

**Thermal Inactivation of Human Norovirus Surrogates
and Hepatitis A Virus in Foods**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
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DEDICATION

To the loving memory of my father, Omer BOZKURT,

&

To my dear husband, OZGUR CEKMER.

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ABSTRACT

Foodborne enteric viruses are the leading cause of gastroenteritis in humans. In particular, human noroviruses and hepatitis A virus (HAV) are the most important foodborne viral pathogens with regard to the number of outbreaks and people affected. Therefore, studies are needed to bridge existing data gaps and determine appropriate parameters for thermal inactivation methods for human norovirus and hepatitis A virus. Due to the absence of appropriate cell culture systems to propagate these viruses, cultivable surrogates (feline calicivirus, FCV-F9 and murine norovirus, MNV-1) are commonly used based on the assumption that they can mimic the viruses they represent. The objectives of this study were to determine thermal inactivation behavior of human norovirus surrogates and hepatitis A virus (HAV) in buffer, mussel, spinach and turkey deli meat, to compare first-order and Weibull models in describing the data in terms of selected statistical parameters, to discuss inactivation mechanism during thermal treatment and to provide insight for future studies and industrial applications. Temperature had a significant effect on both t_D and D-values for the range from 50 to 72°C for all virus surrogates ($p < 0.05$). In general, HAV was more resistant to thermal treatment than FCV-F9 and MNV-1 at all temperatures studied suggesting that it would require a more severe treatment than the tested human norovirus surrogates for inactivation in food. Results also revealed that the Weibull model was more appropriate to represent the thermal inactivation behavior of all tested surrogates. The thermal inactivation of viruses was found to be associated with HAV capsid structural changes and denaturation of proteins. This study provides useful information on the thermal

inactivation behavior of viruses and will contribute to the development of appropriate thermal processing protocols to ensure safety of food for human consumption.

Keywords; thermal inactivation, human norovirus surrogates, hepatitis A virus, D and z value, first-order and Weibull model, buffer, mussel, spinach, turkey deli meat.

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INTRODUCTION

Foodborne enteric viruses are commonly associated with foodborne illnesses and frequently cause non-bacterial acute gastroenteritis in humans (Blanton et al., 2006). In the United States, it is estimated that 31 pathogens cause 9.4 million foodborne illnesses, 128,000 hospitalizations, and 3,000 deaths annually (CDC, 2014a). Viruses alone reportedly cause an estimated 58% (5.5 million) of foodborne illnesses, 26% of hospitalizations, and 11% of the deaths. Viruses that are associated with foodborne and waterborne diseases include human noroviruses, hepatitis A virus, rotaviruses, hepatitis E virus, adenoviruses, sapoviruses, astroviruses, aichi virus, parvoviruses, and other enteroviruses (CDC, 2014b). Even though gastroenteritis caused by viruses is generally ranked as the primary cause of foodborne illness in the United States, viruses are not routinely tested for in food and environmental samples (Grove et al., 2006).

Viruses have properties that are unique to those of bacterial pathogens (D'Souza et al., 2007). They have been described by some scientists as extracellular organelles evolved to transfer nucleic acid from one cell to another (Harrison et al., 1996). The diameter of viruses ranges between 25 and 300 nm, so typically cannot be visualized with a light microscope (Grove et al., 2006). Viruses have no cellular structure and contain either RNA or DNA enclosed in a protein coat or capsid (Madigan et al., 2000). The capsid functions as the primary protective barrier for the viral particle or virion. While the capsid of some viruses is enveloped in an outer lipid membrane, all human enteric viruses are non-enveloped. Since their outer coat primarily comprise of protein, human enteric viruses are more resistant to environmental conditions (Adams and Moss, 2000).

They often have a low infectious dose where as few as 10 virus particles may produce an illness (Koopmans and Duizer, 2004). The ability of viruses to persist in the environment and foods coupled with low infectious doses suggests that even a small amount of contamination may pose a significant health risk to the public.

Viruses can be transmitted through the “fecal-oral route” by contaminated food and water, as well as through person- to person contact and cross contamination from surfaces. It has been also reported that high numbers of viral particles are shed in the stools from infected persons and thus poor personal hygiene is often a responsible for transmission (Koopmans and Duizer, 2004). Foods at risk for the presence of enteric viruses include those primarily subject to environmental contamination, such as seafood, and fresh produce and those primarily subject to handling/transmission via the fecal-oral route such as leafy vegetables, deli items and other ready-to-eat (RTE) foods that do not undergo further processing (Sair et al., 2002). Due to the obligate parasitic nature of viruses, they cannot multiply in the environment or in foods. Therefore, typical methods used to control bacterial growth in food products and current food hygiene guidelines that rely on prevention of bacterial growth are relatively ineffective against viruses (Jaykus, 2000; Koopmans and Duizer, 2004).

Thermal processes are one of the main technologies applied in the food industry for the preservation of food materials (Silva and Gibbs, 2012). One of the primary goals of thermal processing is to inactivate spoilage and pathogenic microorganisms and produce a safe product with enhanced/extended shelf life (Lee and Kaletunc, 2002). The D-value (thermal decimal reduction time) is the time necessary to reduce by 90% a

microbial population present in a well-defined medium, and it is indicative of the thermal resistance of a microorganism at a constant temperature. The z-value is the temperature increase necessary to reduce the D-value by 90% and is indicative of the temperature dependence of microbial inactivation (Houben and Eckenhausen, 2006). Knowledge of the thermal inactivation parameters (D- and z-values) for a particular microorganism makes it possible to design effective thermal processes (Houben and Eckenhausen, 2006; Lee and Kaletunc, 2002). In the current literature, even though thermal inactivation of behavior of foodborne bacterial pathogens has been well studied, there is limited information about thermal inactivation behavior of foodborne enteric viruses (Newell et al., 2010). There is no specific US regulation covering the minimum time-temperature combinations for inactivating virus contaminated food. Thus, determination of the thermal inactivation behavior of foodborne enteric viruses will contribute towards improving strategies for the control of virus contamination in foods using thermal processing. The objective of this study is

(i) to determine thermal inactivation behavior of human norovirus surrogates and hepatitis A virus (HAV) in buffer, mussel, spinach and turkey deli meat, (ii) to compare first-order and Weibull models in describing the data in terms of selected statistical parameters, (iii) to discuss inactivation mechanism during thermal treatment, and (iv) to provide insight on foodborne enteric viruses for future study and industrial applications.

CHAPTER I
A REVIEW OF THERMAL INACTIVATION OF FOODBORNE
ENTERIC VIRUSES IN FOODS

Abstract

Foodborne viruses, in particular noroviruses and hepatitis A virus, are the most common causes of food associated infections and outbreaks around the world. Therefore, they have become an important concern for health authorities. Despite their importance in public health, there is little information on the thermal inactivation characteristics of human noroviruses and wild type strain of HAV due to the lack of appropriate cell culture systems for their propagation. Therefore, viral surrogates have been commonly used based on the assumption that they can mimic the viruses they represent. Since, thermal inactivation of microorganisms is a fundamental operation in the food industry, the precise understanding of the thermal inactivation behavior of human norovirus surrogates, and hepatitis A virus could provide precise determination of the thermal process calculations to prevent foodborne viral outbreaks associated with the consumption of contaminated food. Therefore, the objective of this study was to (i) discuss some common behavior patterns of enteric foodborne viruses, (ii) evaluate viral surrogates used in thermal inactivation studies, (iii) review available thermal data, (iv) discuss mechanisms of inactivation during thermal treatment and (v) provide insight on foodborne enteric viruses for future study and industrial applications. The results of this study should contribute to the development of appropriate thermal processing protocols to ensure safety of food for human consumption.

Keywords; thermal inactivation, D-value, z-value, human norovirus surrogates, hepatitis A virus, food.

Foodborne enteric viruses

Human norovirus

Human noroviruses are the leading cause of outbreaks as well as sporadic cases of acute gastroenteritis worldwide (Blanton et al., 2006; Green et al., 2001). Noroviruses belong to the *Caliciviridae* family that is comprised of five genera: Norovirus, Sapovirus, Lagovirus, Vesivirus, and Nebovirus (Clarke et al., 2012). The first two genera contain primarily human viruses, while the other genera represent animal viruses.

Human noroviruses are non-enveloped RNA viruses, approximately 27 to 38 nm in diameter, which to-date cannot be cultivated in animal cell-culture systems, posing a problem for experimental and foodborne research purposes (Grove et al., 2006).

Noroviruses are icosahedral in shape and contain single-stranded positive-sense RNA genomes ranging in size from 7.4 to 8.3 kb. Excluding the 3' end of the genome which contains a poly A tail, the norovirus genome sequence is 7,642 nucleotides in length (D'Souza et al., 2007). This genome contains three open reading frames (ORF1, ORF2, and ORF3) which encode structural and non-structural genes (Donaldson et al., 2008). ORF1 (nucleotides 146 to 5,359) is the largest genome corresponding to ca. 1,700 amino acids and encodes six non-structural proteins including p48 (responsible for replication), NTPase (nucleoside triphosphatases), p22 (precursor in the proteolytic processing pathway), VPg (binds to 5' end to initiate translation), 3CL^{pro} (protease), RdRp (RNA-dependent RNA polymerase) (Chen et al, 2004; D'Souza et al., 2007; Donaldson et al., 2008). It is speculated that the viral protein (VPg) is covalently linked to the viral RNA and caps the 5' end and may function in transporting to negative strand synthesis sites

(Donaldson et al., 2008). ORF2 (nucleotides 5,346 to 6,935) encodes the major viral structural protein (VP1) of approximately 60 kD that folds into an S (shell) and a P (protruding) domain that P domain is further divided into P1 and P2. Among those, P2 is the most hyper variable region of the genome and is responsible for histoblood group antigen (HBGA) receptor binding (Chen et al, 2004; D'Souza et al., 2007; Donaldson et al., 2008). ORF3 (nucleotides 6,938 to 7,573) encodes a minor structural protein (VP2) with an unknown function but *in vitro* studies have suggested that this gene regulates the expression and stability of VP1 (Bertolotti-Ciarlet et al, 2003; D'Souza et al., 2007; Donaldson et al., 2008, Glass et al., 1993).

Based on the molecular characterization of complete capsid gene sequences, noroviruses are classified into five different genogroups: GI (prototype, Norwalk virus), GII (prototype, Snow Mountain virus) GIII (prototype, bovine enteric calicivirus), GIV (prototypes, Alphanoron and Ft. Lauderdale viruses), and GV (prototype, murine norovirus) (Zheng et al., 2006; D'Souza et al., 2007). Strains of three genogroups, GI, GII, and GIV, are found in humans, and GIII and GV strains are found in cattle and mice, respectively. Among human infecting genogroups, GII is predominant, responsible for the most human norovirus outbreaks. Approximately 73% of human norovirus illnesses are caused by GII viruses (Zheng et al., 2006). The emergence of virulent strains, including GII.4, can cause death in infected elderly and/or immune-compromised persons (Siebenga et al., 2010).

The transmission of human noroviruses occurs by three general routes: person-to-person, foodborne, and waterborne. Person-to-person transmission might occur directly

through the fecal-oral route, by ingestion of aerosolized vomitus, or by indirect exposure via fomites or contaminated environmental surfaces. Foodborne transmission typically occurs by contamination from infected food handlers during preparation or service but might also occur further upstream in the food distribution system through contamination with human feces (Dowell et al., 1995). Drinking water may serve as vehicles of norovirus transmission and result in large community outbreaks (Yoder et al., 2008). These outbreaks often involve water that becomes contaminated from septic tank leakage or sewage or from breakdowns in chlorination of municipal systems (Beller et al., 1997; Kukkula et al., 1999).

Once ingested, human noroviruses bind to the histoblood group antigens (HBGA) in human intestinal epithelial cells and the site of replication is thought to be the upper intestinal tract of small intestine (Huang et al., 2005). Genetic determinants for these blood group antigens play a role in susceptibility of an individual to human noroviruses infection. Human volunteer studies have shown that H type I antigen of HBGA likely serves as the receptor for human noroviruses which is encoded by the fucosyltransferase (*fut2*) gene (Lindesmith et al., 2008). Individuals that are recessive for this gene, lack the Type I antigen, and are inherently resistant to human noroviruses infections (Lindesmith et al., 2008).

Norovirus infection is characterized as a self-limiting gastrointestinal infection in humans with symptoms that include nausea, vomiting, diarrhea, malaise, abdominal pain, muscle pain, anorexia, and headache and low grade fever. Symptoms generally begin one to two days following consumption of contaminated foods or water and persist for one to

eight days (Glove et al., 2006). There is no specific anti-viral treatment against human noroviruses. Infections can be treated by oral or intravenous fluid therapy. There is no vaccine currently available against human noroviruses, the major barrier being the inability to culture them *in vitro*. Human volunteers reinfected with human noroviruses after exposure were found to be susceptible to the same strain as well as heterologous strains (Johnson et al., 1990; Parrino et al., 1977). To prevent virus transmission, the proper application of food hygiene practices are recommended by the US Centers for Disease Control and Prevention (CDC, 2014c).

Hepatitis A virus

Hepatitis A virus (HAV) is the second largest cause of viral enteric gastroenteritis. HAV belongs to the *Picornaviridae* family that is comprised of five genera: *Hepatoviruses*, *Enteroviruses*, *Rhinoviruses*, *Cardioviruses*, and *Aphthoviruses* (Sattar et al., 2000).

HAV is non-enveloped RNA virus ca. 27-32 nm in diameter. HAV is icosahedral in shape and contains single-stranded positive-sense RNA genomes 7.5 kb in size (D'Souza et al., 2007). Unlike human noroviruses, the HAV genome consists of only one ORF (ORF1) which is divided into three regions designated P1, P2 and P3. The P1 region encodes for three major viral capsid proteins VP1, VP2, and VP3. P2 and P3 regions encode for non-structural proteins required for RNA replication and virion formation (Jeong et al., 2010; Nainan et al., 2006).

Based on the molecular characterization of capsid gene sequences HAV are classified into seven different genogroups. Strains of four genogroups, GI, GII, GIII, and

GVII, are found in humans, and GIV, GV, and GVI are found in simians (Robertson et al., 1992). The most prevalent genotype, genotype I, and its sub-genotypes, encompass 80% of human strains (Kokkinos et al., 2010). Only one serotype of HAV has been identified to date and a single exposure can render life-long immunity in an individual (Arauz-Ruiz et al., 2001).

Hepatitis A virus as well as other enteric viruses are transmitted directly from person to person by the fecal-oral route or indirectly through contaminated food, water or environmental surfaces. As many as 1 billion viral particles can be shed in 1 g of stool and direct or indirect contact with feces, emesis or their aerosolized droplets are important routes of transmission (Chan et al, 2006). Since the infectious dose is very low (between 10 to 100 virus particles), hands or surfaces that appear clean can still harbor infectious material, contributing to virus spread (Teunis et al, 2008). Contamination of food can occur anywhere along the farm to fork continuum that involves human contact, or indirectly through fecal contamination of water that comes in contact with foods. Since the capsids of enteric viruses have properties that promote survival for long periods of time under harsh conditions such as desiccation, freezing and extremes in pH, they are well adapted to survival in and on foods (Abad et al, 1994; Abad et al, 2001; D'Souza et al, 2006; Hollinger and Emerson 2007; Mbithi et al, 1992).

