THE STABILITY OF *CLOSTRIDIUM BOTULINUM* TOXIN TYPE A ON FRESH AND FRESH-CUT PRODUCE

by

ROBIN GWEN SALINSKY BROEKER

(Under the Direction of Mark Harrison)

ABSTRACT

Foodborne botulism is a potentially lethal neuroparalytic illness resulting from ingesting *C. botulinum* neurotoxins. There is little information concerning the stability of preformed toxin on fresh produce. This research determined the stability of toxin type A on whole grape tomatoes, pre-cut lettuce, and pre-sliced cantaloupe. Different combinations of toxin inoculum pH (5.5 or 7.0) and storage temperature (4, 15, or 25^{o}C) were analyzed to determine their relationship to the rate of toxin decay. The toxin was measured using the DIG-ELISA method, which screens for the presence of botulinal toxin type A. For tomatoes, the toxin inoculum at pH 5.5 and storage temperature of 25^{o}C provided the most stability for the toxin. For lettuce, the toxin inoculum at pH 7.0 and storage temperature of 15^{o}C provided the most stability. For cantaloupe, only the temperature of 4^{o}C provided stability to the toxin.

INDEX WORDS: *Clostridium botulinum*, toxin type A, tomatoes, lettuce, cantaloupe, ELISA, pH

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ROBIN GWEN SALINSKY BROEKER

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ROBIN GWEN SALINSKY BROEKER

Major Professor: Mark Harrison

Committee: Joseph F. Frank
Robert Shewfelt

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

INTRODUCTION

Increased consumer awareness of the nutritional benefits of fruits and vegetables, busier lifestyles, and more spending power (Rajkowski and Baldwin, 2003) have helped fresh-cut produce to become one of the fastest growing convenience foods in history (Zhuang et al., 2003). Fresh-cut produce is defined as any fresh fruit or vegetable or any combination thereof that has been physically altered from its original form yet remains in a fresh state (IAFP, 2005). Fresh-cuts receive no thermal or other preservative treatments designed to reduce or eliminate microbial load. New technologies, such as modified atmosphere packaging (MAP), have extended the shelf-life of fresh-cut produce while at the same time reducing decay and spoilage organisms. This has resulted in a shift in microbial population dynamics that favor growth of human pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Clostridium botulinum* (Rajkowski and Baldwin, 2003).

Spores of *C. botulinum* are ubiquitous in the environment (Hauschild, 1989) and may be found on many types of raw vegetables (Hao et al., 1998, 1999). However, botulism associated with the consumption of fresh produce has not been a major food safety concern since *C. botulinum* requires anaerobic conditions for growth. With the advent of MAP, concerns have been raised about the potential for production of botulinal toxin in these products. Challenge studies using both proteolytic and nonproteolytic strains of *C. botulinum* have been conducted to determine the safety of minimally processed fruits and vegetables in modified atmospheres (Hotchkiss et al., 1992; Larson et al., 1997; Austin et al., 1998; Hao et al., 1998, 1999; Larson and Johnson, 1999). These studies indicated that produce might become toxic but only after

becoming spoiled beyond the point of being organoleptically acceptable. Only butternut squash and onion remained acceptable after detection of toxin (Austin et al., 1998).

Ingesting the neurotoxins of the anaerobic, spore-forming bacterium, *C. botulinum*, causes foodborne botulism. Although seven immunologically distinct toxins exist, designated type A through G, types A, B, and E are most frequently associated with foodborne botulism (Smith, 1977). Botulism neurotoxins act preferentially on peripheral cholinergic nerve endings to block acetylcholine release thus producing paralysis of the motor system (Simpson, 2004). Clinical illness is characterized by symmetric descending paralysis (proximal to distal) first appearing in the cranial nerve area. If left untreated, death will result from respiratory failure, airway obstruction, or failure of the heart muscles (CDC, 1998; Lund and Peck, 2001). Clinical recovery can take weeks to months (Shapiro et al., 1998).

There is little information concerning the stability of preformed toxin on fresh produce. This should be of concern because not only can botulism occur naturally as a form of accidental food poisoning but it can also occur unnaturally as a product of malice. Various countries and terrorist groups have developed botulinum toxin as a biological weapon that could be disseminated by deliberate contamination of the food supply (Arnon et al., 2001). Contamination of a widely distributed food product could affect large numbers of persons.

The only validated *in vivo* method to detect *C. botulinum* toxin is the AOAC (2000) mouse bioassay, which is usually performed in a sequence of related analysis. The samples are first screened for the presence of toxin by i.p. injections into pairs of mice. If the mice die with botulinum symptoms, then the end point of toxicity is determined. Finally, a neutralization assay is performed to determine the toxin type(s) present (AOAC, 2000). The disadvantages of the mouse bioassay are that each step requires two days of analysis and can only be conducted in

facilities that have mice available for the test. There is a need for a more rapid method to detect botulinum toxins along with the ability to examine large numbers of samples. The enzyme-linked immunosorbent assay (ELISA) satisfies these requirements.

This research focused on the rate of decay of *C. botulinum* toxin type A on fresh-cut lettuce, fresh-cut cantaloupe, and whole grape tomatoes. For this research, the toxin was measured using the DIG-ELISA method (Ferreira et al., 2002), which has shown considerable promise as a rapid screening tool for *C. botulinum* toxin in food.

REFERENCES

Austin, J.W., K.L. Dodds, B. Blanchfield, and J.M. Farber. 1998. Growth and toxin production by *Clostridium botulinum* on inoculated fresh-cut packaged vegetables. *J. Food Prot.* 61:342-328.

AOAC: Association of Analytical Chemists. 2000. *Clostridium botulinum* and its toxins in foods, p. 68-70. *In* W. Horwitz (ed.), Official methods of analysis of AOAC international, 17th ed. AOAC, Inc., Gaithersburg, MD.

Arnon, S.S., R. Schechter, T.V. Inglesby, D.A. Henderson, J.G. Bartlett, M.S. Ascher, E. Eitzen, A. D. Fine, J. Hauer, M. Layton, S. Lillibridge, M.T. Osterholm, T. O'Toole, G. Parker, T.M. Perl, P.K. Russell, D.L. Swerdlow, and K. Tonat. 2001. Botulinum toxin as a biological weapon. *JAMA.* 285:1059-1070.

CDC: Centers for Disease Control and Prevention. 1998. Centers for Disease Control and Prevention: Botulism in the United States, 1899-1996. Handbook for epidemiologists, clinicians, and laboratory workers. Atlanta, GA.

Ferreira, J.L., S. Maslanka, and J. Andreadis. 2002. Detection of type A, B, E, and F *Clostridium botulinum* toxins using digoxigenin-labeled IgGs and the ELISA. FDA/ORA Laboratory Information Bulletin, vol. 18, number 10. 4292:1-10.

Hao, Y.Y., R.E. Brackett, L.R. Beuchat, and M.P. Doyle. 1998. Microbiological quality and the inability of proteolytic *Clostridium botulinum* to produce toxin in film-packaged fresh-cut cabbage and lettuce. *J. Food Prot.* 61:1148-1153.

Hao, Y.Y., R.E. Brackett, L.R. Beuchat, and M.P. Doyle. 1999. Microbiological quality and production of botulinal toxin in film-packaged broccoli, carrots, and green beans. *J. Food Prot.* 62:499-508.

Hauschild, A.H.W. 1989. *Clostridium botulinum,* p. 111-189. *In* M.P. Doyle (ed.), Foodborne bacterial pathogens. Marcle Dekker, Inc. New York.

Hotchkiss, J.H., M.J. Banco, F.F. Busta, C.A. Genigeorgis, R. Kociba, L. Rheaume, L.A. Smoot, J.D. Schuman, and H. Sugiyama. 1992. The relationship between botulinal toxin production and spoilage of fresh tomatoes held at 13 and 23°C under passively modified and controlled atmospheres and air. *J. Food Prot.* 55:522-527.

IAFP: International Fresh Cut Association. 2005. http://www.fresh-cuts.org Accessed 1 March 2005.

Larson, A.E., E.A. Johnson, C.R. Barmore, and M.D. Hughes. 1997. Evaluation of the botulism hazard from vegetables in modified atmosphere packaging. *J. Food Prot.* 60:1208-1214.

Larson, A.E. and E.A. Johnson. 1999. Evaluation of botulinal toxin production in packaged fresh-cut cantaloupe and honeydew melons. *J. Food Prot.* 62:948-952.

Lund, B.M. and M.W. Peck. 2001. *Clostridium botulinum*, p. 69-85. *In* R.G. Labbé and S. García (ed.), Guide to foodborne pathogens. John Wiley & Sons, Inc. New York.

Rajkowski, K.T. and E.A. Baldwin. 2003. Concerns with minimal processing in apple, citrus, and vegetable products, p. 35-52. *In* J.S. Novak, G.M. Sapres and V.K. Juneja (ed.), Microbial safety of minimally processed foods. CRC Press. Boca Raton.

Shapiro, R.L., C. Hatheway, and D.L. Swerdlow. 1998. Botulism in the United States. *Ann. Intern. Med.* 129:221-228.

Simpson, L. 2004. Identification of the major steps in botulinum toxin action. *Annu. Rev. Pharmacol. Toxicol.* 44:167-93.

Smith, L.D. 1977. Botulism: the organism, its toxins, the disease. Charles C. Thomas. Springfield, IL.

Zhuang, H., M.M. Barth, and T.R. Hankinson. 2003. Microbial safety, quality, and sensory aspects of fresh-cut fruits and vegetables, p. 255-278. *In* J.S. Novak, G.M. Sapres and V.K. Juneja (ed.), Microbial safety of minimally processed foods. CRC Press. Boca Raton.

CHAPTER 1

LITERATURE REVIEW

Consumption of fresh fruits and vegetables in the United States has increased during the last two decades as Americans try to maintain healthier lifestyles (Pollack 2001). Public information campaigns, such as *Five-a-Day for Better Health* by the National Cancer Institute (NCI) and the United States government's *Dietary Guidelines for Americans* promote good health through increased consumption of fresh fruits and vegetables (HHS, 2002, 2005; USDA, 2005). The Five-a-Day for Better Health Program, a national initiative founded in 1991, encourages all Americans to increase consumption of fruits and vegetables to between 5 and 9 servings a day, which may reduce the risk for major chronic diseases such as high blood pressure, heart disease, diabetes, stroke, many cancers, and other chronic diseases (HHS, 2002).

In 2005, the United States Department of Health and Human Services (HSS) along with the U.S. Department of Agriculture (USDA) released the latest version of *Dietary Guidelines for Americans*. This guideline provides science-based advice using diet and exercise to promote health and to reduce the risk for major chronic diseases. A key diet recommendation included consuming more fruits and vegetables along with whole grains, while limiting meats and foods with a high fat content (HHS, 2005; USDA, 2005).

This dietary shift from a predominately meat-and-potato diet to a diet rich in fruits and vegetables has altered the epidemiology of foodborne disease in the United States (Hedberg et al., 1994; Sivapalasingam et al., 2004). An increasing number of outbreaks caused by foodborne pathogens reported to the Centers for Disease Control and Prevention (CDC) has been associated with fresh produce consumption. This represents a change from the vehicles traditionally associated with outbreaks of foodborne illness, generally foods of animal origin, e.g., meat, poultry, seafood, eggs, milk, and dairy products (Hedberg et al., 1994), to foods previously thought to be safe (Tauxe et al., 1997).

Microorganisms should be expected on fresh produce since it is a raw agricultural commodity (Tauxe et al., 1997). Although most pathogens are not native to fresh produce, evidence shows that fresh produce of all types can harbor pathogens (Brackett, 1999; Zhuang et al., 2003). The lack of a microbial intervention treatment during production, processing, or preparation intensifies the difficulty of eliminating the risk associated with consuming fresh produce. By identifying and controlling risk from the farm to the table, it is possible to reduce the risk of illness (Tauxe et al., 1997).

Under most circumstances, the exterior of produce acts as a physically barrier preventing bacteria from penetrating into the interior. Pathogens may adhere tightly to the surface of produce, which makes removing them difficult. Once the surface integrity is compromised, bacterial growth can be rapid. Mechanical processing, e.g., cutting, shredding or juicing, of the produce increases the risk of bacterial growth. Consequently, foodborne diseases associated with fresh produce often involve produce that has undergone some kind of minimal, nonthermal processing, e.g., fresh-cut fruits and vegetables and fresh-squeezed juice (Tauxe et al., 1997).

Fresh-cut produce is defined as any fresh fruit or vegetable or any combination thereof that has been physically altered from its original form yet remains in a fresh state. Regardless of commodity, it has been trimmed, peeled, washed, and/or cut into 100% usable product that is subsequently bagged or prepackaged to offer consumers high nutrition, convenience and value while maintaining freshness (IAFP, 2005). New technologies, such as modified atmosphere packaging (MAP), have extended the shelf-life of fresh-cut produce while at the same time reducing decay and spoilage microorganisms. This has shifted the microbial population dynamics that favor growth of human pathogens such as *Escherichia coli*, *Listeria monocytogenes*, and *Clostridium botulinum* (Rajkowski and Baldwin, 2003).

Clostridium botulinum

Between 1815 and 1828, Justinius Kerner documented cases of "sausage poisoning" (botulism) in Germany, identifying blood and liver sausages as the predominant cause (Smith, 1977; Hauschild, 1989). Emile Pierre Marie van Ermengem in 1897 first isolated and characterized *Clostridium botulinum* after his investigation of a foodborne outbreak involving ham, in Ellezells, Belgium; three people died (Smith, 1977; Smith and Sugiyama, 1988; Hatheway, 1993; CDC, 1998). In 1904, 11 people died from eating wax-bean salad in Darmstadt, Germany. This was the first time a vegetable had been implicated in an outbreak of botulism (Sakaguchi, 1983; Hauschild, 1989). Today, incidences of botulism in the United States are primarily associated with home processed vegetables (Sobel et al., 2004).

C. botulinum is an anaerobic, gram-positive, spore-forming, rod-shaped bacterium producing a potent toxin known as botulinum neurotoxin (BoNT) (FDA/CFSAN, 1992; Solomon et al., 2001; Solomon and Lilly, 2001). The resulting neuroparalytic illness is known as botulism (CDC, 1998; Solomon et al., 2001). The strains of *C. botulinum* produce seven antigenically distinct neurotoxins, designated A, B, C, D, E, F, and G (Smith, 1977; ICMSF, 1996).

