 80°C for 60 min or at 85°C for 5 min could completely inactivate Stx1. Their warm-up and cool-down times were measured with a thermocouple and a calibrated recorder and were reported to be negligible compared to the incubation time. However, the warm-up time could affect the final result easily with such a large sample size and the tube size used to contain samples in the inactivation studies was not mentioned, which could affect the come-up time. No kinetics calculation was done in this study. The half life calculated according to their data was 4.5 min, as shown in Figure 2-4.

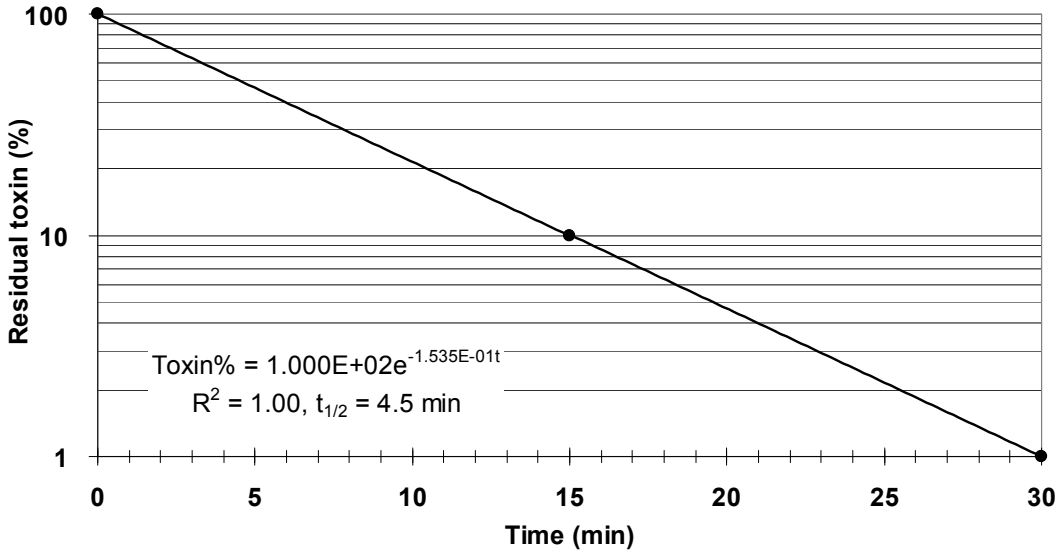


Figure 2-4. Inactivation of Shiga toxin 1 in buffer at 80°C from Kittell and others (37).

Tesh and others (67) determined the inactivation effect of Shiga toxins at different temperatures and at different pH. They found that heating at 65°C at pH 7.0 could reduce 99% of the cytotoxic activity of purified Shiga toxin 1 while it could not reduce Stx2 to a comparable level until heating at 85°C. Heating at 60°C and an extreme pH of 3.0 could completely reduce the cytotoxic activity of Shiga toxin 1 while heating to 95°C was required for a comparable loss in activity of Shiga toxin 2. However, neither the heating time nor how much toxin was left after the treatment was mentioned in their study. Furthermore, no sample size or any consideration of the come-up time was mentioned in this study.

Babu and others (4) studied the stability of Shiga toxins (Stx1 and Stx2) in fruit punch at different temperatures and pH values and flow cytometry was utilized to quantify apoptosis to determine the stability of Shiga toxins. Toxin-punch mixtures were heated at 95°C for 10 min or adjusted to pH ranging from 2 to 9

and incubated at 4 or 20°C for 0 to 90 days. However, no detailed inactivation methods were mentioned in their study. The best inactivation was achieved with the combination of acidic pH and storage temperature (20°C) at the 30 day time point. Nonetheless, no detailed data were presented about the kinetics of the heat resistance of the toxin.

Rasooly and Do (58) investigated the ability of Shiga toxin to inhibit the LDH activity of Vero cells and protein synthesis. They spiked whole milk with 1, 10 or 100 ng/mL Shiga toxin 2 (100 µL) and heated at the suggested temperatures and times by the U.S. Food and Drug Administration (63°C for 30 min, 72°C for 15 s or 89°C for 1 s) as well as 100°C for 5 min. The Stx2 activity was tested with a GFP assay and confirmed by a MTT test. The results suggested that Stx2 was heat stable and could not be inactivated by conventional pasteurization of milk. However, the thermal treatment at 100°C for 5 min was able to completely inactivate the toxin.

2.3 Terminology

2.3.1 High temperature short time (HTST) pasteurization

High temperature short time (HTST) pasteurization is the most widely used treatment for preserving the quality and extending the shelf life of dairy products and orange juice. It is a continuous pasteurization process using a continuous-flow heat exchanger. The typical heating temperature for milk is 72 to 74°C with a holding time of 15 to 30 s compared with 62 to 65°C for at least 30 min in low temperature long time pasteurization (LTLT) (32). Standard HTST pasteurization conditions are heating at 72°C for 15 s for milk and commercially orange juice is pasteurized at 90 to 95°C for 15 to 30 s. The HTST process has many advantages as compared to the LTLT process including less heat damage and flavor changes as well as higher throughput.

2.3.2 Inactivation kinetics

The rate law is an equation that incorporates the reaction rate constant and the concentrations of reactants in a chemical reaction (40). A reaction rate is given by:

$$\pm\frac{dA}{dt} = k[A]^a[B]^b \tag{2.1}$$

Where $[A]$ = concentration of reactant A

$[B]$ = concentration of reactant B

a = stoichiometric coefficient of A in the balanced equation

b = stoichiometric coefficient of B in the balanced equation

t = time

The overall order = sum of exponents= $a+b$

An apparent zero order reaction has a reaction rate independent of reactant concentration, thus the concentration of the reactant will not change the reaction rate. The loss in A is given by:

$$-\frac{dA}{dt} = k[A]^0 = k \tag{2.2}$$

Or $A = A_0 - kt$ (2.3)

Where A = amount at time t

A_0 = initial amount

k = rate constant in amount per unit time

t = time

The reaction rate of a first order reaction is dependent on the concentration of only one reactant and it is given by:

$$A = A_0 e^{-kt} \tag{2.4}$$

$$\text{Or } \text{Ln}\left[\frac{A}{A_0}\right] = -kt \tag{2.5}$$

Where k = the slope of $\text{Ln}\left[\frac{A}{A_0}\right]$ versus time and k = 2.3 × slope if using a semi-$\log_{10}$ scale. In Microsoft Excel, one can use the trendline exponential function on a semi-log plot of A versus time to get the rate constant directly.

2.3.3 D value

The D value (59), decimal reduction time, is the time required to reduce the toxicity of a toxin by a factor of 10 fold (one log cycle) or to 90% loss of the original at some constant condition (e.g. temperature, pH, ionic strength). It represents the resistance of a toxin to a specific temperature. The D value is not influenced by the initial amount of toxin since its magnitude is directly related to the slope of the straight line of N versus time in a semi-log plot as seen in Figure 2-5.

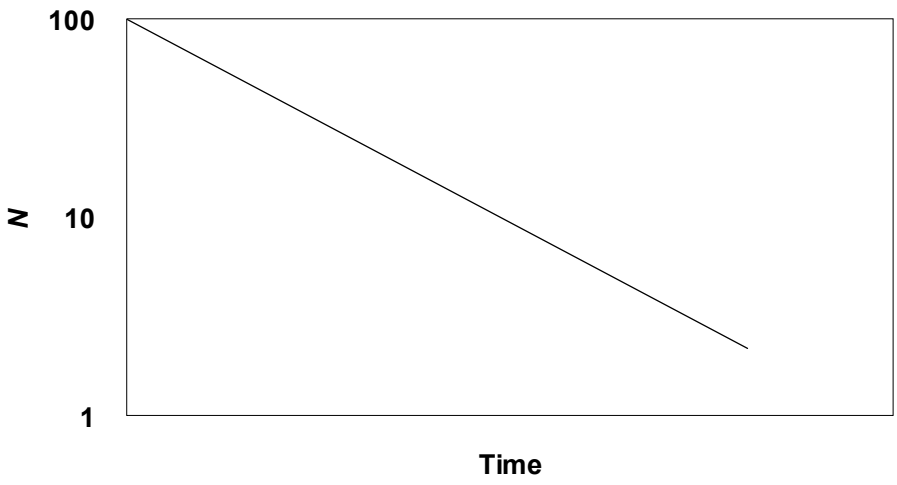


Figure 2-5. Theoretical inactivation curve for calculation of D value.

$$N = N_0 e^{-kt} \tag{2.6}$$

Where N_0 = initial amount of toxin

N = final amount of toxin

k = rate constant in amount per unit time

Since Equation 2.6 is an exponential decay, the slope of the line is the rate constant k (on a semi-log plot it is 2.3 × slope) and the D value is the time for one log cycle reduction. Thus,

$$\frac{N_0}{N} = 10 = e^{kt} \tag{2.7}$$

$$Ln\left(\frac{N_0}{N}\right) = 2.3 = kt = kD \tag{2.8}$$

Where D = the time for one log reduction.

Thus,

$$D = \frac{2.303}{k} \tag{2.9}$$

2.3.4 Half life

Half life ($t_{1/2}$) is the time needed to reduce the concentration of active toxin to 50% of original value. It describes the thermal resistant of toxins at a specific temperature measured in time units. The half life is equal to:

$$t_{1/2} = \frac{-\text{Ln}\frac{A}{A_0}}{k} = \frac{-\text{Ln}\frac{50}{100}}{k} = \frac{0.693}{k} \tag{2.10}$$

The half life could be also calculated as follows:

$$N_2 = N_1\left(\frac{1}{2}\right)^{\frac{t}{t_{1/2}}} = N_1(0.5)^n \tag{2.11}$$

Where N_1 = initial amount of toxin

N_2 = final amount of toxin at time t

$t_{1/2}$ = half life of the inactivation (time)

n = number of half lives ($\frac{t}{t_{1/2}}$)

Thus we can calculate the final concentration percentage left for any number of half lives:

$$\%\text{left} = 100 \times (0.5)^n \tag{2.12}$$

Where n = number of half lives

Conversely we can determine the number of log reductions equivalent to the number of half lives (n):

$$Ln\left(\frac{N_0}{N}\right) = 2.3\times\log\left(\frac{N_0}{N}\right) = kt = knt_{1/2} = \frac{0.693}{t_{1/2}}nt_{1/2} = 0.693\times n \tag{2.13}$$

Thus,

$$\frac{N_0}{N} = 10^{\left(\frac{0.693}{2.3}n\right)} \quad \text{or} \quad \log\left(\frac{N_0}{N}\right) = \frac{0.693}{2.3}n \tag{2.14}$$

For example, for 7 half lives:

$$\frac{N_0}{N} = 10^{\frac{0.693}{2.3}\times 7} = 10^{2.11} = 129 \tag{2.15}$$

So, the amount left is $N = \frac{N_0}{129} = 0.78\%$.

For 12 log reduction then:

$$Ln\left(\frac{N_0}{N}\right) = kt_{12} = 2.3\log\left(10^{12}\right) = 2.3\times 12 = 27.6$$

Therefore, the time to achieve 12 log reduction is $t_{12} = \frac{27.6}{k} = \frac{27.6}{0.693}t_{1/2} \approx 40t_{1/2}$.

$$\%\text{left} = 100\times\left(0.5\right)^{40} = 9\times 10^{-11}\%$$

Some examples of calculation results are listed in Table 2-3 and Table 2-4.

Table 2-3. Examples of calculation results of residual amount of toxin through number of half lives.

No. of half lives	Residual amount
7	0.78%
12	0.02%
13	0.01%

Table 2-4. Examples of calculation results of residual amount of toxin through number of log reduction.

No. of log reduction	Equivalent No. of half lives	Residual amount
5	16.6	0.001%
6	20	0.000095%
12	40	9×10^{-11}%

2.3.5 Activation energy

The Arrhenius equation (Equation 2.16) was developed by Svante Arrhenius (2) in the late 1800s. In chemical kinetics, Arrhenius equation is used to describe the influence of temperature on the rate constant which consequently shows the temperature dependence or sensitivity of the reaction.

$$k = k_0 e^{-E_a / RT} \tag{2.16}$$

Where k_0 = theoretical value of k at T = infinity intercept on plot and related to the probability of collision frequency

E_a = activation energy (calories/mole or joules/mole)

R = gas constant = 8.314 joules/mole K = 1.9869 calories/mole K

According to Equation 2.16, the influence of temperature on the rate constant k is expressed by the magnitude of the activation energy constant E_a. The activation energy E_a measures the threshold or energy barrier that must be overcome or surpassed to permit the transformation of reactants into products. The Arrhenius equation could be manipulated to an equation in the form of

y = m × x + b. Thus:

$$\ln k = -\frac{E_a}{R} \bullet \frac{1}{T} + \ln k_0 \qquad (2.17)$$

E_a could be determined by an Arrhenius plot of ln k versus 1/T which gives a straight line whose slope (b) is equal to $-E_a/R$, as illustrated in Figure 2-6. Therefore, E_a could be calculated as follows:

$$E_a = -(b \times R) \qquad (2.18)$$

In addition, activation energy can be set from a plot of Ln D values versus 1/T (k), as the slope has the same magnitude but the curve is in opposite direction.

Typical values of activation energy for reactions that occur in foods and drugs are listed in Table 2-5.

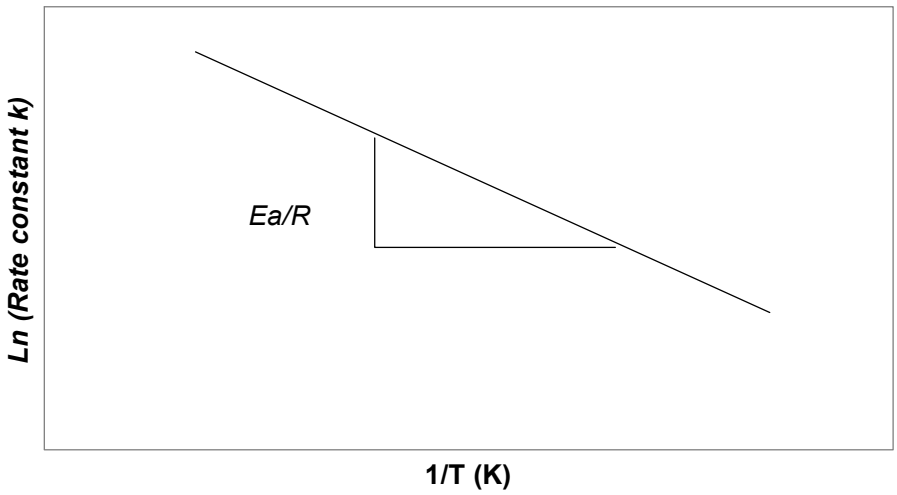


Figure 2-6. Hypothetical Arrhenius plot of the reaction rate constant *k* versus the inverse of absolute temperature 1/*T.*

Table 2-5. Typical values of activation energy for reactions in foods and drugs (39).

Reaction	Activation energy	
	kcal/mole	kJ/mole
Diffusion	0-12	0-50.4
Enzymatic	10-15	42-63
Hydrolytic	10-20	42-84
Lipid oxidation	10-25	42-105
Non enzymatic browning (NEB)	20-45	84-189
Spore death	60-80	252-336
Vegetative cell death	50-150	210-630
Protein denaturation	80-120	336-504

2.3.6 Z value

The Z value is the change in temperature that causes a 10-fold change in D value. It represents the thermal sensitivity of a toxin to a change in temperature. The smaller the Z value, the smaller change in temperature needed to get a 10 fold change in rate. It is a different measure of temperature sensitivity. The Z value can be determined by plotting the D values against corresponding temperatures on a semi-logarithmic scale and doing a linear regression to get the best fit to the data. The absolute value of the reciprocal of the slope of this line is the Z value. The Z value could be calculated as follows:

$$Z = T_1 - T_2 \tag{2.19}$$

Where D_1 = D value at temperature T_1

D_2 = D value at one log cycle difference found at temperature T_2

2.3.7 Q_{10}

The temperature sensitivity can also be expressed as the Q_{10}, or the increase in rate for a 10°C increase in temperature. It could be calculated by analyzing rate constants at a temperature interval of 10°C:

$$Q_{10} = \frac{k_{T+10}}{k} = \frac{D_{T_2}}{D_{T_1}} \tag{2.20}$$

Where k = rate constant at temperature T

k_{T+10} = rate constant at temperature T+10

D_{T_2} = D value at temperature T_2

D_{T_1} = D value at temperature T_1 which is 10°C different than T_2

Also, Q_{10} could be calculated by analyzing the rate increases for temperature intervals other than 10°C:

$$Q_A = \frac{k_2}{k_1} = Q_{10}^{\frac{\Delta T}{10}} \tag{2.21}$$

Where k_1 = rate constant at temperature T_1

k_2 = rate constant at temperature T_2

ΔT = difference between the two test temperatures (T_2-T_1)

2.4 Orange juice

2.4.1 Orange juice definition

Orange juice has the largest consumption in the United States among juices at 5.1 gal/year/person (70). There are a variety of orange juice products including fresh, pasteurized, frozen concentrated and canned orange juice. Different products have different definitions according to the Code of Federal Regulations (CFR) although processing steps are similar.

Orange juice has a standard of identity defined in Title 21, CFR Chapter 1 Section 146.135 (22), as “the unfermented juice obtained from mature oranges of the species *Citrus sinensis* or of the citrus hybrid commonly called “Ambersweet” (1/2 *Citrus sinensis* x 3/8 *Citrus reticulata* x 1/8 *Citrus paradisi* (USDA Selection:1-100-29: 1972 Whitmore Foundation Farm)). Seeds (except embryonic seeds and small fragments of seeds that cannot be separated by current good manufacturing practice) and excess pulp are removed. The juice

may be chilled, but it is not frozen."

Pasteurized orange juice is defined in CFR, Title 21, Chapter 1 Section 146.140 as "orange juice (defined above) that is treated by heat as to reduce substantially the enzymatic activity and the number of viable microorganisms. Either before or after such heat treatment, all or a part of the product may be frozen. The finished pasteurized orange juice contains not less than 10.5% by weight of orange juice soluble solids, exclusive of the solids of any added optional sweetening ingredients, and the ratio of the Brix hydrometer reading to the grams of anhydrous citric acid per 100 mL of juice is not less than 10 to 1."

Frozen concentrated orange juice is regulated in 21 CFR 140.146 and defined as "the food prepared by removing water from the general orange juice defined in 21 CFR 146.135, to which may be added unfermented juice obtained from mature oranges of the species *Citrus reticulate*, other *Citrus reticulate* hybrids, or of *Citrus aurantium*, or both. However, the volume of juice from *Citrus reticulate* or *Citrus reticulate* hybrids shall not exceed 10% (with the exception of the Ambersweet) and from *Citrus aurantium* shall not exceed 5% in the unconcentrated blend. The finished product of frozen concentrated orange juice is of such concentration that when diluted according to label directions the diluted article will contain more than 11.8% by weight of orange juice soluble solids, exclusive of the solids of any added optional sweetening ingredients and the dilution ratio shall be not less than 3 plus 1."

Canned orange juice is defined in 21 CFR 146.141 as the food prepared complying with 21 CFR 146.135, which is "sealed in containers and so processed by heat, either before or after sealing, as to prevent spoilage. The finished canned orange juice tests not less than 10° Brix, and the ratio of the Brix hydrometer reading to the grams of anhydrous citric acid per 100 mL of juice is not less than 9:1". Orange juice, no matter fresh, pasteurized, frozen concentrated or canned orange juice, has similar nutritional value and

composition.

2.4.2 Orange juice processing

Orange juice processing begins with harvesting mature oranges from the orchards and shipping by truck to juice plants. Following arrival at the orange juice plant, the fruit initially goes through inspection and grading. Samples are tested for titratable acidity, °Brix and juice yield. Fruits are then conveyed to washers where sanitizers are applied and then they enter a juicer (squeezer) where juice is produced and the pulp and seed are separated from the liquid. Once general orange juice is produced, the products are processed differently according to the definitions utilizing different steps or technologies such as pasteurization, ultrafiltration, deoiling, deaeration (64). Orange juice and orange juice products are regulated in the juice labeling rule (21 CFR 101.17) and juice Hazard Analysis and Critical Control Point (HACCP) (23), as they have to be processed to attain a 5-log reduction in the pertinent pathogen as stated in the performance standard.

2.4.3 Vulnerability of the orange juice processing system

Similar to other food systems, the orange juice processing system is vulnerable at a certain degree to special weapons attack such as the introduction of biological agents such as ricin and Shiga toxins. A number of points in the processing operation are potential targets for a bioterrorist where large volume of orange juice could be contaminated. This is critical as the aim of bioterrorists is to have mass destruction rather than 10-12 people getting ill or dying. Since the supply chain begins with a vast number of widely distributed small farms and numerous transportation, processing and distribution facilities to the

consumers' table, orange juice could be contaminated intentionally in every and each of the steps. The three most vulnerable points in the orange juice system include the pesticides applied before harvest in the orchard, which could be washed into the juice later in the plant, storage in the silos (typically 7,000 gal) in the plant and the tanker truck after pasteurization where agents could be added deliberately (64). Although it is less possible to evenly contaminate a large amount of orange juice during storage or in trucks, it can still cause intended harm effectively. Scenarios for deliberate contamination events are still likely although possible vulnerability in processing orange juice is recognized.

2.5 Pasteurization processing

The goal of pasteurization in orange juice processing is to inactivate both spoilage organisms and pathogenic organisms as well as the enzyme pectin methylesterase (PME) which is responsible for loss of cloud stability and discoloration in juice. Pasteurized orange juice has an extended shelf life and ensured safety. Commercially, orange juice is rapidly heated to ~92°C and the exact heating temperature depends on the type of equipment that is used in the plant and also on the rate of juice flow. Pasteurizers such as tube in tube and plate and frame heat exchangers are used in pasteurization using steam or hot water as the heating agent as well as using the hot juice to warm up the cold juice in a regeneration step (32, 44).