After infection, HAV illness spans four phases. The first phase is characterized by viral replication in the body without symptoms and lasts an average of 28 to 30 days (D'Souza et al. 2007; Grove et al., 2006). The second phase, prodromal or pre-icteric, is characterized by an onset of symptoms including anorexia, vomiting, fatigue, and

jaundice and lasts an average of 5 to 7 days (Brundage and Fitzpatrick, 2006). The third phase is characterized by the onset of jaundice and an enlarged liver lasting up to 28 days. During the final phase, symptoms resolve and liver enzymes returns to normal.

There is no specific treatment available for hepatitis A infection. However, symptoms can be alleviated by appropriate patient care. For prevention, immunoglobulin (Ig) therapy is effective when administered to individuals within two weeks of viral exposure (through passive immunity). Also, inactivated and heat-killed vaccines against HAV are commercially available, that provide immunity against HAV for >20 years or lifetime (van Damme et al., 2003). The CDC recommends routine vaccination against HAV for children aged between 12 to 23 months (CDC, 2014d). When economically feasible, vaccination of food-handlers is recommended to prevent transmission of HAV and to prevent HAV outbreaks.

Other foodborne enteric viruses

Adenovirus, rotavirus, and Aichi virus have also been implicated with outbreaks of foodborne illnesses but much less frequently than outbreaks of human norovirus and HAV (le Guyader et al., 2008; Yamashita et al., 2000). The general characteristics of these enteric viruses are given in Table 1.1

Thermal inactivation of foodborne enteric viruses

Viral surrogates

Traditionally, the use of “surrogate” microorganisms is as a substitute for a pathogenic microorganism in validating the efficacy of a food process, most often

thermal processes. Methods for inactivating microorganisms in foods using thermal treatments are targeted at pathogenic and/or spoilage microorganisms. Once a processing method is developed, it must be validated or verified in an actual food processing system or environment. The use of surrogates derives from the need for validation or verification of a microbial control step in a food process and, at the same time, the need to prevent introduction of pathogenic microorganisms into an industrial food processing operation. Thus, the use of surrogates is of great importance to ensure microbiological safety of the process (Busta et al., 2003, Hoeltzer et al., 2013).

In contrast to the traditional uses, surrogates for enteric viruses are used for a very different reason. To date, all attempts to propagate human norovirus and wild type strains of HAV in routine laboratory cell culture or primary tissue cultures have been unsuccessful (Duizer et al, 2004; Richards 2012). Straub et al. (2007) did report that, using a 3-D cell culture vessel, they were able to demonstrate passage of both GI and GII NoV *in vitro*. However, this model still awaits confirmation in subsequent studies. Thus, viral surrogates have been played an important role as indicators for inactivation of foodborne enteric viruses to aid design and validation of food processing systems (Black et al., 2010). The ideal surrogate should have a similar structure and size to the target; be cultivable in the lab; be more resistant to treatments; be nonpathogenic; mimic the survival and persistence characteristic; be transmitted by fecal oral route (Busta et al., 2003). The surrogates for foodborne enteric viruses including feline calicivirus, murine norovirus, bacteriophage MS2, tulane virus, sapovirus, poliovirus, hepatitis A virus have

been used in inactivation studies. Characteristic of these viral surrogates are shown in Table 1.2.

Feline calicivirus (FCV) is a respiratory virus and was the first animal virus surrogate used in laboratories to mimic human noroviruses (Doulton et al., 1999). It is a member of the genus *Vesivirus* in the *Caliciviridae* family and is a non-enveloped RNA virus that is approximately 35 to 39 nm in diameter. FCV is icosahedral in shape and contains single-stranded positive-sense RNA genomes 7.5 kb in size. Similar to human noroviruses, FCV also has 3 ORFs. Since it is a respiratory virus, and sensitive to low pH (2.0-4.0), it may not adequately mimic the survival of human noroviruses in the environment or food (Cannon et al., 2006).

Murine norovirus (MNV), also a member of the *Caliciviridae* family. It has greater genetic similarities to norovirus than FCV since it is within the *Norovirus* genus (Hirneisen et al., 2013; Hutson et al., 2004). MNV has immunological, biochemical, genetic and molecular properties, which are very similar to human noroviruses. MNV is an icosahedral, non-enveloped, single-stranded RNA virus with 3 ORFs, 28-35 nm in diameter, with a genome containing three ORFs (Wobus et al., 2006). Although it causes a different disease in mice, it is transmitted via the fecal oral route similar to human noroviruses (Cannon et al., 2006). Unlike FCV, it is less sensitive to pH within the range of 2.0 to 10.0. In one of the first studies on thermal inactivation parameters of MNV, processes such as pasteurization (63°C) were reported to be similar for FCV and MNV (Cannon et al., 2006).

Another potential human enteric virus surrogate is the bacteriophage MS2. A bacteriophage is a virus that only infects bacterial cells and MS2 infects *Escherichia coli* ATCC 15597B in particular. MS2 is a single-stranded RNA virus with icosahedral symmetry. It belongs to the *Leviviridae* family that is group 1 of the RNA coliphages (Calender, 1988; Dawson et al., 2005). MS2, commonly found in sewage, is between 27-34 nm in diameter and is adapted to the intestinal tract (Dawson et al., 2005).

A recently discovered calicivirus with potential for use as a surrogate is the Tulane virus (TV). It was isolated from the stools of rhesus macaques (*Macaca mulatta*) and represents a new genus, *Recovirus* (Farkas et al., 2008). Even though TV does not belong to the genus Norovirus, sequence analysis has revealed that TV is closely related to the GII noroviruses (Farkas et al., 2010). Similar to human noroviruses, TV also bind to histo-blood group antigens (Farkas et al., 2008). This characteristic could make TV structurally more similar than MNV to human noroviruses and potentially a good surrogate.

Sapovirus (SaV) is a member of the genus Sapovirus in the Caliciviridae family, and is a non enveloped RNA virus of approximately 27 to 35 nm. SaV is also icosahedral in shape and contain single-stranded positive-sense RNA genomes 7.5 kb in size. Similar to human noroviruses, SaV is also is transmitted through the ingestion of fecally contaminated material; however it cause gastroenteritis only in gnotobiotic pig (Wang et al., 2012).

Virus like particles (VLPs) have also been used as surrogates to understand virus behavior in inactivation studies. Coexpression of viral capsid proteins in the baculovirus

expression systems results in the assembly of virus-like particles (VLPs) that maintain the structural and functional characteristics of the native particles, i.e., they resemble a real virus but they are non-infectious (Loisy et al., 2005). VLPs have been used as surrogates for viruses in environmental persistence and inactivation studies (Caballero et al., 2004, Loisy et al., 2005; Ausar et al., 2006). It has been reported that VLPs are highly stable over a pH range of 3–7 and up to 55 °C. However, temperature above 55 °C, they undergo distinct phase transitions arising from secondary, tertiary, and quaternary level protein structural perturbations (Ausar et al., 2006). Thus, they may not adequately mimic the survival of human noroviruses in food.

There are few strains of HAV (HM-175, HAS-15, MBB 11/5) that are cell-culture adaptable and maintained using fetal rhesus monkey kidney (FRhK-4) and/or human fetal lung fibroblast (MRC-5) cells. Due to their resistance to environmental stresses such as acid, heat, drying, pressure, disinfectants and UV, they have been used as surrogates in inactivation studies (Martin et al., 2006; Reiner et al., 1992).

Evaluation of thermal inactivation data for foodborne enteric viruses

Thermal inactivation data for foodborne enteric viruses and their surrogates in cell culture media, seafood, fruits and vegetables, dairy, and meat products is shown in Tables 1.3-1.7. The temperature ranges studied were 37-100°C, 50-100°C, 4-85°C, 62.8-85°C, and 50-72°C for viruses in cell culture medium, seafood, fruits and vegetables, dairy products, and meat products, respectively.

The viruses used in these studies belonged to two families (*Picornaviridae* and *Caliciviridae*), and five genera (Hepatitis virus, vesivirus, norovirus, reovirus, sapovirus and enterovirus).

Cell culture media:

FCV was the most commonly used viral surrogates in thermal inactivation studies involving cell culture media. The effect of thermal treatment on inactivation of FCV in cell culture media has been investigated in the temperature range of 37 to 80°C (Table 1.3). Surviving viruses have been enumerated using either the Tissue Culture Infective Dose (TCID₅₀), the plaque assay (Plaque Forming Unit: PFU)/ml or RT-PCR techniques.

The TCID₅₀ method is performed to determine the dilution of viruses required to infect 50% of a series of inoculated cell cultures, and like the plaque assay it relies on the presence and detection of cytopathic effect (CPE). Host cells are grown in confluent healthy monolayers, in the wells of a multi-well (24, 48, or 96 well) tissue culture plate, to which aliquots of virus are added. During incubation, the virus replicates and releases progeny virions into the culture medium of each well, which in turn infect other healthy cells in the monolayer. The CPE is allowed to develop over a period of time, at which the cell monolayers are observed microscopically, directly, or following fixing and/or staining. Each well is scored for the presence or absence of CPE, and marked as positive or negative, accordingly (Baker et al., 2011). The number of positive wells at each dilution tested are used to calculate TCID₅₀ which represents the dilution of virus that would give CPE in 50% of the monolayers inoculated (Eq. 1.1).

$$TCID_{50} = \frac{(\% \text{ of wells infected at dilution above } 50\% - 50\%)}{\% \text{ of wells infected at dilution above } 50\% - \% \text{ of wells infected at dilution below } 50\%} \quad (1)$$

The basis of plaque assay technique is to measure the ability of a single infectious virus to form a plaque on a susceptible confluent monolayer culture of cells. Plaque assays offer the specific advantage of producing a countable event, i.e. plaque formation, versus $TCID_{50}$ (Darling et al., 1998). The virus titer (PFU/ml) is determined by dividing the total number of plaques by the total volume of original sample tested.

RT-PCR technique has been used to quantify virus by determining the number of copies of given virus sequence (the target) that are present after a known number of cycles, and subsequently estimating the number of target sequences that were present in the original sample.

In general, the D-values determined by Bozkurt et al. (2013), Cannon et al. (2006), Doultree et al. (1999) and Duizer et al. (2004) were much lower than those of Gibson and Schwab (2011) (Fig. 1A). Since the volume of the sample (15 ml) used by Gibson and Schwab (2011) was much higher than other studies, this difference between D-values might be associated with heat transfer rate and heating system. As stated by Chung et al. (2007), the differences in container size can potentially lead to differences in heat transfer rate and thus differences in apparent D-value.

The reported and/or calculated z-values for FCV in cell culture media were in the range of 9.29°C to 11.54°C but there were no significant differences observed between the studies ($p > 0.05$). (Doultree et al., 1999; Duizer et al., 2004; Cannon et al., 2006; Bozkurt et al., 2013; Bozkurt et al., 2014d). However, there was a significant difference

between the thermal inactivation data by Croci et al. (2012) and the other studies. In their study, Croci et al. (2012) evaluated thermal inactivation behavior of FCV in cell culture media by comparing plaque assay and molecular detection (rRT-PCR). In terms of TCID₅₀ assay, they observed the same amount of inactivation (3.5 log) at both 60 and 80°C after 3 min of thermal treatment and thus the same D-value (1.16 min) for both temperatures (60, and 80°C) (Table 1.3). Compared to other studies where viral inactivation was related to time and temperature as it is with other microorganisms, Croci et al. (2012) found no such relationship. They also determined the D-value based on rRT-PCR data for which they calculated a $D_{60^{\circ}\text{C}} = 0.13$ min and a $D_{80^{\circ}\text{C}} = 0.12$ min. While the rRT-PCR may be useful for viral nucleic acid destruction, it does not provide information on virus infectivity and might be the reason for differences observed between plaque assays in other studies and rRT-PCR.

The second most commonly studied viral surrogate during thermal treatment in cell culture media is MNV (29%) (Table 1.3). The reported D-values for MNV in the range of 50 to 80°C were 0.15 to 36.28 min. The first reported thermal inactivation data generated for MNV was by Cannon et al. (2006) who studied survival at 56, 63, and 72°C using the capillary tube method (50 µl). At 56, 63, and 72°C the D-values for MNV-1 were 3.47, 0.44, and 0.17 min, respectively (Cannon et al., 2006). Bozkurt et al. (2013) also investigated the thermal inactivation behavior of MNV-1 in cell culture media at 50, 56, 60, 65, and 72°C using a capillary tube method (50 µl) and their reported D-values were consistent with those of Cannon et al. (2006). Bozkurt et al. (2014d) also evaluated the contribution of sample volume (2 ml) on thermal inactivation behavior of MNV, and

their reported D-values were higher than those obtained in their previous study at 60, 65, and 72°C ($p < 0.05$) but no statistical differences were observed at 50 and 56°C ($p > 0.05$). The difference in the results may be explained by different heat transfer rates (Bozkurt et al., 2013, 2014d). In the capillary tube method, temperature reaches the desired level almost instantly, while in the 2 ml vial, there is a short come up time to achieve desired temperature. Even though Hirneissen and Kniel (2013) also investigated thermal inactivation behavior of MNV-1 at the same temperature range (50, 55, 60, and 65°C), their reported D-values were not consistent with those of Cannon et al. (2006), Bozkurt et al. (2013) or Bozkurt et al. (2014d). Differences between these studies might be related to the heating systems. In their studies, Cannon et al. (2006), Bozkurt et al. (2013), and Bozkurt et al. (2014d) used a water bath. However, Hirneissen and Kniel (2013) performed heat treatment in PCR thermo cycler and they did not consider to time to reach desired temperature. The reported and/or calculated z-values for MNV for the studies with consistent D-values (Cannon et al., 2006; Bozkurt et al., 2013; Bozkurt et al., 2014d) were 9.31 to 12.23°C and there were no significant differences between the studies ($p > 0.05$) (Fig. 1B).

Another commonly used surrogate in thermal inactivation studies was HAV which represented 21% of the studies. The reported and/or calculated D-values for 50 to 72°C ranged from 0.88 to 385 min for HAV (Table 1.3). Similar to FCV and MNV, the highest values were reported by Gibson and Schwab (2011) where D-values were 385, 74.6, and 3.84 min at 50, 60, and 70°C, respectively. As discussed above the use of larger heating vessels (15 ml) is a likely reason for the observed differences. Unfortunately,

most of studies on the thermal resistance of HAV in cell culture media cover only one or two temperatures (Croci et al., 1999; Hewitt et al., 2009; Cappellozza et al., 2012). While valuable empirical information was gathered in these studies, there was no thermal kinetic information generated and thus prediction of thermal inactivation data outside the limits of the studies is impossible. As an example, Cappellozza et al. (2012) reported D-values at 60, and 70°C of 2.19 and 1.09 min which were consistent with the 2.67, and 1.27 min at the same temperatures reported by Bozkurt et al. (2014d) (Fig 1C). Since Bozkurt et al. (2014d) covered a wide temperature range (50-72°C), and their data could be helpful to generate precise thermal process conditions for HAV. The calculated z-values for HAV were 9.99°C by Gibson and Schwab (2011) based on three temperatures and 12.51°C by Bozkurt et al. (2014d) based on five temperatures. The only study that reported z-value and activation energy for HAV was those of Bozkurt et al. (2014d) who reported z-value for HAV in cell culture media was 12.51°C, and an activation energy of 171 kJ/mol.