According to *Bergey's Manual of Systematic Bacteriology*, the strains of *C. botulinum* are classified into four groups (designated Groups I through IV) based on physiological differences (Cato et al., 1986). In addition, neurotoxin-producing strains of *Clostridium butyricum* and *Clostridium baratii* have been identified as two additional genomic groups that produce BoNT distinct from *C. botulinum* (Austin, 2001).

Group I strains are proteolytic (i.e., activated by endogenous proteases which enables the decomposition or digestion of proteins, peptides, and/or amino acids), grow at temperatures ranging from 10-48^{o}C (optimal growth temperature is 37^{o}C), and have spores with high heat

resistance ($D_{100°C}$ value ~ 25 min.). This group produces toxin of either type A, B, or F. Types A and B strains generally produce 10^6 mouse lethal doses (LD_{50}) of toxin per ml in cultures whereas type F strains produce between $10^3 – 10^4$ LD_{50}/ml (Hatheway, 1993; Austin, 2001). *C. sporogenes* appears to be a nontoxigenic variant of this group (Cato et al., 1986; Hatheway, 1993; ICMSF, 1996).

Group II strains are nonproteolytic, grow optimally at 30°C although they can grow at refrigerated temperatures (as low as 3°C), and have spores with low heat resistance ($D_{100°C}$ values less than 0.1 min). This group produces toxin types B, E, or F. Since these bacteria lack proteolytic enzymes, their toxicity is increased through trypsinization (addition of trypsin to activate the neurotoxin). In cultures, Type E strains generally produce 10^5 LD_{50}/ml after activation. There appears to be no named nontoxigenic variants of this group (Hatheway, 1993; ICMSF, 1996).

Group III strains are nonproteolytic, grow optimally at 40°C although they can grow as low as 15°C, and have spores with intermediate heat resistance ($D_{100°C}$ values ~ 0.1-0.9 min). This group produces toxin types C and D, which are not involved in human botulism but rather animal botulism (Hatheway, 1993). *C. novyi* appears to be a nontoxigenic variant of this group (Cato et al., 1986; Hatheway, 1993; ICMSF, 1996).

Group IV strains, which are also classified as a different species, *C. argentinense* (Suen et al., 1988), are proteolytic, grow optimally at 37°C although they can grow as low as 10°C, and have spores with fairly low heat resistance ($D_{104°C}$ values ~ 0.8-1.12 min). This group produces toxin type G; approximately 50 LD_{50}/ml is produced in chopped meat broth with higher toxin levels (10^4-10^5 LD_{50}/ml) obtained through dialysis cultures. *C. subterminale* appears to be a nontoxigenic variant of this group (Cato et al., 1986; Hatheway, 1993).

Botulinum Neurotoxin

As stated, there are seven serologically different neurotoxins that are produced by various strains of *C. botulinum*. All seven are similar in structure, mode of action (Austin, 2001) and molecular weight (approximately 150kDa) (Hatheway, 1993; Lund and Peck, 2001). Botulinum neurotoxins are produced during growth (between 24 and 48 h), within the clostridial cell (Duda and Slack, 1969). They generally accumulate in the culture fluids, largely through cell lysis but the toxin can also be released during the logarithmic phase, long before any significant lysis has occurred (Siegel and Metzger, 1979).

Botulinum neurotoxins initially form as single-chained polypeptides with relatively low toxicity (Lund and Peck, 2001). The single-chained form becomes activated when it is "nicked" by extracellular bacterial proteases or by an added enzyme such as trypsin into a dichain molecule held together by a disulfide bond (Sakaguchi et al., 1984; Hatheway, 1993; Lund and Peck, 2001). This dichain molecule consists of a light chain (L) and a heavy chain (H) having molecular weights of approximately 50 and 100 kDa, respectively (DasGupta, 1981). This dichain molecule has been termed S (small), and is referred to as the "derivative toxin" (Lamanna and Sakaguchi, 1971; Sakaguchi et al., 1984) or "neurotoxin" (Sugiyama, 1980). With this conformational change, the botulinum neurotoxin becomes one of the most toxic biological substances known (FDA/CFSAN, 1992).

Botulinum neurotoxins form complexes with nontoxic proteins in foods and culture supernatant fluids to form progenitor toxins (Lamanna and Sakaguchi, 1971; Sakaguchi et al., 1981). Three forms of progenitor toxins have been identified: M (medium, sediments at 12S, 300 kDa), L (large, sediments at 16S, 500 kDa), and LL (extra-large, sediments at 19S, ~900 kDa) (Sakaguchi et al., 1981; Sakaguchi et al., 1984; Sakaguchi, 1990). The M form consists of

the S or “derivative toxin” with an atoxic, nonhemagglutinin protein. It is the most common progenitor toxin (Hauschild, 1989), found in all but type G (Sakaguchi, 1990). The L form consists of the M toxin with the addition of a hemagglutinin protein and is found in types A-D, and G. The LL form consists of the M toxin with the addition of a larger hemagglutinin protein than the one found in the L form and is found only in type A (Sakaguchi et al., 1981; Sakaguchi et al., 1984; Sakaguchi, 1990). Under mild alkaline conditions, the progenitor toxins will dissociate into the S form and atoxic components (Hauschild, 1989).

Once ingested orally and absorbed by the small intestine, botulinum neurotoxin acts by blocking the exocytic release of the neurotransmitter acetylcholine from synaptic vesicles at the neuromuscular junction (Hatheway, 1993; Austin, 2001) by three main steps. They are (a) binding, (b) productive internalization, and (c) intracellular poisoning. Each of these main steps can be further subdivided into two steps.

The receptor for the botulinum toxin to bind to the neuromuscular junction has not been clearly identified. There is a proposal that binding may actually be a two-step process. In the first step, the toxin associates with the plane of the membrane creating a low-affinity complex. This complex migrates laterally until it interacts with a high-affinity binding site. This would then allow for subsequent events such as receptor-mediated endocytosis. Although this model has appeal, it cannot be fully evaluated until the high-affinity binding site(s) have been identified. Work on identifying the receptor(s) for botulinum toxin is moving forward. One advance has been the crystallization of botulinum toxin and a determination of its three-dimensional structure, which has been achieved for serotypes A and B (Simpson, 2004).

Productive internalization has been divided into two steps, receptor-mediated endocytosis and pH-induced translocation. Receptor-mediated endocytosis has been the least examined by

botulinum toxin workers. By default, investigators have assumed that the process is essentially the same as that of most ligands that are internalized by cells. However, there is one other possibility that warrants consideration. Exocytosing nerves have a well-developed mechanism for membrane retrieval, which is thought to be part of an overall mechanism in which synaptic vesicle membrane melds with plasma membrane. This membrane is later retrieved to reform vesicles. Therefore, it is plausible that the retrieval phase of the vesicle recycling mechanism is the route for toxin entry into nerve endings (Simpson, 2004).

The concept that the botulinum toxin is productively internalized by pH-induced translocation is now accepted universally. However, the exact nature of the membrane-penetrating event has proved somewhat elusive. There is agreement that the translocation step can be fractioned into at least six distinct events. These are (a) pH-induced change in toxin structure that results in exposure of previously occult hydrophobic domains; (b) insertion of the toxin into the endosome membrane; (c) translocation of the light chain from the luminal to the cytosolic surface of the membrane; (d) reduction of the single disulfide bond that links the heavy chain and light chain; (e) uncoupling of the noncovalent forces that bond the heavy and light chains with subsequent separation of chains; and (f) restoration of light-chain structure associated with movement from an acidic environment (endosome) to a more neutral environment (cytosol) (Simpson, 2004).

Intracellular poisoning occurs when botulinum toxin blocks one of the final steps in exocytosis, which affects both spontaneous and evoked transmitter release. This is believed to be a two-step process where the toxin binds to and cleaves one of three intracellular substrates needed for exocytosis. It was discovered through sequencing the toxin's genome and deducing the amino acid sequence that serotypes A and E cleave SNAP-25, serotype C cleaves syntaxin as

well as SNAP-25, and serotypes B, D, F, and G cleave VAMP, also known as synaptobrevin. Furthermore, it was discovered that each serotype cleaves a unique peptide bond in two senses: (a) serotypes that act on the same substrate cleave different peptide bonds, and (b) each serotype cleaves only one peptide bond in its substrate even though the sequence of the scissile bond may be repeated elsewhere in the substrate. Progress continues to be made in defining the interaction between the toxin and substrate. It is still not known whether all points of contact on each substrate have been identified. As for the toxin, none of the light chains has been fully mapped for the domains that bind to and cleave substrate (Simpson, 2004).

The duration of the botulinum toxin varies with serotype. Serotype A has the most sustained action. However, no one knows the mechanism that accounts for termination of toxin action. To date, there is no evidence that the light chain is transported across the plasma membrane to reach the extracellular space making intracellular disposition of the molecule most likely. This would suggest diffusion, proteolysis, or a combination of the two contributing to the loss of activity (Simpson, 2004).

Botulism

Botulism comes from the Latin word "botulus" meaning sausage. When botulism was first recognized in Europe, many cases were associated with home-fermented sausages. Today plant rather than animal products are the most common vehicles for botulism. Human botulism has been classified into four types based on the mode of acquisition: foodborne, wound, infant, and adult (from intestinal colonization) (CDC, 1998).

Foodborne botulism has been recognized since the first half of the 20th century and results from the ingestion of food containing preformed toxin produced by *C. botulinum* (CDC, 1998; Shapiro et al., 1998). *C. botulinum* toxin types A, B, and E are most commonly associated

with foodborne botulism, type F is rare (Smith, 1977), and type G has yet to be demonstrated in, or isolated from, foods (Hatheway, 1993; ICMSF, 1996). Although one laboratory has reported isolation of organisms capable of producing type G from autopsy specimens, to date, there is no direct evidence of type G causing illness (Hatheway, 1993; Austin, 2001).

In 1943, the first case of wound botulism appeared (CDC, 1998). It was not until 1951 that wound botulism was first described in the literature (Davis et al., 1951; Hampson, 1951; Thomas et al., 1951). To date, only types A and B have been implicated (ICMSF, 1996). Essentially, once *C. botulinum* becomes established in the wound, toxin forms and circulates throughout the body via the blood stream. Gastrointestinal symptoms, which are associated with foodborne, infant and adult botulism, do not occur with wound botulism (CDC, 1998). Since 1980, it has been most often associated with drug abuse, especially with so-called "black tar heroin" (CDC, 1998; Lund and Peck, 2001).

It was not until 1976 that infant botulism was first described in the literature (Smith and Sugiyama, 1988; CDC, 1998). It is caused by the endogenous production of toxin by germinating spores in the infant's intestine (CDC, 1998; Lund and Peck, 2001). In the United States, the number of infant botulism cases reported annually is greater than that of foodborne botulism in adults (CDC, 1998; Lund and Peck, 2001).

In 1986, Chia et al., and in 1988, McCroskey and Hatheway, described adults having their gastrointestinal tract colonized by *C. botulinum*. This colonization is analogous to the pathogenesis of infant botulism (McCroskey and Hatheway, 1988) and appears to be associated with abnormal gastrointestinal function (MacDonald et al., 1986). In all cases, no food vehicle was identified nor any evidence of wound botulism (CDC, 1998), thus the designation as adult botulism.

Occurrence of *Clostridium botulinum*

Environment

C. botulinum is widely distributed throughout the land and coastal waters (Hauschild, 1989; Dodds, 1993). The factors affecting the distribution of the different types of *C. botulinum* in nature are poorly understood. However, some salient points have emerged. For example, soils in the western United States contain predominately type A spores whereas type B spores predominate in the eastern United States. Most of the type B strains found here are proteolytic (Smith, 1977). Type E predominates in northern regions, such as Alaska and Canada, and in aquatic environments and their surroundings (Hauschild, 1989; Dodds, 1993).

It has been suggested that *C. botulinum* in the marine environment may be of terrestrial origin (Johannsen, 1963). However, it is believed to be more likely from fresh water sediments (Smith and Sugiyama, 1988). River runoffs will deposit spores of terrestrial origin into the marine environment. However, *C. botulinum* is capable of developing within the marine environment because it grows well in the carrion of fish, marine mammals, and invertebrates. Since these animals carry the botulinum spores within their gut, growth could be initiated upon the animals' death and well before the microorganisms are inhibited by salt equilibration (Hauschild, 1989).

Why a specific serotype prevails in any given area is not well understood. One suggestion is that type A is favored by neutral to alkaline soil with low organic content (Smith, 1977) and low rainfall (Smith and Sugiyama, 1988). Currently, the factors governing the incidence of type B are not so clear. It seems that proteolytic type B favors drier environments, while non-proteolytic type B shows a tendency to grow in moist habitats. However, proteolytic strains of

type B can be established in areas were it was not originally present (Smith and Sugiyama, 1988). Type E is most often associated with moist or wet soil (Smith, 1977).

Food

Since *C. botulinum* is ubiquitous in the environment, it is not surprising to find it in food (Smith, 1977). However, far fewer surveys of foods contaminated with *C. botulinum* have been conducted than environmental surveys (Dodds, 1993). Hauschild (1989) noted that most food surveys focused primarily on fish, meats, and honey.

Results of food surveys indicate that fish have the highest level of contamination. This is expected since aquatic environments often show high levels of *C. botulinum* contamination. Meat contamination is low when compared to fish, since fish tend to be contaminated with spores prior to slaughter. Also, the farm environment has a lower amount of contamination when compared to the aquatic environment. These results are also in agreement with data on foodborne botulism (Hauschild, 1989; Dodds, 1993).

Far fewer food surveys focused on fruits and vegetables even though the incidences of botulism in the United States are primarily associated with home processed vegetables (Sobel et al., 2004). Dodds (1993) reviewed the incidence of *C. botulinum* in fresh vegetables and found it varied from 0% to 43%. Lilley et al. (1996) indicated a low overall incidence rate, 0.36%, of *C. botulinum* spores in commercially available fresh-cut MAP vegetables. Nevertheless, the presence of spores in fresh produce is not a public health threat unless they can germinate, outgrow, and multiply into toxin-producing vegetative cells (Juneja, 2003).