Tube in tube heat exchangers are used for a variety of products from low to high viscosity, of course including fruit juices. It consists of one or multiple tubes fixed inside a larger tube with either current flow (same direction inside and outside) or countercurrent flow (opposite directions). The fluid in the larger outer tube functions as the heating or cooling medium while the smaller tubes carry the juice (18). Tube in tube heat exchangers have many advantages including

suitability over a wide product range, high pressure resistance, simple design and low maintenance costs (although high cleaning costs). However, tubular heat exchangers tend to form thermal cracks especially at joints due to the changes in temperature.

A plate and frame heat exchanger is generally used to pasteurize less viscous liquids, such as fruit juices on a large scale. The plate heat exchanger is composed of several thin vertical stainless steel corrugated plates to form parallel channels, which are separated by rubber gaskets to produce a watertight seal. Plate heat exchangers have a number of advantages over other heat exchangers, such as saved space per unit area of contact, increased flexibility and the better heating profile (57).

2.6 ELISA

The enzyme linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA), is a biochemical technique based on the specific affinity between antibody and antigen, which could be used to determine the presence or amount of antigen or antibody in a sample. An enzyme covalently linked to one of the antibodies is the key component of ELISA. The antigen-antibody enzyme conjugate complex catalyzes a specific reaction with a chromogenic substance used to develop color after addition of substrate for quantification of antigen or antibody at the appropriate wavelength (43).

There are many types of ELISA but four main methods form the basis to all ELISAs including direct ELISA, indirect ELISA, sandwich ELISA and competitive ELISA (33, 43). The simplest form of ELISA is the direct ELISA, illustrated in Figure 2-7. In direct ELISA, the antigen protein, i.e. the toxin in this study or protein on the surface of a pathogenic microbe, after extraction from the food or directly is first attached to a solid surface by hydrophobic bonding.

After washing, an enzyme-linked antibody is attached to the antigen. After incubation and washing again, the substrate is added and reacts with the enzyme to develop color. The direct ELISA method has the disadvantage that the antibodies against all different antigens have to be labeled with an enzyme, so it is not suitable to detect crude samples which contain a high concentration of contaminating substances. It is mainly used to estimate the titer of the enzyme-linked antibody conjugates (13, 14).

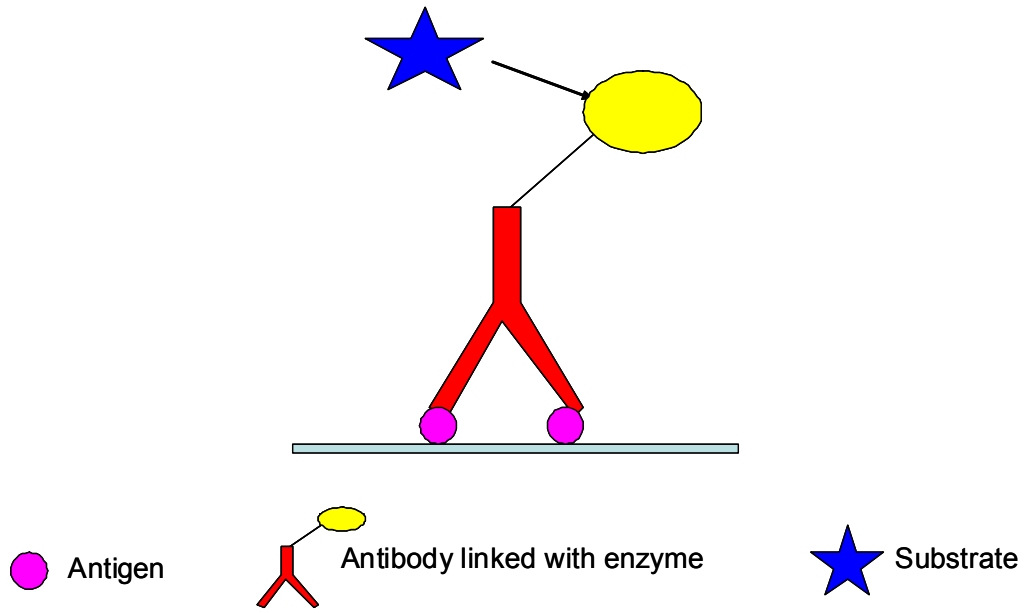


Figure 2-7. Cartoon depicting the principle of a direct ELISA method.

The indirect ELISA method is mainly used to detect the presence of a type of antibody. Steps in indirect ELISA are similar to the direct ELISA system but it involves the addition of an unlabeled detecting antibody which binds to an enzyme-linked antibody conjugate, which is added later. Substrate is then added to the conjugate and develops color which is ready to be read, as shown in Figure 2-8. It has a strong advantage over the direct ELISA method since it only needs one single enzyme-linked antibody which allows great flexibility in

use of antibody conjugates. Indirect ELISA has been widely used in diagnosis when screening large numbers of samples. However, due to the varying degree of nonspecific binding of individual antibodies, indirect ELISA tends to widen the dispersion in results (13).

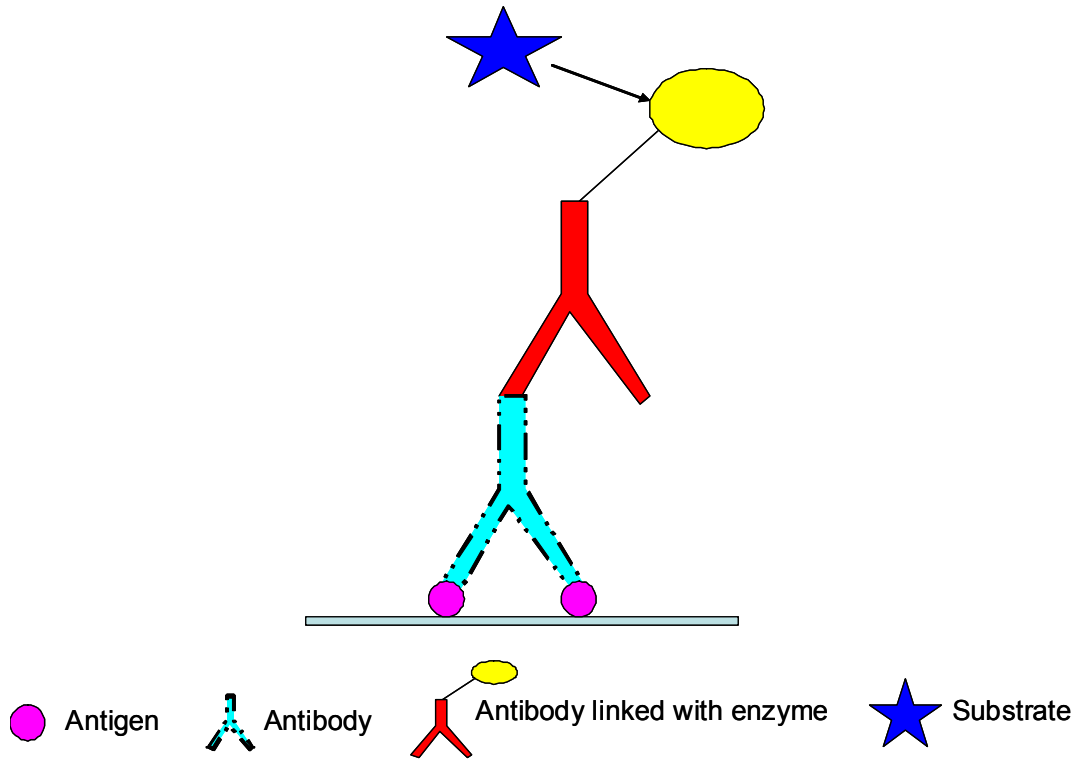


Figure 2-8. Illustration of the principle of an indirect ELISA method.

The sandwich ELISA is commonly used to detect antigens in samples. This type of ELISA is called “sandwich” ELISA because it forms an antibody-antigen-antibody format. There are two types of sandwich ELISA, direct and indirect sandwich ELISA. In direct sandwich ELISA, an antibody is bound to a solid phase and the antigen in sample is added to bind to the antibody (33). Then enzyme-linked antibody and substrate are added in order to develop color so that the amount of antigens could be quantified, as shown in Figure 2-9. The two antibodies used in this ELISA format can be from the same or different

species. In indirect sandwich ELISA, there are capture antibodies, detection antibodies and enzyme-linked antibodies. The detection antibody is typically raised from a different animal species than the capture antibody otherwise the enzyme-linked antibody is not able to bind to the detection antibody specifically, as shown in Figure 2-10. Sandwich ELISA is sensitive and specific in detection of antigens and is widely used and commercially available.

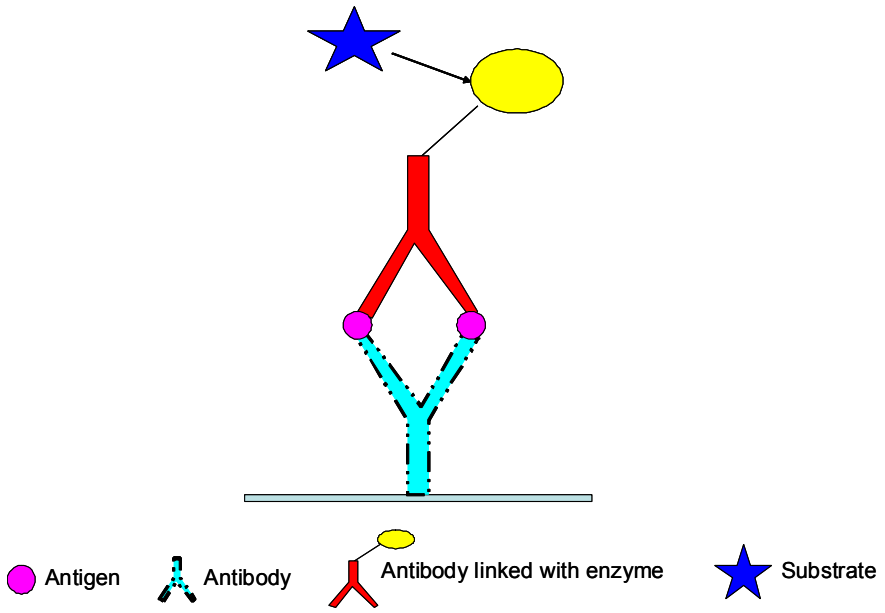


Figure 2-9. Cartoon that illustrates the principle of a direct sandwich ELISA method.

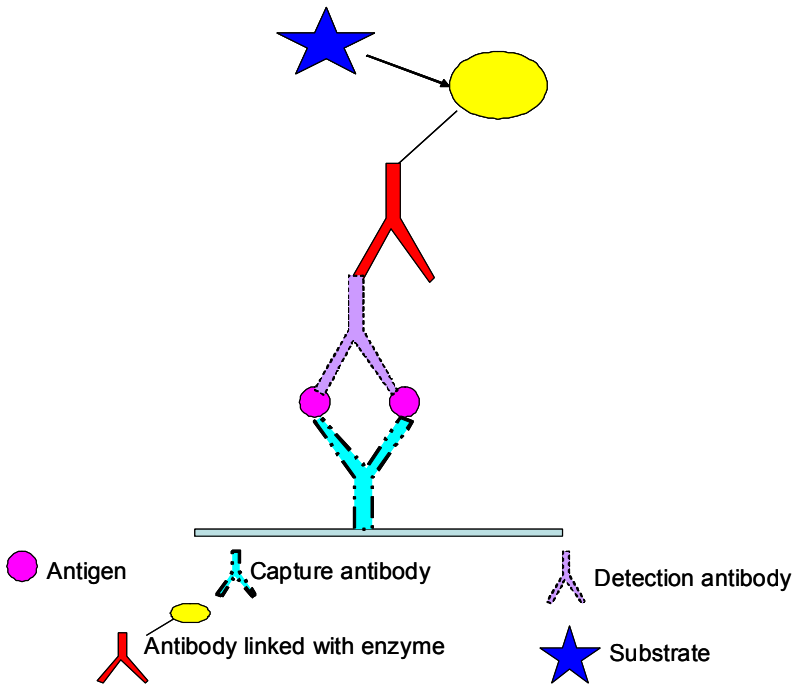


Figure 2-10. Illustration that depicts the principle of an indirect sandwich ELISA method.

Competitive ELISA can be used to measure antigen or antibody, but is most commonly used in detecting small molecules (less than 5000 g/mol) linked to a carrier (such as albumin proteins). In a competitive ELISA sequence, labeled antigen competes for the antibody binding sites with the antigens in the sample which is unlabeled with enzyme (Figure 2-11). The greater the absorption in the well, the more labeled antigen is retained and the less the presence of antigen in the sample. The major advantage of competitive ELISA is its ability to detect crude or impure samples specifically and selectively (14).

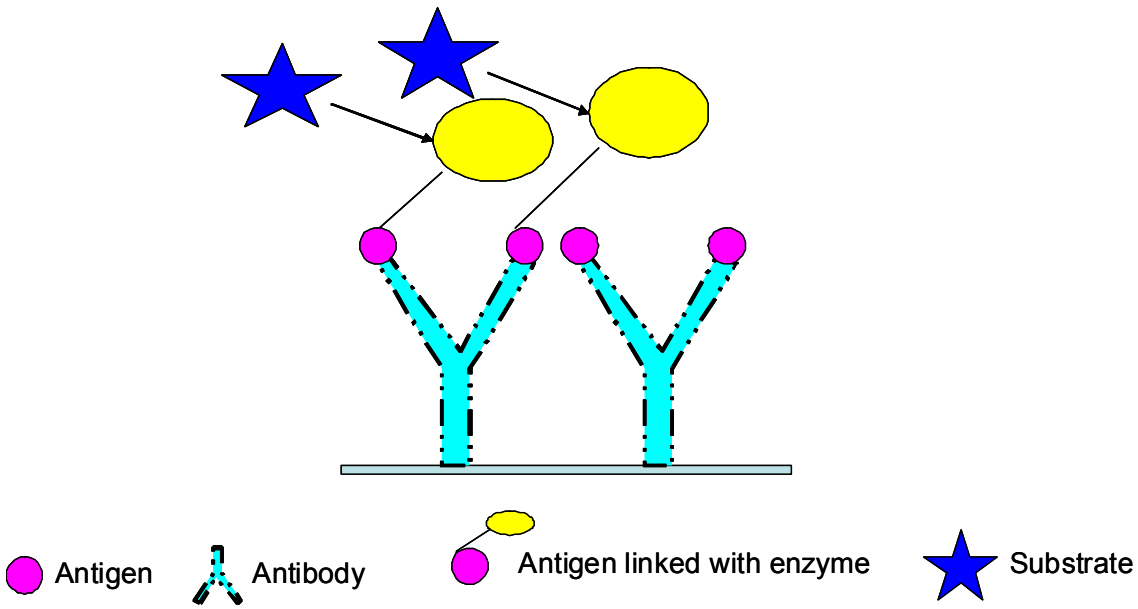


Figure 2-11. Cartoon that depicts the principle of a competitive ELISA method.

Since each ELISA method is able to distinctively determine the presence of antigen or antibody depending on the type of ELISA, it is a very useful tool in industry, medical community or in the academic area. The ELISA methods are widely used in hospitals for detection of serum antibody concentrations, such as in the HIV (Human Immunodeficiency Virus) test (68). In the food industry, the ELISA methods are usually used to detect potential food allergens such as peanut protein, egg protein etc. (52). Furthermore, ELISA has many applications in toxicology to screen for the presence of toxins or drugs (10). Since it is easy, cheap and rapid with good sensitivity and specificity, ELISA methods are widely used and commercial available for many different targets. It should also be noted that the surface capture mechanism can be made into a magnetic bead (42). These beads can be mixed with the food made into a dilute slurry thus increasing the potential capture of the antigens on the surface (indirect ELISA method) or the antibody can be bounded first on the surface (all other ELISA methods) and then a powerful magnet is used to separate out the

beads from the food, which are then washed and the other ELISA methods used.

2.7 EPA regulations

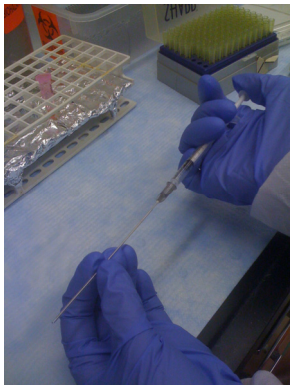
In order to meet the goal of this study, ricin and Shiga toxin have to be inactivated to a specific level so that it could meet the disposal standards of the Environmental Protection Agency (EPA). However, no such limit is present in the EPA standard although the EPA should have published rules to address the potential public health effects from the possible presence of ricin or Shiga toxin in water. In addition, in an event of deliberate delivery of toxins in a food supply, we have no idea of what level of toxins are added, which would be based on how much of a given toxin is available for delivery by the bioterrorists. Therefore, it is hard to know what the target level for reduction of the toxin is in the intentionally contaminated food so as to safely dispose of the toxins. However, this could be extrapolated from other toxic compounds that have EPA regulations. For instance, EPA has issued a Maximum Contaminant Level (MCL) or Maximum Tolerated Dose (MTD) of 0.01 mg/L for arsenic (21). The NOAEL (No Observable Adverse Effect Level) for arsenic is 0.009 mg/L (20), which is almost the same as the MCL. Once we have the NOAEL for ricin or Shiga toxin, it could be used as the MCL. Due to the lack of a NOAEL, which must be obtained through 90 day acute feeding studies with at least 2 animal species (such as mice and rabbits), the LD_{50} was divided by a factor of 1000 to get an estimation of NOAEL. Therefore, an estimated 1000 fold inactivation could reach a presumable safe level when there is a deliberate delivery of ricin or Shiga toxin at the concentration of 1 human LD_{50}. Further data is needed to get a more exact value and we need to adjust the value for infant, children or adults.

3 Inactivation of ricin

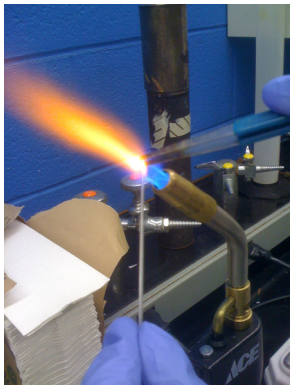
3.1 Materials and methods

3.1.1 Thermal inactivation of ricin

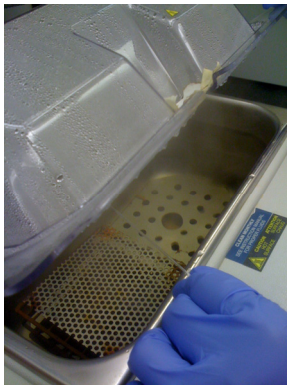
R. communis agglutinin II, a purified non-toxic form of ricin, was obtained from Vector Laboratories, Burlingame, CA with a concentration of 5 mg/mL in phosphate buffered saline (PBS). This research was exempted from select agent category since our laboratory always had less than 100 mg in stock. The capillary tube method of heating with the agent in a water bath was used to minimize the come-up time when determining the heat resistance of ricin and its steps are shown in Figure 3-1. Ricin was diluted in PBS to a concentration of 50 µg/mL and used for the inactivation studies. Volumes of 90 µL of orange juice were inoculated with 10 µL of ricin solution. Aliquots of 50 µL of ricin-containing orange juice samples were transferred into capillary tubes (0.8-1.1 × 90 mm; No. 34507, Kimble, Vineland, NJ). Tubes were heat-sealed and then completely submerged into a water bath at different temperatures from 72 to 90°C. At each sampling time, capillary tubes were removed from the water bath and cooled in an ice-water bath. After cooling, the capillary tubes were rinsed with distilled water and crushed by mixing in test tubes (15 mL) containing 5 mL PBS and a magnetic bar using a vortexer. Mixtures were then collected and transferred into a microcentrifuge tube (1.5 mL, Fisherbrand) and stored at 4°C until the residual toxin concentration was quantified by an ELISA kit on the next day. Treatments and measurements of each sample were performed at least in duplicate.



A. Injecting mixture into capillary tubes

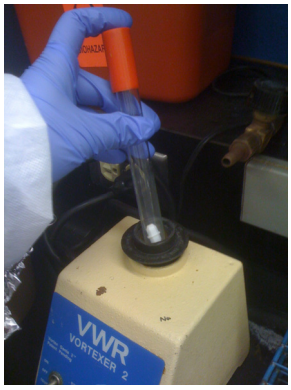


B. Flame sealing capillary tubes

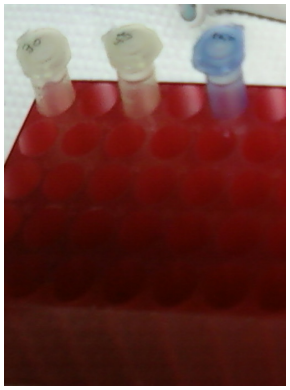


C. Heating capillary tubes

D. Rinsing capillary tubes



E. Crushing capillary tubes



F. Storage in microcentrifuge tubes prior to analysis

Figure 3-1. Illustration of the protocol steps for toxin inactivation studies.

3.1.2 Chemical inactivation of ricin

To be consistent with the thermal inactivation study, the capillary tube method was also used for chemical inactivation of ricin in orange juice. In order to decontaminate orange juice containing ricin, chemicals that are used for sanitation in food processing plants were evaluated for the inactivation of ricin. These chemicals included 13% sodium hypochlorite (Ecolab Inc., St. Paul, MN),

1 N sodium hydroxide (Sigma-Aldrich Inc, St. Louis, MO), and 0.32% peracetic acid (Sigma-Aldrich Inc, St. Louis, MO).

Ricin was diluted in phosphate buffered saline (PBS) to a concentration of 50 µg/mL and used for inactivation. Ricin and sanitizers were added into orange juice in that order at the desired concentration. Volumes of 50 µL of ricin-containing orange juice samples were then transferred into capillary tubes. The tubes were flame-sealed and then left at room temperature. At each sampling time, capillary tubes were transferred into an ice-water bath for cooling and then rinsed with distilled water and crushed in test tubes containing 5 mL PBS. The mixture was then collected and transferred into a microcentrifuge tube and stored at 4°C until the residual toxin concentration was quantified. Treatments and measurements of each sample were performed at least in duplicate. The sanitizers were used separately or in combination in different concentrations.