Other surrogates used in inactivation studies were TV, and SaV (Table 1.3). For TV, the calculated D-values (50-65°C) ranged from 0.65 to 1.12 min (Hirneissen and Kniel, 2013). Based on their thermal data, the calculated z-value for TV was 55.4°C. The only reported D-value for SaV at 56°C was 12.60 min (Wang et al., 2012).

Seafood:

As might be expected since it is associated with many of the outbreaks, seafood is the most commonly used food sample in thermal inactivation studies for foodborne enteric viruses representing 25% of the studies identified (Table 1.4). Surrogates used in

these studies included HAV (50%), FCV (21%), and MNV (14%). Since human noroviruses cannot be cultivated *in vitro*, thermal inactivation studies with actual human norovirus are rare at this time, but it was used in two studies using RT-PCR to determine survival (Hewitt and Greening, 2006; Croci et al., 2012).

There is limited information about thermal inactivation behavior of FCV in seafood (Slomka and Appleton, 1998; Croci et al., 2012; Bozkurt et al., 2014a) (Fig. 2A). The only foods that were used in inactivation studies were cockles (Slomka and Appleton, 1998) and mussel (Croci et al., 2012; Bozkurt et al., 2014a). Slomka and Appleton, (1998) investigated the inactivation of FCV in cockles only at 100°C, and the calculated $D_{100^{\circ}\text{C}}$ -values was 0.26 min. The calculated $D_{60^{\circ}\text{C}}$ and $D_{80^{\circ}\text{C}}$ value of MNV in mussel were 6.82, 1.36 min, respectively (Croci et al., 2012). Bozkurt et al., (2014a) reported detailed thermal inactivation kinetics of FCV in blue mussel. The calculated D -values (50-72°C) ranged from 0.07 to 5.20 min for FCV-F9. The reported z -value was 11.39°C (Bozkurt et al., 2014a). This value was consistent with their previous findings in which the reported z -values were 9.29°C in cell culture media (Bozkurt et al., 2013), 9.89°C in spinach (Bozkurt et al., 2014b), and 10.91°C in turkey deli meat (Bozkurt et al., 2014f).

For MNV, the only foods that were used in inactivation studies were clam and blue mussel homogenate (Sow et al., 2011; Bozkurt et al., 2014a) (Fig. 2B). The calculated $D_{90^{\circ}\text{C}}$ value of MNV in clam was 0.55 min (Sow et al., 2011). The reported D -values (50-72°C) ranged from 0.18 to 20.19 min with a z -value of 11.62°C (Bozkurt et

al., 2014b). This finding was in agreement with Bozkurt et al. (2013, 2014b, 2014d) which reported similar z-values (9.31, 10.37, 10.98°C).

Thermal inactivation studies for HAV involved shellfish, mussels, clams and cockles. The most common shellfish used were mussels (Croci et al., 1999, 2005; Hewitt and Greening, 2006; Bozkurt et al., 2014c). Croci et al. (1999) reported that immersion of blue mussels (*Mytilus galloprovincialis*) at 100°C for 2 min were sufficient to achieve complete inactivation (5.6 log) of HAV. In a subsequent study, Croci et al. (2005) investigated the resistance of HAV in blue mussels (*Mytilus galloprovincialis*) subjected to different domestic cooking methods (mussels hors-d'oeuvre, mussels au gratin, mussels in tomato sauce). They reported that a 4 log reduction was achieved only in the mussels in tomato sauce which were cooked for a total of 23 min at boiling (100°C) (Croci et al., 2005). Hewitt and Greening (2006) stated that treatment at 90°C for 3 min (both steaming and immersion) was enough to cause a 3.5 log reduction of HAV in New Zealand greenshell mussels (*Perna canaliculus*). Similarly, Sow et al. (2011) concluded that application of 90°C for 3 min was sufficient to obtain 5.5 log reduction in soft shell clams (*Mya arenaria*). While valuable empirical data was generated in these studies, no thermal inactivation kinetics were established. In a recent study, Bozkurt et al. (2014c) investigated the thermal inactivation kinetics of HAV in blue mussel homogenate (50-72°C). They reported D-values of 54.17, 9.32, 3.25, 2.16, and 1.07 min at 50, 56, 60, 65, and 72°C, respectively (Fig. 2C).

The reported and/or calculated z-values for HAV in mussels was 12.97°C (Bozkurt et al., 2014c) and in clams 68°C (Cappellozza et al., 2012). According to the

study of Cappellozza et al. (2012), the z-values of HAV in cell culture media in a PCR thermocycler and clams in an industrial gas oven were 33, and 68°C, respectively. As the z-value is not a function of the heating environment but rather is a characteristic of the microorganism it should not be greatly different using different heating environments. Thus the reason for the large differences in z-values in this study are unknown but may relate to inactivation in wet and drying conditions. The reported z-value for HAV in mussels as determined by Bozkurt et al. (2014c) was 12.97°C and was consistent with previous findings for the z-value of 12.51°C in cell culture media (Bozkurt et al., 2014d). The calculated activation energy for HAV in blue mussel was 165 kJ/mole (Bozkurt et al., 2014c). From this study, it was determined that a process time necessary to achieve a 6 log reduction of HAV in boiling water (100°C) was 2.7 min. Thus, kinetic information was determined which will be useful designing thermal processes to eliminate HAV.

As stated above, there is very limited thermal inactivation data on actual human noroviruses. Mussels are the only food sample used in thermal inactivation studies of human noroviruses (Crocì et al., 2012; Hewitt and Greening, 2006). The reported D-values at 60 and 80°C were 25 and 4.84 min, and the two-point z-value based on these two data was 28°C (Hewitt and Greening, 2006). The calculated D-value for human norovirus at 100°C was 0.93 min by Crocì et al. (2012) and this value was consistent with those of Hewitt and Greening (1.3 min) (2006).

Fruits and vegetables:

Fruits and vegetables used to determine the thermal inactivation of enteric viruses have included spinach (33% of the data), basil, chives, mint and parsley (25%),

strawberry (17), raspberry puree (8%), lettuce (8%), and cabbage (8%). The surrogates used in studies with fruits and vegetables were FCV (36% of the data), MNV (27%), HAV (27%). As with seafood, the use of actual human norovirus in studies involving thermal inactivation on produce remains very limited (9% of the data).

Thermal inactivation behavior of FCV in basil, chives, mint and parsley was investigated only at 75°C and D-values based on the reported thermal data ranged from 0.63 to 0.68 min for FCV (Butot et al., 2009) (Fig. 3A). For FCV, the other food/produce sample that was used in inactivation studies was spinach (Bozkurt et al., 2014b) (Fig. 3A). They investigated the thermal inactivation behavior of FCV in spinach at temperatures 50, 56, 60, 65, and 72°C for different treatment times (0-6 min). The reported D-values (50-72°C) ranged from 0.15 to 17.39 min for FCV-F9 (Bozkurt et al., 2014b). The reported z-value for FCV in spinach was 9.89°C (Bozkurt et al. (2014c) and this value was consistent with their previous findings in which the reported z-values were 9.29°C in cell culture media (Bozkurt et al., 2013) and 11.39°C in blue mussel homogenate (Bozkurt et al., 2014c).

For MNV, the only foods that were used in inactivation studies were spinach and raspberry puree (9.2° brix) (Baert et al., 2008a, 2008b; Bozkurt et al., 2014) (Fig. 3B). Baert et al. (2008a) investigated the efficiency of blanching at a constant temperature (80°C) on the survival of MNV-1 during spinach processing. However, the researchers did not consider come up time for the blanch process and they did not specify the final temperature of the spinach after their treatment. Thus, no thermal inactivation kinetics was established. The only study that reported detailed thermal inactivation kinetics of

MNV in spinach was Bozkurt et al. (2014b). They investigated thermal inactivation kinetics of MNV in spinach at temperatures 50, 56, 60, 65, and 72°C. The reported D-values (50-72°C) ranged from 0.16 to 14.57 min with a z-value of 10.98°C (Bozkurt et al., 2014b). This finding was in agreement with Bozkurt et al. (2013, 2014c, 2014d) which reported similar z-values (9.31, 11.62, 10.37°C).

The other commonly used surrogates was HAV (27%) (Fig. 3C). The reported D-values for strawberry mashes with different brix values (28, and 52° brix) at 85°C were 0.96 and 8.94 min for HAV. Their results indicated the increased amount of brix content had a protective effect on thermal resistance of HAV. For strawberry mash with 52° brix, they also reported D-value at 80°C, and it was 4.98 min (Deboosere et al., 2004). Based on these data available for strawberry mash with 52° brix at 80, and 85°C, the calculated z-value was 19.67°C. Butot et al. (2009) investigated thermal inactivation of HAV in basil, chives, mint, and parsley only at 75°C. Thermal resistance of HAV in basil, chives, mint and parsley was higher than FCV at 75°C (Butot et al., 2009). Thermal inactivation of HAV (50-72°C) in spinach was investigated by Bozkurt et al. (2014e) and their reported D-values were 34.4, 8.43, 4.55, 2.3, and 0.91 min at temperatures 50, 56, 60, 65, and 72°C, respectively. Based on reported thermal data, the z-value of HAV in spinach was 13.92°C. The findings of this study was consistent with those of Bozkurt et al. (2014c, 2014d) who reported similar z-values (12.51, and 12.97°C).

The only reported thermal data for human norovirus was at 75°C (Butot et al., 2009). The reported D-value data for basil, chives, mint and parsley were 1.71, 1.85, 1.58, and 1.64 min, respectively.

Dairy products:

The only dairy product in which foodborne enteric viruses have been tested for their thermal resistance is milk (Table 1.6). The surrogates used in these studies included HAV (66%), MNV (16%), and poliovirus (16%) (Fig. 4A, B). Bidawid et al. (2000) investigated the effect of fat content (1%, 3.5% and 18%) of milk on the thermal resistance of HAV at 71°C in milk. $D_{71^{\circ}\text{C}}$ -values were 1.64, 2.08, and 3.08 min, respectively (Fig. 4B). They concluded that increasing the fat content of milk provided a protective effect against thermal inactivation of HAV. For milk at 63°C, the normal temperature for vat pasteurization, the D-values ranged from 1 to 10 min (Mariam and Cliver, 2000; Parry and Mortimer, 1984; Hewitt et al., 2009). At 72°C, the normal pasteurization temperature for high temperature-short time pasteurization, the D-values for HAV in milk were 7.8 sec and < 18 sec (Parry and Mortimer, 1984; Hewitt et al., 2009). Due to the survival curves at different temperatures, it was not possible to calculate a z-value. There was only one study that dealt with thermal inactivation of MNV in milk (Hewitt et al., 2009). D-values were 0.7 and 0.5 min at 63, and 72°C, respectively (Fig. 4A). Additionally, only one study was completed on the thermal inactivation of poliovirus in milk where the $D_{72^{\circ}\text{C}}$ was 0.44 min. With the exception of the study by Bidawad et al. (2000), evidence exists that the current practice of pasteurizing milk at 63°C for 30 min or 72°C for 15 sec should inactivate HAV, MNV or poliovirus. It is important to note that these are extrapolated values and that use of different foods and/or heating conditions may result in altered heating characteristics.

Therefore, validation of calculated process conditions must be carried out before actually applying a process.

Meat products:

Very limited information about thermal inactivation behavior of human norovirus surrogates and HAV in meat products is available, with only one reported study on turkey deli meat (Table 1.7). The calculated D-values (50-72°C) ranged from 0.14 to 9.94 min for FCV-F9, 0.22 to 21.01min for MNV-1, and 1.01 to 42.08 min for HAV, respectively (Bozkurt et al., 2014f) (Fig. 5A,B,C). The z-values determined for FCV-F9, MNV-1, and HAV were 11.90°C, 10.91°C, and 12.83°C for FCV-F9, MNV-1, and HAV, respectively (Bozkurt et al., 2014f). Their reported z-values for FCV-F9, MNV-1, and HAV were consistent with their previous findings (Bozkurt et al., 2014d). In general, HAV was more resistant to thermal treatment than FCV and MNV at all temperatures studied suggesting that it would require a more severe treatment than human norovirus surrogates for inactivation in turkey deli meat.

Factors affecting efficiency of thermal treatment:

There are several factors that could affect the apparent thermal resistance of foodborne enteric viruses including intrinsic properties of the food matrix, heat transfer rates and the heating system used. Thermal inactivation data available in the literature revealed that the apparent thermal resistance of foodborne enteric viruses was highly dependent on the food matrix as there were significant differences among food types and between food and cell culture media. The differences in inactivation results between different food matrix results may be explained by the compositional differences of cell

culture media and food samples (seafood, dairy products, fruits and vegetables, meat products), because the environment in which viruses are found influences their sensitivity to thermal inactivation. To understand thermal inactivation of viruses in food, temperature and matrix interaction should be considered together (Bertrand et al., 2012). The presence of certain food components in the heating medium, such as protein and fat, may play a protective role against heat inactivation (Millard et al., 1987; Croci et al., 1999; Bidawid et al., 2000; Croci et al., 2012). In particular, the effects of fat and protein on foodborne enteric virus inactivation by thermal treatment have been reported (Bidawid et al., 2000; Parry and Mortimer, 1984; Croci et al., 1999; Croci et al., 2012). Bidawid et al. (2000) investigated the effect of fat content (1, 3.5, and 18.5%) on the heat resistance of HAV in milk and they concluded that increasing fat content played a protective role and increased the stability of viruses. This finding was in agreement with Parry and Mortimer, 1984) findings which observed similar protective effect of milk on poliovirus inactivation. It has been suggested that the presence of fat and protein in the heating environment medium influences the heat inactivation rate by protecting the cell receptors or formation of viral aggregates (Croci et al. (2012).

The differences in results may be explained by the compositional differences of buffer solution and spinach, because the environment in which viruses are found influences their sensitivity to thermal inactivation. Bertrand et al., (2012) concluded that the presence of a complex matrix will lead to faster protein denaturation for virus inactivation

Another potential factor that could affect efficacy of thermal inactivation behavior of foodborne enteric viruses is heat transfer rate. The change in container size might cause differences in heat transfer rate, and affects time to reach desired temperature (come-up time) and apparent heat resistance (Chung et al., 2007). Therefore the consideration of come-up time during process time calculations is important. The contribution of sample size on apparent thermal resistance of human norovirus surrogates (FCV and MNV) was investigated by Bozkurt et al. (2013) and Bozkurt et al. (2014d) and they reported that especially at high temperatures, the increase in container size might contribute to differences in the D-value due to increased come up time. Hence, the reduction in number of survivors during come up time is important to determine precise thermal process conditions, the consideration of come up time is needed to achieve the desired amount of reduction and to design appropriate thermal system.

Various methods for heat treatment have been used in the studies reported in this review. The preponderance of the experiments have been done in a controlled temperature water bath. The exceptions were usage of immersion in boiling water (Millard et al., 1987; Hewitt and Greening, 2006; Slomka and Appleton (1998) gas-powered steam oven (Cappellozza et al., 2012), conventional oven (Butot et al., 2009), and glycerol bath (Deboosere et al., 2004). The usage of different heating system might have different heating behavior. In the oven method with clams, convectional heat transfer occurs between the heating medium and the food sample, then conductional heat transfer takes place throughout the sample. Therefore, a definite temperature gradient was observed throughout clam during heat treatment. However, in the water bath studies, both

conduction and convectional heat transfer take place and the temperature throughout the sample could be considered to be uniform. Since, the primary objective of inactivation studies is to investigate the interaction of the virus and heat, the use of homogenized samples is useful to obtain a uniform food matrix, and a more homogenous temperature distribution. To obtain good thermal inactivation data it is important to use a method of heat treatment that avoids local temperature variations (Stringer et al., 2000).