Control in Foods

Preventing food and the food-contact environment from being contaminated with *C. botulinum* during harvesting, processing, and storage would be ideal. However, since C.

botulinum is widely distributed in the environment (Hauschild, 1989), it is almost impossible to keep the organism from contaminating food (Kim and Foegeding, 1993). Nonetheless, contamination levels depend on the concentration in the soil (Notermans, 1993). Once the food is harvested, *C. botulinum* may be transferred from soil to the processing plant (Notermans, 1993). Therefore, during processing, two main strategies are used for controlling *C. botulinum*: (1) inactivation and (2) preventing growth and toxin production (Kim and Foegeding, 1993).

Inactivation of *C. botulinum* vegetative cells and/or spores involves using heat (thermal inactivation), gas (e.g., ethylene oxide), ionizing radiation, and/or chemicals (e.g., chlorine based sanitizers and hydrogen peroxide). However, it is not always practical or desirable to inactivate *C. botulinum* spores to prevent botulism. Such harsh treatments may reduce the sensory and/or nutritional quality of the food while increasing the processing cost (Kim and Foegeding, 1993). In these situations, preventing germination, growth, and toxin production is desired. This can be achieved by controlling one or more environmental factors such as temperature (i.e., strictly controlled and kept below 3^{o}C, otherwise it will not prevent growth and toxin formation by non-proteolytic strains), pH (i.e., pH of 4.6 or below), redox potential (i.e., increasing the E_h), water activity (i.e., maintain a_w at or below 0.94), food preservatives (e.g., nitrite, sorbic acid, parabens, nisin, phenolic antioxidants, polyphosphates, and ascorbates), and competitive non-pathogenic microorganisms (e.g., lactic acid bacteria) which may produce unfavorable growth conditions within the food matrix (Kim and Foegeding, 1993; Austin, 2001).

In contrast to the thermo-resistant spores, the toxins produced by *C. botulinum* are heat-labile and may be destroyed by heating at 80^{o}C (176^{o}F) for 10 minutes (Jay, 2000). However, this is not practical with regard to minimally processed fruits and vegetables. Therefore, preventing toxin production is necessary.

Foodborne Botulism in the United States

Since 1899, 2,368 cases of foodborne botulism have been reported (CDC, 1998). From 1990-2000, 160 botulism cases were recorded in the United States affecting 263 persons. For the contiguous states and Hawaii, there were 102 reported foodborne botulism cases affecting 160 people while Alaska had 58 botulism cases affecting 103 people. While no seasonal pattern was observed in the contiguous states and Hawaii, most cases in Alaska occurred in the spring and throughout the fall with a sharp peak in July. Toxin type A overall caused 131 cases (50%) with 7 deaths. Toxin type B caused 27 cases (10%) with 1 death. Toxin type E caused 97 cases (37%) with 3 deaths. Home-processed foods remained the leading cause of foodborne botulism in the United States (Sobel et al., 2004).

For the contiguous states and Hawaii, food was implicated by either laboratory detection of the toxin or by epidemiological investigation without the laboratory confirmation in 77 (76%) cases. Of these, 68 (67%) were caused by home-processed foods. The remaining 9 events were caused by non-homemade foods: five events were caused by commercial foods, two events were caused by restaurant-prepared foods, and two were not specified. In Alaska, the contaminated food was identified in 49 (84%) of the events, all of which were home-processed Alaska Native foods (Sobel et al., 2004).

A wide variety of foods have been involved in outbreaks including fish, meats, and vegetables. In the botulinum events involving non-canned homemade foods, commercially produced foods, and restaurant-prepared foods, improper food-handling practices were identified (e.g., improper storage temperature, insufficient heating of product) (Sobel et al., 2004).

Clinical Symptoms and Treatment of Foodborne Botulism

Foodborne botulism may range from a mild illness, which may be disregarded or misdiagnosed, to a serious disease that can be fatal within 24 h. Typically, the onset of symptoms occurs 18-36 h after ingestion of toxin and ranges from a few h to 8 d (Hughes et al., 1981). Generally, the quicker symptoms appear, the more serious the disease (CDC 1998; Shapiro et al., 1998; Austin, 2001). The initial symptoms may be gastrointestinal and include nausea, vomiting, abdominal cramps, or diarrhea. This may be caused by other products of *C. botulinum's* metabolism, other bacteria and their toxins present in the improperly preserved food, or by changes in bowel motility, not necessary the neurotoxin (CDC, 1998; Lund and Peck, 2001). After the onset of neurological symptoms, constipation is more typical (Shapiro et al., 1998). It should be noted that these gastrointestinal symptoms might not occur if purified botulinum toxin were to be intentionally placed in food (Arnon et al., 2001).

The neurological signs and symptoms include but are not limited to visual impairments (blurred or double vision, ptosis or drooping eyelids, fixed and dilated pupils), dry mouth, difficulty in speaking clearly (dysphonia), inability to swallow (dysphagia), general fatigue and lack of muscle coordination, and respiratory impairment (CDC, 1998; Lund and Peck, 2001). Symmetric descending paralysis is characteristic of botulism. The paralysis begins with the cranial nerves, moves to the upper extremities, then the respiratory muscles, and finally the lower extremities in a proximal-to-distal pattern. If left untreated, death will result from respiratory failure, airway obstruction, or failure of the heart muscles (CDC, 1998; Lund and Peck, 2001).

Although clinical symptoms are similar for each toxin type differences have been noted. Nausea and vomiting appear more often in cases associated with type B and type E rather than type A (Smith, 1977). Dysphagia and muscle weakness are more common in outbreaks

involving types A and B than of type E (Austin, 2001). Type E tends to have the most rapid onset of symptoms; type A tends to be a more severe disease and have a higher fatality rate that type B or type E (Woodruff et al., 1992).

Botulism is oftentimes confused with other illnesses including other forms of foodborne poisoning (especially staphylococcal), myasthenia gravis, and carbon monoxide poisoning (Smith, 1977). However, it is most commonly confused with Guillain-Barré syndrome (CDC, 1998; Shapiro et al., 1998; Austin, 2001). Whereas botulism's neurological signs and symptoms first appear in the cranial nerve area and descend, Guillain-Barré begins in the extremities and progresses in an ascending fashion (Smith, 1977; Cherington, 1981). The initial diagnosis of foodborne botulism is based on the clinical signs and symptoms exhibited by the patient (Hatheway, 1995). It is generally confirmed by detecting botulinal toxin or viable *C. botulinum* in the suspected food in addition to the clinical symptom(s), (Hauschild, 1989).

Initial treatment of botulism deals with the removal or inactivation of the botulinal toxin by (a) neutralizing the circulating toxin with anitiserum, (b) removing unabsorbed toxin in the gastrointestinal tract either by treatment with cathartics or an enema, or (c) in the absence of vomiting, use of a gastric lavage, or treatment with emetics (Smith, 1977; Hauschild, 1989). Treatment with antiserum is common and effective, especially in the early stages of the illness (Dack, 1956). This should be expected considering that equine antiserum neutralizes only toxin molecules unbound to nerve endings (Sugiyama, 1980). Subsequent treatments deal primarily with counteracting paralysis of the respiratory muscles and diaphragm by artificial ventilation (Hauschild, 1989). Clinical recovery can take weeks to months (Shapiro et al., 1998).

Methods for Detecting Toxin in Food

Currently, the mouse acute toxicity and neutralization bioassay is the only official method for botulinal toxin detection and identification (CDC, 1998), which is described in both the Association of Analytical Chemists (AOAC) guide and the Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM) (AOAC, 2000; Solomon and Lilly, 2001). *In vitro* methods (e.g. ELISA) for detecting botulinum toxin are under development (CDC, 1998). In November 2004, the FDA, through their online BAM, updated their methods on detecting toxins in food to include three *in vitro* methods: amplified-ELISA (amp-ELISA), digoxigenin-ELISA (DIG-ELISA), and polymerase chain reaction (PCR) (Solomon and Lilly, 2001). All three still require positive samples to be confirmed using the mouse bioassay.

In Vivo

In the mouse bioassay procedure, each test is conducted in duplicate using trypsin treated and untreated materials (e.g., food supernate) that are diluted 1:2, 1:10, and 1:100, respectively, with gel-phosphate buffer (pH 6.2). Each pair of mice is injected intraperitoneally (i.p.) with 0.5 ml of the original, diluted trypsin treated and untreated material, using a syringe. For a negative control, 1.5 ml of the original untreated test material is heated for 10 min at 100^{o}C. A pair of mice is injected with 0.5 ml of the cooled material. Since heat inactivates toxin, these mice should not die (AOAC, 2000).

The mice are observed for 48 h to determine if the toxin is present, although most symptoms and death occur within 24 h. Typical symptoms of botulism in mice include ruffling of fur, pinching of the waist, labored breathing, weakness of limbs, and total paralysis before death. If death should occur without the development of these symptoms, one cannot assume that the samples were contaminated with botulinal toxins since their death may have had other

causes (e.g., presence of other toxic components, trauma, poor health). Another 48 h is required to establish the minimum lethal dose (MLD) (AOAC, 2000), which is the amount that causes all injected animals to die (Hatheway and Ferreira, 1996). The amount that causes 50% of the injected animals to die is known as the median lethal dose (MLD_{50} or LD_{50}) (Hatheway and Ferreira, 1996).

Since the acute toxicity test is non-specific, neutralization tests are carried out along with the toxicity test to identify the specific toxin type present. To type the toxin present in the sample, several groups of paired mice are injected i.p. with each mouse receiving 0.5 ml of one of the diluted monovalent antitoxins (either type A, B, E, or F). After approximately 30-60 min, each pair of mice is injected i.p., with each dilution of the toxic supernate (AOAC, 2000). Another method involves "*in vitro* neutralization" where antitoxin is added to the test material and then injected into the mice (Hatheway, 1988). Over the next 48 h, the mice are observed for clinical symptoms of botulism and death. The mice that survive do so by the neutralization of the corresponding serological type of toxin (AOAC, 2000).

In Vitro

Alternative *in vitro* assays have been developed due to the limitations posed by animal testing (e.g., cost, time, public unease about the use of animals) (Bell and Kyriakides, 2000). Since small quantities of the highly potent botulinum toxins may be present, only sensitive immunoassays are of value such as hemagglutination, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), and latex agglutination (Notermans and Nagel, 1989; Hatheway and Ferreira, 1996). Although different variants of these assays have been described, all techniques are based on a quantitative reaction of the antigen (toxin) with its antibody (antitoxin) (Notermans and Nagel, 1989). The general disadvantage of these assays is that only the

antigenicity is determined (Notermans and Nagel, 1989), not the biological activity (Notermans and Kozaki, 1981).

One of the most promising and popular immunoassays, the ELISA uses an enzyme label to help detect the amount of toxin present (Notermans and Kozaki, 1981; Kozaki et al., 1989). This enzyme label may be conjugated to the toxin. However, due to the hazard involved with attaching an enzyme label to toxin, researchers prefer to conjugate the enzyme label to the antibody (Notermans and Kozaki, 1981). There are three main ELISA methods that utilize the enzyme label being conjugated to the antibody: direct, indirect, and sandwich. The sandwich ELISA, which is preferred among the three methods due to its sensitivity, can be performed as either a "direct" sandwich or an "indirect" (double) sandwich (Crowther, 2001).

Engvall and Perlmann originally described the direct sandwich ELISA in 1972. As a general rule, it is carried out using polystyrene or polyvinyl tubes coated with antibotulinum IgG (e.g., from rabbits) and then incubated with the toxin. The amount of adsorbed toxin is measured using antibotulinum IgG conjugated to an enzyme. A suitable substrate is added and the amount of enzyme adsorbed is determined spectrophotometrically (Engvall and Permlmann, 1972; Notermans et al., 1982).

In 1978, Notermans et al. used an indirect (double) sandwich ELISA to detect toxin type A. With this assay, polystyrene tubes are coated with an antibotulinum IgG originating from a species other than rabbit (e.g., horse) and then incubated with the toxin. The adsorbed toxin is labeled with rabbit serum against botulinum toxin type A. Sheep anti-rabbit serum conjugated with enzyme measures the amount of rabbit serum adsorbed. A specific substrate for the enzyme is added and the amount of enzyme adsorbed is determined spectrophotometrically. The sensitivity was determined to be 50-100 LD_{50} (Notermans et al., 1978). In 1979, Kozaki et al.

and Notermans et al. used the indirect (double) sandwich ELISA to detect botulinum toxin type B and E, respectively.

Whereas the previously described ELISA tests were based on the use of polyclonal antibodies, Shone et al. (1985) developed a monoclonal antibody-based amplified (amp) ELISA for type A toxin. This type of direct sandwich ELISA, which used an amplified substrate, proved to be almost as sensitive as the mouse bioassay (10-20 LD_{50}/ml).

In 2001, Ferreira demonstrated that a polyclonal antibody-based amp- ELISA was effective for detecting toxin types A, B, E, and F in culture media. In 2003, Ferreira et al. conducted a collaborative study on the performance of the amp-ELISA with the mouse bioassay to detect botulinal neurotoxins A, B, E, and F in culture media. The data showed that this indirect sandwich ELISA utilizing an amplified substrate would be useful as a preliminary test for the detection and typing of botulinal toxins produced in culture media with a toxicity > 10 MLD/ml. However, due to cross-reactivity with nonbotulinal cultures, confirmation must still be done by the mouse bioassay (Ferreira et al., 2003). Since ELISA can be run in one day, this ability to screen suspect cultures for botulinal toxins would have numerous advantages if botulinum toxin was to be used as a biological weapon.

In 2002, Ferreira et al. modified the amp-ELISA by substituting digoxigenin-labeled IgG for biotin-labeled IgGs and anti-digoxigenin horse-radish peroxidase conjugate (HRP) for the streptavidin-alkaline phosphatase. An appropriate substrate, hydrogen peroxide, along with an appropriate chromophore, tetramethlybenzidine (TMB), is used along with the HRP enzyme for optimal spectrophotometric readings. When compared to the amp-ELISA, this substitution resulted in an equivalent sensitivity while simplifying the ELISA procedure by using a one-step substrate.

REFERENCES

AOAC: Association of Analytical Chemists. 2000. *Clostridium botulinum* and its toxins in foods, p. 68-70. *In* W. Horwitz (ed.), Official methods of analysis of AOAC international, 17th ed. AOAC, Inc., Gaithersburg, MD.

Arnon, S.S., R. Schechter, T.V. Inglesby, D.A. Henderson, J.G. Bartlett, M.S. Ascher, E. Eitzen, A. D. Fine, J. Hauer, M. Layton, S. Lillibridge, M.T. Osterholm, T. O'Toole, G. Parker, T.M. Perl, P.K. Russell, D.L. Swerdlow, and K. Tonat. 2001. Botulinum toxin as a biological weapon. *JAMA.* 285:1059-1070.