3.1.3 Combined inactivation of ricin

Thermal and chemical inactivation methods were combined to determine any synergistic effect. Combined inactivation was similar with the chemical inactivation method, except for the submerging of the ricin-containing orange juice samples in the capillary tubes into a water bath at desired temperature instead of holding at room temperature. Duplicates were performed. Sanitizers were used separately or in combination at different concentrations and temperatures.

3.1.4 ELISA test for ricin

The residual ricin concentration was quantified using a pre-coated indirect

capture ELISA kit (Tetracore Inc., Rockville, MD). Multi-point standard curves as well as positive and negative controls were established for each ELISA plate to reduce the variations among ELISA testing. The cut-off concentration calculated using the instruction of kit was 5 ng/mL.

The Tetracore ELISA kit utilizes the indirect capture ELISA format, i.e. wells coated with positive capture antibody (goat anti-ricin) and with negative capture antibody (normal goat). When testing, each well in the plate (96 total) was first blocked with 150 µL of ELISA dilution/blocking buffer made from dry skim milk and incubated at 37°C for 1 h. PBST (phosphate buffered saline with Tween-20) made prior to the ELISA test was used to wash the wells for four times. Aliquots of 100 µL positive control, negative control as well as testing samples were added into corresponding wells and incubated at 37°C for 1 h. After washing with PBST according to the manufacturer's instructions, volumes of 100 µL of detector antibody (monoclonal antibody, 10 µg/mL) were then added into each well and the plate was incubated again at 37°C for 1 h. Wells were washed again with PBST to remove unbound detector antibodies. Volumes of 100 µL of conjugate antibodies (goat anti-mouse IgG (H+L)-HRP) with a concentration of 0.08 µg/mL were added to each well and the plate was incubated again at 37°C for 1 h. After washing to remove unbound conjugate antibodies, 100 µL aliquots of substrate (ABTS 2-part peroxidase) were added to each individual well and the plate was incubated again at 37°C for 30 min. Absorbance was measured at 405 nm using a Bio-Tek Kinetics microplate Reader (Bio-Tek Instruments Inc., Winooski, VT) and software (KC 4, Bio-Tek). According to the manufacture, the kit is able to detect the holotoxin, A chain and B chain but no other information on epitope is available.

3.1.5 Data analysis

The residual ricin percentage was calculated from the concentration of ricin

detected in the treated orange juice versus the amount of ricin at time zero as determined by ELISA. The kinetics of heat treatments were determined by first-order regression plots of concentration over time. The inactivation rate constant (k), half-life ($t_{1/2}$) (time for a toxin to be inactivated by half) were calculated using a spreadsheet developed by Dr. Ted Labuza (University of Minnesota) and the D value (time required to reduce the toxicity of a toxin by a factor of 10) were estimated by linear regression. Activation energy (E_a) (the threshold or energy barrier that must be overcome or surpassed to permit the transformation of reactants into products), Q_{10} (increase in rate for a 10°C increase in temperature) and Z value (change in temperature that accompanies a 10-fold change in D value) were also calculated using these models. In addition, the times for a 12 fold half-life destruction ($12 \times t_{1/2}$) were calculated assuming that the final concentration has to be reduced to ~ 0.02% of the initial amount to meet EPA standards for disposal.

All experiments were replicated at least twice with duplicate samples at each time interval for each treatment. The lack of convergence of the upper and lower 95% confidence limits were calculated in each plot of residual ricin (%) versus treatment time and semi-log plots of D value versus temperature (41). Note that all original data are available in an electronic appendix.

3.2 Results

3.2.1 Establishment of ELISA standard curve

The product inserts of each ELISA kit included quality control standard curves provided by the manufacturer with a linear range from 1.2 to 20 ng/mL of ricin (Figure 3-2). However, the experimental standard curve rarely matched the kits' standard curves and each standard curve was different with each determination. Figure 3-3 shows four different repetitions for standard curves with different ELISA plates for ricin. The results indicated that there were marked variations in the ELISA kits and individual standard curves had to be

performed for each ELISA plate.

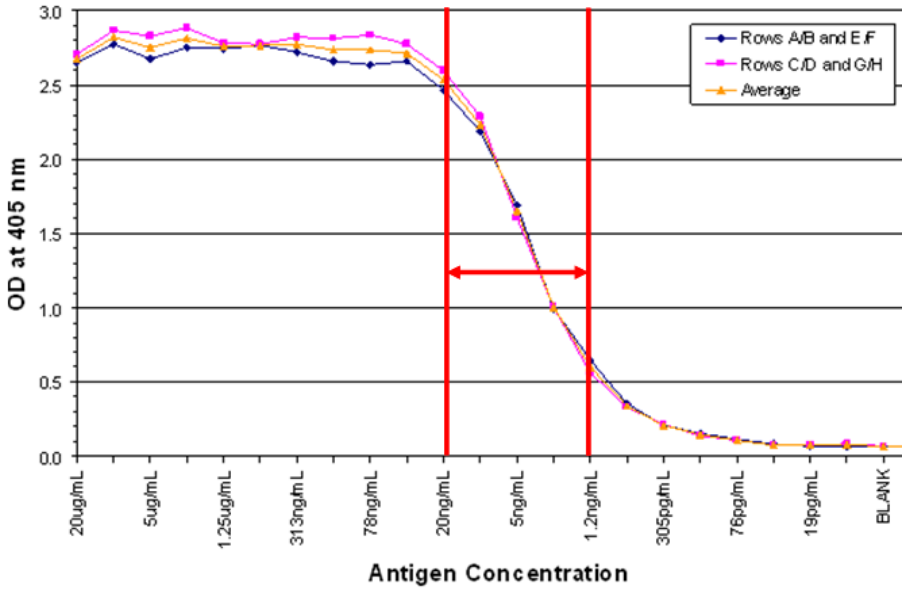


Figure 3-2. Example of a quality control standard curve included in an ELISA kit insert for ricin by the manufacturer.

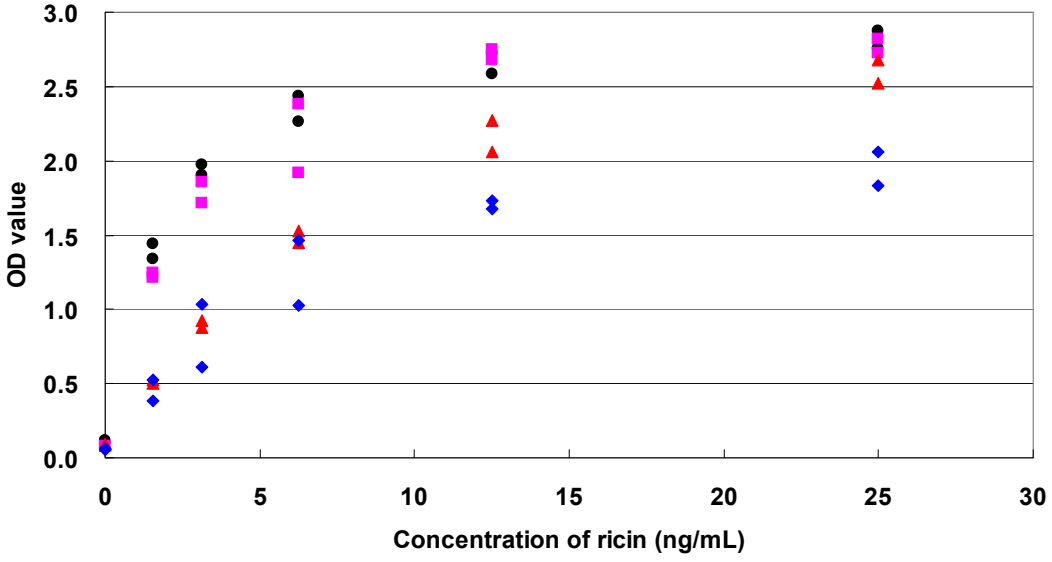


Figure 3-3. Standard curves plots from four different ELISA plates for ricin detection. Note that each symbol represents the results for the standardization of different 96 well plates.

In order to reduce the variability from different ELISA kits, a number of preliminary experiments were performed for establishing standard curves. In addition, the effect of media (buffer or orange juice) and dilution ratios on standard curve variability were also evaluated. Figure 3-4 shows a typical ELISA standard curve of ricin inactivation in orange juice (See all of the standard curve plots in the Appendix). The plot had a very good R^2 of 0.99 indicating good correlation between the concentration of ricin and the OD (optical density) value. Table 3-1 shows the results of four different standards of ricin at 10 ng/mL and 5 ng/mL. The standards included ricin in buffer only and ricin with juice in buffer at dilution ratios of 1:500, 1:100 and 1:50 without any chemical agents. There were six repeats in each concentration and the statistics of the results as well as the ratios of OD values at 5 ng/mL to those at 10 ng/mL are listed in Table 3-1. The ratios ranged from 0.48 to 0.64 and decreased when samples were less diluted.

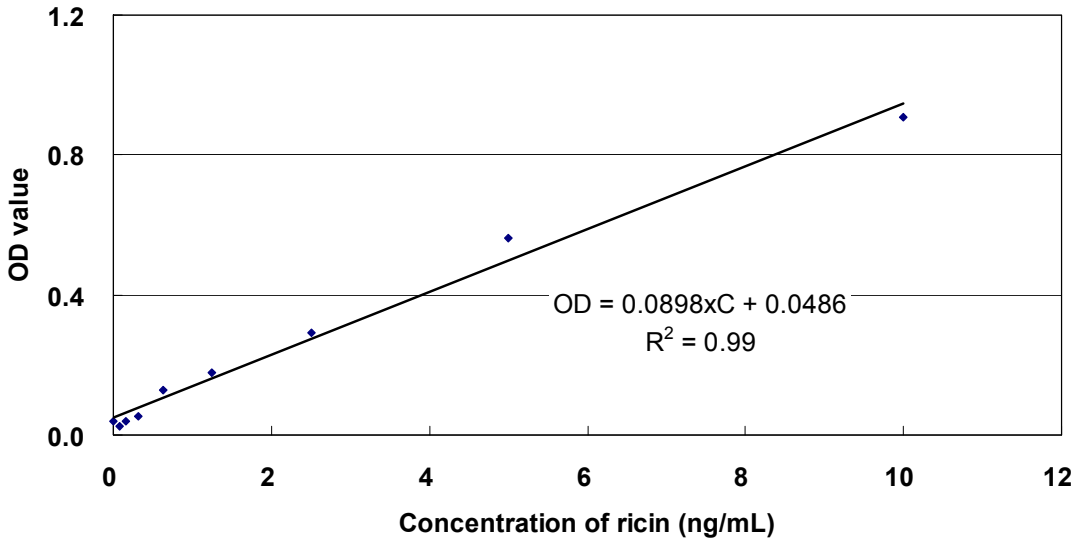


Figure 3-4. A representative ELISA standard curve of ricin in orange juice.

Table 3-1. The effect of dilution ratio of juice and buffer on the variability of standards used for detection of ricin in orange juice by ELISA.

With orange juice	Dilution ratio	OD*** value						OD ratio of 5:10 ng/mL
		10 ng/mL of ricin			5 ng/mL of ricin			
		Mean	STDV*	95% CL**	Mean	STDV	95% CL	
No	1:500	0.987	0.036	0.020	0.629	0.018	0.010	0.637
Yes	1:500	0.864	0.069	0.039	0.506	0.033	0.019	0.586
Yes	1:100	0.725	0.052	0.029	0.424	0.039	0.022	0.585
Yes	1:50	0.889	0.070	0.039	0.426	0.049	0.028	0.479

*STDV: Standard Deviation

**CL: Confidence Limit

***OD: Optical Density

3.2.2 Thermal inactivation results of ricin

The time/temperature combinations that were capable of delivering an effective inactivation for ricin in orange juice were identified. The effect of processing temperature and time on ricin in orange juice was determined at 72, 80, 85 and 90°C (Figure 3-5). Thermal inactivation of ricin in orange juice followed first-order kinetics as is expected for denaturation of proteins. At each temperature, R^2 values obtained from linear regression using apparent 1^{st} order kinetics were greater than 0.87 indicating a relatively good correlation. As shown the rate of inactivation (k) increased as expected with an increase in inactivation temperature. Ricin in orange juice would reach the detection limit of the ELISA kit within 75 s at 90°C.

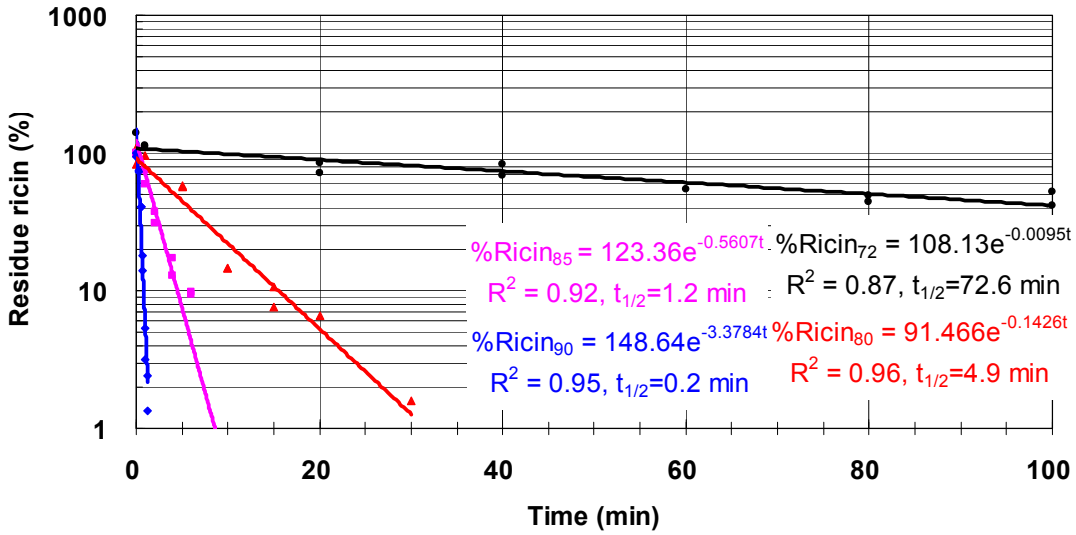


Figure 3-5. The effect of temperature on inactivation of ricin in orange juice at 72°C (●), 80°C (▲), 85°C (■), and 90°C (◆).

Despite such a high degree of correlation for each individual inactivation experiment (R^2 values >0.84), the kinetic parameters of the inactivation of ricin were variable among replicates (Table 3-2 and Table 3-3). The largest variation was observed at 90°C when one of the replicates was more than 5-fold smaller than the largest value due to the variances between the ELISA plates. The half life values decreased from 72 min at 72°C to slightly less than 1 min at 90°C. When 12 × $t_{1/2}$ values were estimated, only at 85°C or larger temperatures a complete inactivation could be accomplished in less than 30 seconds. In the determination of D values, smaller variations were observed among replicates and this parameter declined from approx. 350 min at 72°C to slightly more than 3 min at 90°C. From those primary kinetic values, an average Z value of 8.8°C and an E_a of 64.7 Kcal/mol were calculated. Figure 3-6 shows the Arrhenius plots for the determination of E_a of ricin in orange juice in triplicate.

Table 3-2. The effect of temperature on inactivation rates (k) and half life ($t_{1/2}$) of ricin in orange juice measured in triplicate.

Temp. (°C)	Rep	R^2	k (95% CL)* (min^{-1})	$t_{1/2}$ (95% CL) (min)	12 × $t_{1/2}$ (95% CL) (min)
72	1	0.87	0.0095 (0.0071, 0.0119)	72.6 (58.1, 97.0) [a]**	871.4 (696.6, 1163.4)
80	1	0.94	0.06 (0.05, 0.08)	11.0 (9.2, 13.7) [b]	132.5 (110.8, 164.8)
	2	0.96	0.14 (0.12, 0.16)	4.9 (4.3, 5.7) [c]	58.3 (51.2, 67.8)
	3	0.95	0.06 (0.05, 0.07)	11.1 (9.6, 13.4) [b]	133.7 (114.6, 160.4)
85	1	0.95	0.32 (0.25, 0.38)	2.2 (1.8, 2.7) [d]	26.4 (22.1, 32.8)
	2	0.92	0.56 (0.43, 0.68)	1.2 (1.0, 1.6) [e]	14.9 (12.1, 19.1)
	3	0.96	0.26 (0.21, 0.31)	2.7 (2.2, 3.4) [d]	32.2 (26.8, 40.2)
90	1	0.87	2.67 (1.85, 3.48)	0.3 (0.2, 0.4) [f]	3.1 (2.4, 4.6)
	2	0.95	3.30 (2.73, 3.87)	0.2 (0.2, 0.3) [f]	2.5 (2.2, 3.0)
	3	0.84	0.65 (0.39, 0.91)	1.1 (0.8, 1.8) [g]	12.8 (9.2, 21.4)

*Lower and upper value of half life

**Different letters indicate statistical difference at the $p<0.05$ level

Table 3-3. D, Z and activation energy (E_a) values of thermal inactivation of ricin in orange juice in as measured in triplicate.

Temp. (°C)	D value (min)				
	Rep 1	Rep 2	Rep 3	Mean	STDV*
72	346.1	NT**	NT	346.1 [a***]	0.0
80	56.0	48.4	60.9	55.1 [b]	6.3
85	13.7	15.7	14.2	14.6 [c]	1.1
90	2.2	2.2	4.8	3.1 [d]	1.5
Z value (°C)	8.3	8.5	9.6	8.8	0.7
E_a (Kcal/mole)	69.0	67.6	59.9	64.7	4.9

*STDV: Standard Deviation

**Not tested

***Different letters indicate significantly difference at the $p<0.05$ level

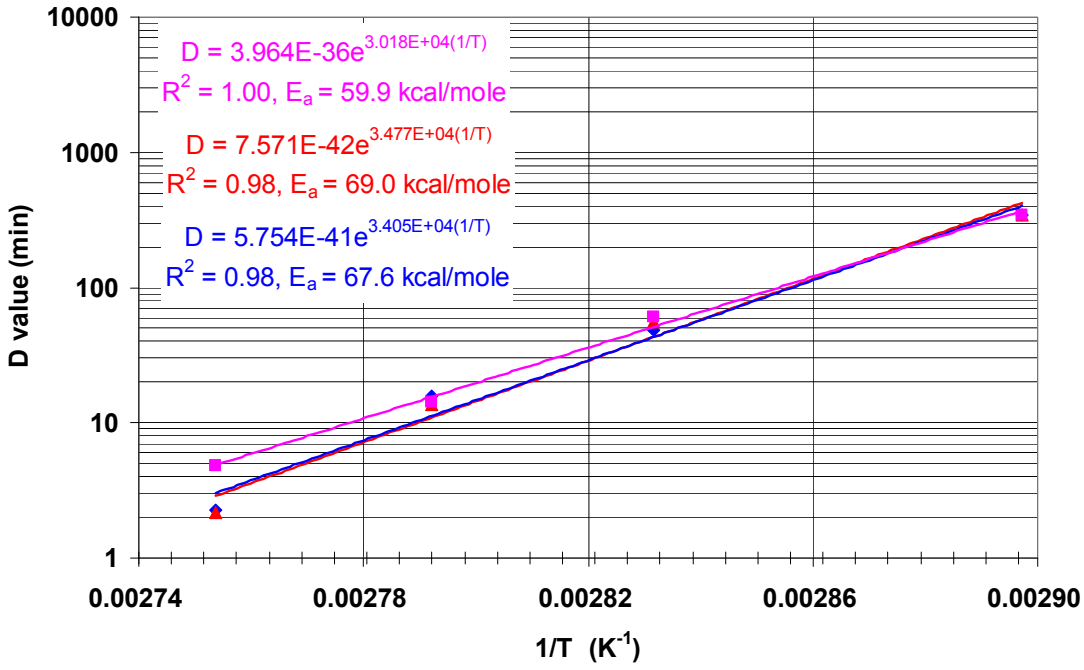


Figure 3-6. Arrhenius plots of inactivation of ricin in orange juice used to determine activation energy (E_a).

3.2.3 Chemical inactivation results for ricin

The effects of sanitizing chemicals on inactivation of ricin in orange juice were evaluated at room temperature (approximately 23°C). The ricin concentration was significantly reduced immediately after addition of 0.18 to 0.20 N NaOH (Figure 3-4), but at 0.16 N or less little to no effect was observed. When NaOCl was used, a concentration dependent inactivation of ricin was detected (Figure 3-8). Almost 70% of the ricin concentration was inactivated after addition of 1% of NaOCl. The inactivation effect with peracetic acid (PA) also followed the similar trend as the other two chemicals (Figure 3-9). The residual ricin immediately after addition of increased concentration of PA was markedly decreased. Reductions to <10% residual ricin occurred at approx. 0.02% PA making it the most effective sanitizer capable of inactivating ricin by simple addition. All three chemicals shared the same concentration dependent trend, however, no further inactivation effect was observed after a certain period of time. When the concentration of each chemical was increased to a sufficient amount, the detection limit of the ELISA kit was attained when measuring ricin inactivated within 5 s at room temperature.