Mechanisms of inactivation of viruses during thermal treatment

Foodborne enteric viruses are non-enveloped, positive stranded RNA virus that are surrounded by protein shell (capsid) formed by units known as capsomers (Dimmock et al., 2001). Since, the virus capsid encloses the viral genome and any other components necessary to virus structure or function and also responsible for binding to the host, the mechanism of thermal inactivation of viruses is associated with the changes in the capsid of the virus. Pollard (1960) discussed the theory of virus inactivation during thermal treatment and he concluded that structural alterations in viral protein occur due to the differential expansion of the various parts of the virus under the action of heat. Heat disrupts the hydrogen bonding and destroys the space relationship that is necessary to keep the structural integrity of viral proteins. He stated that it is quite possible that the various components of the virus such as capsid, and nucleic acid have widely different values of entropy and enthalpy. Therefore, the degradation rate of these component would be different (Pollard, 1960). Similarly, Song et al. (2010) concluded that the mechanisms of thermal inactivation include denaturation of viral proteins, as well as disassembly of virus particles into noninfectious viral subunits and single proteins. He

also concluded that the mode of action during thermal treatment depends on the temperature. At mild temperatures ($<56^{\circ}\text{C}$), the destruction of the viral receptor and structural changes in the capsid might cause the inactivation by disrupting the specific structures needed to recognize and bind the host cells (Wigginton et al., 2012). It has been reported that the quaternary structure of the capsid was unaffected up to 60°C ; however, above 60°C , an alteration of tertiary structure occurs and facilitate access of thermal energy to nucleic material. Therefore, the capsid ceases to play a protective role and inactivation of nucleic material results (Katen et al., 2013). The increased inactivation rate at higher temperatures ($>65^{\circ}\text{C}$) could be associated with the changes in tertiary structures of the viruses (Ausar et al., 2006; Bertrand et al., 2012; Bozkurt et al., 2013; Bozkurt et al., 2014a; Bozkurt et al., 2014b; Croci et al., 1999; Croci et al., 2012; Sow et al., 2011; Volking et al., 1997).

Industrial applications:

The Codex Alimentarius Commission (WHO, 2008) has proposed providing guidance for the control of viruses in food which will include the development of a general guidance document concerning the control of human noroviruses and HAV in foods. Using the thermal data in this review, an industrial thermal process for clams, cockles, mussels, strawberry mashes (28, and 52° brix), raspberry puree, spinach, cabbage, basil, chives, mint, parsley, milk, and turkey deli meat could be estimated. Since each of these food samples have different thermal conductivity, it is also essential to consider the come up time during thermal design calculations of any process. According to Stumbo (1973), the contribution of come up time (t_c) to the apparent lethality of a

process can be calculated by addition of $0.4 \cdot t_c$ (in min) to the calculated process time for that specific temperature. It is also important to note that use of different heating medium such as steam, hot water, hot air have different heating characteristics and validation of the recommendation using different heating medium must be carried out before actual application of the process. The data reviewed here should serve as baseline for food processors to effectively determine thermal process conditions to develop control measures for foodborne enteric viruses.

Conclusion

Foodborne enteric viruses are more found to be heat resistant than most other foodborne non-sporeforming bacterial pathogens; thus, processing recommendations based on data for vegetative bacterial pathogens may not eliminate similar numbers of foodborne enteric viruses. Therefore, the correct understanding the thermal inactivation behavior of human norovirus and hepatitis A virus has great importance for integration of thermal processing. Since human noroviruses and HAV are the leading cause of acute gastroenteritis, the correct/accurate characterization of the thermal inactivation behavior of these viruses is essential for the food process industry. The result of this study should contribute to the development of appropriate thermal processing protocols to ensure safety of food for human consumption.

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Appendix

Table 1.1. The general characteristic of common foodborne enteric viruses.

Characteristic	NoV	HAV	Rotavirus	Adenovirus	Aichi virus
Classification					
Baltimore class	Group IV	Group IV	Group III	Group I	Group IV
Family	<i>Caliciviridae</i>	<i>Picornaviridae</i>	<i>Reoviridae</i>	<i>Adenoviridae</i>	<i>Picornaviridae</i>
Genus	Norovirus	Hepatovirus	Enterovirus	Adenovirus	Kobuvirus
Capsid					
Envelope	No	No	No	No	No
Virion diameter (nm)	27-38	27-32	30	90-100	27-30
Isoelectric point	5.5-6.0	2.8	4-4.5, 6.6-7.5	9.7	3.5
Host receptor	HBGA, heparin sulfate	TIM-1	PVR (CD55)	CD46, CAR	GM1b
Genome					
Composition	(+) ss RNA	(+) ss RNA	ds RNA	ds DNA	(+) ss RNA
Architecture	Linear	Linear	Linear	Linear	Linear
Size (kb)	7.5	7.5	7.5	26-48	8.2

Table 1.1. The general characteristic of common foodborne enteric viruses (continued).

Characteristic	NoV	HAV	Rotavirus	Adenovirus	Aichi virus
Route of transmission	Fecal oral	Fecal oral	Fecal oral	Fecal oral	Fecal oral
Incubation time	24-48 h	4 weeks	2-4 days	3-10 day	24-48 h
Duration time	12-72 h	2-6 months	3-8 days	7 days	12-72 h
Symptoms	Diarrhea, nausea, vomiting, abdominal pain	Malaise, dark urine, nausea, vomiting, jaundice	Vomiting, diarrhea, dehydration, fever	Vomiting, and diarrhea	Diarrhea, abdominal pain, nausea, vomiting
Clinical features	Gastroenteritis	Hepatitis	Gastroenteritis (children)	Gastroenteritis (children)	Gastroenteritis
Current therapeutics	No specific treatment	No specific treatment	No specific treatment	No specific treatment	No specific treatment
Vaccine	No	Yes	Yes	Yes	No
Detection method	RT-PCR, ELISA, NASBA, RT-LAMP	RT-PCR, ELISA, NASBA, RT-LAMP	RT-PCR, ELISA, NASBA, RT-LAMP	RT-PCR, ELISA, NASBA, RT-LAMP	RT-PCR, ELISA, NASBA, RT-LAMP

Table 1.2. Common viral surrogates used in inactivation studies.

Characteristic	HAV	FCV	MNV	MS2	TV	SaV
Classification						
Family	<i>Picornaviridae</i>	<i>Caliciviridae</i>	<i>Caliciviridae</i>	<i>Leviviridae</i>	<i>Caliciviridae</i>	<i>Caliciviridae</i>
Genus	Hepatovirus	Vesivirus	Norovirus	Levivirus	Recovirus	Sapovirus
Capsid						
Envelope	No	No	No	No	No	No
Virion diameter (nm)	27-32	35-39	35-39	27-34	36	27-35
Isoelectric point	2.8			2.2-3.1, 3.3-3.5, 3.9-4.0	2.8	4-4.5, 6.6-7.5
Host receptor	HAVCR1	JAM-1, sialic acid	Sialic acid, glycoproteins	F-pilus	HBGA	bile acid needed for replication
Host	Monkey/human	Cat	Mouse	<i>E. coli</i>	Monkey	Pig
Genome						
Composition	(+) ss RNA	(+) ss RNA	(+) ss RNA	(+) ss RNA	(+) ss RNA	(+) ss RNA
Architecture	Linear	Linear	Linear	Linear	Linear	Linear
Size (kb)	7.5	7.5	7.5	3.5	6.7	7.5
Surrogate for	HAV	NoV	NoV	Enteric viruses*	NoV	NoV

* Human enteric viruses such as noroviruses, hepatitis A virus, enteroviruses and rotaviruses.

Table 1.3. Thermal inactivation of foodborne enteric viruses in cell culture media.

Virus	Enumeration units	Volume	T(°C)	D-value (min)	z-value (°C)	References
Feline calicivirus (FCV-F9)	TCID ₅₀	100 µl	56	8	-	Doultree et al. (1999)
			70	0.49		
			100	0.13		
	TCID ₅₀	250 µl	37	480	9.87	Duizer et al. (2004)
			56	2.7		
			71.3	0.17		
	PFU/ml	50 µl	56	6.40	9.46	Cannon et al. (2006)
			63	0.41		
			72	0.12		
	PFU/ml	100 µl	70	1.5	-	Buckow et al. (2008)
	TCID ₅₀	15 ml	37	599	14.01	Gibson and Schwab (2011)
			50	50.6		
			60	14.1		
	RT-PCR	400 µl	60	0.13	-	Croci et al. (2012)
			80	0.12		
			60	1.16		
	TCID ₅₀	2 ml	80	1.16	-	
			80	1.16		
TCID ₅₀	100 µl	56	6.09	-	Wang et al. (2012)	
PFU/ml	50 µl	50	20.23	9.29	Bozkurt et al. (2013)	
		56	6.36			
		60	0.56			
		65	0.32			
		72	0.11			

Table 1.3. Thermal inactivation of foodborne enteric viruses in cell culture media (continued).

Virus	Enumeration unit	Volume	T(°C)	D-value (min)	z-value (°C)	References
Feline calicivirus (FCV-F9)	PFU/ml	2 ml	50	19.95	10.97	Bozkurt et al. (2014d)
			56	6.37		
			60	0.94		
			65	0.72		
			72	0.21		
Murine norovirus (MNV-1)	PFU/ml	50 µl	56	3.47	12.23	Cannon et al. (2006)
			63	0.44		
			72	0.17		
	PFU/ml	400 µl	80	0.38	-	Baert et al. (2008)
			63	0.9		
	PFU/ml	100 µl	72	<0.3	-	Hewitt et al. (2009)
			56	12.39		
	TCID ₅₀	100 µl	56	12.39	-	Wang et al. (2012)
			50	34.49		
			56	3.65		
			60	0.57		
			65	0.3		
			72	0.15		
	PFU/ml	2 ml	50	36.28	10.37	Bozkurt et al. (2014d)
			56	3.74		
			60	1.09		
			65	0.77		
			72	0.25		

Table 1.3. Thermal inactivation of foodborne enteric viruses in cell culture media (continued).

Virus	Enumeration units	Volume	T(°C)	D-value (min)	z-value (°C)	References
Murine norovirus (MNV-1)	PFU/ml	200 µl	50	2.47	22.83	Hirneissen and Kniel (2013)
			55	1.18		
			60	0.64		
			65	0.56		
Hepatitis A (HAV)	TCID ₅₀	4 ml	60	6.5	-	Croci et al. (1999)
	PFU/ml	100 µl	63	0.6	-	Hewitt et al. (2009)
			72	<0.3		
	PFU/ml	15 ml	50	385	9.99	Gibson and Schwab (2011)
			60	74.6		
			70	3.84		
	TCID ₅₀	50 µl	60	2.19		Cappellozza et al. (2012)
			70	1.09		
	PFU/ml	2 ml	50	56.22	12.51	Bozkurt et al. (2014d)
			56	8.40		
			60	2.67		
			65	1.73		
			72	0.88		
Tulane virus (TV)	PFU/ml	200 µl	50	1.12	55.4	Hirneissen and Kniel (2013)
			55	1.09		
			60	0.69		
			65	0.65		
Sapovirus (SaV)	TCID ₅₀	100 µl	56	12.6	-	Wang et al. (2012)

Table 1.4. Thermal inactivation of foodborne enteric viruses in seafood samples.

Virus	Enumeration unit	Sample	T(°C)	D-value (min)	z-value (°C)	References
Feline calicivirus (FCV-F9)	RT-PCR	Cockles	100	0.26	-	Slomka and Appleton (1998)
	TCID ₅₀	Mussel	60	6.82	-	Croci et al. (2012)
			80	1.36		
	PFU/ml	Mussel	50	5.20	11.39	Bozkurt et al. (2014a)
			56	3.33		
			60	0.77		
			65	0.33		
			72	0.07		
Murine norovirus (MNV)	PFU/ml	Clam	90	0.55	-	Sow et al. (2011)
	PFU/ml	Mussel	50	20.19	11.62	Bozkurt et al. (2014a)
			56	6.12		
			60	2.64		
			65	0.41		
			70	0.18		
Hepatitis A (HAV)	TCID ₅₀	Cockles	85	0.25	-	Millard et al. (1987)
			90	0.25		
	TCID ₅₀	Mussel	60	6.5	-	Croci et al. (1999)
			80	3.2		
	TCID ₅₀	Mussel	76.1	2	-	Croci et al. (2005)
	RT-PCR	Mussel	100	1.58	-	Hewitt and Greening (2006)
	TCID ₅₀		100	0.86	-	

Table 1.4. Thermal inactivation of foodborne enteric viruses in seafood samples (continued).

Virus	Enumeration unit	Sample	T(°C)	D-value (min)	z-value (°C)	References
Hepatitis A (HAV)	PFU/ml	Clam	90	0.55	-	Sow et al. (2011)
			60	3.56		
			70	1.93		
	TCID ₅₀	Clam	80	1.58	68	Cappellozza et al. (2012)
			90	1.23		
			50	54.17		
	PFU/ml	Mussel	56	9.32		Bozkurt et al. (2014c)
			60	3.25	12.97	
			65	2.16		
			72	1.07		
Norovirus (NVo)	RT-PCR	Mussel	100	1.3	-	Hewitt and Greening (2006)
	RT-PCR	Mussel	60	25		Crocì et al. (2012)
			80	4.84	-	

Table 1.5. Thermal inactivation of foodborne enteric viruses in fruits and vegetables.

Virus	Enumeration unit	Sample	T(°C)	D-value (min)	z-value (°C)	References
Feline calicivirus (FCV-F9)	PFU/ml	Spinach	50	17.39	9.89	Bozkurt et al. (2014b)
			56	5.83		
			60	0.78		
			65	0.27		
			72	0.15		
	PFU/ml	Basil	75	0.63	-	Butot et al. (2009)
		Chives		<0.63	-	
		Mint		<0.63	-	
		Parsley		0.68	-	
	PFU/ml	Cabbage	4	1.5	176	Allowood et al. (2004)
			25	1		
			37	1		
	PFU/ml	Lettuce	4	1.5	176	Allowood et al. (2004)
			25	1		
			37	1		
Murine norovirus (MNV)	PFU/ml	Spinach	80	0.74	-	Baert et al. (2008a)
	PFU/ml	Raspberry pure (9.2°brix)	65	0.44	-	Baert et al. (2008b)
			75	0.17		
	PFU/ml	Spinach	50	14.57	10.98	Bozkurt et al. (2014b)
			56	3.29		
			60	0.98		
			65	0.4		
			72	0.16		

Table 1.5. Thermal inactivation of foodborne enteric viruses in fruits and vegetables (continued).