Austin, J.W. 2001. *Clostridium botulinum*, p. 329-349. *In* M.P. Doyle, L.R. Beuchat, and T.J. Montville (ed.), Food microbiology: fundamentals and frontiers, 2nd ed. ASM Press. Washington, D.C.

Bell, C. and A. Kyriakides. 2000. *Clostridium botulinum*: a practical approach to the organism and its control in foods. Blackwell Science Ltd. London.

Brackett, R.E. 1999. Incidence, contributing factors, and control of bacterial pathogens in produce. *Postharvest Biol. Technol.*15:305-311.

Cato, E.P., W.L. George, and S.M. Finegold. 1986. Endospore-forming Gram positive rods and cocci, p. 1141-1160. *In* P.H.A. Sneath (ed.), Bergey's manual of systematic bacteriology, vol. 2. Williams & Wilkins. Baltimore, MD.

CDC: Centers for Disease Control and Prevention. 1998. Centers for Disease Control and Prevention: Botulism in the United States, 1899-1996. Handbook for epidemiologists, clinicians, and laboratory workers. Atlanta, GA.

Cherington, M. 1981. Botulism: Clinical, electrical, and therapeutic considerations, p.327-330. *In* G.E. Lewis, Jr. (ed.), Biomedical aspects of botulism. Academic Press, Inc. New York.

Chia, J.K., J.B. Clark, C.A. Ryan, and M.Pollack. 1986. Botulism in an adult associated with foodborne intestinal infection with *Clostridium botulinum*. *N. Engl. J. Med.* 315:239-241.

Crowther, J. R. 2001. The ELISA guidebook, p. 1-421. *In* Walker, J.M. (ed.), Methods in molecular biology, vol. 149. Humana Press Inc. Totowa, NJ.

Dack, G.M. 1956. Food Poisoning, 3rd ed. The University of Chicago Press. Chicago.

DasGupta, B.R. 1981. Structure and structure function relation of botulinum neurotoxins, p. 1-20. *In* G.E. Lewis, Jr. (ed.), Biomedical aspects of botulism. Academic Press, Inc. New York.

Davis, J.B., L.H. Mattman, and M. Wiley. 1951. *Clostridium botulinum* in a fatal wound infection. *J.A.M.A.* 146:646-648.

Dodds, K. 1993. *Clostridium botulinum* in foods, p. 53-68. *In* A.H.W. Hauschild and K.L. Dodds (ed.), *Clostridium botulinum*: ecology and control in foods. Marcel Dekker, Inc. New York.

Duda, J.J., and J.M. Slack. 1969. Toxin production in *Clostridium botulinum* as demonstrated by electron microscopy. *J. Bact.* 97:900-904.

Engvall, E., and P. Perlmann. 1972. Enzyme-linked immunosorbent assay, ELISA. *J. Immun.* 109:129-135.

FDA/CFSAN: Food and Drug Administration /Center for Food Safety and Applied Nutrition. 1992. "*Clostridium botulinum*". Foodborne pathogenic microorganisms and natural toxin handbook. http://vm.cfsan.fda.gov/~mow/chap2.html Accessed 15 February 2005.

Ferreira, J.L. 2001. Comparison of amplified ELISA and mouse bioassay procedures for determination of botulinal toxins A, B, E, and F. *J. AOAC Int.* 84:85-88.

Ferreira, J.L., S. Maslanka, and J. Andreadis. 2002. Detection of type A, B, E, and F *Clostridium botulinum* toxins using digoxigenin-labeled IgGs and the ELISA. FDA/ORA Laboratory Information Bulletin, vol. 18, number 10. 4292:1-10.

Ferreira, J., S. Maslanka, E. Johnson, and M. Goodnough. 2003. Detection of botulinal neurotoxins A, B, E, and F by amplified enzyme-linked immunosorbent assay: collaborative study. *J. AOAC Int.* 86:314-331.

Hampson, C.R. 1951. A case of probable botulism due to wound infection. *J. Bact.* 61:647.

Hatheway, C.L. 1988. Botulism, p. 111-133. *In* A. Balows, W.H. Hausler, Jr., M. Ohashi, and A. Turano (ed.), Laboratory diagnosis of infectious diseases: principles and practice. Springer-Verlag. New York.

Hatheway, C.L. 1993. *Clostridium botulinum* and other clostridia that produce botulinum neurotoxin, p. 3-20. *In* A.H.W. Hauschild and K.L. Dodds (ed.), *Clostridium botulinum*: ecology and control on foods. Marcel Dekker, Inc. New York.

Hatheway, C.L. 1995. Botulism: The present status of the disease, p. 55-75. *In* C. Montecucco (ed.), Clostridial neurotoxins. Springer-Verlag. Berlin.

Hatheway, C.L. and Ferreira, J.L. 1996. Detection and identification of *Clostridium botulinum* neurotoxins, p. 481-498. *In* B.R. Singh and A.T. Tu (ed.), Natural toxins 2: structure, mechanism of action, and detection. Plenum Press. New York.

Hauschild, A.H.W. 1989. *Clostridium botulinum*, p. 111-189. *In* M.P. Doyle (ed.), Foodborne bacterial pathogens. Marcle Dekker, Inc. New York.

Hedberg, C.W., K.L. MacDonald, and M.T. Osterholm. 1994. Changing epidemiology of food-borne disease: a Minnesota perspective. *Clin. Infect. Dis.* 18:671-82.

HSS: United States Department of Health and Human Services (HHS), National Institute of Health, and National Cancer Institute. 2002. http://www.5aday.gov/news/news041602.html Accessed 1 March 2005.

HSS: United States Department of Health and Human Services (HHS). 2005. Dietary Guidelines for Americans. Washington, D.C. http://www.healthierus.gov/dietaryguidelines Accessed 1 March 2005.

Hughes, J.M., J.R. Blumenthal, M.H. Merson, G.L. Lombard, V.R. Dowell, Jr., and E.J. Gangarosa. 1981. Clinical features of types A and B food-borne botulism. *Ann. Intern. Med.* 95:442-445.

IAFP: International Fresh Cut Association. 2005. http://www.fresh-cuts.org Accessed 1 March 2005.

ICMSF: International Commission on Microbiological Specifications for Foods. 1996. Microbiological specifications of food pathogens, p. 66-111. *In* T.A. Roberts, A.C. Baird-Parker, and R.B. Tompkin (ed.), Microorganisms in foods, vol. 5. Blackie Academic & Professional. Toronto.

Jay, J.M. 2000. Modern Food Microbiology, 6th ed. Aspen Publishers, Inc. Gaithersburg, MD.

Johannsen, A. 1963. *Clostridium botulinum* in Sweden and the adjacent waters. *J. Appl. Bact.* 26:43-47.

Juneja, V.K. 2003. Microbial safety, quality, and sensory aspects of fresh-cut fruits and vegetables, p. 97-126. *In* J.S. Novak, G.M. Sapers and V.K. Juneja (ed.), Microbial safety of minimally processed foods. CRC Press. Boca Raton.

Kim, J. and P.M. Foegeding. 1993. Principles of control, p. 121-176. *In* A.H.W. Hauschild and K.L. Dodds (ed.), *Clostridium botulinum*: ecology and control in foods. Marcel Dekker, Inc. New York.

Kozaki, S., J. Dufrenne, A.M. Hagenaars, and S. Notermans. 1979. Enzyme linked immunosorbent assay (ELISA) for detection of *Clostridium botulinum* type B toxin. *Japan. J. Med. Sci. Biol.* 32:199-205.

Kozaki, S., Y. Kamata, M. Takahashi, T. Shimizu, and G. Sakaguchi. 1989. Antibodies against botulinum neurotoxin, p. 301-318. *In* L.L. Simpson (ed.), Botulinum neurotoxin and tetanus toxin. Academic Press, Inc. San Diego.

Lamanna, C. and G. Sakaguchi. 1971. Botulinal toxins and the problem of nomenclature of simple toxins. *Bact. Rev.* 32:242-249.

Lilley, T., H.M. Solomon, and E.J. Rhodehamel. 1996. Incidence of *Clostridium botulinum* in vegetables packaged under vacuum or modified atmosphere. *J. Food Prot.* 59:59-61.

Lund, B.M. and M.W. Peck. 2001. *Clostridium botulinum*, p. 69-85. *In* R.G. Labbé and S. García (ed.), Guide to foodborne pathogens. John Wiley & Sons, Inc. New York.

MacDonald, K., M.L. Cohen, and P.A. Blake. 1986. The changing epidemiology of adult botulism in the United States. *Am. J. Epidemiol.* 124:794-799.

McCroskey, L.M., and C.L. Hatheway. 1988. Laboratory findings in four cases of adult botulism suggest colonization of the intestinal tract. *J. Clin. Microbiol.* 26:1052-1054.

Notermans, S., J. Dufrenne, and M. van Schothorst. 1978. Enzyme-linked immunosorbent assay for the detection of *Clostridium botulinum* toxin type A. *Japan. J. Med. Sci. Biol.* 31:81-85.

Notermans, S., J. Dufrenne, and S. Kozaki. 1979. Enzyme-linked immunosorbent assay for detection of *Clostridium botulinum* type E toxin. *Appl. Environ. Microbiol.* 37:1173-1175.

Notermans, S. and S. Kozaki. 1981. Isolation and identification of botulinum toxins using the ELISA, p. 181-190. *In* G.E. Lewis, Jr. (ed.), Biomedical aspects of botulism. Academic Press, Inc. New York.

Notermans, S., A.M. Hagenaars, and S. Kozaki. 1982. The enzyme-linked immunosorbent assay (ELISA) for the detection and determination of *Clostridium botulinum* toxins A, B, and E. *Methods Enzymol.* 84:223-238.

Notermans, S. and J. Nagel. 1989. Assays for botulinum and tetanus toxins, p. 319-331. *In* L.L. Simpson (ed.), Botulinum neurotoxin and tetanus toxin. Academic Press, Inc. San Diego.

Notermans, S.H.W. 1993. Control in fruits and vegetables, p. 233-260. *In* A.H.W. Hauschild and K.L. Dodds (ed.), *Clostridium botulinum*: ecology and control in foods. Marcel Dekker, Inc. New York.

Pollack, S. 2001.Consumer demand for fruit and vegetables: the U.S. example, p. 49-54. *In* A. Regmi (ed.), Changing structure of global food consumption and trade. Market and Trade Economics Division, Economic Research Service, U.S. Department of Agriculture, Agriculture and Trade Report, WRS-01-1. Washington, D.C.

Rajkowski, K.T. and E.A. Baldwin. 2003. Concerns with minimal processing in apple, citrus, and vegetable products, p. 35-52. *In* J.S. Novak, G.M. Sapers and V.K. Juneja (ed.), Microbial safety of minimally processed foods. CRC Press. Boca Raton.

Sakaguchi, G., I. Ohishi, and S. Kozaki. 1981. Purification and oral toxicities of *Clostridium botulinum* progenitor toxins, p. 21-34. *In* G.E. Lewis, Jr. (ed.), Biomedical aspects of botulism. Academic Press, Inc. New York.

Sakaguchi, G. 1983. *Clostridium botulinum* toxins. *Pharmac. Ther.* 19:165-194.

Sakaguchi, G. S. Kozaki, and I. Ohishi. 1984. Structure and function of botulinum toxins, p. 435-443. *In* J.E.Alouf, F.J. Fehrenback, J.H. Freer, and J. Jeljaszewicz (ed.), Bacterial protein toxins. Aademic Press. London.

Sakaguchi, G. 1990. Molecular structure of *Clostridium botulinum* progenitor toxins, p. 173-180. *In* A.E. Pohland, V.R. Dowell, Jr., and J.L. Richard (ed.), Microbial toxins in foods and feeds: cellular and molecular modes of action. Plenum Press. New York.

Shapiro, R.L., C. Hatheway, and D.L. Swerdlow. 1998. Botulism in the United States. *Ann. Intern. Med.* 129:221-228.

Shone, C., P. Wilton-Smith, N. Appleton, P. Hambleton, N. Modi, S. Gatley, and J. Melling. 1985. Monoclonal antibody-based immunoassay for type A *Clostridium botulinum* toxin is comparable to the mouse bioassay. *Appl. Environ. Microbiol.* 50:63-67.

Siegel, L.S., and J.F. Metzger. 1979. Toxin production by *Clostridium botulinum* type A under various germination conditions. *Appl. Environ. Microbiol.* 38:606-611.

Simpson, L. 2004. Identification of the major steps in botulinum toxin action. *Annu. Rev. Pharmacol. Toxicol.* 44:167-93.

Sivapalasingam, S., C.R. Freidman, L. Cohen, and R.V. Tauxe. 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *J. Food Prot.* 67:2342-2353.

Smith, L.D. 1977. Botulism: the organism, its toxins, the disease. Charles C. Thomas. Springfield, IL.

Smith, L.D. and H. Sugiyama. 1988. Botulism: the organism, its toxins, the disease, 2nd ed. Charles C. Thomas. Springfield, IL.

Sobel, J., N. Tucker, A. Sulka, J. McLaughlin, and S. Maslanka. 2004. Foodborne botulism in the United States, 1990-2000. *Emer. Infect. Dis.* 10:1606-1611.

Solomon, H.M., E.A. Johnson, D.T. Bernard, S.S. Arnon, and J.L. Ferreira. 2001. *Clostridium botulinum* and its toxins, p. 317-324. *In* F.P. Downs and K. Ito (ed.), Compendium of Methods for the Microbiological Examination of Foods, 4th ed. American Public Health Association, Washington, D.C.

Solomon, H. and T. Lilly. 2001. FDA Bacteriological Analytical Manual, Chapter 17, *Clostridium botulinum*. http://www.cfsan.fda.gov/~ebam/bam-17.html Accessed 30 April 2005.

Sugiyama, H. 1980. *Clostridium botulinum* neurotoxin. *Microbiol. Rev.* 44:419-448.

Suen, J.C., C.L. Hatheway, A.G. Steigerwalt, and D.J. Brenner. 1988. *Clostridium argentinense* sp. nov.: a genetically homogeneous group composed of all strains of *Clostridium botulinum*

toxin type G and some nontoxigenic strains previously identified as *Clostridium subterminale* or *Clostridium hastiforme*. *Intern. J. Systematic Bacteriol.* 38:375-381.