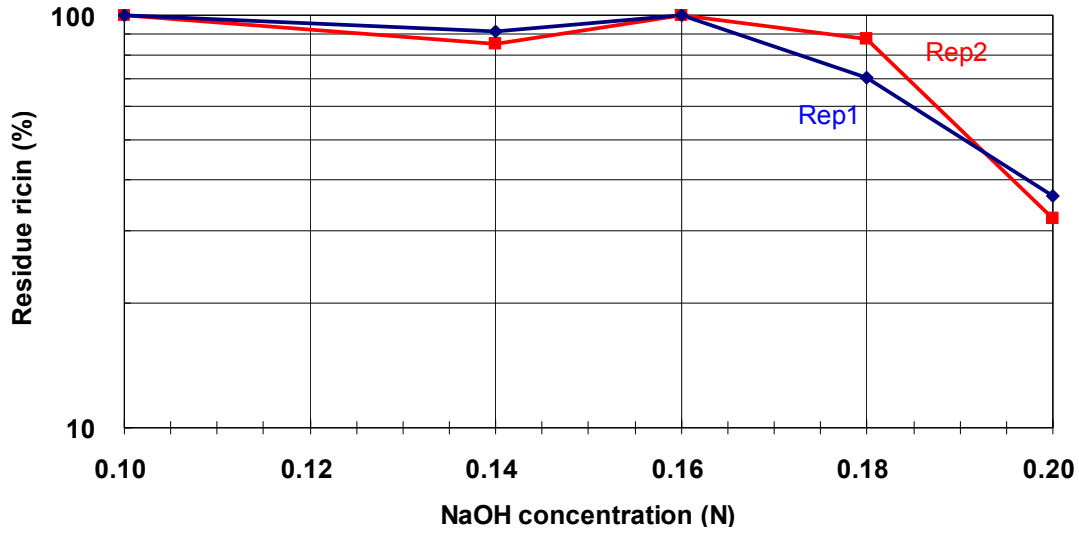


Figure 3-7. Chemical inactivation effects of ricin in orange juice by different concentrations of NaOH at room temperature without any incubation time.

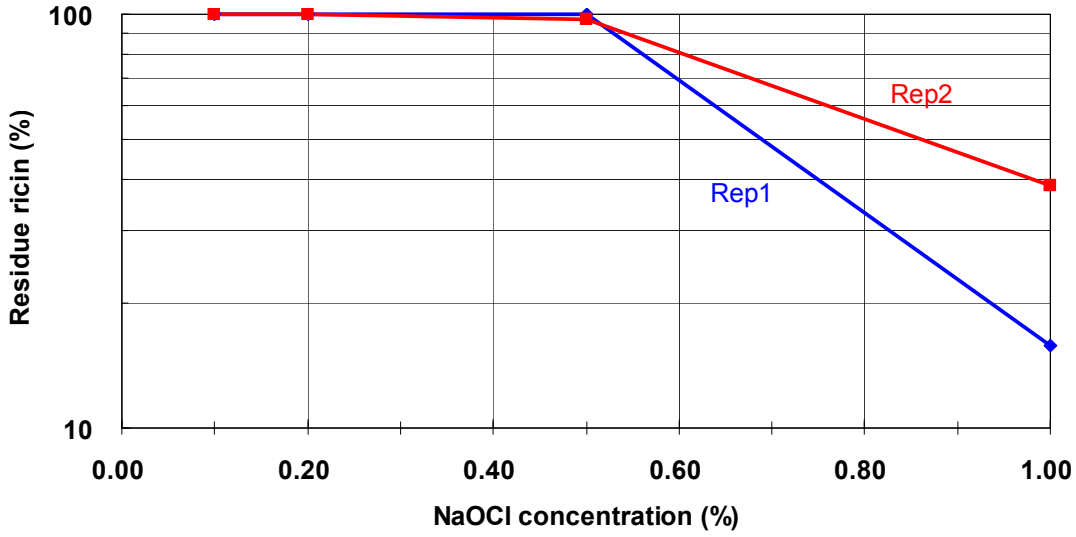


Figure 3-8. Chemical inactivation effects of ricin in orange juice by different concentrations of NaOCl at room temperature without any incubation time.

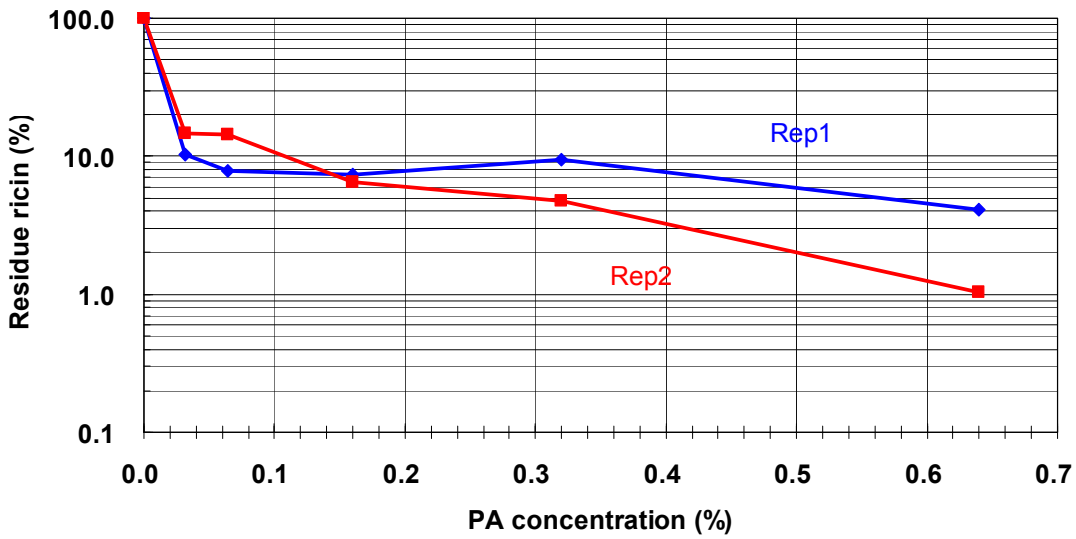


Figure 3-9. Chemical inactivation effects of ricin in orange juice by different concentrations of PA at room temperature without any incubation time.

Combinations of NaOH and NaOCl were tested for inactivation of ricin in orange juice (Table 3-4). The degree of inactivation was not significantly different if held for 0 or 2 min until the highest levels of the NaOH-NaOCl mixture were used, i.e. 0.3% NaOCl/0.15 N NaOH and 0.2% NaOCl/0.17 N NaOH. Compared to inactivation by 0.2% NaOCl or 0.17 N NaOH alone, depicted in Figure 3-7 and Figure 3-8, a significant synergism between NaOH and NaOCl was observed.

Table 3-4. Inactivation of ricin by combinations of NaOH and NaOCl.

NaOCl (%)	NaOH (N)	0 min		2 min	
		Residual Ricin (%)	STDV*	Residual Ricin (%)	STDV
0.10	0.10	87	17	88	17 [a**]
0.20	0.10	88	16	81	27 [a]
0.10	0.15	84	21	80	28 [a]
0.20	0.15	74	37	75	35 [a]
0.30	0.15	65	17	67	18 [b]
0.20	0.17	54	11	48	35 [b]

*STDV: Standard Deviation

**Rows with same letters are not significantly different

3.2.4 Combined thermal and chemical inactivation of ricin

Combination of heat and chemical treatments were evaluated for inactivation of ricin in orange juice. The treatments included NaOH, NaOCl and/or PA at 72, 80, 85 and 90°C and the results are shown in Figure 3-10 and Figure 3-11.

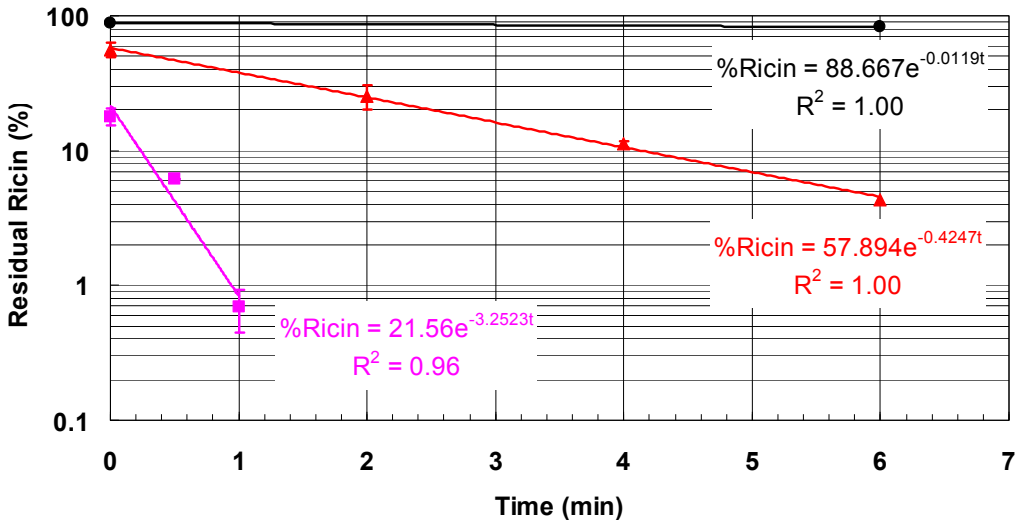


Figure 3-10. Inactivation of ricin in orange juice at 72°C by addition of NaOH and NaOCl. Mixtures included ricin-containing orange juice without chemical addition (●), with 0.2% NaOCl/0.15 N NaOH (▲), and with 0.2% NaOCl/0.2 N NaOH (■).

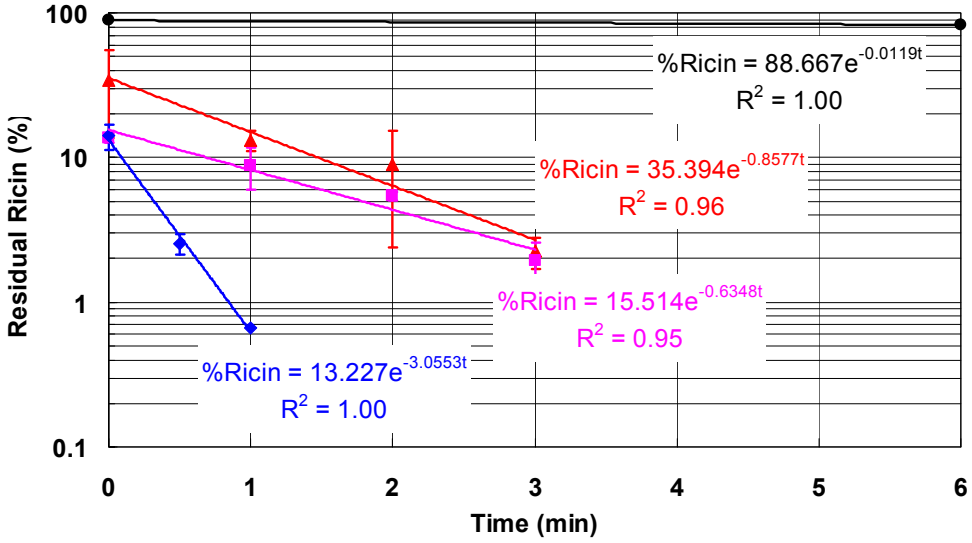


Figure 3-11. The effect of peracetic acid (PA) on inactivation of ricin in orange juice at 72 and 80°C. Mixtures included ricin containing orange juice alone at 72°C (●), 0.032% at 72°C (▲), 0.064% at 72°C (■), and 0.032% at 80°C (◆).

The thermal inactivation of ricin was markedly enhanced by the presence of sanitizers. Inactivation using either NaOH and NaOCl or PA at high temperatures followed a two-step kinetics process. There was an initial drop after the addition of chemicals similar to the results from chemical inactivation without any incubation time. This was followed by first order destruction kinetics. R^2 values obtained from exponential regression at all combinations were >0.95 indicating a relatively good correlation (Table 3-5).

Table 3-5. Half life ($t_{1/2}$) of combined inactivation of ricin in orange juice.

Temp. (°C)	First chemical	Second chemical	R^2	$t_{1/2}$ (min)
72	None	None	1.00	72.6
72	0.2% NaOCl	0.15 N NaOH	1.00	1.6
72	0.2% NaOCl	0.2 N NaOH	0.96	0.2
72	0.032% PA	None	0.96	0.8
72	0.064% PA	None	0.95	0.9
80	0.032% PA	None	1.00	0.2

The loss of activity rate of ricin was highly dependent on the concentration of added chemicals, and the thermal inactivation effect on ricin was significantly increased with greater concentrations of NaOCl and NaOH or PA. Inactivation by 0.2% NaOCl and 0.15 N NaOH was significantly faster than the inactivation by 0.2% NaOCl and 0.2 N NaOH at 72°C. Increasing the concentration of PA and temperature from 0.032% at 72°C to 0.064% at 72°C or 0.032% at 80°C also yielded higher inactivation rates. These half lives were calculated based on the starting point of the first order reaction. For instance, when ricin was inactivated by 0.064% PA at 72°C, the residual concentration of ricin was 13.7% after addition of PA (Figure 3-9), and the half life for this treatment to

reduce 50% of the starting point of the first order reaction (13.7% residual ricin) was 0.9 min. Ricin was “completely” inactivated at 80°C with lower concentrations of NaOCl and NaOH or PA which means the ELISA kit can not detect any residual ricin at this condition.

3.3 Discussion

3.3.1 Establishment of a standard curve for evaluation of ricin concentration in orange juice

Different dilution media and dilution ratios as well as addition of sanitizing chemicals were first tested to determine their impact on ELISA detection. Data from Figure 3-2 and Table 3-1 indicated that the response of ELISA measurement to ricin concentrations were not necessarily proportional to the dilution factor. Different OD ratios suggested that the amount of orange juice in terms of dilution ratio was affecting the final ricin concentration through OD value tested by ELISA. As a result of those preliminary experiments, standard dilutions in orange juice of 1:500 were utilized for later experiments to eliminate dilution effects.

3.3.2 Thermal inactivation of ricin

The inactivation of ricin in orange juice in the HTST pasteurization temperature range has not been reported in the literature. Although the stability of ricin had been studied in thermal treatments, there was little information about its inactivation in complex food matrices. This study determined the thermal inactivation kinetics of ricin at HTST pasteurization conditions including 72, 80, 85 and 90°C. A series of factors including D value, Z value, half life, and E_a

were determined from the inactivation kinetics.

The results of this study indicate that thermal treatment could be a relatively effective method for inactivating ricin in orange juice. Although heat could not ensure complete inactivation of ricin present in orange juice at standard HTST pasteurization conditions (72°C, 15 s; 90°C, 0.5 s), the thermal resistance of ricin is remarkably reduced with increased temperature likely due to the denaturation of the toxin protein. The half life of ricin decreased approximately 140-fold when the heating temperature was increased from 72 to 90°C.

Xu and others (unpublished) studied the thermal inactivation effect of ricin in milk in the HTST temperature range. The results in milk as compared to the results of this study in juice are shown in Table 3-6. The current study found that the half life at each temperature and the Z value were higher than the corresponding ones found in milk while the activation energy was lower than the one found in milk. Since the inactivation steps were almost the same, the main difference between these two studies were likely due to the difference of the composition of orange juice and milk in terms of pH, ionic strength as well as the presence of protein. Furthermore, to the author's acknowledge, Xu and others chose a wider range of ricin concentration to establish the standard curves and data points with OD values greater than 2.5 also considered in the standard equation. These would all contribute to the significant difference between the results of the current study and the ones from Xu and others.

Table 3-6 also compares the Z value obtained here to the milk and soy based infant formulas from Jackson and others (29). Although the FDA values were in the same order of magnitude, they had lower activation energy and higher Z value compared to the inactivation studies of ricin in orange juice. Possible reasons include the different characteristics between infant formulas and orange juice such as the composition and pH values. Inactivation methods were very different since larger glass test tubes (size not specified) were used in the

FDA study while capillary tubes were used in the current study. One mL aliquots of samples in glass test tubes would have been difficult to heat evenly and the come-up time (less than 2 min) was not considered in their final calculation. These factors could have contributed to the differences in these kinetics values.

Table 3-6. Comparison of thermal inactivation parameters of ricin in milk and orange juice.

	Half life $t_{1/2}$ (min)			
Medium	**Infant formula**		**Milk**	**Orange juice**
	Soy based	**Milk based**		
Temp. (°C)	**Jackson and others, (29) (FDA)**		**Xu and others, (unpublished)**	**Current work**
60	99.0	115.5	NT*	NT
70	16.1	9.9	NT	NT
72	NT	NT	32.1	72.6
75	8.7	5.8	NT	NT
80	6.9	5.0	3.0	9.0
85	3.0	3.2	0.5	2.0
90	2.0	1.8	0.2	0.5
E_a (kcal/mole)	30.6	30.9	72.9	64.7
Z value (°C)	18	18	7.8	8.8

*Not tested

Losikoff (45) determined the Z value for inactivation of *Clostridium botulinum* 62A protein toxin in beef broth using 1.5 mL of samples in thermal death time tubes (9 by 150 mm). The effect of lethality of mice after intraperitoneal injection of treated toxin was evaluated. The come-up time was considered in this study although they didn't mention how they measured it. Z values obtained for *Clostridium botulinum* 62A protein toxin were 5.4°C and 4.6°C in beef broth at pH 6.8 and pH 5.0, respectively. These Z values were somewhat less than the Z value obtained for ricin in orange juice. A smaller Z value equals to a steeper slope of D value versus temperature, so it indicates that the reaction is more sensitive to temperature, i.e. it has a higher activation energy. Similarly, characteristics of beef broth and orange juice as well as volumes of samples and the detection methods were different. The nature of the *Clostridium botulinum* 62A protein toxin should be the main contributor to the huge difference.

Many inactivation studies on *Staphylococcus aureus* enterotoxin have been done previously. Read and Bradshaw (60), Hilker and others (27) and Denny and others (16) conducted studies on the inactivation of *Staphylococcus aureus* enterotoxin A or B, respectively. Studies on enterotoxin A considered the come-up time while the study on enterotoxin B did not mention any information about the come-up time. Their results showed that the Z value for *Staphylococcus aureus* enterotoxin A was 27.8°C in Veronal buffer (pH 7.2) and beef bouillon (pH 6.2) the one for enterotoxin B was 25.9°C in milk. These values are significantly greater than the Z values obtained for ricin in orange juice. The protein toxin structure and the characteristics of food matrices could be the two main reasons for the huge differences, but one would expect smaller Z values for any type of protein denaturation.

3.3.3 Chemical inactivation of ricin

Since thermal inactivation alone did not seem to be sufficient to inactivate ricin at HTST temperatures, the addition of chemical sanitizers for inactivating ricin in orange juice was studied. NaOH and NaOCl have been widely used as sanitizers in food processing plants and have been routinely used in research laboratories to destroy biotoxins, such as ricin (8). PA has been recognized as an ideal antimicrobial agent as well as a sanitizing agent due to its high oxidizing potential. The effect of these three chemicals was to be very sensitive to changes in concentration for inactivation of ricin in orange juice. For instance, 0.18 N NaOH had little effect on the inactivation of ricin but 0.20 N NaOH had a relatively stronger effect to inactive ricin. Since these chemicals could interact with a variety of substances, lower concentrations of chemicals would be consumed rapidly and depleted while no or little amount left for the inactivation of ricin. Therefore, higher concentrations were necessary for inactivation.

NaOH and NaOCl are widely used as disinfectants. The alkali ion from NaOH plays a key role in destruction of ricin protein. Previous studies recommended using 0.1 to 0.5% or 0.1 N NaOH for the inactivation of biological toxins on surfaces (49). NaOH was also studied in the inactivation of ricin in castor cake (1) (Table 3-7). Similar results were found that increasing the concentration of NaOH could increase the inactivation of ricin. However, there was a huge difference between that and the current study when inactivating ricin by 0.18 N NaOH (18% versus ~85%). This may be due to the methodology as well as the characteristics of castor bean cake and orange juice. The extraction efficiency of ricin from the castor bean cake played an important role in the estimation of ricin. The study (62) that used 0.5 M NaOH for inactivation of viruses has extremely rapid rates of inactivation similar as the initial drop in the present study.

Table 3-7. Inactivation results of ricin in processed castor bean cake samples (1) .

NaOH (N)	Ricin (mg/kg)	Residual ricin (%)
0.18	69	18
0.38	53	14
0.75	36	8

In addition, after NaOCl is dissolved in water, extremely active hypochlorous acid ($HOCl^-$) and the less active hypochlorite ion (OCl^-) would form. They play an important role in oxidation and disinfection (55). NaOCl could interact with the amino acid residues from ricin and oxidize ricin damaging the ricin structure and therefore its activity. Table 3-8 indicated the concentration dependence of NaOCl on the inactivation of toxins, which is also shown in the present study. However, the inactivation effect of 0.1% NaOCl with a 30 min exposure time for ricin was not found in our study. Further information from the previous study is needed to confirm the effect. A significant synergism effect with the combination of NaOH and NaOCl was found due to oxidation and alkali effects. Therefore, a mixture of NaOH and NaOCl at specific concentrations would be much more effective on the inactivation of ricin in orange juice. Previous published data also confirmed the synergism effect with the combination of NaOH and NaOCl for T-2 mycotoxin (Table 3-8).

Table 3-8. Complete inactivation of different toxins with a 30 min exposure time to varying concentrations of sodium hypochlorite (NaOCl) and/or sodium hydroxide (NaOH) (49).

Toxin	2.5% NaOCl + 0.25 N NaOH	2.5% NaOCl	1.0% NaOCl	0.1% NaOCl
T-2 mycotoxin	Yes	No	No	No
Brevetoxin	Yes	Yes	No	No
Microcystin	Yes	Yes	Yes	No
Tetrodotoxin	Yes	Yes	Yes	No
Saxitoxin	Yes	Yes	Yes	Yes
Palytoxin	Yes	Yes	Yes	Yes
Ricin	Yes	Yes	Yes	Yes
Botulism toxin	Yes	Yes	Yes	Yes

PA is also used widely as a strong oxidant. The thermodynamically unstable hydroxyl radical (OH·) from PA could oxidize and damage the amino acids from ricin and ultimately lead to the disruption of the ricin structure and reduction of its activity (9, 36). Since 0.65% PA was able to reduce ricin to 1% of its initial amount while a combination of 0.20% NaOCl and 0.17 N NaOH was only able to decrease ricin to 54% left, PA is a more effective strategy to remarkably enhance the inactivation of ricin. In comparing to other disinfectants, a much smaller amount of peracetic acid (PA) is needed to decontaminate ricin. Previous studies (5, 56) on the inactivation of proteins in *Lactobacillus helveticus* and *Streptococcus thermophilus* bacteriophages using PA both confirmed its strong effect as an effective inactivation agent since only 0.15% of PA was able to completely inactivate all phages after 5 min of exposure. PA was also used to inactivate *B. anthracis* spores in milk (78). Increasing concentration of PA was able to decrease the D values. Apparently, the

inactivation of spores by PA at a specific temperature was time-dependent, according to the reported data. This was different with the initial drop, which was non time dependence, found in the present study which may be explained by the differences in the nature of spores or toxins. In summary, PA is a very potent inactivation agent that could be used for decontamination in a deliberate delivery of ricin in a food system by bioterrorists.