Virus	Enumeration unit	Sample	T(°C)	D-value (min)	z-value (°C)	References
Hepatitis A (HAV)	PFU/ml	Strawberry mashes (28°brix)	85	0.96	-	Deboosere et al. (2004)
		Strawberry mashes (52°brix)	80	4.98	-	
			85	8.94	-	
	PFU/ml	Basil	75	1.34	-	Butot et al. (2009)
		Chives		<0.83	-	
		Mint		1.46	-	
		Parsley		1.21	-	
	PFU/leaf	Spinach	4	28.9 day	-	Shieh et al. (2009)
	PFU/ml	Spinach	50	34.4	13.92	Bozkurt et al. (2014e)
			56	8.43		
			60	4.55		
			65	2.30		
			72	0.91		
Norovirus (NVo)	RT-PCR	Basil	75	1.71	-	Butot et al. (2009)
		Chives		1.85	-	
		Mint		1.58	-	
		Parsley		1.64	-	

Table 1.6. Thermal inactivation of foodborne enteric viruses in dairy products.

Virus	Enumeration unit	Sample	T(°C)	D-value (min)	z-value (°C)	References
Murine norovirus (MNV)	RT-PCR	Milk	63	0.7	-	Hewitt et al. (2009)
			72	0.5		
Hepatitis A (HAV)	TCID ₅₀	Milk	62.8	10	-	Parry and Mortimer (1984)
			71.6	0.13		
	PFU/ml	Milk	85	0.01	-	Bidawid et al. (2000)
		1% fat Milk.	71	1.64	-	
		3.5% fat Milk	71	2.08	-	
		18% fat Milk/Cream	71	3.16	-	
	PFU/ml	Milk	63	10	-	Mariam and Cliver (2000)
Poliovirus	PFU/ml	Milk	63	1.1	-	Hewitt et al. (2009)
			72	<0.3		
			72	0.44	-	Strazynski et al. (2002)

Table 1.7. Thermal inactivation of foodborne enteric viruses in meat products.

Virus	Enumeration unit	Sample	T(°C)	D-value (min)	z-value (°C)	References
Feline calicivirus (FCV-F9)	PFU/ml	Turkey deli meat	50	9.94	10.91	Bozkurt et al. (2014f)
			56	3.03		
			60	0.82		
			65	0.43		
			72	0.14		
Murine norovirus (MNV)	PFU/ml	Turkey deli meat	50	21.01	12.83	Bozkurt et al. (2014f)
			56	7.3		
			60	2.74		
			65	0.94		
			72	0.22		
Hepatitis A virus (HAV)	PFU/ml	Turkey deli meat	50	42.08	11.90	Bozkurt et al. (2014f)
			56	20.62		
			60	5.91		
			65	2.27		
			72	1.01		

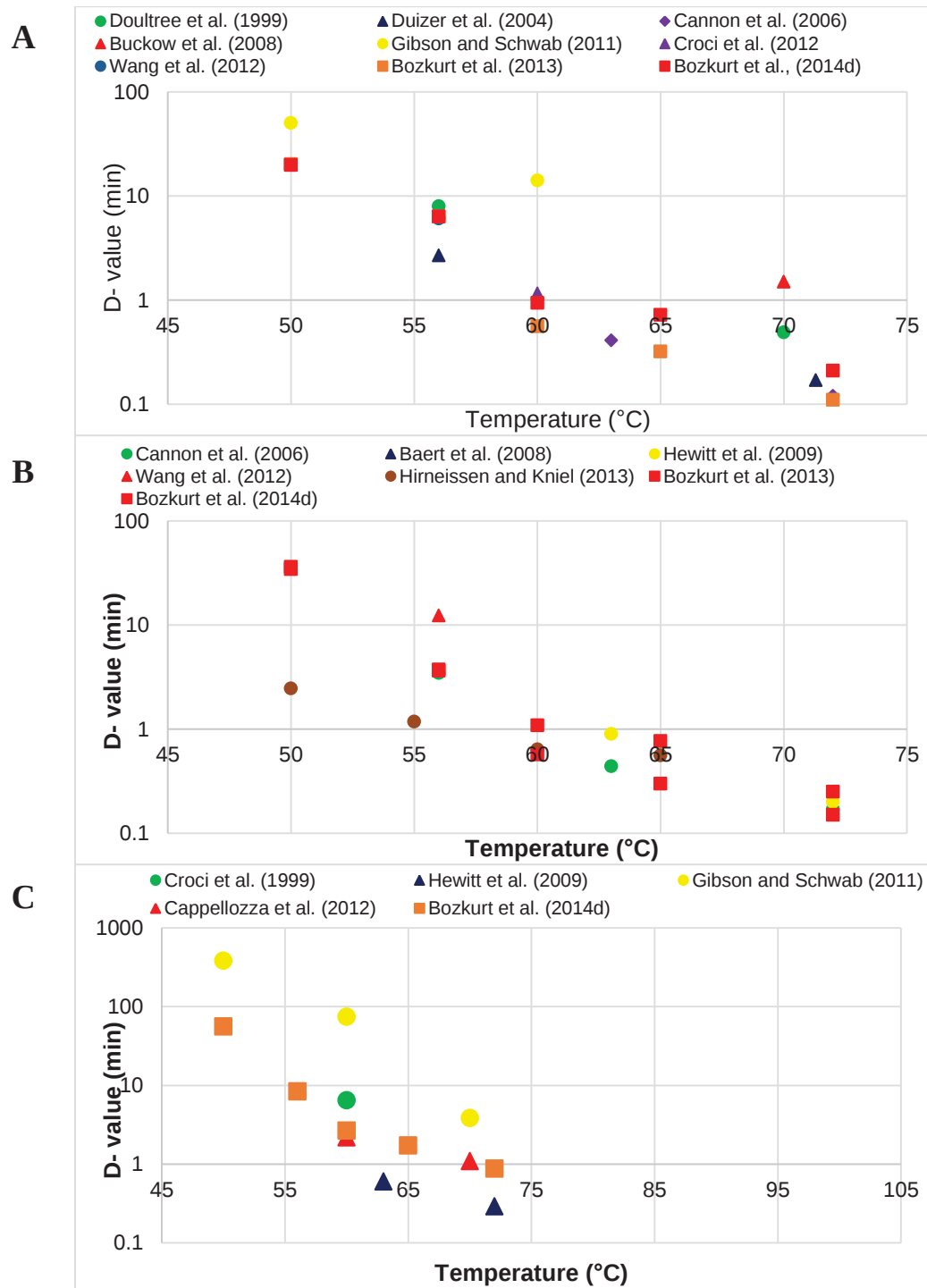


Figure 1.1. [A] FCV-F9, [B] MNV-1, [C] HAV inactivation in cell culture medium.

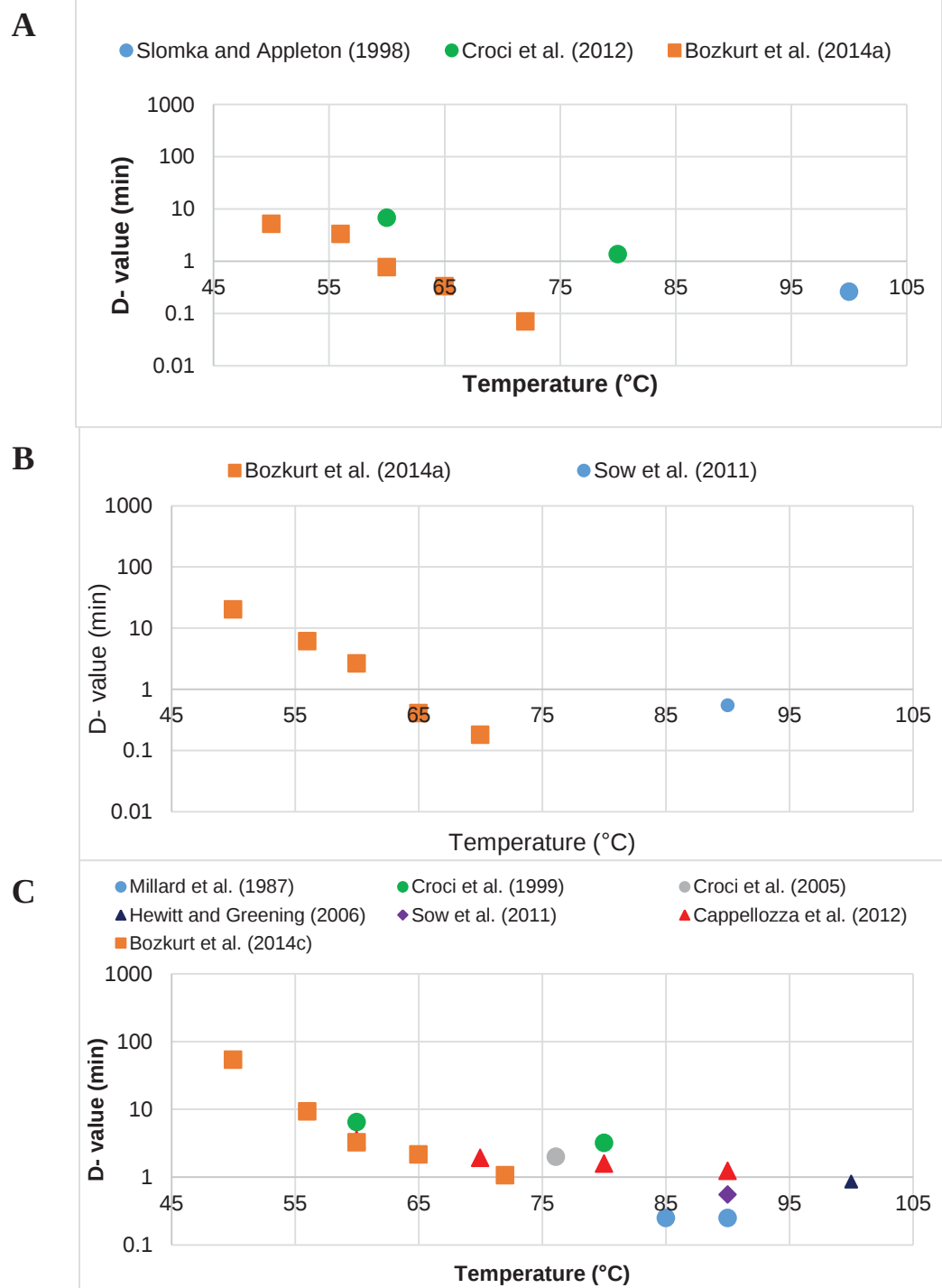


Figure 1.2. [A] FCV-F9, [B] MNV-1, [C] HAV inactivation in seafood.

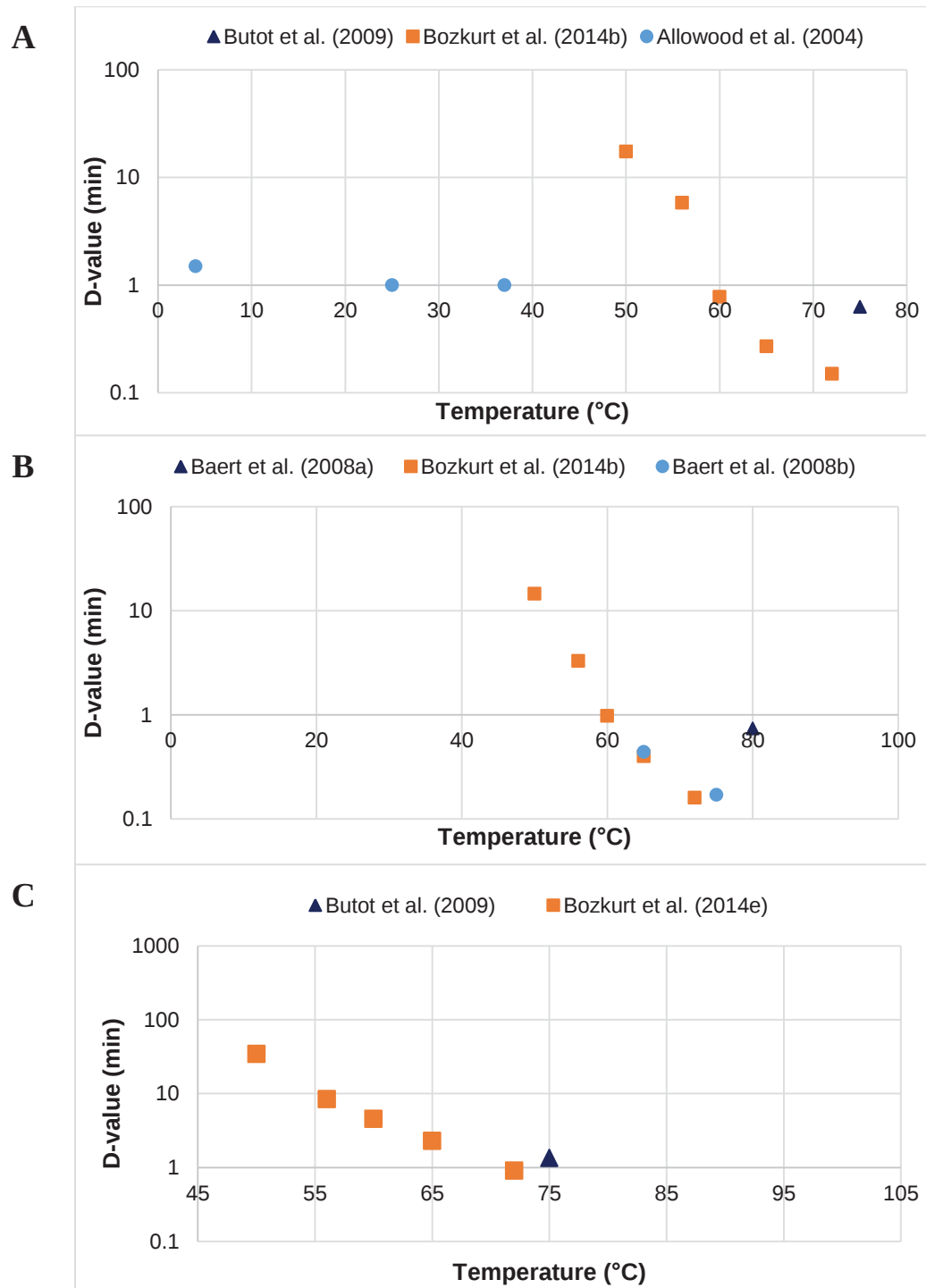


Figure 1.3. [A] FCV-F9, [B] MNV-1, [C] HAV inactivation in fruits and vegetables.