Tauxe, R., H. Kruse, C. Hedberg, M. Potter, J. Madden, and K. Wachsmuth. 1997. Microbial hazards and emerging issues associated with produce a preliminary report to the national advisory committee on microbiologic criteria for foods. *J. Food Prot.* 60:1400-1408.

Thomas, C.G. Jr., M.F. Keleher, and A.P. McKee. 1951. Botulism, a complication of *Clostridium botulinum* wound infection. *Arch. Pathol.* 51:623-28.

United States Department of Agriculture (USDA). 2005. Dietary Guidelines for Americans. Washington, D.C. http://www.healthierus.gov/dietaryguidelines Accessed 1 March 2005.

van Ermengem, E. 1897. Ueber einen neuen anaëroben Bacillus und seine Beziehungen zum Botulismus. Z. Hyg. Infektionskrankh. 26:1-56. (English translation) 1979. *Rev. Infect. Dis.* 1:701-719.

Woodruff, B.A., P.M. Griffin, L.M. McCroskey, J.F. Smart, R.B. Wainwright, R.G. Bryant, L.C. Hutwagner, and C.L. Hatheway. 1992. Clinical and laboratory comparison of botulism from toxin types A, B, and E in the United States, 1975-1988. *J. Infect. Dis.* 166:1281-1286.

Zhuang, H., M.M. Barth, and T.R. Hankinson. 2003. Microbial safety, quality, and sensory aspects of fresh-cut fruits and vegetables, p. 255-278. *In* J.S. Novak, G.M. Sapers and V.K. Juneja (ed.), Microbial safety of minimally processed foods. CRC Press. Boca Raton.

CHAPTER 2

THE STABILITY OF *CLOSTRIDIUM BOTULINUM* TOXIN TYPE A ON FRESH AND FRESH-CUT PRODUCE[1]

[1] Broeker, R.G.S., M.A. Harrison, and J.L. Ferreira. To be submitted to J. Food Prot.

ABSTRACT

Foodborne botulism is a potentially lethal neuroparalytic illness resulting from ingesting *C. botulinum* neurotoxins. There is little information concerning the stability of preformed toxin on fresh produce. This research determined the stability of toxin type A on whole grape tomatoes, pre-cut lettuce, and pre-sliced cantaloupe. Different combinations of toxin inoculum pH (5.5 or 7.0) and storage temperature (4, 15, or 25^{o}C) were analyzed to determine their relationship to the rate of toxin decay. The toxin was measured using the DIG-ELISA method, which screens for the presence of botulinal toxin type A. For tomatoes, the toxin inoculum at pH 5.5 and storage temperature of 25^{o}C provided the most stability for the toxin. For lettuce, the toxin inoculum at pH 7.0 and storage temperature of 15^{o}C provided the most stability. For cantaloupe, only the temperature of 4^{o}C provided stability to the toxin.

INTRODUCTION

Foodborne botulism is a potentially lethal neuroparalytic illness resulting from ingesting *C. botulinum* neurotoxins. *C. botulinum* is an anaerobic, spore-forming bacterium. Although seven immunologically distinct toxins exist, designated type A through G, types A, B, and E are most frequently associated with foodborne botulism (Smith, 1977). Botulism neurotoxins act preferentially on peripheral cholinergic nerve endings to block acetylcholine release resulting in paralysis of the motor system (Simpson, 2004). Clinical illness is characterized by symmetric descending paralysis (proximal to distal) first appearing in the cranial nerve area. Clinical recovery can take weeks to months (Shapiro et al., 1998). If left untreated, death will result from respiratory failure, airway obstruction, or failure of the heart muscles (CDC, 1998; Lund and Peck, 2001).

Spores of *C. botulinum* are ubiquitous in the environment (Hauschild, 1989) and may be found on many types of raw vegetables (Hao et al., 1998, 1999). However, botulism associated with the consumption of fresh produce has not been a major food safety concern since *C. botulinum* requires anaerobic conditions for growth. New technologies, such as modified-atmosphere packaging (MAP), have been used to extend the shelf life of fresh produce and have raised concerns about the potential for production of botulinal toxin in these products. Challenge studies using both proteolytic and nonproteolytic strains of *C. botulinum* have been conducted to determine the safety of MAP minimally processed fruits and vegetables (Hotchkiss et al., 1992; Larson et al., 1997; Austin et al., 1998; Hao et al., 1998, 1999; Larson and Johnson, 1999). These studies indicated that produce might become toxic but only after becoming spoiled beyond the point of being organoleptically acceptable. Only butternut squash and onion remained acceptable after detection of toxin (Austin et al., 1998).

There is little information concerning the stability of preformed toxin on fresh produce. This should be of concern because not only can botulism occur naturally as a form of accidental food poisoning but it can also occur unnaturally as a product of malice. Various countries and terrorist groups have developed botulinum toxin as a biological weapon, which could be disseminated by deliberate contamination of the food supply (Arnon et al., 2001).

This research focused on the rate of decay of *C. botulinum* toxin type A on fresh-cut lettuce, fresh-cut cantaloupe, and whole grape tomatoes. Currently, the AOAC (2000) mouse bioassay method is the only approved method for detection of botulinal toxin during investigations of botulism. This method is highly sensitive but the method is limited because it requires up to 6 days to obtain final results and can only be conducted in facilities that have mice available for the test. Recently, rapid, alternative, *in-vitro* procedures, such as enzyme-linked immunosorbent assay (ELISA), which can be run in a day, have been developed to detect toxin producing organisms and their toxins (Ferreira, 2001). For this research, the toxin was measured using the DIG-ELISA method (Ferreira et al., 2002), which has shown considerable promise as a rapid screening tool for *C. botulinum* toxin in food.

MATERIALS AND METHODS

***Clostridium botulinum* toxin inoculum.** *Clostridium botulinum* toxin complex type A, from Metabiologics, Inc, Madison, WI, was used. The toxin complex was stored at 4^{o}C in a solution of sodium phosphate, pH 7.0 or sodium citrate, pH 5.5.

Preparation and inoculation of minimally processed fresh produce. Grape tomatoes, pre-packaged cut lettuce, and pre-sliced cantaloupe were purchased from a local grocery store in Athens, GA. No wash treatment was applied prior to inoculation. Each produce item received four treatments: (1) toxin inoculum pH 7.0, stored at 4^{o}C; (2) toxin inoculum pH 5.5,

temperature storage of 4°C; (3) toxin inoculum pH 7.0, temperature storage of 15 or 25°C; and (4) toxin inoculum pH 5.5, temperature storage of 15 or 25°C. All treatments were run in triplicate.

Grape Tomatoes. Grape tomatoes, free of visible cuts or ruptures, each weighing 5.0 ± 0.5 g, were surface inoculated with 10 µl inoculum per tomato. The tomatoes were allowed to dry in a laminar flow biosafety hood (class II, type A2) for 30 min at room temperature. The tomatoes were then either stored at 4°C or 25°C for 10 d. On sampling days, tomatoes were individually placed in 50 ml centrifuge tubes and then washed with 5 ml of casein buffer (Pierce; Rockford, IL). The wash was adjusted to pH 7.4-7.6, if necessary, using 1 N sodium hydroxide (NaOH) and then analyzed.

Lettuce. Pre-washed, pre-cut bagged lettuce was used. Each piece had a surface area of 7.62 ± 1.50 cm^2 and was surface inoculated with 10 µl of inoculum. The lettuce was allowed to dry in a laminar flow biosafety hood (class II, type A2) for 30 min at room temperature. The lettuce was then either stored at 4°C or 15°C for 10 d. On sampling days, the lettuce was individually placed in 50 ml centrifuge tubes and then washed with 5 ml of casein buffer. The wash was adjusted to pH 7.4-7.6, if necessary, using 1 N sodium hydroxide (NaOH) and then analyzed.

Pre-sliced cantaloupe. Pre-cut cantaloupe was used. Each piece weighed 5.0 ± 0.5 g and was surface inoculated with 10 µl of inoculum. The cantaloupe pieces were allowed to dry in a laminar flow biosafety hood (class II, type A2) for 30 min at room temperature. The cantaloupe pieces were then either stored at 4°C or 15°C for 8 d. On sampling days, cantaloupe were individually placed in 50 ml centrifuge tubes and then washed with 5 ml of casein buffer. The cantaloupe and casein buffer were then ground into a paste using mortar and pestle the resulting

mixture was centrifuged at 7,000 x g at 4°C using an Allegra ™ X-22R Centrifuge (Beckman Coulter, Inc.; Fullerton, CA). The supernate was adjusted to pH 7.4-7.6, if necessary, using 1 N sodium hydroxide (NaOH) and then analyzed.

ELISA to detect toxin in samples. An amplified ELISA procedure for botulinal toxins (Ferreira et al., 2002) was modified by substituting digoxigenin-labeled IgG for biotin-labeled IgGs and anti-digoxigenin horse-radish peroxidase conjugate (HRP) for the streptavidin-alkaline phosphatase (Ferreira, 2001).

Capture antibodies (FDA; Atlanta, GA) (100 µl/well) were diluted in bicarbonate buffer (Sigma-Aldrich; St. Louis, MO) and placed in the number of needed microtiter plate wells (Dynex Immulon II, Thermo Labsystems; Franklin, MA). The plates were stored overnight at 4°C (Lab Research Products; Summerville, SC), covered with a plastic seal (Thermo Labsystems; Franklin, MA) to prevent evaporation.

The following day, the plates were removed from the 4°C storage and washed five times using the EL_x50™ automated strip washer (BIO-TEK Instruments, Inc.; Winooski, VM), with phosphate buffered saline with tween 20 (PBS-T; Sigma-Aldrich). The plate was blocked with casein buffer by filling all wells to the top of the plate (~300 µl/well) and incubated (Innova 4230; Edison, NJ) for 1 h at 35°C. While the plate was being blocked, the controls (positive, negative, and food inhibition) and samples (diluted in casein buffer) were prepared. The casein buffer was discarded from the plate and the plate was tamped on paper towels to remove any remaining liquid from the wells. The controls and toxic samples were added to the plate in duplicate (100 µl/well). The plate was covered with a plastic seal and incubated for 2 h at 35°C. The plate was washed five times with PBS-T. The digoxigenin-labeled antibody (FDA) was diluted (1:200) in casein buffer, added to the plate (100 µl/well) and incubated for 1 h at 35°C.

The plate was washed five times with PBS-T. The anti-digoxigenin horseradish peroxidase (HRP) conjugate (Roche Diagnostics Corporation; Indianapolis, IN) was diluted (1:5000) in casein buffer, added to the plate (100 µl/well) and incubated for 1 h at 35°C. The plate was washed five times with PBS-T. The 1-Step™ Ultra TMB-ELISA substrate solution was added to the plate (100 µl/well) and incubated for 20 min at 25°C in the dark. The stopping reagent, 1 N sulfuric acid (H_2SO_4), was added (100 µl/well). The color changed from blue to yellow when detectable toxin was present. The plate was read immediately after the addition of the stopping reagent. The plate reader, ELx800 universal microplate reader (BIO-TEX Instruments, Inc.) was set to read at 450 nm.

Enumeration of aerobic microflora. To help determine spoilage, aerobic plate counts (APC) were performed (Morton, 2001). French legislation specifies a maximum of 7.7 $\log_{10}$ CFU/g at the use-by date for fresh-cut produce (Nguyen-the and Carlin, 1994). In the U.S., the meat industry uses 8.0 $\log_{10}$ CFU/g to indicate product spoilage. Some food service companies have informally adopted these recommendations (Zhuang et al., 2003). It should be noted that APCs of 6-7 $\log_{10}$ CFU/g are common on ready to eat vegetables (Jay, 2000).

Samples were plated onto plate count agar (PCA; BBL/Difco; Sparks, MD) for bacterial enumeration. Twenty-five g portions of each sample were aseptically weighed into 225 ml of 0.1% buffered peptone water (pH 7.0; BBL/Difco) and hand massaged for 2 min. Serial 10-fold dilutions were prepared in 0.1% buffered peptone water and duplicate 0.1 ml samples of appropriate dilutions were spread onto PCA for enumeration. Plates were incubated at 4, 15, or 25°C for 7, 3, and 3 days, respectively, before counting colony-forming units.

Measurement of produce pH. A Beckman pH meter (model 350, Beckman Instruments, Inc.; Fullerton, CA) with a flat surface electrode was used to measure the surface pH of the product.

Statistical Analysis. Three replicate experiments were conducted for each of the four treatments (pH 7.0, temperature 4^{o}C; pH 7.0, temperature 15 or 25^{o}C; pH 5.5, temperature 4^{o}C; pH 5.5, temperature 15 or 25^{o}C) per produce item. After a logarithmic transformation, the data was analyzed to determine the effect pH and/or temperature had on the rate of toxin decay using the linear regression with common intercepts model on SAS software (Statistical Analysis Systems Institute; Cary, NC), which determined significant differences ($P < 0.01$) between slopes.

RESULTS

ELISA

Standard curves for each of the six batches of toxin are shown in Figs. 2.1 – 2.3, as determined by serial dilutions of the toxin from 20 to 0.05 LD_{50}/well. The threshold absorbance for a positive ELISA reading was determined by calculating the mean plus two times the standard deviation of 10 controls (Feldkamp and Smith, 1987). Numbers are expressed as optical density at A_{450}. The threshold readings for tomatoes when the toxin inoculum was at pH 7.0 and pH 5.5 were 0.113 and 0.152, respectively. For lettuce, the readings for the toxin inoculum at pH 7.0 and pH 5.5 were 0.178 and 0.089, respectively. For cantaloupe, the readings for the toxin inoculum at pH 7.0 and pH 5.5 were 0.063 and 0.091, respectively.

Tomatoes

The rates of decay slopes for the four different treatments are shown in Table 2.1. The intercept was based on the original concentration (350,000 LD_{50}/10 µl) inoculated onto the tomatoes and was identical for all four treatments. The pH 5.5 and temperature 25^{o}C treatment resulted in the slowest rate of decay for the toxin whereas the pH 7.0 and temperature 4^{o}C treatment resulted in the greatest rate of decay. It appears overall that the toxin inoculum of pH 5.5 and the temperature of 25^{o}C both significantly ($P < 0.01$) slowed the rate of decay.