3.3.4 Combined inactivation of ricin with heat and chemicals

The combination of heat and the three sanitizers studied including NaOH, NaOCl or PA was an effective method to inactivate ricin in orange juice at HTST pasteurization temperatures. It included the inactivation effect from heat or chemical treatment and a combination with two-step inactivation.

The inactivation rate of ricin in milk and orange juice both were highly dependent on the concentration of the chemical substances as well as the heating temperature and time. Significant synergistic effects between NaOH and NaOCl were observed in orange juice. Different food matrices have to be studied individually to determine feasible inactivation conditions.

The estimated lethal dose for human ingestion of ricin is approx. between 1 to 20 mg/kg body weight (6, 75), estimated from consumption of whole castor beans. Using the higher value, the estimated lethal dose for a 20 kg child using an uncertainty factor of 100 (10 for the most sensitive population and 10 for the maximum contaminant level in water) would be 4 mg. A 7,000 gal (26,409 kg) silo of orange juice plant is capable to produce 106,000 servings of orange juice with 0.25 kg/serving. A hypothetical deliberate delivery of ricin by bioterrorists would need 106,000 × 4 mg = 424 g of pure ricin to get a lethal dose in one serving of orange juice. It thus would be easy for bioterrorists to accomplish this in an orange juice plant due to the minimal amount of ricin needed. Using the

lethal dose for mice ingestion of ricin (5 μg/kg) instead, the estimated lethal dose for a 20 kg child using an uncertainty factor of 100 (10 for interspecies and 10 for the most sensitive population) would be 1 μg. A hypothetical deliberate delivery of ricin by bioterrorists would need 106,000 × 1 μg = 0.1 g of pure ricin to get a lethal dose in one serving of orange juice. It is extremely easy for bioterrorists to accomplish this in an orange juice plant due to the minimal amount of ricin needed. Thus, the fact is that one is likely able to use ricin as a bioterror weapon since it is quite effective. In the event of an intentional delivery of ricin, assuming that an 8 oz glass of orange juice is contaminated with ricin, 0.064% of peracetic acid (PA) could be added into the silos and orange juice could go through the pasteurization equipment at 72°C. To achieve a 1000 fold reduction, a 10 half life process (10 × 0.9) of 9 min would be required.

In summary, this project generated the procedures of thermal and chemical inactivation conditions at HTST pasteurization temperatures to decontaminate ricin in an orange juice plant. In case of a deliberate delivery of ricin into an orange juice plant, a feasible and effective procedure could be applied in the plant to decontaminate ricin with small concentrations of chemicals and the typical pasteurization equipment available in the plant.

4 Inactivation of Shiga toxins

4.1 Materials and Methods

4.1.1 Production of Shiga toxins

Escherichia coli O157:H7 strains were used as the source of Shiga toxins. The following strains were tested for their ability to produce toxins: Shiga toxin 1 (Stx1) producers: ATCC 43890 and O29 (an isolate originally obtained from cattle); Shiga toxin 2 (Stx2) producers: O14, O42, O47 (cattle isolates) and EK-1 TW08609 (provided by the Minnesota Department of Health and originally isolated from a patient); and producers of both toxins: ATCC 31350 and ATCC 43895. Bacterial cultures of these strains were grown separately in 3 mL of Luria-Bertani (LB) broth and incubated at 37°C overnight. Stock cultures were stored at 4°C. Working cultures were cultivated in 3 mL LB broth with 10 µg/L norfloxacin at 37°C overnight. The best producers of Shiga toxins were chosen for future studies and were cultivated in a larger amount of broth for stock and working cultures.

Cultures were centrifuged at 1000 × g for 20 min at 4°C. The supernatants were filtered using a 0.2 µm pore size with a low protein binding filter (VWR Scientific Inc.) (71). The retentate was frozen in a centrifuge tube (50 mL, Corning Inc.) at -20°C and then freeze dried in a 2 L flask at 0 millitorr/microns for ~3 days (ATR, Inc.) and then stored over desiccant (Drierite) at 4°C for future use. Toxin concentrations in the dried supernatant were determined using an immunoassay test (see below) and freeze-dried supernatants were produced once a month to ensure the freshness of toxins.

4.1.2 Thermal Inactivation of Shiga toxins

A capillary tube method was used to minimize the come-up time when determining the heat resistance of toxins in a water bath (78). Freeze dried culture supernatant powder was weighed and dissolved in phosphate buffered saline (PBS) as in the ricin study. Volumes of 90 µL of PBS or orange juice (Minute Maid, The Coca-Cola Company, Atlanta, GA) were mixed with 10 µL of the supernatant solution containing Shiga toxin. Shiga toxin-containing PBS and orange juice samples (50 µL) were transferred into capillary tubes (0.8-1.1 × 90 mm; No. 34507, Kimble, Vineland, NJ). Tubes were heat-sealed and then completely submerged into a water bath (High-Temp™ Bath 160A, Fisher Scientific) at different temperatures from 72 to 90°C. At each sampling time, capillary tubes were removed from the water bath and cooled in an ice-water bath. After cooling, tubes were rinsed with distilled water to remove any possible contaminants and crushed by mixing in test tubes (10 mL, 16 × 125 mm) containing 0.25 mL PBS and a magnetic stirrer using a vortexer (Mini Vortexer, VWR). Mixtures were transferred into a 1.5 mL mirocentrifuge tube and stored at 4°C until the residual toxin concentration was quantified by an immunoassay on the same day. Treatments and measurements for each sample were performed at least in duplicate.

In addition to the capillary tube method, inactivation of toxins was also conducted in 1.5 mL microcentrifuge tubes containing mixtures of 0.45 mL of PBS or orange juice and 0.05 mL of supernatant-PBS solutions. Shiga toxin-added PBS or orange juice samples were then completely submerged into a water bath at different temperatures. At each sampling time, two tubes were removed from the water bath, cooled in an ice-water bath and stored at 4°C until testing was performed as described above.

4.1.3 ELISA Test for Shiga Toxins

The concentration of residual Shiga toxins after heat treatment was determined by using ELISA, the Premier^{TM} EHEC ELISA kit (Meridian Bioscience, Cincinnati, OH). Multi-point standard curves as well as positive and negative controls were performed to reduce variations among ELISA testings. The cut-off concentration according to the manufacturer's instructions was OD=0.18 where the concentrations of Stx1 and Stx2 were 0.07 and 0.15 ng/mL respectively.

The Premier EHEC test kit utilizes monoclonal anti-Shiga toxin capture antibodies absorbed onto the bottom of microwells. When testing, 100 µL samples were added to each well, mixed thoroughly with the pipette and the 96-well plates were incubated at room temperature (RT) for 1 h. Wells were then washed according to manufacturer's instructions. Volumes of 100 µL of polyclonal anti-Shiga toxin antibodies provided by the kit were added into each well, mixed well as before, and incubated at RT for 30 min. Wells were washed again to remove unbound antibody. Aliquots of 100 µL of enzyme-conjugated anti-IgG polyclonal antibody were added into each well, mixed and incubated at RT for 30 min. After washing to remove unbound conjugate, 100 µL of substrate were added to each well and plates were incubated for 10 min at RT followed by addition of 100 µL of stop solution into each well. Absorbance was then measured at 450 nm using a Bio-Tek Kinetics Reader (Bio-Tek Instruments Inc., Winooski, VT) and software (KC 4, Bio-Tek). The steps of this ELISA method are shown in Figure 4-1.

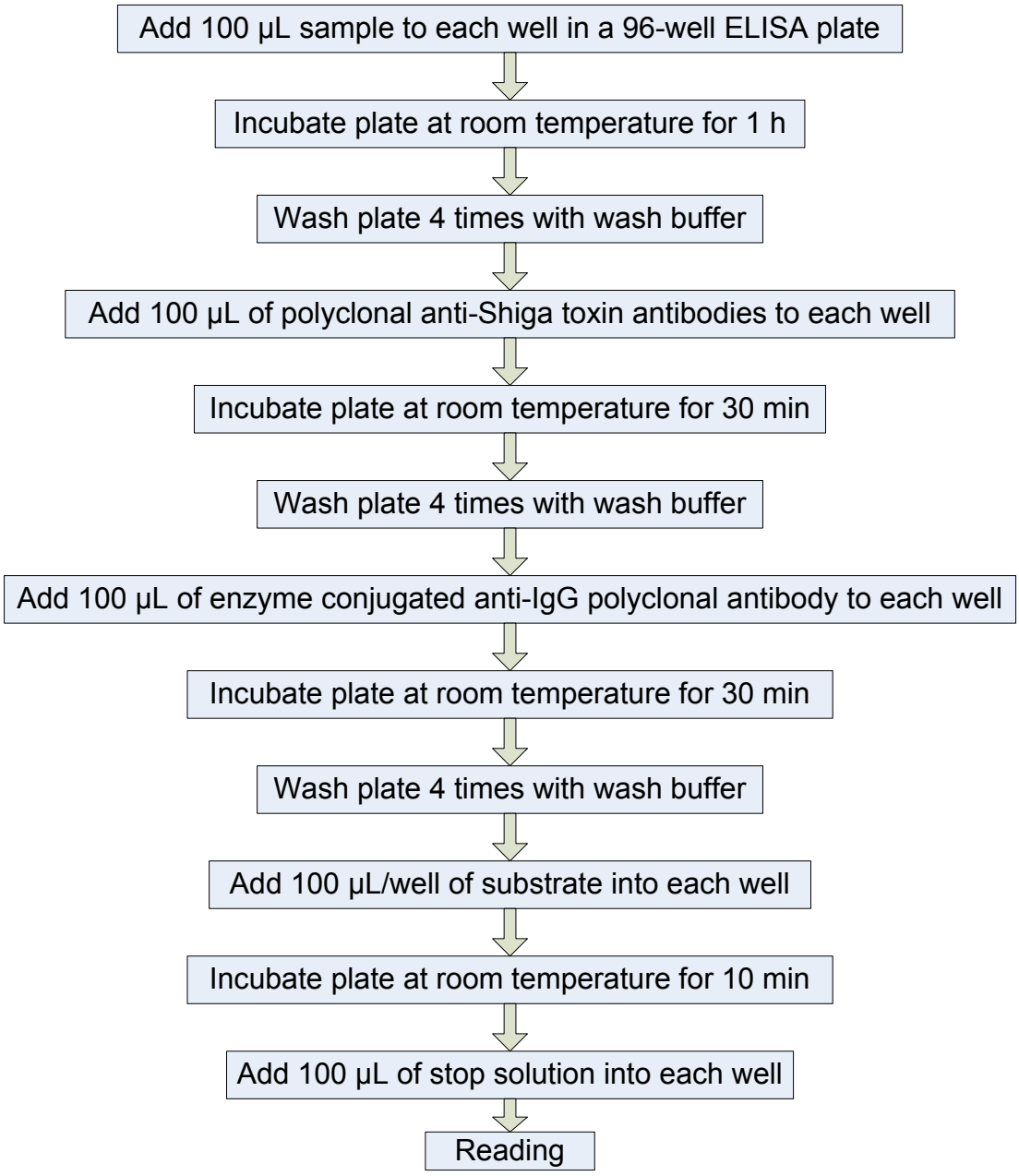


Figure 4-1. Steps of ELISA method to determine Shiga toxins.

4.1.4 Cytotoxicity Assay for Shiga Toxins

A cytotoxicity assay that measured the amount of lactate dehydrogenase (LDH) released by Vero cells exposed to Shiga toxin-containing fractions using a lactate dehydrogenase (LDH) detection kit (Roche Applied Science, Indianapolis, IN) was used to confirm the reduction of activity of Shiga toxins after heat treatment. In order to perform the LDH assay, Vero cells (African green monkey kidney tissue; ATCC No. CCL-81^{TM}) were cultured in Dulbecco's modified Eagles medium (DMEM; ATCC No. 30-2003) with 10% fetal bovine serum (FBS; Fisher Scientific Inc., Hanover Park, IL) at 37°C with 5% CO_2 under 90% humidity in a 96-well tissue culture plate (Sarstedt Inc., Newton, NC) stored in a commercial CO_2 incubator. They were subcultured periodically every 2 to 3 days according to research needs.

The optimum cell concentration for a specific cell type was determined in a preliminary experiment. In this study, an entire 96-well tissue culture plate (Sarstedt Inc., Newton, NC) was filled with 100 µL/well assay medium (DMEM with 1% bovine serum albumin (BSA; Fisher Scientific Inc., Hanover Park, IL)). Vero cells were washed with assay medium (~3 mL) and the cell suspensions were adjusted to a concentration of ~2 × 10^6 cells/mL counted by Trypan blue (0.4%) staining. Serial dilutions were performed into wells and the plate was then incubated in an incubator (37°C, 5% CO_2, 90% humidity) overnight. Supernatants (100 µL per well) were transferred into corresponding wells of an optically clear 96-well flat bottom microplate (Sarstedt Inc., Newton, NC). Volumes of 100 µL of LDH reaction mixture according to the manufacturer's instructions (freshly prepared) were then added into each well and the plate was incubated for 30 min in the dark at RT. Absorbance was measured at 490 nm as described above.

Due to laboratory facilities and biosafety limitations, alternative incubation methods in addition to the regular CO_2 incubator (model 3154, Forma Scientific,

Inc.) were tested. A regular CO_2 incubator was used as a control (method A). Method B consisted in leaving a sealable plastic container open in a regular CO_2 incubator for some time to reach an equilibrium condition with the incubator so that it would have a similar condition when sealed and incubated in air incubator. Method C was accomplished by using an anaerobic jar injected with an approximate amount of CO_2 and incubated in a standard incubator. Method D was incubation in air with its own cover. Four experiments were conducted using the LDH assay and absorbance was measured at 490 nm.

To measure the cytotoxic potential of Shiga toxins, Vero cells were first washed in assay medium made according to the instructions from the manufacture of the LDH kit. Aliquots of 100 µL/well cell suspensions in assay medium were added to sterile 96-well tissue culture plate (No. 82.1581.001, Sarstedt Inc., Newton, NC) and incubated overnight at 37°C, 5% CO_2 and 90% humidity to allow cells to adhere tightly. Immediately before use, the assay media from the adherent cells was removed with pipette and 100 µL/well fresh assay media was added. Samples (toxin in orange juice) of 100 µL were transferred into wells as well as background control (200 µL assay medium), low control (100 µL/well cell suspensions with 100 µL/well assay medium) and high control (100µL/well Triton X-100 solution with 100 µL/well cells). Plates were incubated under the same conditions as before for 2 h. Volumes of 100 µL of supernatant were transferred into corresponding wells of an optically clear 96-well flat bottom microplate. The reaction mixture (100 µL freshly prepared) was added into each well and incubated for 30 min in the dark at RT. The absorbance was determined as described before. Note that all the original data are available in an electronic appendix. Steps are shown in Figure 4-2.

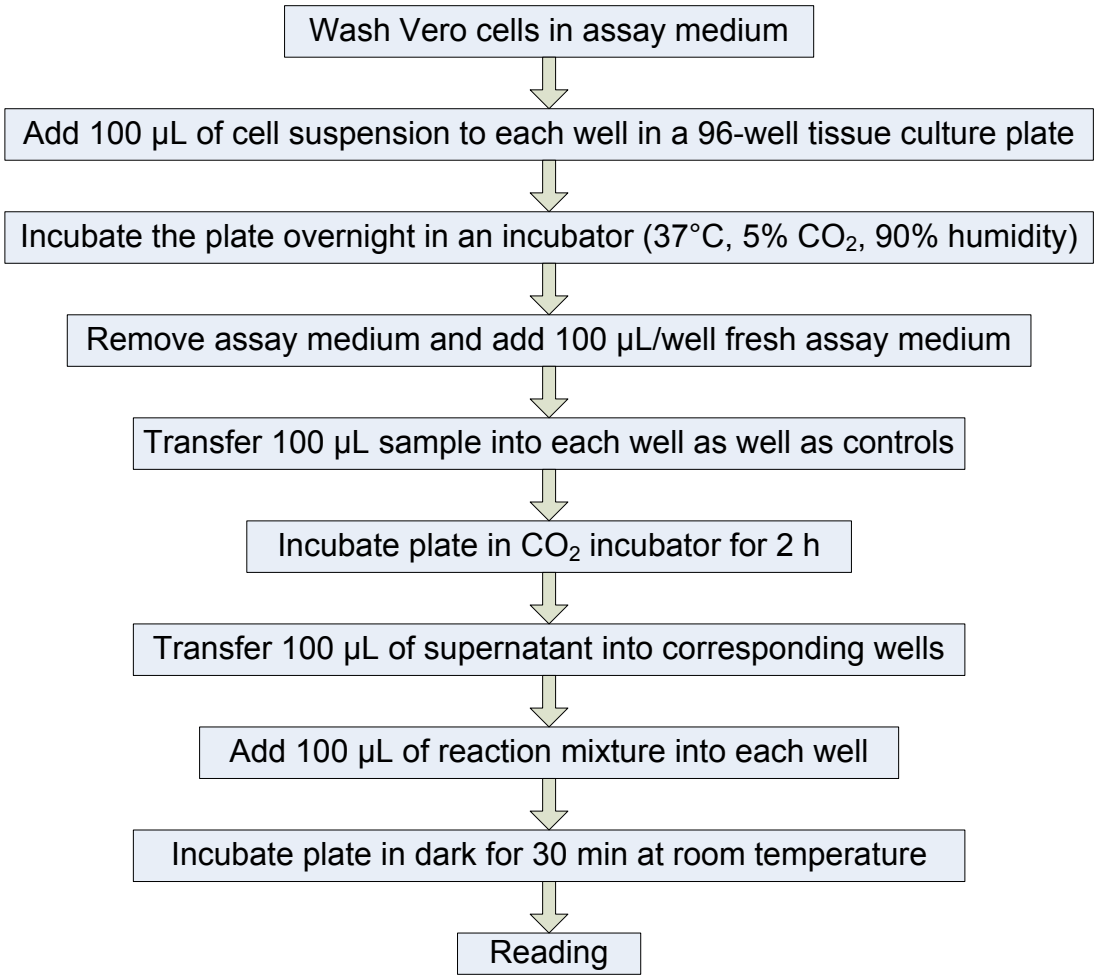


Figure 4-2. Steps of LDH assay for detection of Shiga toxins.

4.1.5 Data Analysis

As noted above, the ELISA test and LDH assay were used to determine the residual concentration of Shiga toxins after thermal treatment. In the ELISA test, residual Shiga toxin (%) was the percentage of Shiga toxins detected in the treated PBS or orange juice with respect to the amount of Shiga toxin detected at zero time. The kinetics of heat treatment was determined by making first-order plots of concentration with time. A spreadsheet developed by Dr. Ted Labuza (University of Minnesota) was used to set the inactivation rate (k), half-

life ($t_{1/2}$) (time for a toxin to be inactivated by half) and D value (time required to reduce the toxicity of a toxin by a factor of 10) through linear regression. The activation energy (E_a) (the threshold or energy barrier that must be overcome or surpassed to permit the transformation of reactants into products), Q_{10} (increase in rate for a 10°C increase in temperature) and Z value (change in temperature that accompanies a 10-fold change in D or k value) were also calculated with these spreadsheets. In addition, the time equivalent to destruction of 12 half lives was calculated assuming that the final concentration was reduced to approximately 0.02% of the initial level. In the LDH assay, the absorbance from treated samples was determined to qualitatively confirm whether there was inactivation of the Shiga toxins.

All experiments were replicated at least two times with duplicate samples at each time interval for each treatment. Confidence interval limits of 95% were calculated from semi-log plots of residual Shiga toxin (%) versus time and D value versus temperature. The difference of D values between Shiga toxins or media (PBS or juice) was analyzed by using analysis of variance procedure (ANOVA R version 2.8.1, 2008) with a significance level of 0.05. In addition, the lack of convergence of the upper and lower 95% confidence limit of each key value was used to confirm the significance (41). Note again that all original data are available in an electronic appendix.

4.2 Results

4.2.1 Production of Shiga toxins

All *Escherichia coli* O157:H7 strains were capable of producing Shiga toxins, but the amount of toxin detected was quite variable (Table 4-1). For Stx1 producing strains, ATCC 43890 was the only strain capable of producing significant amounts of toxin. Strain EK-1 TW08609 produced more than 100-

fold Stx2 than O14, O42 and O47. The two strains capable of producing Stx1 and Stx2 had the highest titer of Shiga toxin.

Table 4-1. Concentration of Shiga toxins in the supernatant of *Escherichia coli* O157:H7 cultures after incubation at 37°C overnight.