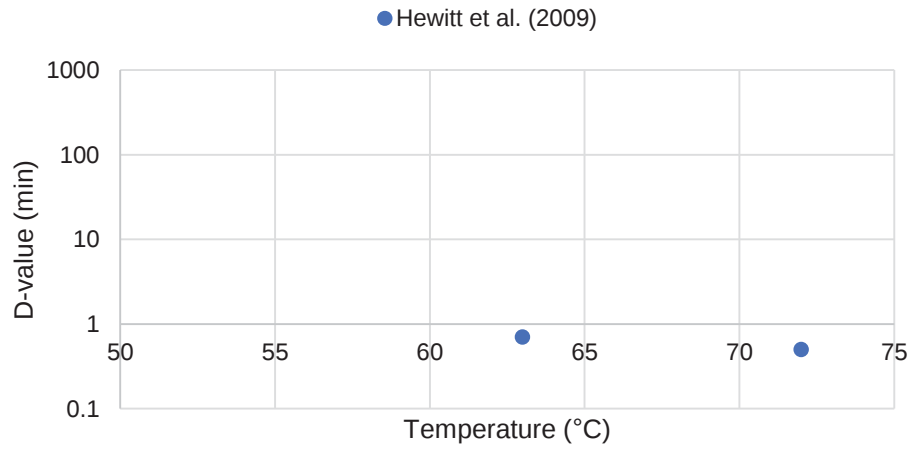
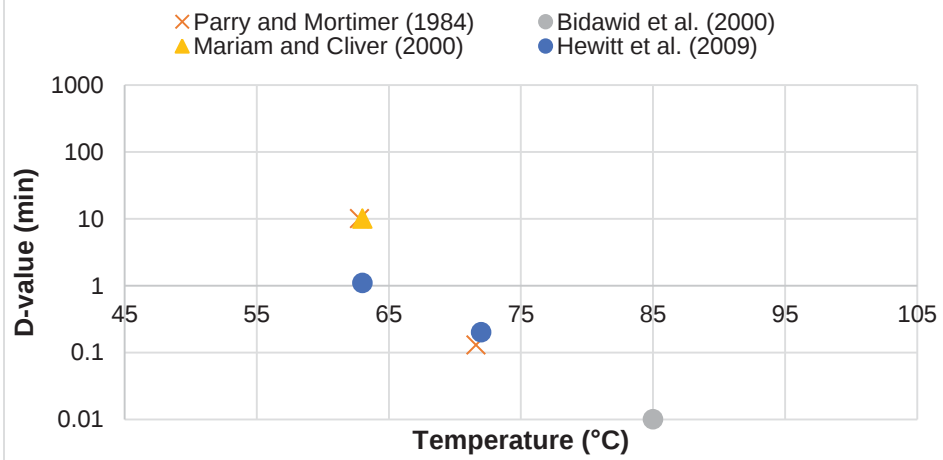
A**B**

Figure 1.4. [A] MNV-1, [B] HAV inactivation in dairy products.

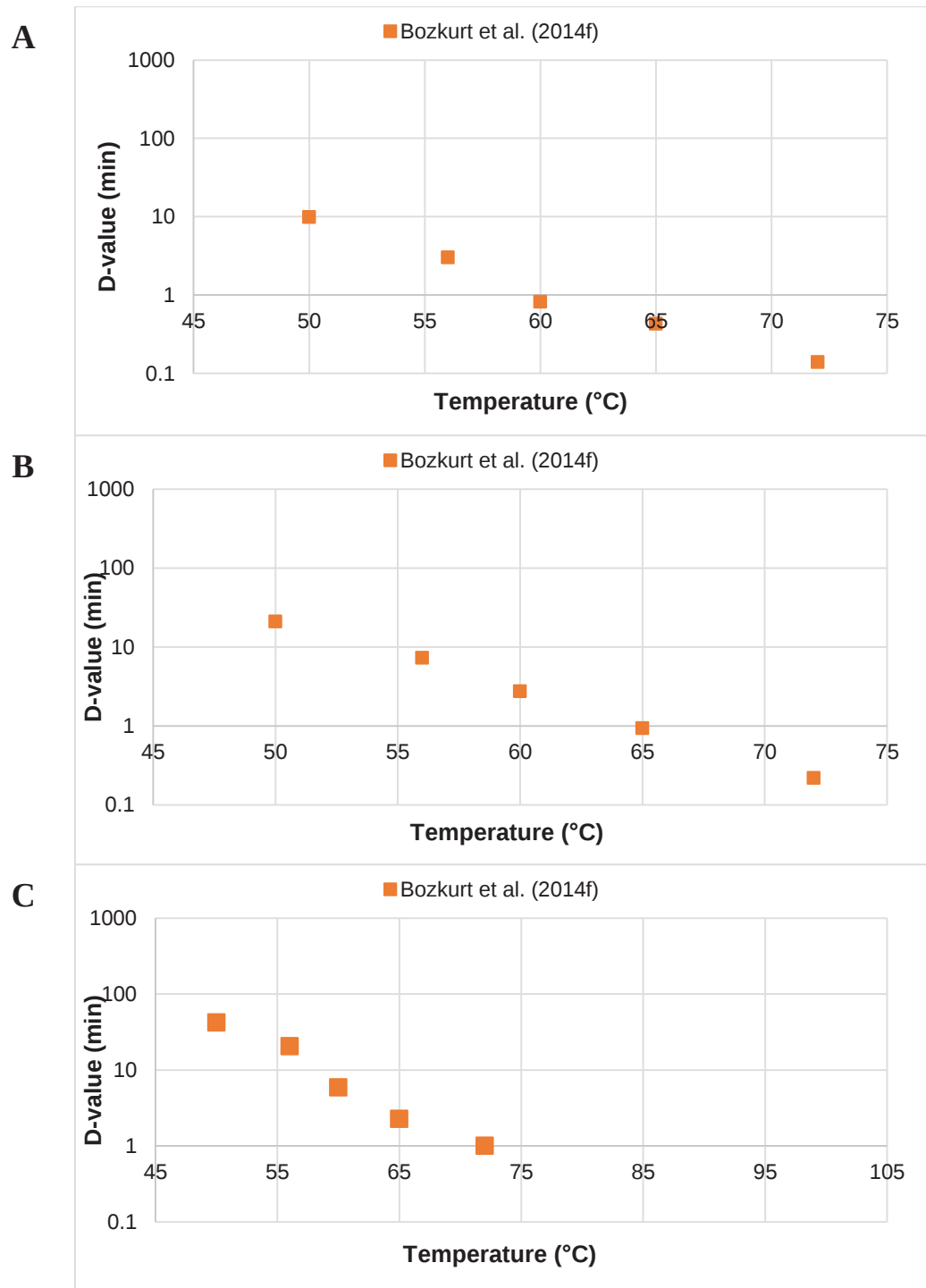


Figure 1.5. [A] FCV-F9, [B] MNV-1, [C] HAV inactivation in meat products.

CHAPTER II
DETERMINATION OF THE THERMAL INACTIVATION
KINETICS OF MURINE NOROVIRUS AND FELINE CALICIVIRUS

Reproduced with permission from the Journal of Food Protection: “Bozkurt, H., D’Souza, D.H., Davidson, P.M., 2013. Determination of the thermal inactivation kinetics of murine norovirus and feline calicivirus, J Food Protect 76(1):79-84.”

Abstract

Studies are needed to bridge existing data gaps and determine appropriate parameters for thermal inactivation methods for human norovirus. Cultivable surrogates, such as feline calicivirus (FCV-F9) and murine norovirus (MNV-1), have been used in the absence of human norovirus infectivity assays. This study aimed to characterize the thermal inactivation kinetics of MNV-1 and FCV-F9 at 50, 56, 60, 65 and 72°C for different treatment times (0-60 min). Thermal inactivation was performed using the capillary tube method with titers of 4.0×10^7 (MNV-1) and 5.8×10^8 (FCV-F9) plaque forming units (PFU)/ml in triplicate experiments, followed by standard plaque assays in duplicate for each experiment. Weibull and first-order models were compared to describe survival curve kinetics. Model fitness was investigated by comparing regression coefficient (R^2), chi square (χ^2), and root mean square error (RMSE) values. The D values calculated from the first-order model (50-72°C) were in the range of 0.15 to 34.48 min for MNV-1 and 0.11 to 20.23 min for FCV-9. Using the Weibull model, the t_D for MNV-1 and FCV-F9 to destroy 1 log ($D=1$) at the same temperatures were in the range of 0.11 to 28.26 and 0.06 to 13.86 min, respectively. In terms of thermal resistance, MNV-1 was more sensitive than FCV-F9 up to 65°C. At 72°C, FCV-F9 was slightly more susceptible to heat inactivation. Results revealed that the Weibull model was more appropriate to represent the thermal inactivation behavior of both tested surrogates. z values were calculated using D-values for the first-order model and t_d values for the

Weibull model. z values were 9.31 and 9.19°C for MNV-1 and 9.36 and 9.31°C for FCV-F9 for first-order and Weibull models, respectively. This study provides more precise information on thermal inactivation kinetics of norovirus surrogates for use in thermal process calculations than previous reports.

Key words: Murine norovirus, feline calicivirus, Weibull model, first-order model, D value, t_D value, z-value, capillary tube method, thermal inactivation.

Introduction

Human noroviruses are commonly associated with foodborne illnesses and frequently cause non-bacterial acute gastroenteritis in humans (Blanton et al., 2006; Green et al., 2001). In the US, it is estimated that human noroviruses are responsible for up to 58% of all foodborne illnesses, 26% of hospitalizations and 11% of deaths (Scallan et al., 2011). Viral foodborne illnesses are highly contagious and have low infectious doses. Because human norovirus are not yet cultivable under laboratory conditions, murine norovirus (MNV-1) and feline calicivirus (FCV-F9) have been used as surrogates. As with any human pathogen transmitted by foods, knowledge about the inactivation kinetics is a prior step for the development of a thermal food process as well as correction (and incorporation into) of existing system parameters. There are limited studies published on the thermal inactivation of human norovirus surrogates (Buckow et al., 2008; Cannon et al., 2006; Duizer et al., 2004; Gibson et al., 2011; Hewitt and Greening, 2009). In all the published studies, survivor curves were described using first order models to generate D-values for different temperatures. No alternative models were evaluated in any of the studies. Thus, in the current literature, there is no study on kinetic modeling of human norovirus surrogates during thermal inactivation.

Temperature is considered the essential parameter for the inactivation studies. To characterize the effect of temperature during the thermal inactivation, mathematical tools are needed. For this purpose, mathematical modelling has been used with different thermal processes to predict number of survivors during thermal processing and to give detailed information about inactivation kinetics during treatments (Peleg and Cole 1998).

The use of a first-order model (for a constant temperature an exponential decrease in the number of survivors within the treatment time) is more common in the food processing industry (Peleg, 1999). However, this behavior may not always be applicable and non-linear behavior may also be observed. In recent years, to address this non-linear behavior, the Weibull model has been widely used to describe thermal inactivation of several foodborne pathogenic bacteria (van Boekel, 2002). van Boekel (2002) reviewed 55 thermal inactivation studies on microbial vegetative cells and concluded that use of a non-linear model, such as the Weibull model, better represented data than traditional models. While there are many studies describing bacterial inactivation using the Weibull model, to date there were no studies found on the application of this model to thermal inactivation data of food-related viruses.

To provide data for inactivation studies in the thermal food processing industry, it is also essential to determine a reliable z-value for the studied viruses. In the current literature, there appears to be a lack of z-values reported for the norovirus surrogates. Thus, considering the lack of published information, the purpose of this study was (i) to characterize the thermal inactivation behavior of Murine norovirus (MNV-1) and Feline calicivirus (FCV-F9), (ii) to compare first-order and Weibull models in describing the data in terms of selected statistical parameters, and (iii) to calculate and compare z-values obtained from each model.

Material and Methods

Viruses and cell lines

Feline calicivirus (FCV-F) and its host Crandell Reese Feline Kidney (CRFK) cells were obtained from ATCC (Manassas, VA). Murine norovirus (MNV-1) was kindly

provided by Dr. Skip Virgin (Washington Univ., St Louis, MO) and its host cells (RAW 264.7) were obtained from the University of Tennessee, Knoxville.

Propagation of viruses

FCV-F9 and MNV-1 stocks were prepared by inoculating FCV-F9 or MNV-1 onto confluent CRFK or RAW 264.7 cells, respectively in 175 cm² flasks and incubating at 37°C and 5% CO₂ until >90% cell lysis was observed. The methods for the propagation of the viruses were described with detail in Su et al (2010). Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic-antimycotic were used for cell culture medium. The inoculated flasks were freeze thawed and centrifuged at 5000 x g for 10 min at room temperature. The supernatant was filtered through a 0.2 micron filter, aseptically aliquoted and stored in a -80°C freezer. The recovered FCV-F9 and MNV-1 viruses were plaque assayed as described below to determine the titer and used as viral stocks for the entire study.

Thermal treatment of viral strain

Glass capillary tubes (100 µl) were filled with 50 µl of virus stock using capillary force. They were flame sealed, immersed in a thermostatically controlled water bath. An open bath circulator (Haake model V26, Karlsruhe, Germany) was used to maintain a constant temperature at the water bath during each experiment. Water bath temperature was also confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA) and by placing type-T thermocouples (Omega Engineering, Inc., Stamford, CT) in the geometric center of the water bath. The thermocouples were connected to MMS3000-T6V4 type portable data recorder (Commtest Ins., New Zealand) to monitor temperature.

The samples were heated at 50, 56, 60, 65, and 72°C for different treatment times (0-60 min). Triplicate tubes were used for each time. After the thermal treatment, the tubes were cooled immediately in water/ice bath, and both ends were clipped off under sterile conditions. The contents were poured into a tube which contained 450 µl maintenance media Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum. Un-heated virus suspensions were enumerated as controls.

Enumeration of survivors

Thermally inactivated and control viral suspensions were diluted 1:10 in DMEM containing 10% fetal bovine serum. Plaque assays for MNV-1 and FCV-F9 were carried out as described in Su et al. (2010) and is briefly summarized below. Viral survivors were enumerated as plaque forming units/ml (PFU/ml).

Infectious plaque assays

Infectivity of each treated virus was evaluated in duplicate using a standardized plaque assay in comparison to untreated virus controls. For MNV-1 and FCV-F9, the plaque assay used was previously described by Su et al. (2010). CRFK and RAW 264.7 cells were cultivated and used for FCV-F9 and MNV-1 plaque assays, respectively. The cell suspension was added to six-well plates and incubated with 5% CO₂ at 37°C and until >90% confluency. Media was aspirated and cells were infected with 0.5 ml of treated and untreated virus that was serially diluted in cell culture medium. After incubation for 2 h for FCV-F9 and 3 h for MNV-1 at 37°C and 5% CO₂, the virus suspension was aspirated and the cells were overlaid with 2 ml Dulbecco's Modified Eagle Medium (DMEM) containing fetal bovine serum (10% for MNV-1 and 2% for FCV-F9) and 1% antibiotic-antimycotic. After incubation (72 h for MNV-1 and 48 h for

FCV-F9), 1 ml of a secondary overlay medium containing neutral red (0.02% for MNV-1 and 0.01% for FCV-F9) was added to stain the plates and plaques were counted after incubation for 5 h at 37°C.

Modeling of inactivation kinetics

First-order kinetics

The first-order kinetic model assumes a linear relationship between the decreases in logarithmic reduction of the number of survivors over treatment time:

$$\log_{10}S(t) = -\frac{t}{D} \quad (1)$$

Where $S(t)$ is the survival ratio which is defined as the ratio between the number of survivors after an exposure time (t), $N(t)$ (PFU/ml) and the initial number of survivors N_0 , (PFU/ml). D is the decimal reduction time in min (time required to kill 90% of microorganism) and t is the treatment time (min).