Lettuce

The rates of decay slopes for the four different treatments are shown in Table 2.1. The intercept was based on the original concentration (350,000-380,000 LD_{50}/10 µl) inoculated onto the lettuce and was identical for all four treatments. The pH 7.0 and temperature 15^{o}C treatment resulted in the slowest rate of decay for the toxin whereas the pH 5.5 and temperature 4^{o}C treatment resulted in the greatest rate of decay. Overall, it appears that toxin inoculum of pH 5.5 significantly ($P < 0.01$) increased the rate of decay whereas the temperature of 15^{o}C significantly ($P < 0.01$) slowed the rate of decay.

Cantaloupe

The rates of decay slopes for the four different treatments are shown in Table 2.1. The intercept was based on the original concentration (380,000 LD_{50}/10 µl) inoculated onto the cantaloupe and was identical for all four treatments. The pH 7.0 and temperature 4^{o}C treatment resulted in the slowest rate of decay for the toxin whereas the pH 5.5 and temperature 15^{o}C treatment resulted in the greatest rate of decay. It appears overall that the toxin inoculum of pH 5.5 did not significantly affect the rate of decay. However, the temperature of 15^{o}C significantly ($P < 0.01$) increased the rate of decay.

Aerobic Plate Counts

Aerobic plate counts (APCs) were obtained for tomatoes, lettuce, and cantaloupe for each day an ELISA test was conducted (Table 2.2-2.4). Microbial populations increased in number in all three produce types during incubation at the two temperatures. As expected, microbial populations increased faster at the higher temperatures. Initial APCs at 4^{o}C were < 2, 6.02, and 5.88 $\log_{10}$ CFU/g, respectively, for grape tomatoes, lettuce, and cantaloupe. For lettuce and cantaloupe, APCs increased to > 8 $\log_{10}$ CFU/g after spoilage. For tomatoes, APCs remained at

< 2 $\log_{10}$ CFU/g and no spoilage was observed. Initial APCs at 15^{o}C were 6.15 and 5.34 $\log_{10}$ CFU/g, respectively, for lettuce and cantaloupe. APCs increased to > 9 and > 8 $\log_{10}$ CFU/g, respectively, for lettuce and cantaloupe after spoilage. The initial APCs at 25^{o}C for tomatoes was < 2 $\log_{10}$ CFU/g and increased to > 3 $\log_{10}$ CFU/g. No spoilage was observed for the tomatoes.

Visual and olfactory senses were used alongside aerobic plate counts to judge whether or not the produce item was spoiled or grossly spoiled. Spoilage was indicated by brown discoloration of cut surface of lettuce and loss of texture in the cantaloupe slices. The APCs were between 7-8 $\log_{10}$ CFU/g. Gross spoilage was indicated by gassy packages, turbid liquid in the bottom of the packages, severe off odor, and discoloration of the produce in addition to the APCs being > 9 $\log_{10}$ CFU/g. Attempts have been made to correlate spoilage and the number of microorganisms on the produce. However, total bacterial counts at the end of storage are unrelated to sample quality in many instances (Nguyen-the and Carlin, 1994; Zhuang et al., 2003). It should be noted that toxin still remained on all three produce items even after gross spoilage of lettuce and cantaloupe occurred.

Surface pH

The initial pH values of tomatoes, lettuce, and cantaloupe were 4.91, 4.87, and 6.82, respectively. Regardless of the temperature, the pHs for both tomatoes (Table 2.2) and lettuce (Table 2.3) increased during storage while the pH for cantaloupe (Table 2.4) decreased. The final pH values of tomatoes, lettuce, and cantaloupe at 4^{o}C were 5.51, 5.30, and 4.96, respectively. For tomatoes at 25^{o}C, the final pH was 6.1. For lettuce and cantaloupe at 15^{o}C, the final pHs were 6.85 and 4.32, respectively.

DISCUSSION

Grecz et al. (1965) noted that there was a remarkable dearth of knowledge concerning the stability of *C. botulinum* toxin in food products when they conducted a study on the storage stability of *C. botulinum* toxin and spores in processed cheese. The results showed that the toxin was remarkably stable at 2 to 4^{o}C whereas the stability at 30^{o}C was not as great. For pH, there was no easily detectable relationship to toxin stability. The cheese had initial pHs of 6.0-6.5, 5.7-6.4, and 7.1-7.3. The cheese with the initial pH of 7.1-7.3 had the least amount of residual toxin remaining after 60 d. It was suggested that the pH may have been alkaline enough to contribute to the instability of the toxin (Grecz et al., 1965). It is known that alkaline pH leads to deterioration of toxin activity (Spero, 1958) whereas acidic conditions stabilize the toxin (Smith, 1977). Forty years later there is still a lack of knowledge concerning the stability of *C. botulinum* toxin in foods products, especially in foods that receive no thermal treatment.

This research tried to determine the stability of toxin in whole grape tomatoes, pre-cut lettuce, and pre-sliced cantaloupe using different combinations of pH (5.5 or 7.0) for the toxin inoculum and storage temperature (4, 15, or 25^{o}C). The pHs of the toxin inoculum and storage temperatures were analyzed to determine if there was any relationship to the rate of toxin decay. A baseline condition (pH 7.0; temperature 4^{o}C) was used to create a slope and pH (5.5) and temperature (15 or 25^{o}C) were investigated to determine their effects on this slope. The data from all 3 produce types were not consistent with each other. Even though the 3 produce items have different physiologies, one would expect consistent patterns.

It is known that botulinum toxins are most stable at pH 4.5-6.5 (Spero, 1958). It was expected that the toxin inoculum at pH 5.5 would have slowed the rate of toxin degradation more so than the inoculum at pH 7.0. This occurred for tomatoes but not for lettuce or cantaloupe.

Lettuce showed that pH 7.0 slowed the rate of toxin degradation more so than pH 5.5. One reason may be due to the lettuce being wounded during cutting creating a different environment for the toxin than the surface of a whole tomato. Other factors such as enzymatic activity, absorption of toxin, the surface pH, or the natural microflora of the lettuce may have contributed to stabilizing the toxin better at the neutral pH rather than the acidic pH. For cantaloupe, the rate of decay was not significantly affected by either pH. The same factors for lettuce that provided stability may have had no effect on the pre-sliced cantaloupe. It is interesting to note that the cheese with the initial surface pH 6.0-6.5 provided the best stability for the toxin, which was not the most acidic of the three cheeses in the Grecz et al. (1965) study. There was no mention of the toxin's inoculum pH used in the study.

It is interesting to note that the surface pH of tomatoes and lettuce increased during storage instead of decreasing regardless of temperature. This was not expected. It did not correspond to previous studies (Draughon et al., 1988; Hotchkiss et al., 1992; Larson et al., 1997; Austin et al., 1998). However, the pH of cantaloupe decreased during storage with 15^{o}C showing a slightly lower pH than 4^{o}C on equivalent days. This was expected and corresponded to a previous study conducted by Larson and Johnson. Normally, background microflora helps to reduce the pH of produce by the production of acids. Also, the deterioration of the produce and the dissolution of CO_2 (carbonic acids) contribute to reducing the pH. It is possible that the components of these particular tomatoes and lettuce somehow offered a buffering capacity thereby compensating for pH changes due to acids produced by bacteria (Hao et al., 1999). Regardless, the surface pH of the produce did not appear to provide any advantage for stability to either inoculum pH.

Grecz et al. (1965) noted that the toxin appeared to be protected somewhat from degradation by the actively growing microorganisms in the cheese. The aerobic plate count numbers increased faster at the higher temperature for both tomato and lettuce and therefore may have provided the toxin protection from degradation. This may explain why a higher storage temperature appeared to stabilize the toxin better than the lower storage temperature of 4^{o}C for tomatoes and lettuce. This was not observed for cantaloupe. This may be due to the type of microorganisms found on the cantaloupe.

The results cannot be simply explained by the sample parameters of surface pH and aerobic plate counts collected for each produce item at the two different temperatures. There may be other physiological factors, e.g., enzymes, water loss, maturity of the produce, affecting the results or it simply may be the small sample size tested. It should be noted that for lettuce, a large percentage error (0.56) was indicated by the SAS output. This could indicate that the pre-cut lettuce has more variability from sample to sample than tomatoes or cantaloupe.

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REFERENCES

Austin, J.W., K.L. Dodds, B. Blanchfield, and J.M. Farber. 1998. Growth and toxin production by *Clostridium botulinum* on inoculated fresh-cut packaged vegetables. *J. Food Prot.* 61:342-328.

AOAC: Association of Analytical Chemists. 2000. *Clostridium botulinum* and its toxins in foods, p. 68-70. *In* W. Horwitz (ed.), Official methods of analysis of AOAC international, 17th ed. AOAC, Inc., Gaithersburg, MD.

Arnon, S.S., R. Schechter, T.V. Inglesby, D.A. Henderson, J.G. Bartlett, M.S. Ascher, E. Eitzen, A. D. Fine, J. Hauer, M. Layton, S. Lillibridge, M.T. Osterholm, T. O'Toole, G. Parker, T.M. Perl, P.K. Russell, D.L. Swerdlow, and K. Tonat. 2001. Botulinum toxin as a biological weapon. *JAMA.* 285:1059-1070.

CDC: Centers for Disease Control and Prevention. 1998. Centers for Disease Control and Prevention: Botulism in the United States, 1899-1996. Handbook for epidemiologists, clinicians, and laboratory workers. Atlanta, GA.

Draughon, F.A., S. Chen, and J.O. Mundt. 1988. Metabiotic association of *Fusarium*, *Alternaria*, and *Rhizoctonia* with *Clostridium botulinum* in fresh tomatoes. *J. Food Sci.* 53:120-123.

Feldkamp, C.S., and S.W. Smith. 1987. Practical guide to immunoassay method evaluation, p. 49-96. *In* D.W. Chan (ed.), Immunoassay a practical guide. Academic Press, Inc. Orlando.

Ferreira, J.L. 2001. Comparison of amplified ELISA and mouse bioassay procedures for determination of botulinal toxins A, B, E, and F. *J. AOAC Int.* 84:85-88.

Ferreira, J.L., S. Maslanka, and J. Andreadis. 2002. Detection of type A, B, E, and F *Clostridium botulinum* toxins using digoxigenin-labeled IgGs and the ELISA. FDA/ORA Laboratory Information Bulletin, vol. 18, number 10. 4292:1-10.

Grecz, N., R.O. Wagenaar, and G.M. Dack. 1965. Storage stability of *Clostridium botulinum* toxin and spores in processed cheese. *Appl. Microbiol.* 13:1014-1022.

Hao, Y.Y., R.E. Brackett, L.R. Beuchat, and M.P. Doyle. 1998. Microbiological quality and the inability of proteolytic *Clostridium botulinum* to produce toxin in film-packaged fresh-cut cabbage and lettuce. *J. Food Prot.* 61:1148-1153.

Hao, Y.Y., R.E. Brackett, L.R. Beuchat, and M.P. Doyle. 1999. Microbiological quality and production of botulinal toxin in film-packaged broccoli, carrots, and green beans. *J. Food Prot.* 62:499-508.

Hauschild, A.H.W. 1989. *Clostridium botulinum*, p. 111-189. *In* M.P. Doyle (ed.), Foodborne bacterial pathogens. Marcle Dekker, Inc. New York.

Hotchkiss, J.H., M.J. Banco, F.F. Busta, C.A. Genigeorgis, R. Kociba, L. Rheaume, L.A. Smoot, J.D. Schuman, and H. Sugiyama. 1992. The relationship between botulinal toxin production and spoilage of fresh tomatoes held at 13 and 23^{o}C under passively modified and controlled atmospheres and air. *J. Food Prot.* 55:522-527.

Jay, J.M. 2000. Modern Food Microbiology, 6th ed. Aspen Publishers, Inc. Gaithersburg, MD.

Larson, A.E., E.A. Johnson, C.R. Barmore, and M.D. Hughes. 1997. Evaluation of the botulism hazard from vegetables in modified atmosphere packaging. *J. Food Prot.* 60:1208-1214.

Larson, A.E. and E.A. Johnson. 1999. Evaluation of botulinal toxin production in packaged fresh-cut cantaloupe and honeydew melons. *J. Food Prot.* 62:948-952.

Lund, B.M. and M.W. Peck. 2001. *Clostridium botulinum*, p. 69-85. *In* R.G. Labbé and S. García (ed.), Guide to foodborne pathogens. John Wiley & Sons, Inc. New York.

Morton, D.R. 2001. Aerobic plate count, p. 63-67. *In* F.P. Downs and K. Ito (ed.), Compendium of Methods for the Microbiological Examination of Foods, 4th ed. American Public Health Association, Washington, D.C.

Nguyen-the, C. and F. Carlin. 1994. The microbiology of minimally processed fresh fruits and vegetables. *Crit. Rev. Food Sci. Nutr.* 34:371-401.

Shapiro, R.L., C. Hatheway, and D.L. Swerdlow. 1998. Botulism in the United States. *Ann. Intern. Med.* 129:221-228.

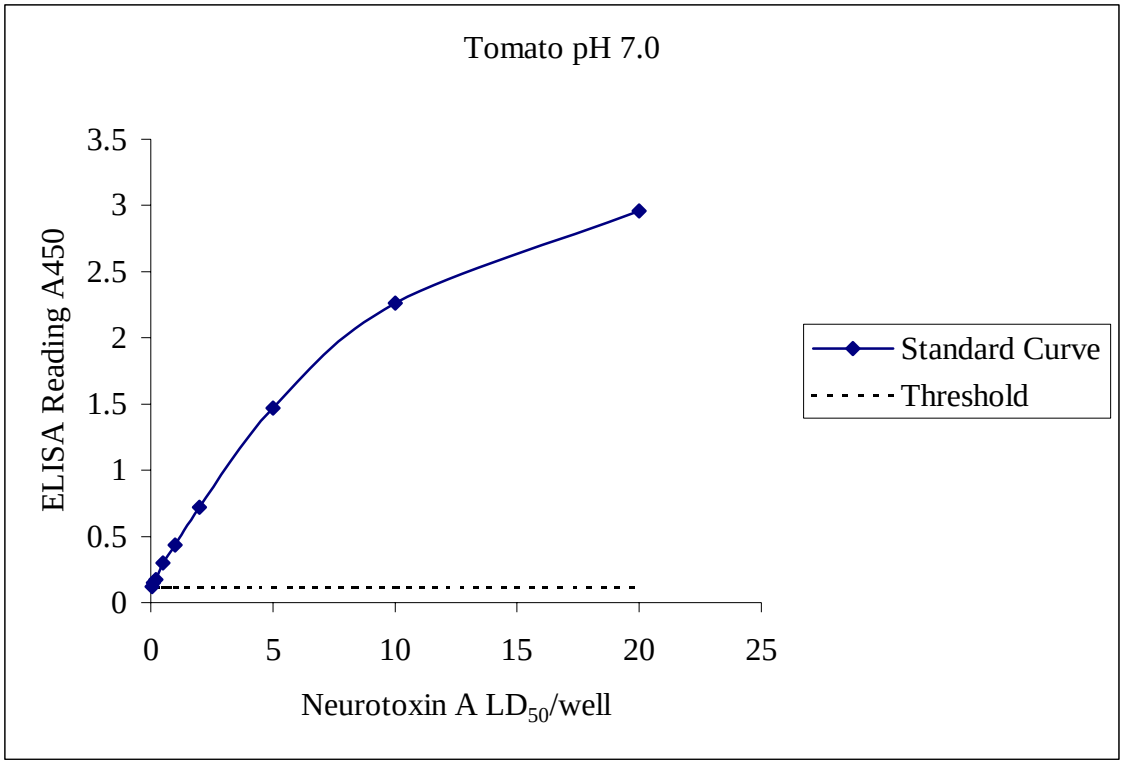
Simpson, L. 2004. Identification of the major steps in botulinum toxin action. *Annu. Rev. Pharmacol. Toxicol.* 44:167-93.