Shiga toxin type	Strain	Toxin concentration (ng/mL)	
		Rep 1	Rep 2
Stx1	O29	0	0
	ATCC 43890	2.7	7.8
Stx2	O14	0.7	1.5
	O42	1.7	1.1
	O47	2.6	0.9
	EK-1 TW08609	72.0	120.0
Stx1&2	ATCC 31350	780.0	660.0
	ATCC 43895	430.0	1,300.0

4.2.2 Thermal Inactivation

1) Shiga toxins in buffer

The capillary tube method was used to determine the kinetics of heat inactivation of Shiga toxins in phosphate buffered saline (PBS). The PBS suspension containing Stx1 or Stx2 culture supernatants were inactivated at 72, 80, 85 and 90°C in triplicate. Figure 4-3 shows a typical ELISA standard curve conducted with each experiment (See all of the standard curve plots in the

Appendix). Both plots had R^2 values greater than 0.99 although they represented different concentration ranges for Stx1 and Stx2. Residual Shiga toxin concentrations after heat treatment were determined and calculated according to the corresponding standard curves.

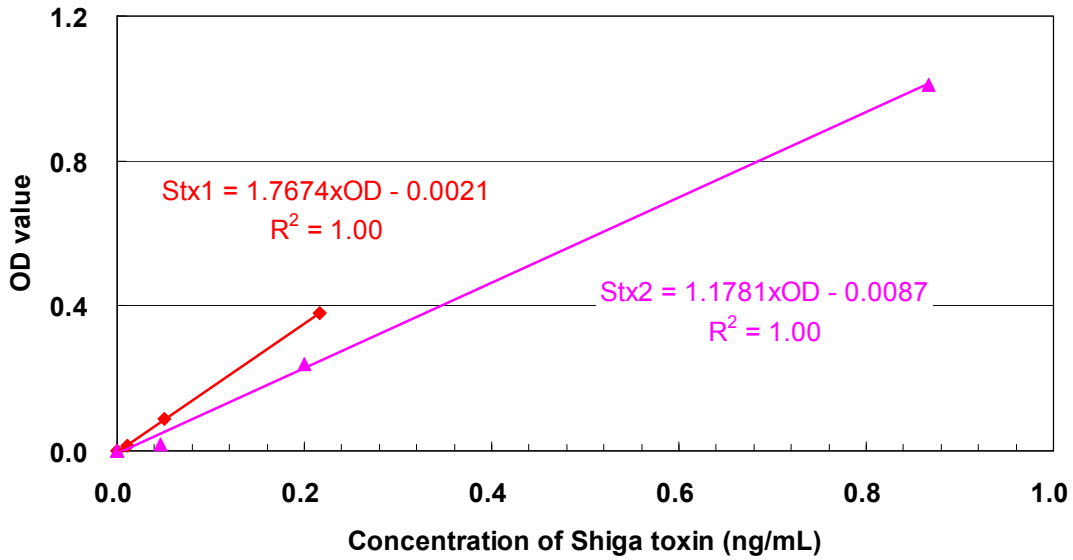


Figure 4-3. A representative ELISA standard curve of Shiga toxins in PBS.

The inactivation of Shiga toxins in PBS was determined at 72, 80, 85 and 90°C. The reduction in detectable Shiga toxins in PBS exhibited clearly a first-order reaction (Figure 4-4). It can be observed that the first order model provided a good fit of the experimental data through exponential regression at all temperatures ($R^2 \geq 0.93$). The residual concentration of Shiga toxins was reduced at a faster rate as temperature increased. For both Shiga toxins in PBS the detection limit of the ELISA assay was reached within 10 s at 90°C and 30 s at 85°C.

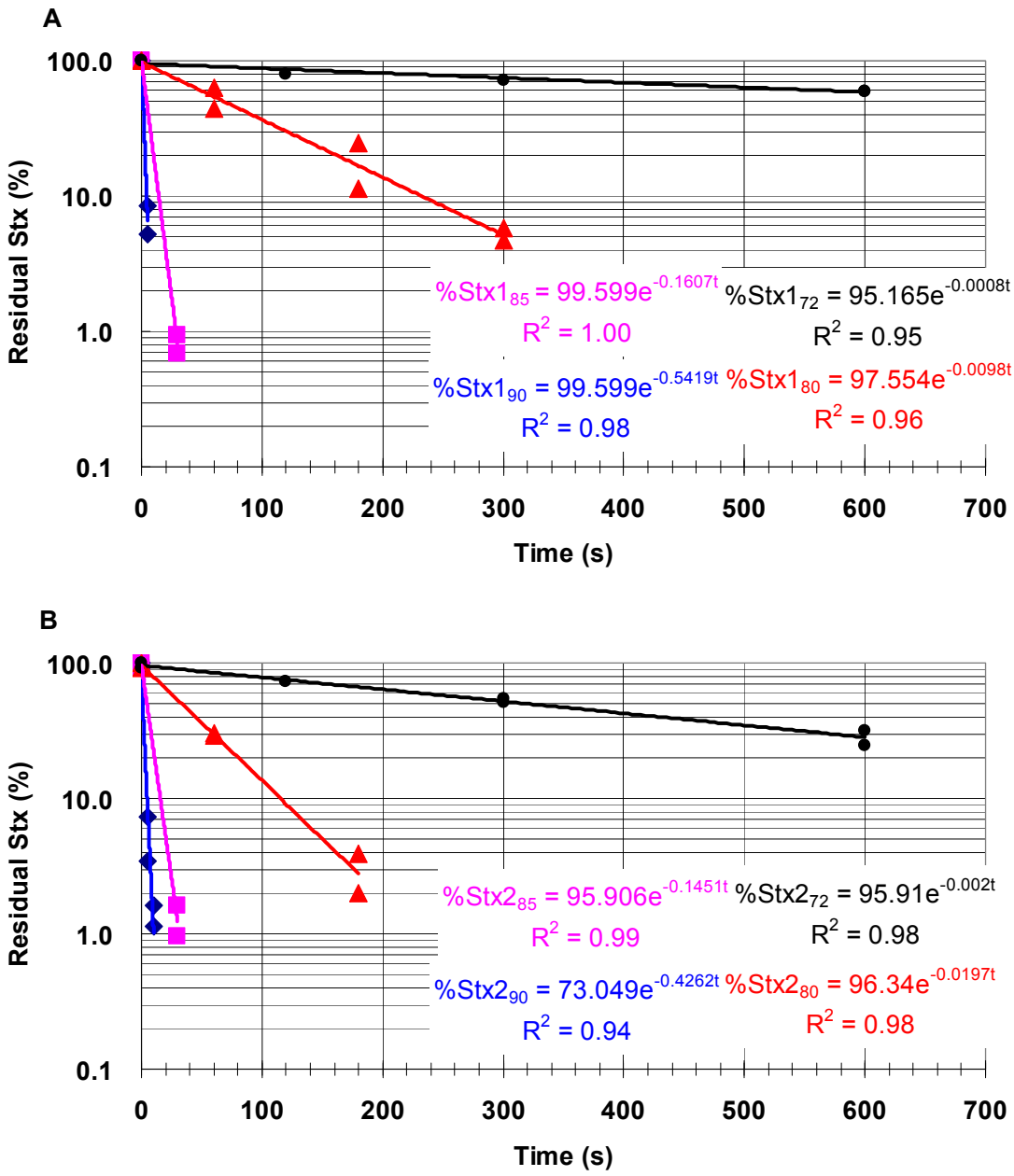


Figure 4-4. The effect of temperature on inactivation of Shiga toxin 1 (A) and Shiga toxin 2 (B) in PBS at 72°C (●), 80°C (▲), 85°C (■), and 90°C (◆).

Based on the rate of inactivation, D values at each temperature were calculated (Table 4-2 and Table 4-3). Z values and activation energy (E_a) were determined

based on those D values. The correlation between processing temperatures and corresponding D values for Stx1 and Stx2 in PBS is depicted in Figure 4-5 with the lower and upper 95% confidence limits.

Table 4-2. D values, Z value and activation energy (E_a) of thermal inactivation kinetics of Shiga toxin 1 in PBS.

Temp. (°C)	D value (s)				
	Rep 1	**Rep 2**	**Rep 3**	**Mean**	**STDV***
72	3,278	3,158	2,722	3,053	293
80	161	176	249	196	47
85	24	29	56	37	17
90	4	5	13	7	5
Z value (°C)	6.1	6.4	7.7	6.7	0.9
E_a (Kcal/mole)	93.7	89.8	80.1	87.9	7.0

*STDV: Standard Deviation

Table 4-3. D values, Z value and activation energy (E_a) of thermal inactivation kinetics of Shiga toxin 2 in PBS.

Temp. (°C)	D value (s)				
	Rep 1	**Rep 2**	**Rep 3**	**Mean**	**STDV***
72	1,050	1,825	1,351	1,409	391
80	91	130	104	108	20
85	20	25	21	22	3
90	4	5	4	4	0
Z value (°C)	7.5	7.0	7.2	7.2	0.3
E_a (Kcal/mole)	76.2	66.6	80.5	74.4	7.1

*STDV: Standard Deviation

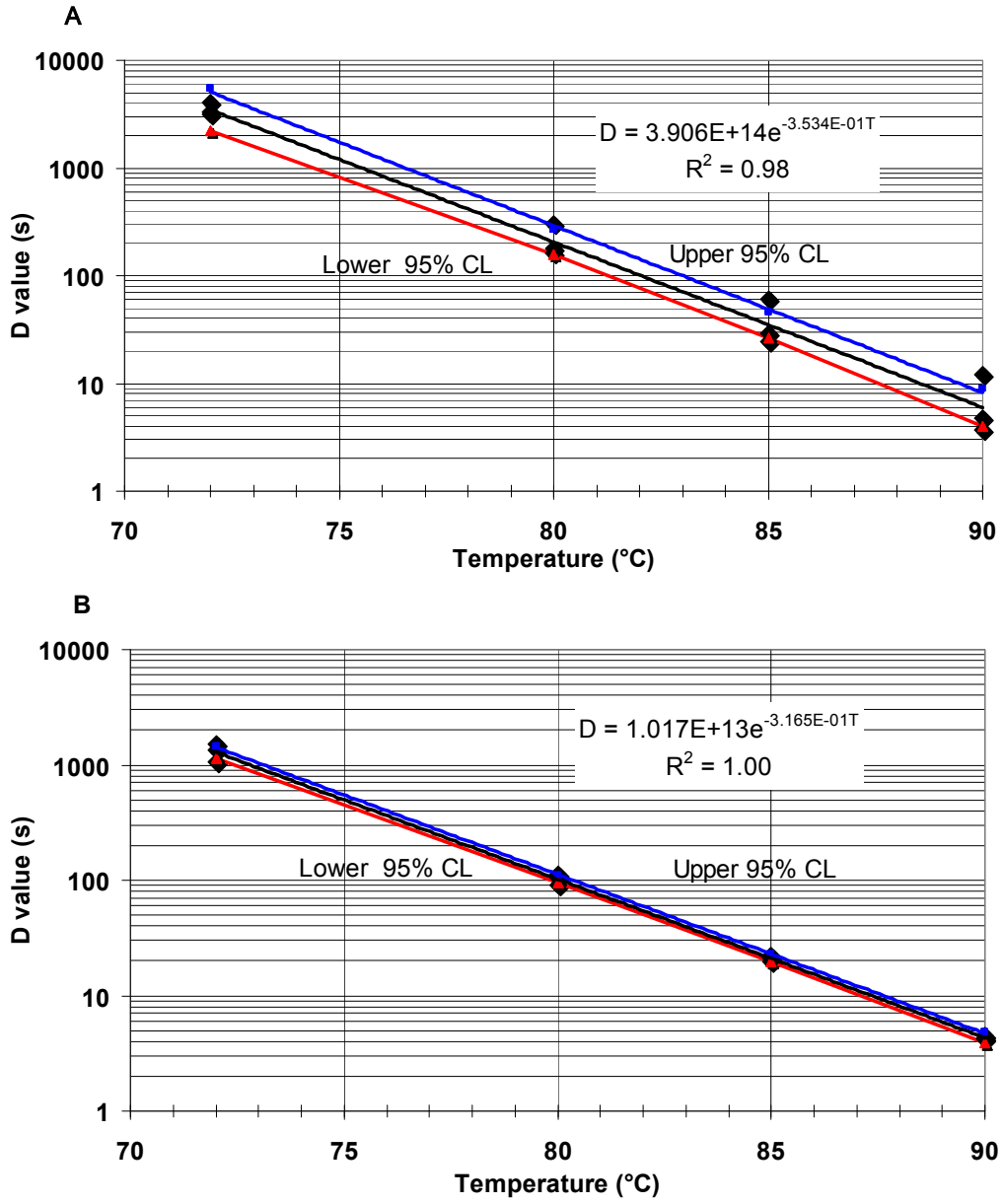


Figure 4-5. Relationship of D values of Shiga toxin 1 (A) and Shiga toxin 2 (B) with temperature when toxin preparations were suspended in PBS.

The first-order rate constants for Shiga toxin inactivation in PBS were fitted to the Arrhenius relationship between log reaction rate (constant) versus 1/absolute temperature in Kelvin (R^2 values greater than 0.98). Figure 4-6

shows the plots for Rep 1 in Table 4-2 and Table 4-3. The calculated average E_a of Stx1 was 10 kcal/mole more than that of Stx2.

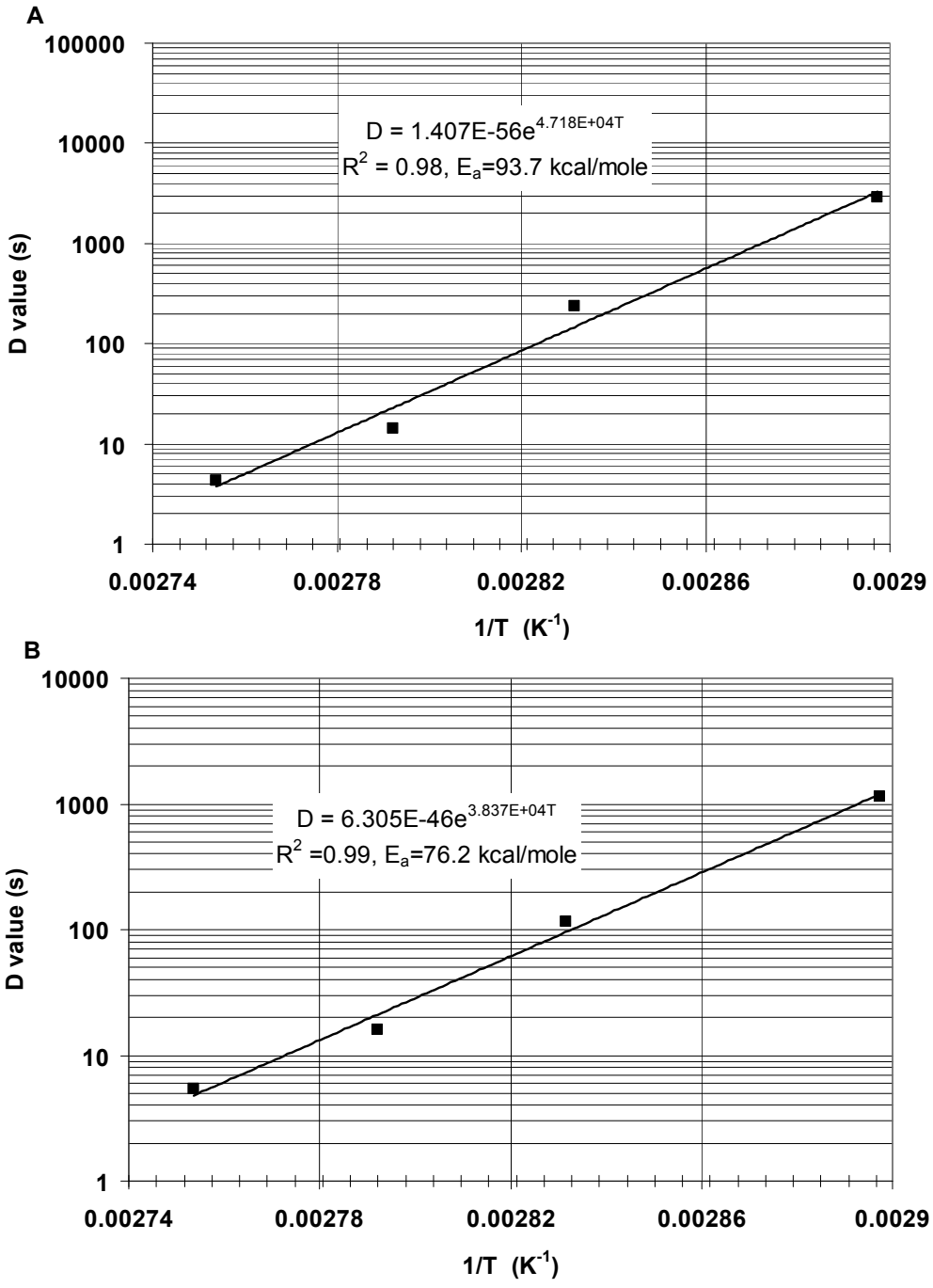


Figure 4-6. Arrhenius plots for inactivation of Shiga toxin 1 (A) and Shiga toxin 2 (B) in PBS used to determine activation energy (E_a).

The inactivation rates (k) and half life ($t_{1/2}$) kinetic parameters were also calculated (Table 4-4 and Table 4-5). In addition, an extrapolation equivalent to 12 half lives (reduction to 0.025%) was also determined for both toxins. Similar to previous kinetic parameters, R^2 values greater than 0.88 indicated a good fit.

Table 4-4. The effect of temperature on inactivation rates (k) and half life ($t_{1/2}$) of Shiga toxin 1 in PBS. Results of triplicate runs are shown for each temperature.

Temp. (°C)	Rep #	R^2	k (95% CL) (s^{-1})	$t_{1/2}$ (95% CL) (s)	12 × $t_{1/2}$ (95% CL) (s)
72	1	0.95	0.0008 (0.0005, 0.0011)	855 (642, 1283)	10263 (7699, 15390) [a*]
	2	0.89	0.0008 (0.0003, 0.0011)	827 (608, 1290)	9918 (7297, 15480) [a]
	3	0.96	0.0007 (0.0005, 0.0009)	930 (757, 1207)	11161 (9078, 14486) [a]
80	1	0.96	0.0098 (0.0079, 0.0117)	71 (59, 88)	849 (712, 1051) [b]
	2	0.96	0.0101 (0.0081, 0.0121)	69 (57, 69)	823 (686, 823) [b]
	3	0.90	0.0052 (0.0035, 0.0069)	134 (100, 201)	1607 (1205, 2410) [c]
85	1	1.00	0.16 (0.14, 0.18)	4 (4, 5)	52 (45, 61) [d]
	2	1.00	0.14 (0.08, 0.20)	5 (3, 9)	59 (41, 106) [d]
	3	0.95	0.03 (0.02, 0.05)	21 (15, 37)	254 (178, 441) [e]
90	1	0.98	0.54 (0.34, 0.74)	1 (1, 2)	15 (11, 25) [f]
	2	0.93	0.40 (0.25, 0.56)	2 (1, 3)	21 (15, 33) [f]
	3	0.97	0.24 (0.17, 0.30)	3 (2, 4)	35 (28, 48) [f]

*Same letter indicates not statistically different at $p \leq 0.05$

Table 4-5. The effect of temperature on the inactivation rates (k) and half life ($t_{1/2}$) of Shiga toxin 2 in PBS. Results of triplicate runs are shown for each temperature.

Temp. (°C)	Rep #	R^2	k (95% CL) (s^{-1})	$t_{1/2}$ (95% CL) (s)	12 × $t_{1/2}$ (95% CL) (s)
72	1	0.98	0.0020 (0.0016, 0.0024)	341 (290, 413)	4088 (3478, 4958) [g]
	2	0.99	0.0014 (0.0013, 0.0015)	489 (457, 527)	5873 (5480, 633) [h]
	3	0.95	0.0015 (0.0012, 0.0018)	468 (379, 611)	5617 (4551, 7333) [g]
80	1	0.98	0.02 (0.02, 0.02)	35 (30, 435)	423 (356, 522) [l]
	2	0.99	0.01 (0.01, 0.01)	66 (58, 77)	792 (693, 924) [i]
	3	0.99	0.02 (0.01, 0.02)	36 (28, 50)	430 (334, 604) [l]
85	1	0.99	0.15 (0.11, 0.18)	5 (4, 7)	57 (46, 78) [k]
	2	0.92	0.10 (0.01, 0.19)	7 (4, 63)	83 (44, 752) [k]
	3	0.95	0.14 (0.04, 0.25)	5 (3, 18)	58 (34, 217) [k]
90	1	0.94	0.43 (0.27, 0.58)	2 (1, 3)	19.6 (14, 31) [m]
	2	0.91	0.47 (0.02, 0.9)	2 (1, 34)	17.5 (9, 410) [m]
	3	0.95	0.47 (0.13, 0.82)	2 (1, 5)	17.5 (10, 64) [m]

* Same letter indicates not statistically different at $p \leq 0.05$

2) Shiga toxins in orange juice

The capillary tube method was also used for inactivation studies in orange juice. Stx1 or Stx2 culture supernatants in orange juice were inactivated at 72, 80, 85 and 90°C, performed in duplicate. An ELISA standard curve for Shiga toxins in orange juice were performed with each experiment and a typical one is shown in Figure 4-7. Both plots had R^2 values higher than 0.95, although the range of concentration of Shiga toxins were different. Standard curve equations shown in the figure were used to calculate the residual Shiga toxin concentration after heat treatment.