Weibull model

The Weibull model assumes that the survival curve is a cumulative distribution of lethal effects

$$S(t) = \exp\left(-\left(\frac{t}{\alpha}\right)^\beta\right) \quad (2)$$

$$\text{and } \log_{10}S(t) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^\beta$$

(3)

where α and β are the scale and shape parameters, respectively. Several authors (Peleg, 1999; Peleg and Cole, 1998, 2000) prefer to write Eq. (3) in the form of Eq. (4):

$$S(t) = -bt^n \quad (4)$$

where $n = \beta$ and

$$b = \frac{1}{2.303} (\alpha)^{-n} \quad (5)$$

Data analysis and model evaluation

The statistical evaluation, linear and non-linear regression analyses were performed using SPSS Ver.11.0.1 Statistical package. The statistical criteria applied to discriminate goodness of the fit of the models to the experimental data were higher R^2 (regression coefficient), lower chi-square (χ^2) and lower root mean square error (RMSE). For each temperature, χ^2 and RMSE values were predicted by using experimental and predicted survival ratio values for each time values;

$$\chi^2 = \frac{\sum_{i=1}^N (S_{exp,i} - S_{pred,i})^2}{N-n} \quad (6)$$

$$RMSE = \left[\frac{1}{N} \sum_{i=1}^N (S_{exp,i} - S_{pred,i})^2 \right]^{1/2} \quad (7)$$

where $S_{exp,i}$ was the i th experimentally observed survival ratio $S_{pred,i}$ was the i th predicted survival ratio, N was the number of observations and n was the number of constants. Standard error (SE) was determined for each coefficient. The effects of the time on survival ratio was analyzed using the comparison test (ANOVA, Post Hoc test). The confidence level used to determine statistical significance was 95%.

Results and Discussion

As expected, as time increased, MNV-1 and FCV-F9 titers were reduced at all tested temperatures ($p < 0.05$). To investigate thermal inactivation behavior of both viruses (MNV-1 and FCV-F9) Weibull and first-order models were evaluated. An example of a survival curve at 60°C illustrating the fitness of first-order and Weibull models on the thermal inactivation of the FCV-F9 and MNV-1 is shown in Figure 2.1. The inactivation

parameters obtained from each model are shown in Table 2.1. For the first-order model, the D value represents the time required to kill 90% of the microbial population whereas, in the Weibull model, the time factor (α) represents the mean of distribution describing the death times of the microbial population, and has a probabilistic interpretation (van Boekel, 2002). The calculated D values for first-order model were significantly different from the time factor (α) values at each temperature for both virus strain (Table 2.1). The time required to achieve a specified logarithmic reduction can be determined using shape and scale parameters as shown in Eq (8);

$$t_D = \alpha - \ln(10^{-D})^{1/\beta} \quad (8)$$

where D is the number of decimal reductions.

The effect of virus type and temperature were found to be significantly important for the time to achieve a given log reduction ($p < 0.05$). The D values calculated from first-order model (50-72°C) were in the range of 0.15 to 34.48 min for MNV-1 and 0.11 to 20.23 min for FCV-9 (Table 2.1). These inactivation times were statistically different than the t_D values ($p < 0.05$). The calculated time requirement for MNV-1 and FCV-F9, to destroy 1 log ($D=1$) at the same temperatures were in the range of 0.11 to 28.26 and 0.06 to 13.86 min, respectively. In terms of thermal resistance, MNV-1 was more sensitive to thermal treatments than FCV-F9 up to 65°C. At 72°C, FCV-F9 became slightly more susceptible to heat inactivation.

The shape factors (β) of the Weibull model indicated that both MNV-1 and FCV-F9 had monotonic upward concave (tailing) curve behavior ($\beta < 1$) and monotonic downward concave (shoulder) behavior ($\beta > 1$) depending on the temperature (Table 2.1). The shoulder behavior ($\beta > 1$) indicates that remaining survivors become increasingly

damaged whereas tailing behavior indicates that sensitive members of the population are destroyed relatively quickly while some others have the ability to survive the applied stress (van Boekel, 2002).

Both the first-order and Weibull models gave a good fit to the experimental data for all tested temperatures (50-72°C) (Table 2.2). It can be concluded that the inactivation behavior of MNV-1 and FCV-F9 is best represented by the Weibull model during thermal inactivation since the regression coefficient was comparatively higher and both the χ^2 and RMSE values were comparatively lower than first-order model (Table 2.2). Further analysis was carried out to evaluate the Weibull model for its validity using the “hazard plot” (Figure 2.2). The hazard plot is a double logarithmic plot of survival ratio $\ln(-\ln S)$ vs time. If the Weibull model fits with the experimental values, a straight line should be obtained. The hazard plot of the survival curve for each virus gave a straight line with regression coefficients (R^2) for MNV-1 and FCV-F9 close to 1. In other words, the appropriateness of the Weibull model was confirmed by the hazard plots. Recently, the Hazard plot analysis has been used to determine model appropriateness for foodborne pathogens in thermal inactivation studies (van Boekel, 2002). Hutchinson (2000) used a Hazard plot to characterize the death of *Escherichia coli* to determine Weibull model appropriateness. To the best of our knowledge, there are no studies reported on the application of the Weibull model for thermal inactivation of viruses.

Cannon et al., (2006) evaluated the stability of norovirus surrogates at 56, 63, and 72°C for applying the capillary tube method. At 56 °C, the D-value for MNV-1 was 3.47 min and for FCV-F9 was 6.71 min. The $D_{63^\circ\text{C}}$ - and $D_{72^\circ\text{C}}$ -values for MNV-1 were 0.43 and 0.17 min and for FCV-F9 were 0.41 min and 0.12 min. The D-values determined in

the present study are very similar to those of Cannon et al., (2006). Gibson and Schwab (2011) also evaluated the thermal inactivation behavior of MNV-1 and FCV-F9 at 50°C for up to 180 min. Unlike Cannon et al., (2006), instead of the capillary tube method, they used 15 ml samples for each heat treatment. The $D_{50^{\circ}\text{C}}$ -values reported for MNV-1 and FCV-F9 were 106 and 50.6 min, respectively. The D-values in the present study are much lower than those of Gibson and Schwab (2011) possibly because of the come-up time and heating system. In another study, Hewitt et al., (2009) evaluated the stability of murine norovirus during thermal treatment (PCR machine) in water for selected times at 63 and 72 °C. They concluded that the D-values in water at 63 and 72°C were 0.9 and < 0.3 min, respectively, which was higher than that found in the present study. As can be seen from the literature on thermal inactivation of norovirus surrogates, there is an inconsistency in the methods and results. In all these studies, linear regression was performed on the survivor data which could be a reason for this inconsistency.

The thermal death time curve for each of the viruses tested was determined by calculating the z-value for each. The z-value is defined as the change in temperature (°C) required to cause a 90% change in the log D-value (or t_D for Weibull) of a population. z-values were calculated using both the first-order and the Weibull models. The z-values for MNV-1 were 9.31 and 9.19°C for the first-order and Weibull models, respectively (Figure 2.3). There was no significant difference between the z-value calculated by the two methods ($p>0.05$). For FCV-F9, the z-values were 9.36, and 9.31°C for the first-order and Weibull models, respectively (Figure 2.4). Again, there was no statistical difference between the z-values calculated by each model. The regression coefficients for

the Weibull and first-order models were 0.962 and 0.941 for MNV-1 and 0.899 and 0.924 for FCV-F9, respectively.

Conclusion

In conclusion, understanding the thermal inactivation behavior of norovirus has great importance for integration of thermal processing. Since human noroviruses are the leading cause of acute gastroenteritis, the correct/accurate characterization of the thermal inactivation behavior of these viruses is essential for the food process industry. In this study, the thermal inactivation kinetics of MNV-1 and FCV-F9 was well characterized by the Weibull model. Since there is a lack of information on the thermal inactivation kinetics of MNV-1 and FCV-F9 in the current literature, this study provides some initial insights. Further studies are needed to investigate and describe thermal inactivation of these viral surrogates in various food commodities.

Acknowledgement

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van Boekel, M., 2002. On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *Int. J. of Food Microbiol.*, 74, 139-159.

Appendix

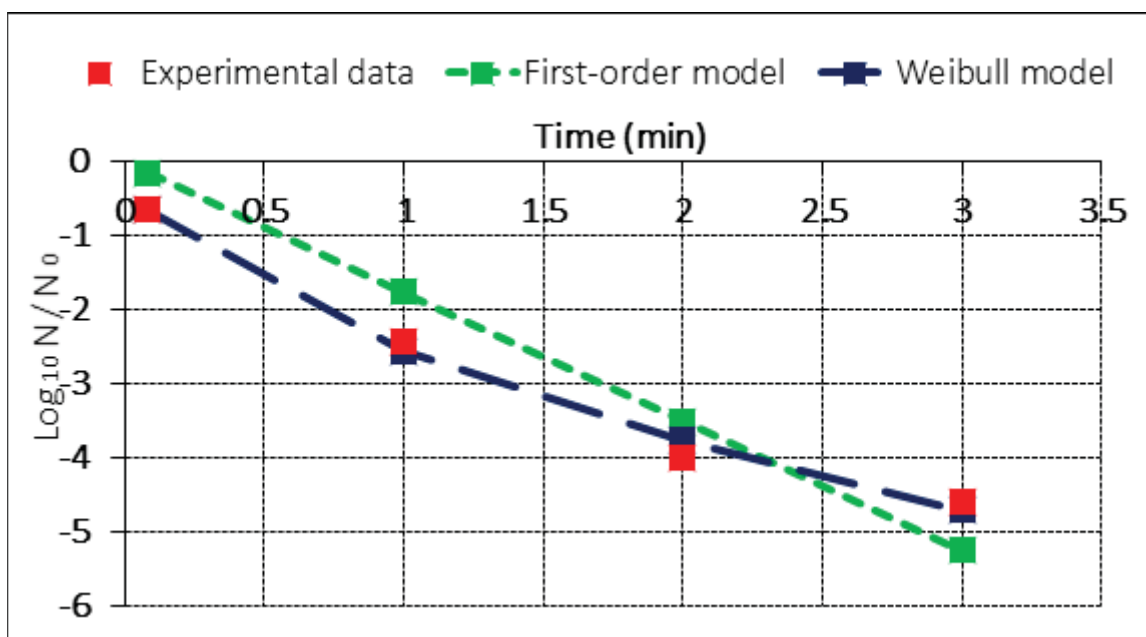
Table 2.1. Coefficients of the first-order and Weibull models for the survivor curves of murine norovirus (MNV-1) and feline calicivirus (FCV-F9).

Virus strain	T(°C)	First-order			
		Weibull distribution			kinetics
		β	α (min)	$t_{D=1}$ (min)	D (min)
MNV-1	50	1.92±0.02	23.59±0.93	28.26±1.47	34.49±2.10
	56	0.83±0.08	1.32±0.10	3.62±0.07	3.65±0.05
	60	0.67±0.02	0.24±0.00	0.83±0.01	0.57±0.01
	65	1.10±0.02	0.18±0.01	0.37±0.01	0.30±0.00
	72	0.85±0.02	0.04±0.00	0.11±0.00	0.15±0.00
FCV-F9	50	0.75±0.06	4.53±0.70	13.86±1.21	20.23±0.69
	56	1.59±0.08	2.79±0.10	4.04±0.09	6.36±0.48
	60	0.74±0.06	0.11±0.03	0.37±0.08	0.56±0.01
	65	1.02±0.12	0.16±0.05	0.34±0.08	0.32±0.01
	72	0.80±0.10	0.02±0.01	0.06±0.01	0.11±0.01

Table 2.2. Statistical comparison of the first-order and Weibull models for the survivor curves of murine norovirus (MNV-1) and feline calicivirus (FCV-F9).

Virus strain	T(°C)	Weibull distribution			First-order kinetics		
		R ²	RMSE	χ^2	R ²	RMSE	χ^2
MNV-1	50	0.9860	0.0106	0.0001	0.912	0.0346	0.0012
	56	0.9637	0.0575	0.0033	0.9618	0.0790	0.0062
	60	0.9833	0.1502	1.39	0.8413	0.5904	0.09
	65	0.9997	0.0783	0.0060	0.9986	0.2884	0.0832
	72	0.9980	0.1051	0.0110	0.9927	0.2957	0.0874
FCV-F9	50	0.9950	0.0260	0.0007	0.975	0.0369	0.0014
	56	0.9840	0.0202	0.0004	0.9327	0.051	0.0026
	60	0.9643	0.2554	0.48	0.9336	0.5691	0.25
	65	0.9797	0.4517	0.2041	0.9786	0.4737	0.2244
	72	0.9815	0.1039	0.0108	0.9830	0.6562	0.4306

A



B

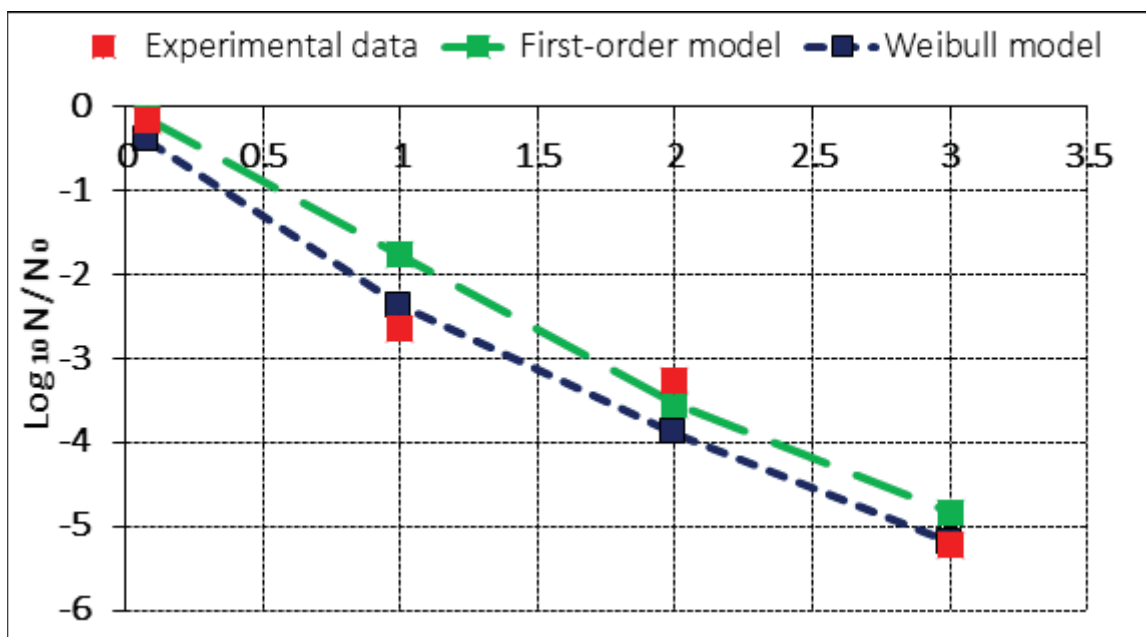
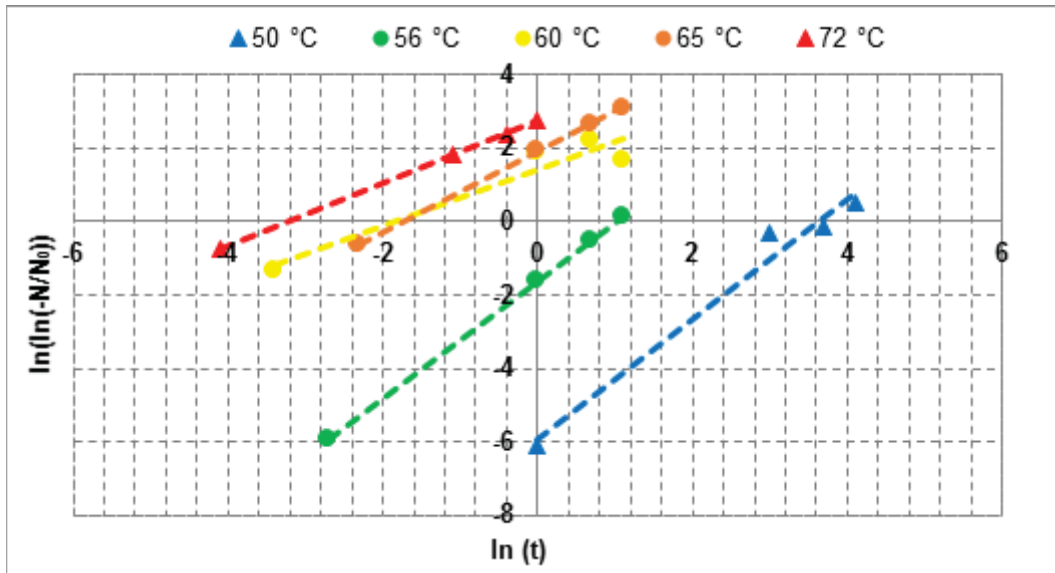


Figure 2.1. Survival curves of [A] murine norovirus (MNV-1) and [B] feline calicivirus (FCV-F9) at 60°C.