Smith, L.D. 1977. Botulism: the organism, its toxins, the disease. Charles C. Thomas. Springfield, IL.

Spero, L. 1958. The alkaline inactivation of botulinum toxin. *Arch. Biochem. Biophysics.* 73:484-491.

Zhuang, H., M.M. Barth, and T.R. Hankinson. 2003. Microbial safety, quality, and sensory aspects of fresh-cut fruits and vegetables, p. 255-278. *In* J.S. Novak, G.M. Sapers and V.K. Juneja (ed.), Microbial safety of minimally processed foods. CRC Press. Boca Raton.

Figure 2.1. Standard curves for botulinum toxin type A inoculum at pH 7.0 and 5.5 for whole grape tomatoes. The toxin inoculum was diluted in casein buffer, pH 7.4. Threshold for positive ELISA (A_{450} 0.112 and 0.151 for pH 7.0 and 5.5, respectively) indicated by dotted line.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3
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1
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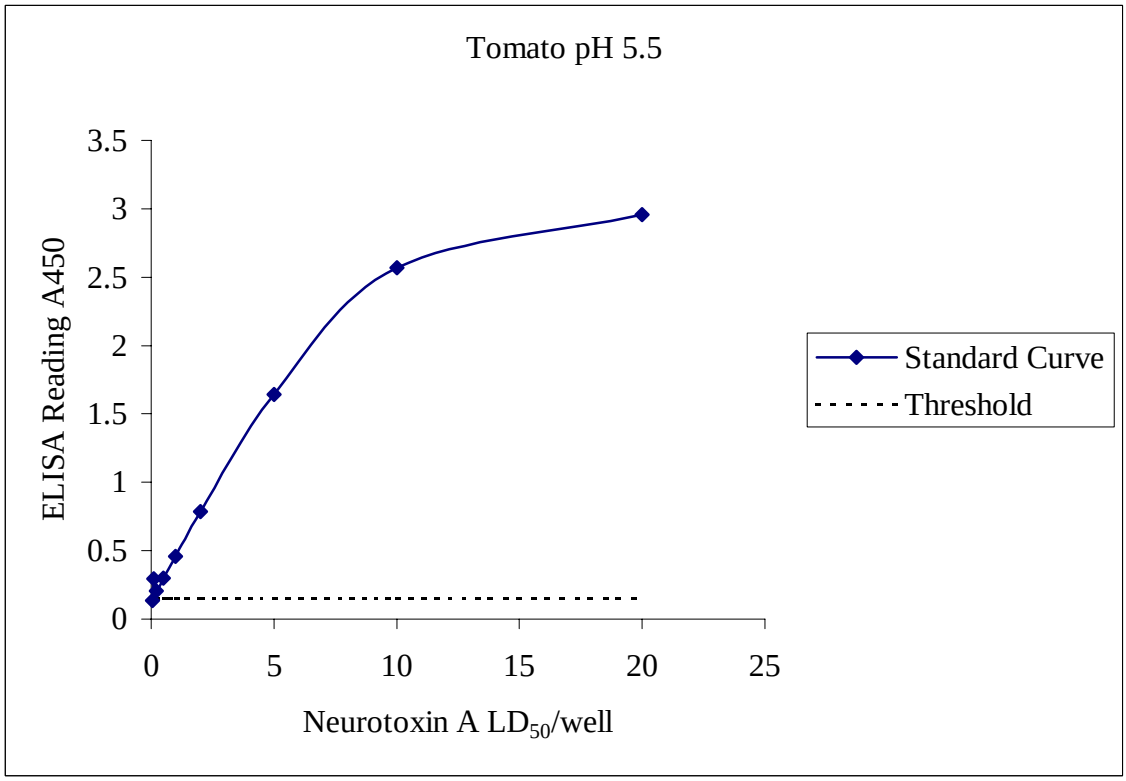
Neurotoxin A LD50/well
Standard Curve
Threshold


Tomato pH 5.5
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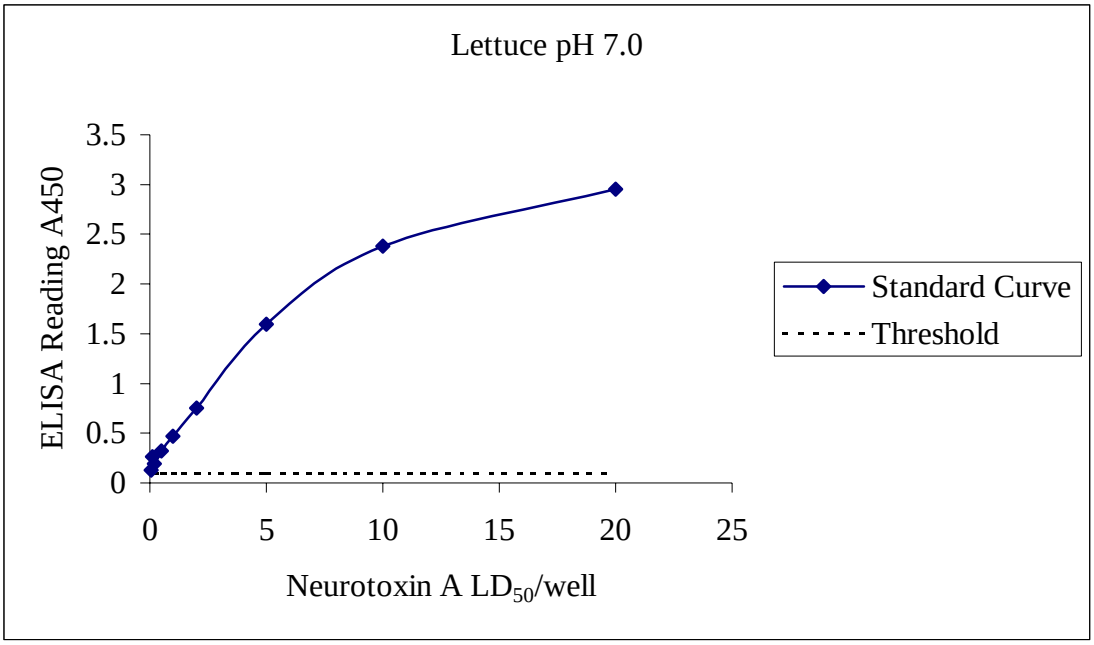
25
Neurotoxin A LD50/well
Standard Curve
Threshold

Figure 2.2. Standard curves for botulinum toxin type A inoculum at pH 7.0 and 5.5 for pre-cut lettuce. The toxin inoculum was diluted in casein buffer, pH 7.4. Threshold for positive ELISA (A_{450} 0.111 and 0.089 for pH 7.0 and 5.5, respectively) indicated by dotted line.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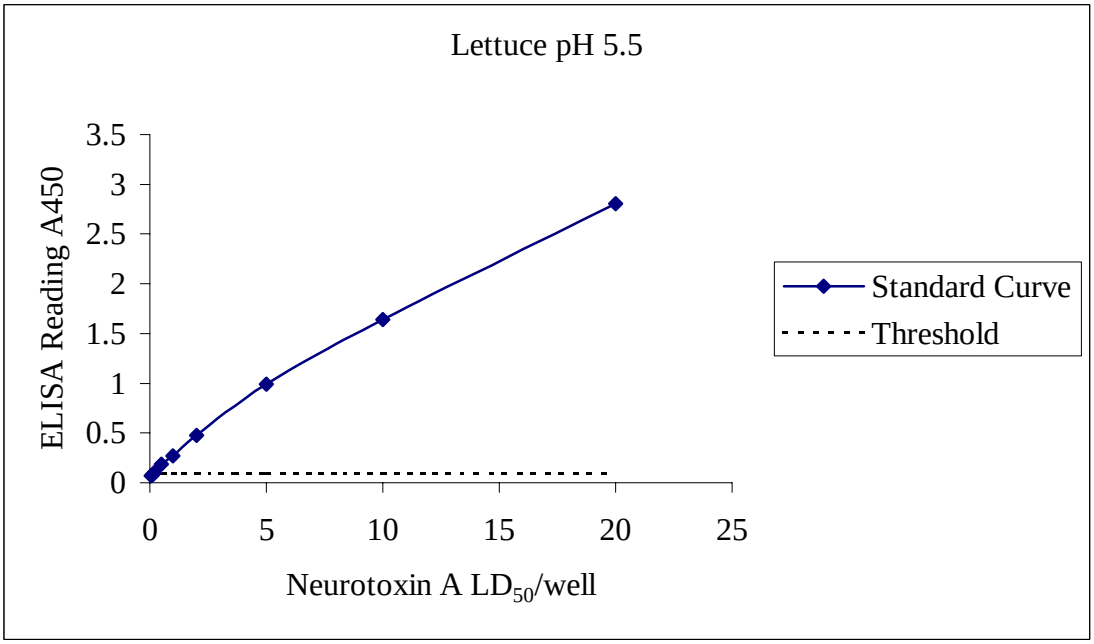
Neurotoxin A LD50/well
Standard Curve
Threshold


Lettuce pH 5.5
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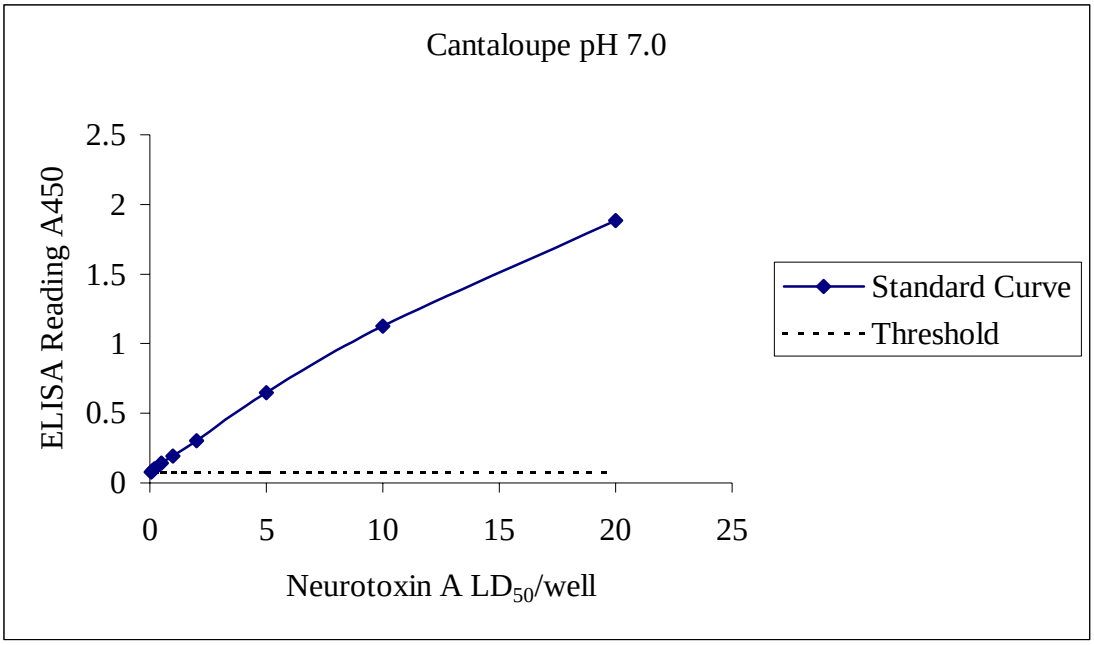
25
Neurotoxin A LD50/well
Standard Curve
Threshold

Figure 2.3. Standard curves for botulinum toxin type A inoculum at pH 7.0 and 5.5 for pre-sliced cantaloupe. The toxin inoculum was diluted in casein buffer, pH 7.4. Threshold for positive ELISA (A_{450} 0.079 and 0.102 for pH 7.0 and 5.5, respectively) indicated by dotted line.