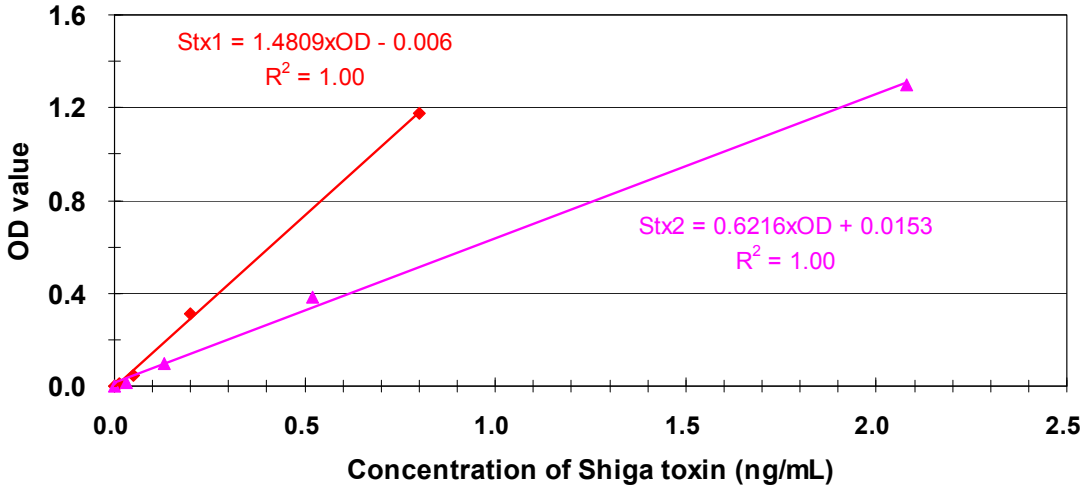


Figure 4-7. A representative ELISA standard curve of Shiga toxins in orange juice.

The inactivation effects of Shiga toxins in orange juice (processing temperature and time) were determined at 72, 80, 85 and 90°C. Measurable inactivation of Shiga toxins in orange juice followed first-order reaction kinetics (Figure 4-8) with distinguishable slopes for each regression line and the rate of loss in ricin activity was highly dependent on temperature. The inactivation rate of Shiga

toxins was increased as temperature increased. All R^2 values of the inactivation curves were greater than 0.90 indicating good fits. In addition, both Shiga toxins in orange juice were inactivated until non-detectable using an ELISA kit within 30 s at 90°C and 120 s at 85°C.

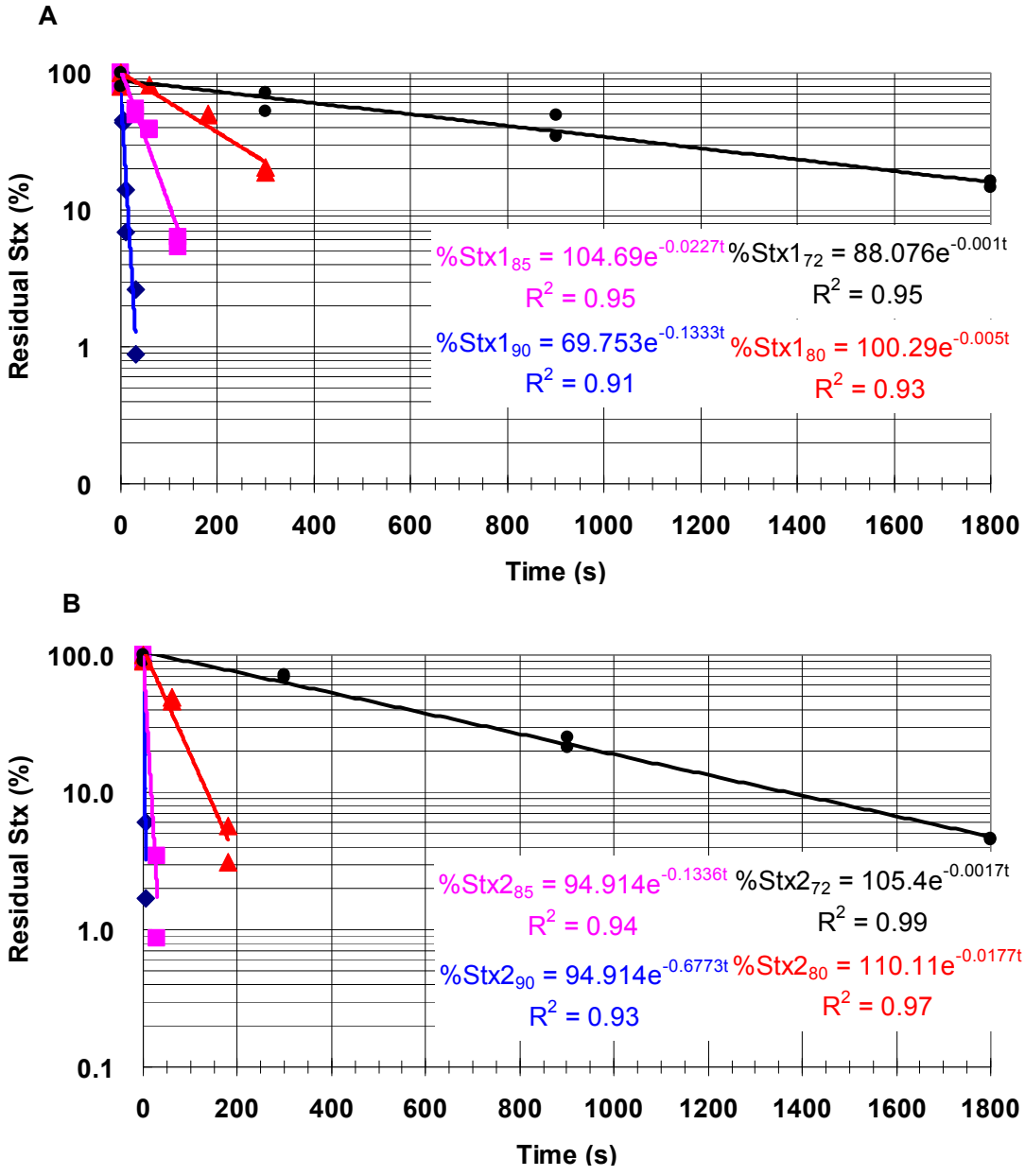


Figure 4-8. The effect of temperature on inactivation of Shiga toxin 1 (A) and Shiga toxin 2 (B) in orange juice at 72°C (●), 80°C (▲), 85°C (■), and 90°C (◆).

The D value at each temperature was calculated according to the rate of inactivation. In addition, the Z value and activation energy (E_a) was determined from the first order rate constants for each temperature treatment using linear regression (Table 4-6 and Table 4-7). Figure 4-9 reveals the linear relationship between D values and corresponding temperatures for Stx1 and Stx2 in orange juice with lower and upper 95% confidence limits.

Table 4-6. D values, Z value and activation energy (E_a) for thermal inactivation of Shiga toxin 1 in orange juice.

T (°C)	D value (s)			
	Rep 1	**Rep 2**	**Mean**	**STDV***
72	2,289	2,929	2,609	452
80	287	338	313	36
85	78	88	83	7
90	21	23	22	1
Z value (°C)	8.9	8.5	8.7	0.3
E_a (kcal/mole)	64.5	67.0	65.8	1.8

*STDV: Standard Deviation

Table 4-7. D values, Z value and activation energy (E_a) for thermal inactivation of Shiga toxin 2 in orange juice.

T (°C)	D value (s)			
	Rep 1	**Rep 2**	**Mean**	**STDV***
72	1,111	1,503	1,307	277
80	77	102	89	18
85	15	19	17	3
90	3	4	3	1
Z value (°C)	6.9	6.8	6.9	0.1
E_a (kcal/mole)	83.0	83.6	83.3	0.4

*STDV: Standard Deviation

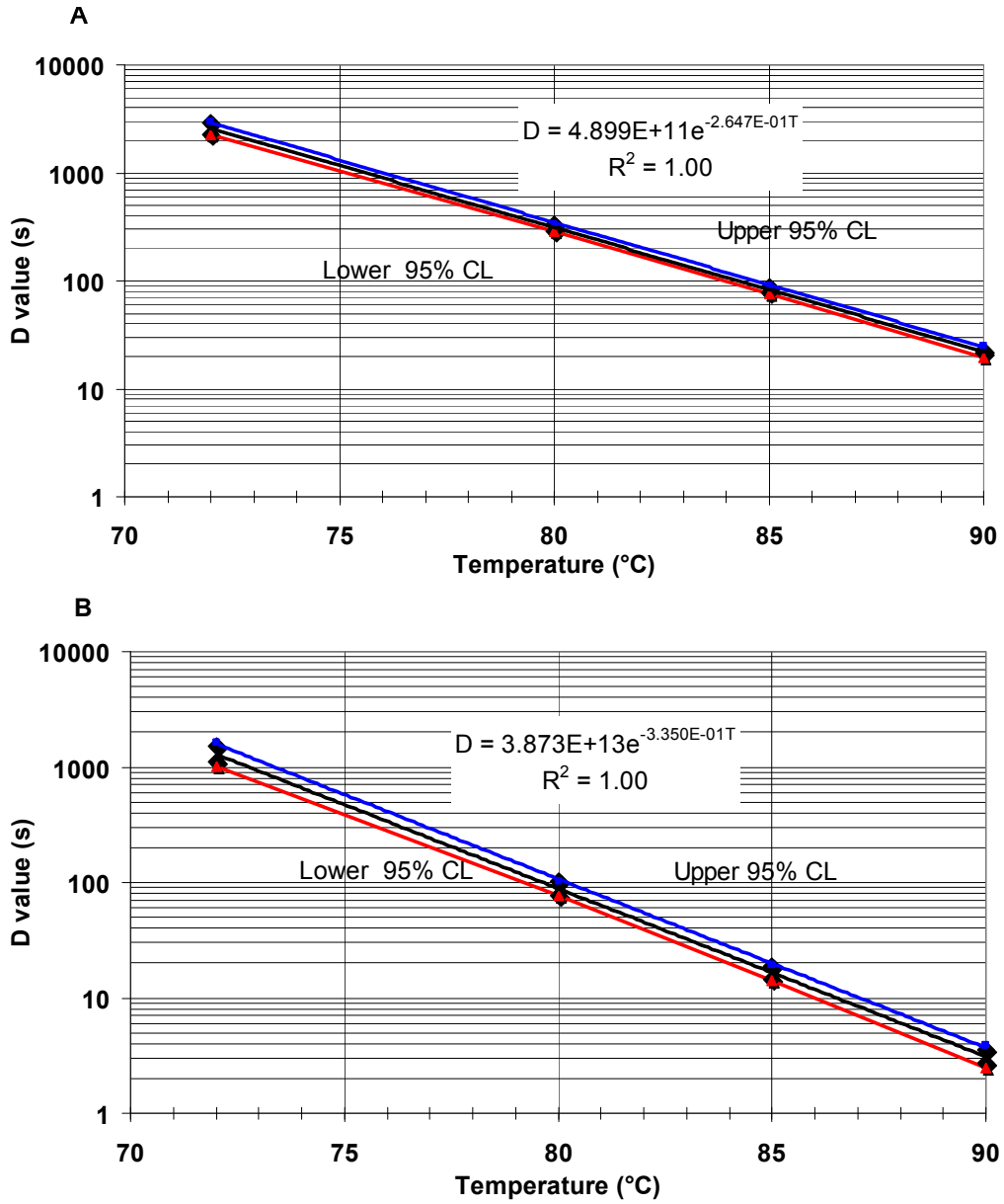


Figure 4-9. Relationship of D values of Shiga toxin 1 (A) and Shiga toxin 2 (B) with temperature when toxin preparations were suspended in orange juice.

The Arrhenius relationship between the inverse of the absolute temperature (Kelvin) and log reaction rate of inactivation of Shiga toxins in orange juice had a good fit with $R^2 > 0.97$. Examples from Table 4-6 and Table 4-7 are shown in

Figure 4-10. The calculated average activation energy E_a of Stx1 was less than that of Stx2 (67 versus 84 kcal/mole).

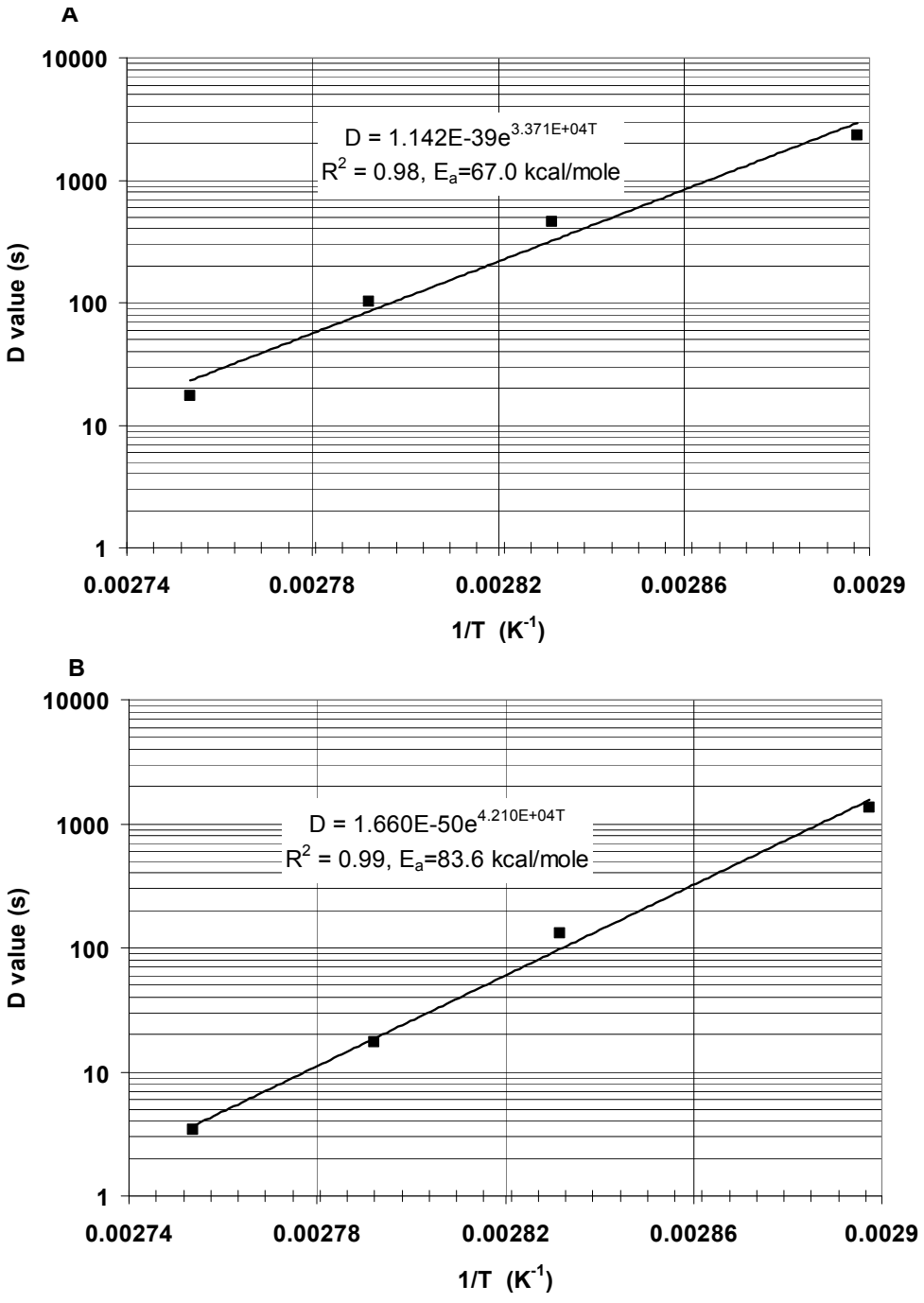


Figure 4-10. Arrhenius plots for inactivation of Shiga toxin 1 (A) and Shiga toxin 2 (B) in orange juice used to determine activation energy (E_a).

Kinetics parameters including inactivation rates (k), half lives, and 12 × half lives for inactivation of Shiga toxins in orange juice, as well as the 95% confidence limits are listed in Table 4-8 and Table 4-9. Correlation is good with $R^2 > 0.86$.

Table 4-8. The effect of temperature on inactivation rates (k) and half life ($t_{1/2}$) of Shiga toxin 1 in orange juice. Results of replicates are shown for each temperature.

Temp. (°C)	Rep #	R^2	k (95% CL) (s^{-1})	$t_{1/2}$ (95% CL) (s)	12 × $t_{1/2}$ (95% CL) (s)
72	1	0.92	0.0012 (0.0008, 0.0016)	555 (412, 846)	6653 (4949, 10149) [a*]
	2	0.95	0.0010 (0.0008, 0.0012)	728 (594, 942)	8739 (7122, 11307) [a]
80	1	0.87	0.0061 (0.0034, 0.0088)	113 (79, 203)	1356 (947, 2441) [b]
	2	0.93	0.0050 (0.0035, 0.0065)	138 (106, 198)	1655 (1269, 2379) [b]
85	1	0.97	0.04 (0.02, 0.06)	18 (12, 37)	215 (142, 440) [c]
	2	0.95	0.02 (0.02, 0.03)	31 (25, 40)	367 (300, 475) [c]
90	1	0.87	0.12 (**, 0.73)	6 (1, **)	67 (11, **) [d]
	2	0.91	0.13 (0.09, 0.18)	5 (4, 8)	62 (47, 92) [d]

* Same letter indicates not statistically different at $p \leq 0.05$

**Not applicable due to poor 95% CL

Table 4-9. The effect of temperature on inactivation rates (k) and half life ($t_{1/2}$) of Shiga toxin 2 in orange juice. Results of replicates are shown for each temperature.

Temp. (°C)	Rep #	R^2	k ± 95% CL (s^{-1})	$t_{1/2}$ (95% CL) (s)	12 × $t_{1/2}$ (95% CL) (s)
72	1	0.91	0.0018 (0.0010, 0.0026)	376 (263, 656)	4509 (3159, 7875) [e*]
	2	0.99	0.0017 (0.0016, 0.0018)	403 (373, 437)	4830 (4478, 5241) [e]
80	1	1.00	0.04 (0.03, 0.04)	19 (17, 22)	232 (204, 267) [f]
	2	0.97	0.02 (0.01, 0.02)	39 (31, 52)	470 (376, 628) [g]
85	1	1.00	0.16 (0.15, 0.16)	5 (4, 5)	53 (50, 57) [h]
	2	0.94	0.13 (0.03, 0.23)	5 (3, 21)	62 (36, 249) [h]
90	1	0.96	0.75 (0.26, 1.23)	1 (1, 3)	11 (7, 32) [j]
	2	0.93	0.68 (0.13, 1.23)	1 (1, 5)	12(7, 65) [j]

* Same letter indicates not statistically different at $p \leq 0.05$

D values obtained from inactivation of Shiga toxins in PBS and orange juice were analyzed by ANOVA. The p values for Shiga toxin type (Stx1 and Stx2), temperature (72, 80, 85 and 90°C) and medium type (PBS and orange juice) were 2.11×10^{-3}, 2.18×10^{-13} and 0.45, respectively (Table 4-10).

Table 4-10. ANOVA table for the inactivation of Shiga toxins in orange juice.

	Df*	Sum of Square	Mean Square	F value	Pr (>F)
Stx	1	2,334,388	2,334,388	11.08	2.11×10^{-3}
Temp.	3	36,249,135	12,083,045	57.3	2.18×10^{-13}
Medium	1	122,759	122,759	0.58	0.45
Residuals	34	0.45	210,702		

*Degrees of freedom

4.2.3 Confirmation of thermal inactivation by indirect enzymatic assay

1) Establishment of lactate dehydrogenase test

A lactate dehydrogenase (LDH) assay was used to confirm the inactivation of Shiga toxins in orange juice by measuring their effect on Vero cells. First, the optimal concentration of Vero cells that released significant LDH was determined (Figure 4-11). The R^2 value obtained from the linear regression indicated a relatively high correlation between concentration of cells and OD resulting from the reduction of tetrazolium dye. The OD values increased as the Vero cells concentration increased. A concentration of 10^6 cell/mL was chosen for additional experiments.

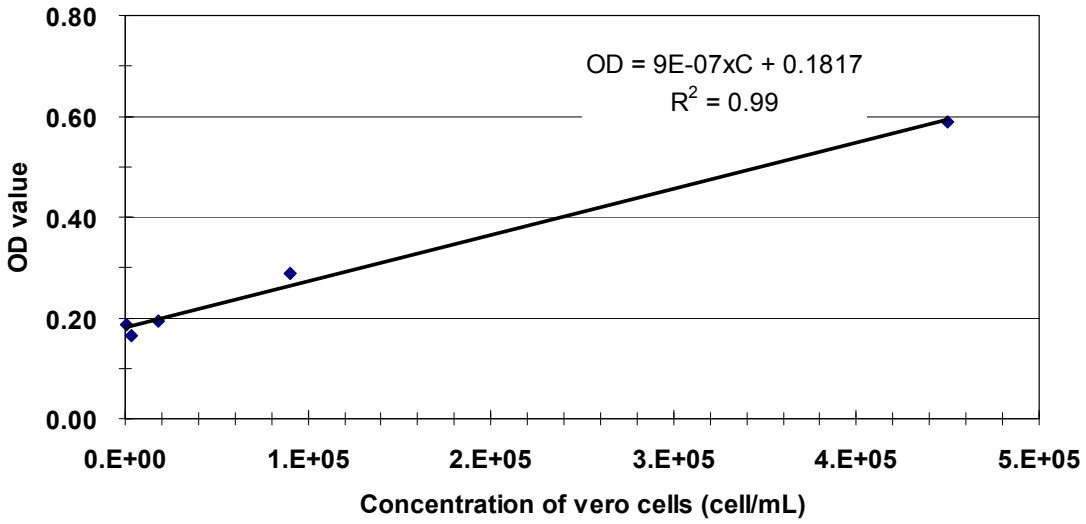


Figure 4-11. Effect of concentration of Vero cells on the amount of lactate dehydrogenase (LDH) released after overnight incubation measured by the change in absorbance (OD) at 490 nm using a commercial LDH assay.

Four incubation methods were tested to determine the best incubation condition for Vero cells and three different incubation times were tested (Figure 4-12). OD value differences between these four incubation conditions were the largest at 10^6 cell/mL Vero cells. At 2, 6 and 24 h, incubation in a tight CO_2 container in an air incubator caused similar LDH amounts to be released into the supernatant as compared incubation in a regular CO_2 incubator. At 6 h and 24 h incubation in air was the only condition that caused significantly greater OD readings. Condition B was selected as the method of incubation for further experiments.

2) Shiga toxin inactivation determined by LDH assay

Once the optimal cell concentration and incubation conditions were determined, the LDH assay with Vero cells was used to determine whether the ELISA test

was practical to determine the inactivation of Shiga toxins. Figure 4-13 shows the results of inactivation using the microcentrifuge tube method at 80, 85 and 90°C and tested by LDH assay using Vero cells. Since active Shiga toxins would attack Vero cells, it would release more LDH which would result in a higher OD value than that caused from normal cell death. At 85°C and 90°C, the OD value of inactive toxins in almost all repetitions for Stx1 and Stx2 were lower than the OD value of active toxins. These qualitative results confirmed the thermal inactivation of Shiga toxins. At 80°C, however, no clear difference trend was observed. Inactivation at 72°C was not done in this study.

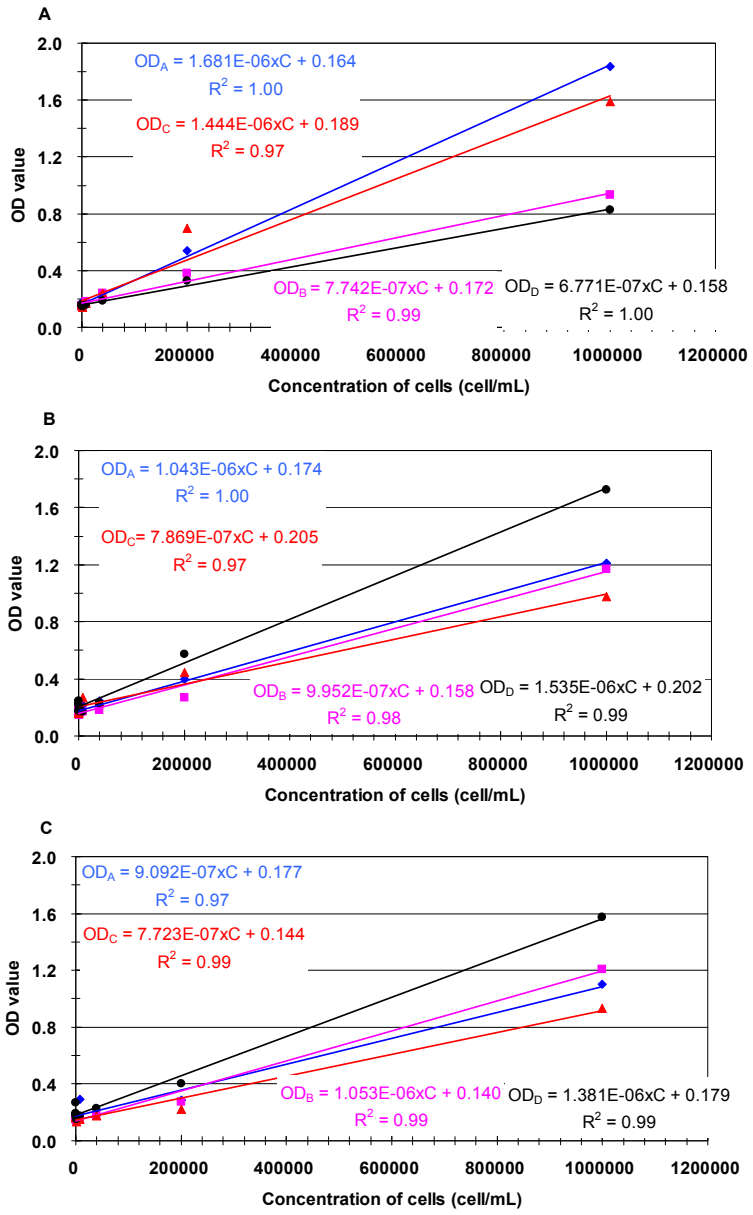


Figure 4-12. Comparison of incubation condition for Vero cells based on the release of lactate dehydrogenase (LDH) as measured by LDH assay at 2 (a), 6 (b) and 24 h (c). Incubation conditions: A, control standard CO_2 incubator (◆), B, tight CO_2 container in air incubator (■), C, CO_2 -flushed container in air incubator (▲), and D, plain air incubator (●).

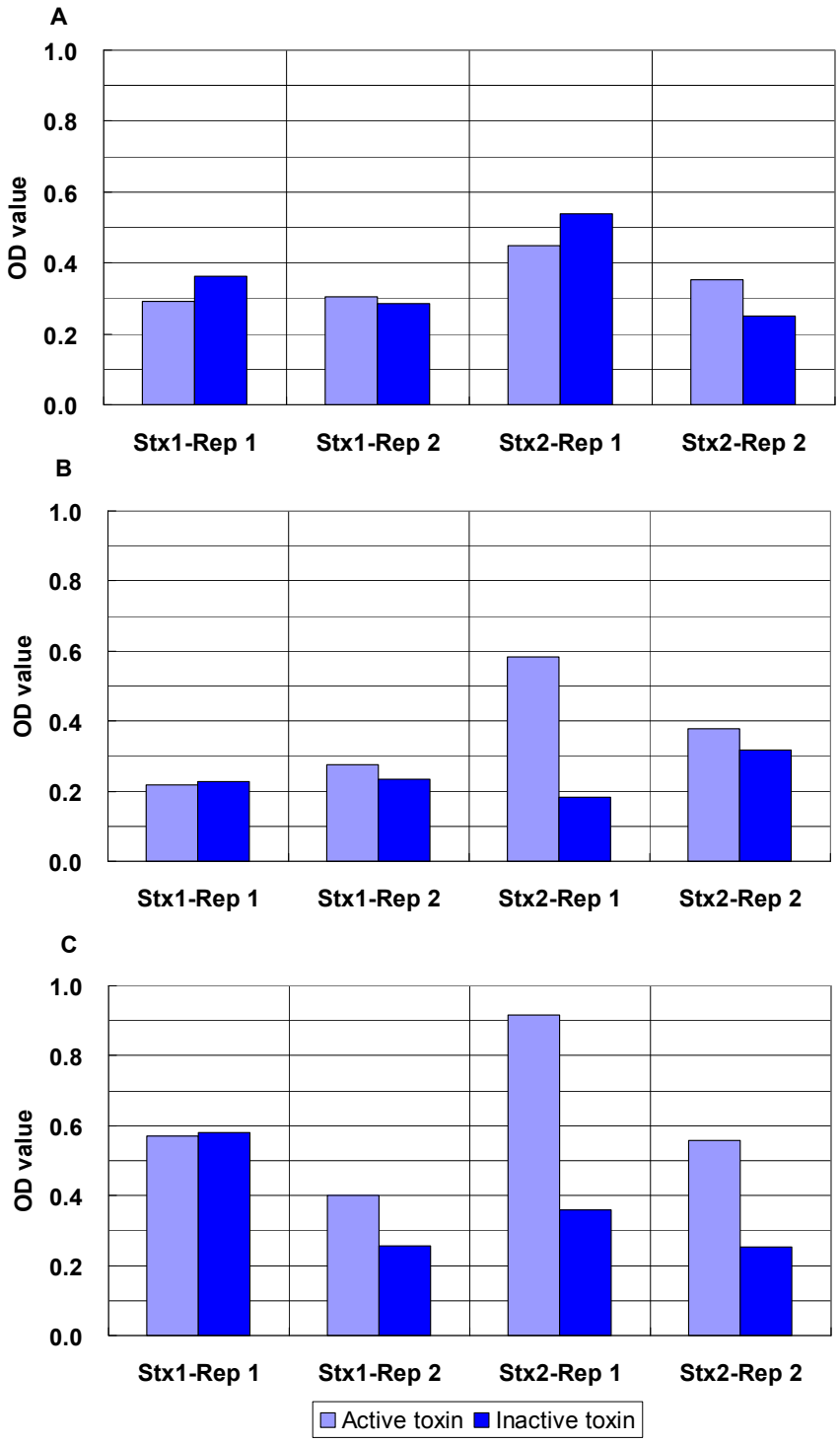


Figure 4-13. Inactivation of Shiga toxins in PBS as indicated by the release of lactate dehydrogenase measured by an LDH assay. Inactivation condition: 80°C for 5 h (A), 85°C for 4 h (B) and 90°C for 2 h (C).

4.3 Discussion

1) Thermal inactivation of Shiga toxins

The inactivation of Shiga toxins in PBS and orange juice at temperatures in the high temperature short time (HTST) pasteurization range has not been reported prior to this work. Although the stability of Shiga toxins as affected by thermal treatments had been studied before (4, 37, 58, 67), there was almost no information about the inactivation condition in a food matrix. This study determined the thermal inactivation kinetics of Shiga toxins at HTST pasteurization temperatures of 72, 80, 85 and 90°C. The kinetic factors including D value, Z value, half life, 12 half life reduction and activation energy (E_a) were determined through inactivation experiments of Shiga toxins in buffer (PBS) and orange juice.

The results of this study indicated that thermal treatment could be an effective method for inactivating Shiga toxins in PBS and orange juice. Although heat could not completely inactivate Shiga toxins at standard HTST pasteurization conditions (72°C, 15 s; 90°C, 0.5 s), the activity of Shiga toxins was remarkably reduced with increased temperature due to denaturation of the toxin protein. The half life of both Shiga toxins decreased at least 100-fold when the heating temperature was increased from 72 to 90°C in both PBS and orange juice.

Previously published studies on thermal inactivation of Shiga toxins in Tris-buffered saline or other pH controlled buffers also confirmed that there was a temperature and time-dependent decrease in the activity of Shiga toxins when heated above 70°C (37, 67). However, due to the different detection and inactivation methods, the inactivation time and temperature results were different. For instance, a previous study (37) reported that Shiga toxin 1 in Tris-buffered saline could be completely inactivated at 80°C for 60 min or 85°C for 5

min while in the present study we found that both Shiga toxins in PBS were completely inactivated 10 s at 90°C and 30 s at 85°C. Detection limits of each method played a key role in the final results.

The intravenous and intraperitoneal LD_{50} of Shiga toxin was determined to be 0.05 µg/kg body weight for mice (48), but there is no information on a similar oral dose. As a risk assessment exercise, we could assume that the oral lethal dose could be 100 times the intraperitoneal dose which would be 5 µg/kg and the lethal dose for a 20 kg child is 100 µg. For instance, a 7,000 gal (26,409 kg) silo of orange juice is able to produce 106,000 servings of orange juice (0.25 kg/serving in a 250 mL juice bottle). A hypothetical intentional attack would require a terrorist to add only 11g of Shiga toxin to get a potential lethal dose in every serving of the orange juice. Under these assumptions, it would then be relatively easy for bioterrorists to deliver sufficient Shiga toxin into tanks in an orange juice plant. Assuming a bottle of orange juice with a volume of 8 oz is deliberately adulterated by addition of 100 µg of Shiga toxin, a 1000 fold reduction to meet a theoretical requirement from EPA would require at least 60 s and 10 s for Stx1 and Stx2, respectively, with a standard treatment at 90°C. Such a reduction is possible in many pasteurizers with the proper hold tube and Shiga toxins would be reduced to 0.1% of the lethal dose which would then be presumably inactive and safe for disposal.

Conventional pasteurization processing requires 30 min at 63°C, 15 s at 72°C, 1.0 s at 89°C and 0.5 s at 90°C. A comparison of the standard processing time versus the D values is listed in Table 4-11. As seen, standard pasteurization would not ensure complete inactivation of ricin or Shiga toxins. However, a 1000 fold reduction required by EPA with longer processing time is possible in many pasteurizers with the proper hold tube.

Table 4-11. Number of D values accomplished within standard processing time under HTST temperatures.

Temp. (°C)	Standard processing time (s)	No. of D values		
		Ricin	Stx1	Stx2
72	15	0.0007	0.006	0.01
90	0.5	0.003	0.02	0.2

2) Comparison of inactivation results in different food matrices

The ANOVA results with a p value of 0.45 in the present study suggested that there was little effect of liquid media on D values. PBS is composed of salts including NaCl, KCl, Na_2HPO_4 and KH_2PO_4 with a pH of 7.2 while orange juice is composed of sugars, salts, citric acid and wide variety of other organic compounds with a pH of ~3.5. The pH difference had little effect on the rate of inactivation and D value at each temperature. These results may suggests that thermal stability of Shiga toxins was not affected by different matrices and no significant difference in inactivation rate was apparent whether determined in PBS or orange juice (Table 4-12).

Table 4-12. Comparison of inactivation rates (k) of Shiga toxins in PBS and juice.

Medium	Temp.(°C)	Stx1	Stx2
PBS	72	0.0008	0.0016
	80	0.01	0.02
	85	0.11	0.13
	90	0.39	0.46
Juice	72	0.0011	0.0018
	80	0.01	0.03
	85	0.03	0.14
	90	0.13	0.71

Nonetheless, a comparison of the results of Shiga toxin inactivation in different buffer systems indicates significant effects from the matrices. The present study showed the half life for inactivation of Shiga toxin 1 in PBS was 91 s (~1.5 min) at 80°C. In contrast to the results of the current study, previous investigation showed the half life of Shiga toxin 1 in Tris-buffered saline was 4.5 min at 80°C (calculated from the reported data). One notable difference between the experiments is that the previous study used 1.5 mL sample size versus 50 µL in the current study. In addition, the difference in methodology may explain this discrepancy, as the previous study only examined the inactivation at 80°C for 5, 15, 30 and 60 min.

3) Comparison of inactivation results for Stx1 and Stx2

The ANOVA results with a p value of 2.11×10^{-3} suggested that the D value of

Shiga toxin was affected by the toxin type. The enzymatic activities of Shiga toxin 1 and Shiga toxin 2 are indistinguishable in cell-free systems as well as their specific activities for Vero cells (48). However, the toxicities of these two types of toxins are significantly different in mice. Since Stx2 binds to the Gb3 receptor in cells less tightly than Stx1, it may be more easily released from the receptor before endocytosis and is exposed to more (48). As a result, Shiga toxin 2 is 400 times more toxic than Shiga toxin 1. In addition, the structural differences of these two Shiga toxins in the length and sequence of amino acids are possible reasons for different D values after heat treatment (48).

4) LDH assay for confirmation of inactivation

An indirect LDH assay that measured the biological effect of Shiga toxins on Vero cells was used to confirm their biological inactivation in this study. Alternative incubation conditions were determined since toxins could not be taken out of the biosafety laboratory where a regular CO_2 incubator was not available. CO_2 and humidity are two key factors for the healthy growth of Vero cells. A CO_2-bicarbonate system is the main buffering system in most media. The interaction of CO_2 derived from the cells or the atmosphere with water leads to a decrease in pH due to the formation of carbonic acid when CO_2 dissolves in water (46).

Shiga toxins were inactivated at 90°C for 2 h, 85°C for 4 h and 80°C for 5 h in microcentrifuge tubes. There was enormous variability apparently due to the unique physiology of Vero cells and the reproducibility of the measurements was markedly affected, especially at 80°C. With increasing inactivation time, some unknown reactions occurred during the inactivation period. Along with the uncertainty of the LDH assay, the OD value after the heat treatment sometimes was higher than the one before. However, the inactivation of Shiga toxins was confirmed based on the reduction of activity at 85°C and 90°C. The

determination of the LDH released by Vero cells was proven to be inconsistent at lower temperatures.

5) Comparison with other agents

The agents which could be used as bioterror weapon, including botulism toxin, ricin, Shiga toxin and *Bacillus anthracis* strain ANR-1 spores, were compared with each other. The amounts of these agents that are lethal to a 20 kg child for an oral dose in one serving of orange juice were calculated and summarized in Table 4-13. Botulism toxin has the lowest amount needed to be lethal as compared to ricin which has the largest amount needed. The amount of Shiga toxin required to result in death is almost 40 fold less than that of ricin. Although these values are significantly different, the maximum amount needed is only 424 g and this is indicating that all of these agents are main concerns as biological warfare in food systems.

Kinetic parameters for these agents were also collected and correlation between safe inactivation time and temperature are compared in Figure 4-14. Generally, botulism toxins needed the shortest heat treatment time to achieve a safe level comparing to any other agents. In contrast, *Bacillus anthracis* spores required the longest time which was significantly different from other agents. The heat treatment time for Shiga toxin in orange juice was approximately one log difference less than that for ricin in orange juice and more than one log difference as compared to that for *B. anthracis* spores to achieve a safe level. Shiga toxin required a shorter time to be inactivated to get a presumable safe level than ricin. In all cases, the protein toxins require less time for inactivation to a safe level as compared to *Bacillus anthracis* spores. The evaluation of the lethality and time required to inactivate these agents to a safe level indicated their great potential as bioterror weapon in a food supply system.

Table 4-13. Hypothetical amount of toxins in a 7,000 gal silo of orange juice lethal to half of the children that would drink the product, assuming 20 kg children with one serving of orange juice.

Toxin	LD_{50}	Amount in one serving	Amount in 7,000 gal silo
Botulism toxin	0.001 µg/kg (77)	0.02 µg	2 mg
Ricin	0.2 mg/kg (29)	4 mg	424 g
Shiga toxin	0.05 µg/kg *	100 µg	11 g
***B. anthracis* spores**	10^6 spores (1 µg) **	10^6 spores (1 µg)	1.06×10^{11} spores (~100 mg)

* approximate oral LD_{50} = 100 × intravenous or intraperitoneal LD_{50}; (48)

**Infectious dose; Jeffrey Bender [College of Veterinary Medicine, University of Minnesota], personal communication.

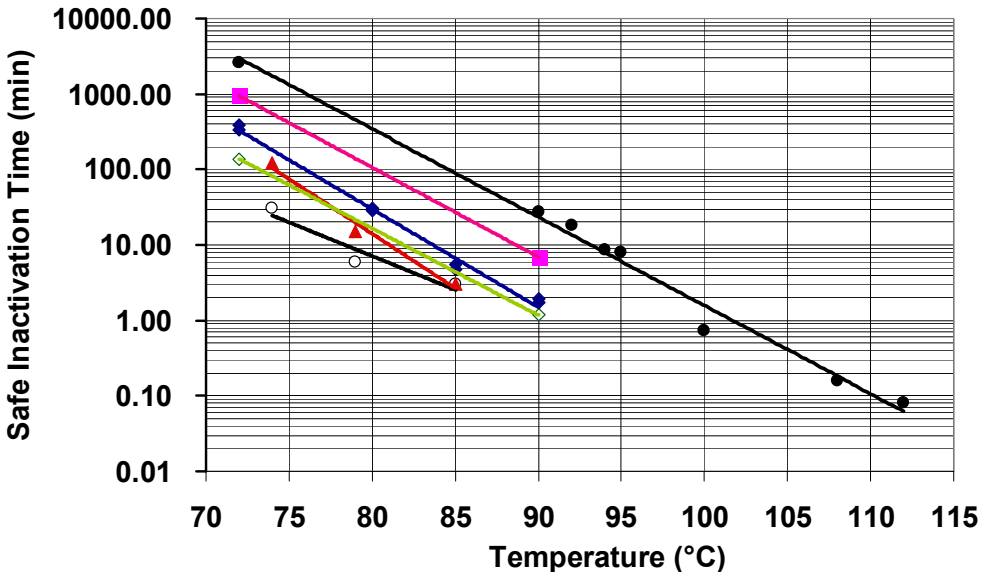


Figure 4-14. Comparison of temperature dependence for safe inactivation time (10 half lives) for *Bacillus anthracis* spores (●) (78), ricin in milk (◆) (Xu and others, unpublished), botulism toxin (BT) (76) in canned corn (○) (pH 6.2) and phosphate buffer (▲) (pH 6.8), ricin in orange juice (■) and Shiga toxin in orange juice (◇).

In summary, this is the first study that reports the thermal inactivation of Shiga toxins in orange juice at temperatures within the HTST pasteurization range. A set of temperature/time processing conditions have been identified that could be useful in the event of a bioterrorist attack. The heat inactivation of Shiga toxins in PBS and orange juice followed first-order reaction kinetics. Both Shiga toxins in PBS and orange juice would reach the concentration that was not detectable by use of ELISA kit within 30 s at 90°C and 120 s at 85°C. The Z value for Stx1 and Stx2 was 6.7 and 7.2 in PBS as well as 8.7 and 6.9 in orange juice. This study generated initial data that could lead to the development of feasible and effective decontamination procedures which could be applied in orange juice plant with typical pasteurization equipment to mitigate the impact of an intentional attack with Shiga toxins. Recommendations for further studies may include animal studies to evaluate the NOAEL and oral

lethal dose of ricin and Shiga toxins, correlation studies of ELISA method and cytotoxicity assay as well as the inactivation studies for other select agents in food systems.

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6 Appendix

1) Permission from Kirsten Sandvig

From Na Wang (wang1127@umn.edu):

Dear Dr. Sandvig,
I am a master student in the Department of Food Science and Nutrition in University of Minnesota. I am writing to ask your permission to cite your figure 1 in "Entry of ricin and Shiga toxin into cells: molecular mechanisms and medical perspectives" in my master thesis. This is for research purpose only and is going to be in my thesis to explain the structure of ricin and Shiga toxin. Please let me know whether I can cite it. Thank you very much!
Have a nice day.
Na Wang

From Kirsten Sandvig (kirsten.sandvig@imbv.uio.no):

Dear Na Wang,
It is fine with me.
Best regards,
Kirsten Sandvig

2) Permission from Jeffrey Pommerville

From Na Wang (wang1127@umn.edu):
Dear Dr. Pommerville,
I am a master student in the Department of Food Science and Nutrition in University of Minnesota. I am writing to ask your permission to cite your figure 4 in "Integrating the agents of bioterrorism into the general biology curriculum II.

mode of action of the biological agents" in my master thesis. This is for research purpose only and is going to be in my thesis to explain the mode of action of ricin. Please let me know whether I can cite it. Thank you very much!
Have a nice day.
Na Wang

From Jeffrey Pommerville (jeffrey.pommerville@gcmail.maricopa.edu)

Mr Wang,
You can certainly cite the figure. Good luck to you!
Dr. Pommerville

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