Cantaloupe pH 7.0
ELISA Reading A450
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
Neurotoxin A LD50/well
Standard Curve
Threshold

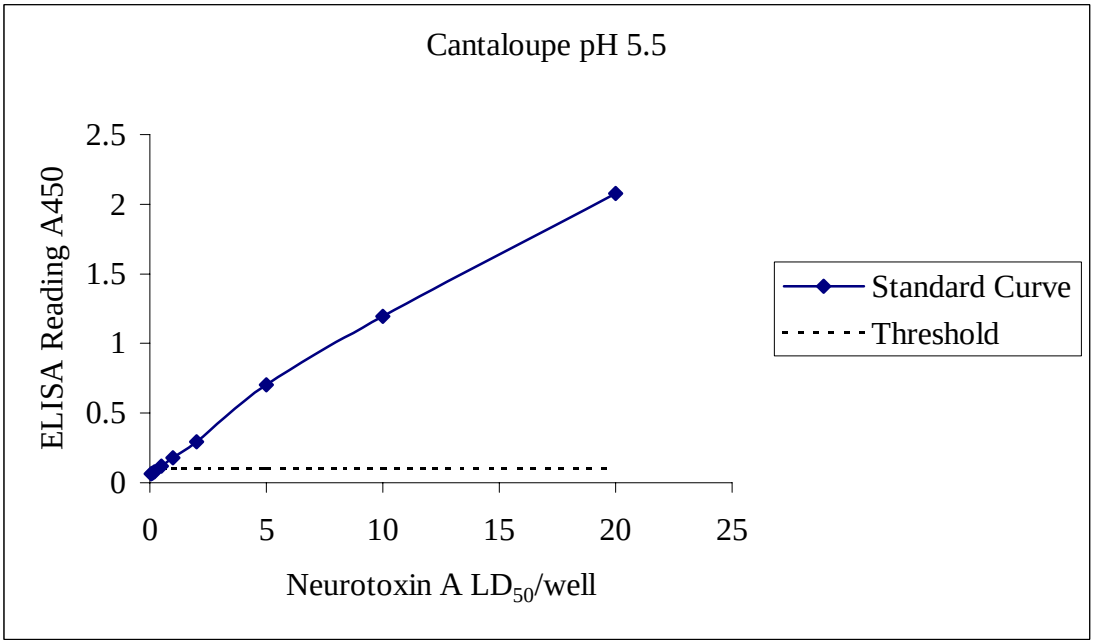

Cantaloupe pH 5.5
ELISA Reading A450
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
Neurotoxin A LD50/well
Standard Curve
Threshold

Table 2.1. *Slopes of the regression lines (defined by the slope parameters of pH and temperature) for tomatoes, lettuce, and cantaloupe.*

Produce	Slope parameters	Slope[a]	RSME[b]
Tomato	pH 7.0, 4°C	-0.0973 [B]	0.12
	pH 5.5, 4°C	-0.0417 [B]	0.12
	pH 7.0, 25°C	-0.0528 [B]	0.12
	pH 5.5, 25°C	0.0028 [A]	0.12
Lettuce	pH 7.0, 4°C	-0.1444 [B]	0.56
	pH 5.5, 4°C	-0.3233 [B]	0.56
	pH 7.0, 15°C	-0.0169 [B]	0.56
	pH 5.5, 15°C	-0.1958 [B]	0.56
Cantaloupe	pH 7.0, 4°C	0.0052 [A]	0.19
	pH 5.5, 4°C	-0.0169 [B]	0.19
	pH 7.0, 15°C	-0.0950 [B]	0.19
	pH 5.5, 15°C	-0.1171 [B]	0.19

[a] The rate of decay slope for each treatment. The value followed by an [A] denotes a slope that is not significantly different from zero ($P > 0.01$). The value followed by a [B] denotes a slope that is significantly different from zero ($P < 0.01$).
[b] RSME, root mean squared errors.

Table 2.2. *Sample pH, aerobic plate count, and sample spoilage analysis of tomatoes inoculated with botulinum toxin type A.*

Incubation Temperature ^{o}C	Incubation (days)	Sample Spoilage	Surface pH	Aerobic plate count (log_{10} CFU/g)
4	0	prespoilage	4.91	<2
	3	prespoilage	5.33	<2
	7	prespoilage	5.76	<2
	10	prespoilage	5.51	<2
25	0	prespoilage	4.91	<2
	3	prespoilage	5.19	3.06
	7	prespoilage	5.51	3.27
	10	prespoilage	6.1	3.73

Table 2.3. *Sample pH, aerobic plate count, and sample spoilage analysis of lettuce inoculated with botulinum toxin type A.*

Incubation Temperature ^{o}C	Incubation (days)	Sample Spoilage	Surface pH	Aerobic plate count (log_{10} CFU/g)
4	0	prespoilage	4.87	6.02
	3	spoilage	4.4	7.85
	7	gross spoilage	4.81	8.91
	10	gross spoilage	5.3	9.64
15	0	prespoilage	4.87	6.15
	3	gross spoilage	4.61	9.12
	7	gross spoilage	6.37	9.73
	10	gross spoilage	6.85	9.98

Table 2.4. *Sample pH, aerobic plate count, and sample spoilage analysis of cantaloupe inoculated with botulinum toxin type A.*

Incubation Temperature ^{o}C	Incubation (days)	Sample Spoilage	Surface pH	Aerobic plate count (log_{10} CFU/g)
4	0	prespoilage	6.82	5.88
	3	spoilage	6.55	7.64
	8	gross spoilage	4.96	8.43
15	0	prespoilage	6.82	5.34
	3	gross spoilage	5.17	8.55
	8	gross spoilage	4.32	8.75

APPENDIX A

STANDARD OPERATING PROCEDURES FOR THE BIOSAFETY LEVEL 3 LABORATORY IN THE FOOD SCIENCE BUILDING ATHENS, GA

S.O.P. 1: Detecting *Clostridium botulinum* toxin type A using ELISA

This procedure utilizes the Enzyme Linked Immunosorbent Assay (ELISA), a variation of the amplified ELISA that was part of an AOAC collaborative study. Adapted from Ferreira et al. (2002)

I. Equipment

a. Dynex Immulon II flat well microtiter plates (Thermolabs)
b. Plate washer
c. Microtiter adhesive plate sealer
d. Microtiter pipettors: range in size from 1µl to 1000 µl
e. Multi-channeled pipettor
f. Microplate reader (viewer)
g. Microtiter pipettor tips
h. Pipettes, disposable 1, 5, 10 ml

II. Materials

a. *Clostridium botulinum* complex neurotoxin standards type A
b. Anti-digoxigenin-horse radish peroxidase (HRP) conjugate
c. Tetramethyl benzidine (TMB)
d. Bicarbonate carbonate buffer
e. Phosphate buffered saline (PBS)
f. PBS with 20% tween (PBS-T) wash buffer
g. Casein buffer
h. Stopping reagent, 1N sulfuric acid
i. ELISA reagents
j. IgG fractions from goat type A hyperimmune sera to botulinal neurotoxins to coat Dynex Immunolon II microtiter plates
k. Digoxigenin labeled goat type A IgGs to bind captured toxin

III. Procedure

All operations are performed in a Class II Laminar Air Flow Cabinet. All wastes are disinfected chemically or autoclaved before disposal. All equipment and containers are disinfected/autoclaved before use.

Day 1

1. Capture antibodies are diluted in bicarbonate buffer and placed in needed microtiter plate wells. Seal plates and store overnight at 4^{o}C.
2. Adjust sample pH to 7.6 ± 0.2 prior to analysis. Need negative and positive controls.

Day 2

1. Remove plates from storage and wash 5 times in a PBS-T wash buffer using a plate washer.

2. Block plate with casein buffer and incubated for 60-90 min at 35°C.
3. Prepare samples and controls while plate was being blocked.
4. Discard casein buffer from plate. Tamp the plate on paper towel. Add the toxic samples diluted in casein buffer. Cover the plate wells with a plastic plate seal. Incubate for 2 h at 35°C.
5. Dilute digoxigenin labeled antibody in casein buffer.
6. Wash plate 5 times in PBS-T. Add diluted digoxigenin labeled goat antibody. Incubate for 1 h at 35°C.
7. Wash plate 5 times in PBS-T. Dilute the anti-digoxigenin HRP conjugate 1:5000 in casein buffer. Incubate for 1 h at 35°C.
8. Wash plate 5 times with PBS-T. Add TMB substrate solution to each well. Incubate for 20-30 min in the dark at 25°C.
9. Add 1N sulfuric acid (stopping reagent) to each well. Color changes from blue to yellow.
10. Read on plate reader set at 450 nm immediately.
11. A positive test has an absorbance value >0.2 above the absorbance of the negative control.

All prep work will be conducted in 358A under biosafety level (BSL) 3 conditions.

Reference:

Ferreira, J.L., S. Maslanka, and J. Andreadis. 2002. Detection of Type A, B, E, and F *Clostridium botulinum* toxins using digoxigenin-labeled IgGs and the ELISA. Laboratory Information Bulletin, FDA, Atlanta, GA.

S.O.P. 2: *Clostridium botulinum* toxin type A

Inventory control, decontamination, autoclaving, and training for *Clostridium botulinum* toxin type A.

Clostridium botulinum toxin type A

I. Receiving

a. Upon receipt by departmental staff, investigators involved in botulinum toxin research will be immediately notified
b. Investigator will relocate toxin to room 358A, a biosafety level (BSL) 3 laboratory
c. Investigator will inspect the contents

II. Inventory Control

a. Initial log in record
    i. Date received
    ii. Amount in container (weight of dry toxin or volume of liquid toxin)
    iii. Manufacturer
    iv. Researcher's initials
b. Each time toxin will be removed from original container record
    i. Date
    ii. Weight of container before and after removing toxin
    iii. Amount of toxin removed (weight or volume)
    iv. Purpose for removal
    v. Researcher's initials

III. Decontamination

a. Decontamination procedure posted in the laboratory (CDC approved procedure, which is attached, will be posted)
b. When a toxin spill occurs, use sequentially (15 to 20 min each; CDC approved procedure)
    i. 0.1N sodium hydroxide (NaOH)
    ii. 10% bleach solution
    iii. 70% alcohol
c. Wipe down all surfaces/areas with 0.1N NaOH and 70%alcohol

IV. Autoclaving

a. All waste, stock, and cultures will be autoclaved for 60 min, 121^{o}C, at 15 to 20 PSI (CDC approved procedure, which is attached)

b. Log book: log in time and day waste autoclaved, estimated amount of toxin disposed of, researcher's initials; log out time and day waste removed from autoclave, researcher's initials
c. Autoclave in room 358A has a built in recorder, the printout will be placed in the autoclave log book

V. Training

a. Read and be familiar with the Centers for Disease Control and Prevention's "Biosafety in Microbiological and Biomedical Laboratories"
    i. Indicate by signing and dating
b. When applicable, the CDC will train proper toxin(s) handling and techniques
c. Log book to indicate training given to researcher
    i. Researcher's name
    ii. Date(s)
    iii. Place trained
    iv. Trainer's name

S.O.P. 3: Security

Access into laboratory room 358A.

I. Who has access

a. Authorized personal only
b. Access permission controlled by Food Science (FS) administrators overseen by Dr. Mark Harrison and Dr. Joseph Frank

II. Access to Laboratory

a. Card Access System (CAS) uses electromagnetic door hardware
b. CAS tracks users who access the lab through the database for personal (University of Georgia's database for faculty, staff, and students)
c. Alarm system in place
d. Emergency release pull station for egress from the lab in case of an emergency

III. Locks

a. Installed on refrigerator
b. Installed on incubators

S.O.P. 4: Chemical Hygiene Plan

Chemical Hygiene Plan is used to identify hazard(s) and how to minimize the risk of exposure.

I. Identify Hazard

a. *Clostridium botulinum* toxin type A

II. Practices to minimize risks

a. Protective wear: disposable lab coats, eyewear, disposable gloves
b. Management of spills with proper decontamination steps (SOP #2)
c. Wash hands after handling toxin, after removing gloves, and when leaving the lab
d. No eating, drinking, smoking, handling contact lenses, and applying makeup in the laboratory
e. No mouth pipetting; only mechanical pipettors are to be used
f. Sharp objects such as syringes are to be placed in a puncture resistant container that is autoclavable
g. Decontaminate lab areas after all spills and after all work is completed for the day
h. All waste, stock, and cultures are autoclaved before disposal (SOP #2)

S.O.P. 5: Laboratory Equipment

Equipment used in the laboratory for experiments with *Clostridium botulinum* toxin(s) to ensure safety of personnel.

I. Biosafety Cabinet

a. NuAire 407-600 Biosafety Cabinet, 6 foot class II, type A2 model
b. Inward airflow of the biosafety cabinet will be verified prior to usage
c. Toxin handling will be conducted within the cabinet
d. Interior of the cabined will be decontaminated before and after every use (SOP #2)

II. Autoclave

a. Primus autoclave, model # PSS5-A-MSDD
    i. Double door
    ii. Pass through

III. Refrigerator(s)/Incubator(s)

a. Contain locks

IV. Protective Laboratory Wear

a. Protective clothing not to be worn outside the laboratory
    i. Disposable lab coats
    ii. Disposable gloves
b. Eye protection

V. Eye wash station

a. Located in the laboratory
    i. Easy access

S.O.P. 6: Laboratory Room 358A

Indicates access and precautionary measures to insure safety of all personnel. Also gives overview of laboratory facility.

I. Access

a. Controlled access through designated doors using stored fingerprint
b. Authorized personal only (SOP # 3)

II. Log Out

a. Log out sheet next to door in laboratory room records
    i. Date
    ii. Time
    iii. Researcher's initials
    iv. Reason(s) for being in the lab room initially

III. Visitors

a. All visitors, at all times, will be accompanied by authorized personnel
b. Visitors will be required to log in and out
    i. Date
    ii. Time in/time out
    iii. Visitor's name
    iv. Name of person visited
    v. Purpose of visit

IV. When room is in use

a. A sign stating that "Toxin in Use Authorized Personnel Only" at the entrance to the room

V. Visibility

a. Window (closed and sealed) in room looks into BSL 2 laboratory room where other individuals can maintain visual contact with the researcher in room 358A

VI. Doors

a. Will be closed at all times during an experiment

VII. Facility

a. Biosafety level (BSL) 3 laboratory
    i. Built in 2002/2003

b. Hand free sink for hand washing located both in room 358A and the vestibule near the exit door
c. Interior of the laboratory: walls, floors, ceilings, which are reinforced fiberglass panels, are easy to clean and decontaminate
d. Bench tops
    i. Impervious to water
    ii. Resistant to heat, organic solvents, acids, alkalis, and chemicals used to decontaminate the work surfaces
e. Chairs covered with a non-fabric material that is easy to decontaminate
f. Eyewash station inside lab
g. Adequate lighting, avoids reflections and glare that could impede vision
h. Annual verification of lab room and equipment will occur

APPENDIX B

INVENTORY CONTROL AND LOGBOOKS

Inventory Control for *Clostridium botulinum* toxin type A:

Date Received: _____

Amount in µl: _____

Manufacturer: _____

Receiver's name:_____

Principle Investigator: _____

Date	Initial Weight of Container	Final Weight of Container	Amount of Toxin Removed in µl	Researcher's Initials

Autoclaving

121^{o}C for 60 min at 15 to 20 PSI

Time In	Date	Estimated amount of Toxin	Researcher's Initials

Log Out

Date	Time	Researcher's Initials	Reason for being in lab

Training received for *Clostridium botulinum*

Name	Signature	Date(s) Trained	Place Trained	Trainer's Name

Visitors

Date	Time In	Time Out	Visitor's Name	Name of Person Visited	Purpose of Visit