A



B

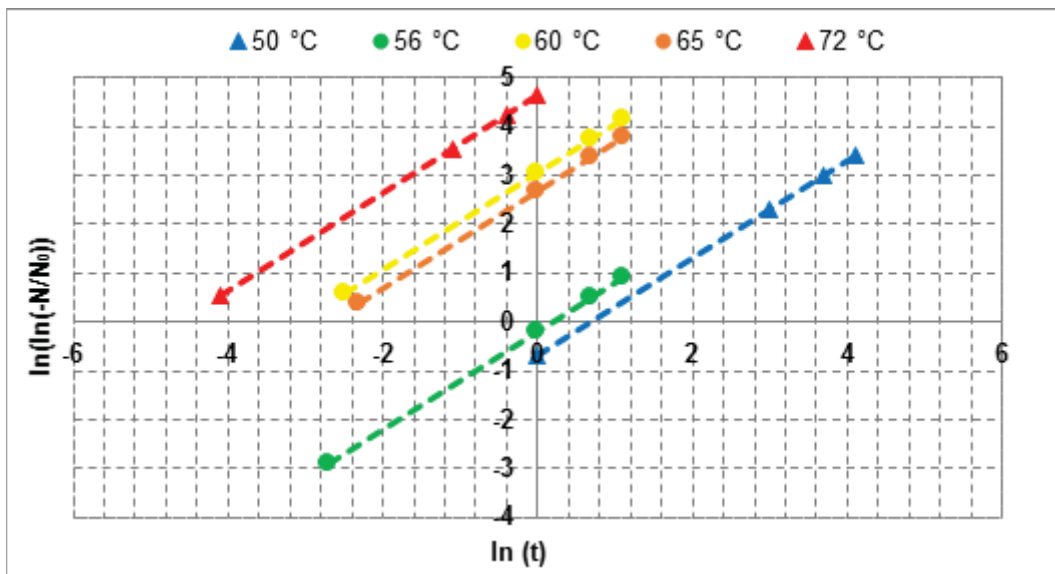
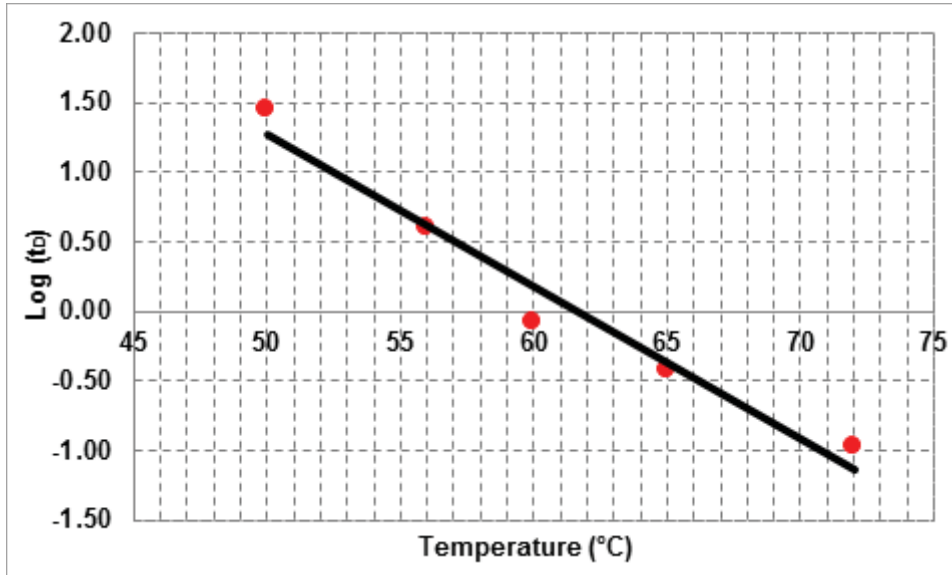


Figure 2.2. Hazard plots of the survival curves for [A] murine norovirus (MNV-1) and [B] feline calicivirus (FCV-F9).

A



B

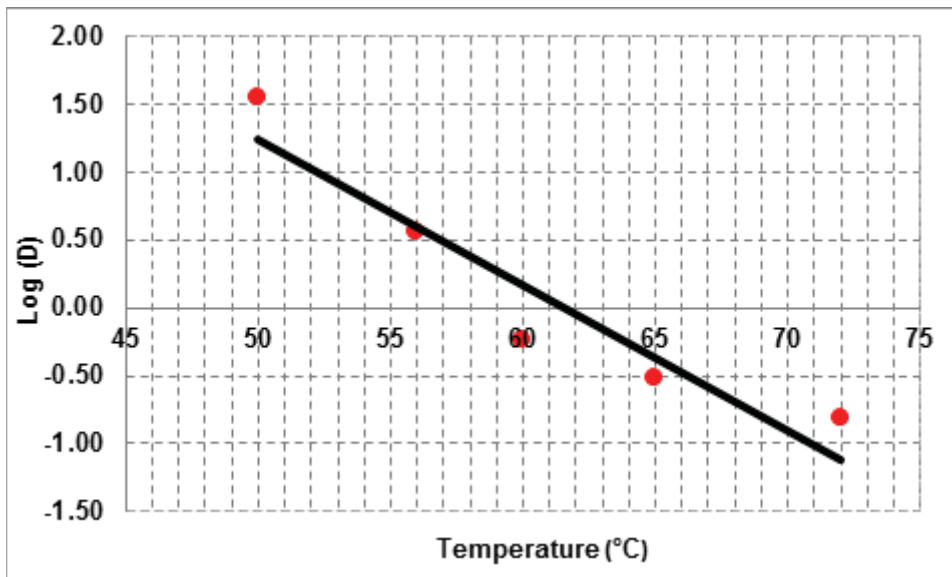


Figure 2.3. Thermal death time curves of murine norovirus (MNV-1) for the [A] Weibull model ($R^2=0.962$) and [B] first-order model ($R^2=0.8987$).

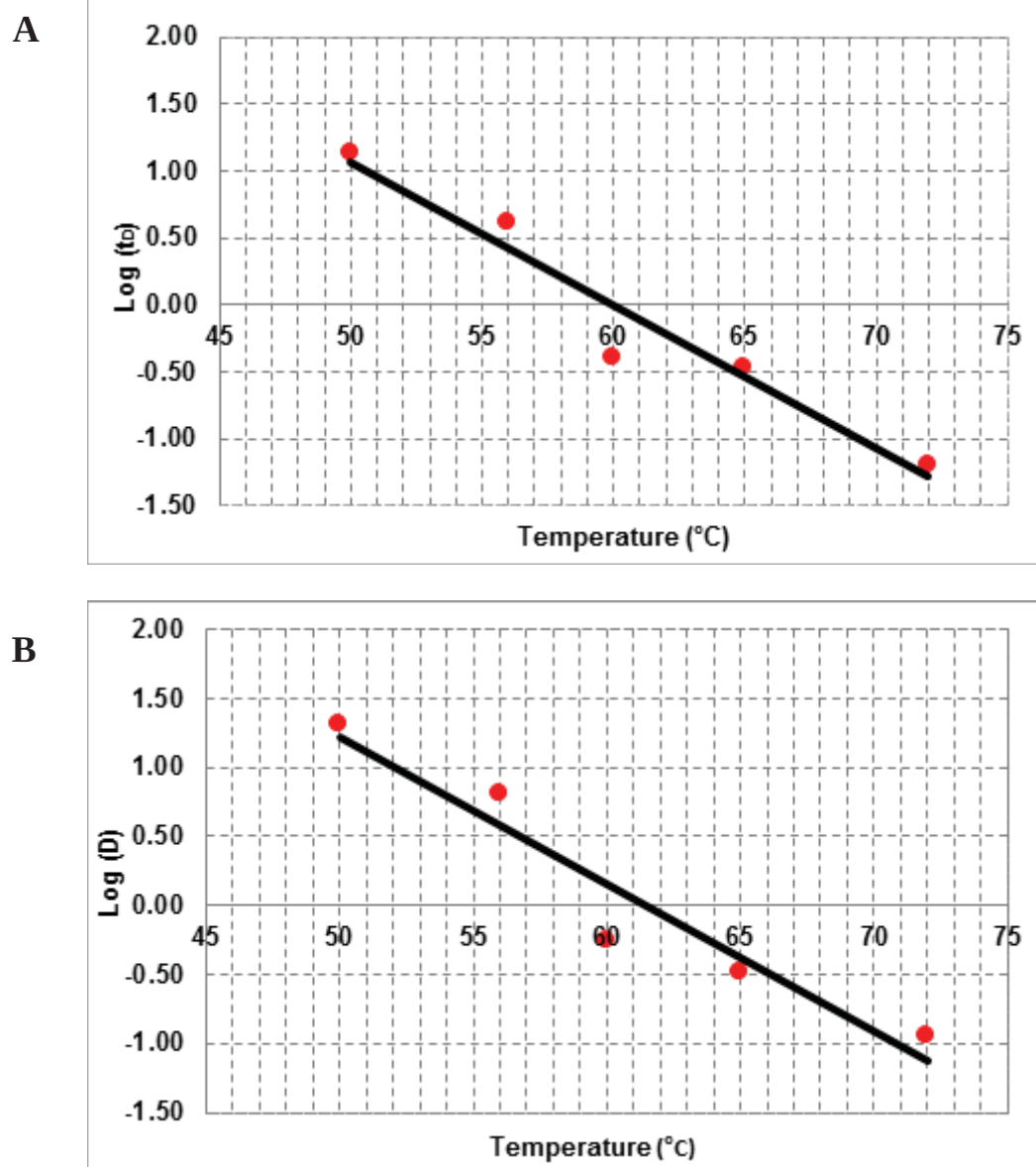


Figure 2.4. Thermal death curves of feline calicivirus (FCV-F9) for the [A] Weibull model ($R^2=0.9409$) and [B] first-order model ($R^2=0.9241$).

CHAPTER III
A COMPARISON OF THE THERMAL INACTIVATION KINETICS
OF HUMAN NOROVIRUS SURROGATES AND HEPATITIS A
VIRUS IN BUFFERED CELL CULTURE MEDIUM

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Abstract

Human noroviruses and hepatitis A virus (HAV) are considered as epidemiologically significant causes of foodborne disease. Therefore, studies are needed to bridge existing data gaps and determine appropriate parameters for thermal inactivation of human noroviruses and HAV. The objectives of this research were to compare the thermal inactivation kinetics of human norovirus surrogates (murine norovirus (MNV-1), and feline calicivirus (FCV-F9)) and HAV in buffered medium (2-ml vials), compare first-order and Weibull models to describe the data, calculate Arrhenius activation energy for each model, and evaluate model efficiency using selected statistical criteria. The D-values calculated from the first-order model (50-72°C) ranged from 0.21 to 19.75 min for FCV-F9, 0.25 to 36.28 min for MNV-1, and 0.88 to 56.22 min for HAV. Using the Weibull model, the $t_{D=1}$ (time to destroy 1 log) for FCV-F9, MNV-1 and HAV at the same temperatures ranged from 0.10 to 13.27, 0.09 to 26.78, and 1.03 to 39.91 min, respectively. The z-values for FCV-F9, MNV-1, and HAV were 9.66°C, 9.16°C, and 14.50°C, respectively, using the Weibull model. For the first order model, z-values were 9.36°C, 9.32°C, and 12.49°C for FCV-F9, MNV-1, and HAV, respectively. For the Weibull model, estimated activation energies for FCV-F9, MNV-1, and HAV were 225, 278, and 182 kJ/mole, respectively, while the calculated activation energies for the first order model were 195, 202, and 171 kJ/mole, respectively. Knowledge of the thermal

inactivation kinetics of norovirus surrogates and HAV will allow the development of processes that produce safer food products and improve consumer safety.

Key words: human norovirus surrogates, hepatitis A virus, Weibull model and first order model, D- value and z-value, activation energy.

Introduction

In recent years, viruses have been increasingly recognized as important causes of foodborne disease. In particular, human noroviruses and hepatitis A virus (HAV) are the most important human foodborne viral pathogens with regard to the number of outbreaks and people affected. Scallan et al., (2011) reported that an estimated 80-90% of all non-bacterial outbreaks of gastroenteritis reported each year are due to human noroviruses and HAV. These viruses are generally environmentally stable, survive adverse conditions and are resistant to extreme pH conditions and enzymes of the gastrointestinal tract (D'Souza et al., 2007; D'Souza et al., 2006). They have low infectious doses; as few as 10 infectious particles can cause illness (CDC, 2012; Teunis et al., 2008). Even though viruses, unlike bacteria, cannot grow in or on foods, foodborne illnesses result via contamination of the fresh produce or processed food by fecal material containing viruses (Atreya et al., 2004). Thus, proper inactivation of foodborne enteric viruses in foods prior to consumption is essential to protect public health.

Despite its importance in public health, there is little information on the thermal inactivation characteristics of human noroviruses because these viruses are currently non-culturable in the laboratory and their infectivity can only be assessed using human dose experiments (i.e., feeding studies). Cultivable surrogates, such as murine norovirus (MNV-1) and feline calicivirus (FCV-F9), have been used as human norovirus surrogates in inactivation studies based on the assumption that they can mimic characteristics of human noroviruses (Hewitt and Greening, 2004; Richards, 2012). For HAV, there are a few strains (HM-175, HAS-15, MBB 11/5) that are cell-culture adaptable and can be

maintained using fetal rhesus monkey kidney (FRhK-4) and/or human fetal lung fibroblast (MRC-5) cells. These strains have been used for inactivation studies (Martin and Lemon, 2006; Reiner et al., 1992).

Thermal processing is still one of the most effective methods for inactivating microorganisms (Silva and Gibbs, 2012). Heat is used to inactivate pathogens to produce safer foods with longer shelf life (Lee and Kaletunc, 2002). In the current literature, limited studies have been performed to investigate thermal inactivation of MNV-1 (Cannon et al., 2006; Hewitt et al., 2009; Gibson and Schwab, 2011; Bozkurt et al., 2013), FCV-F9 (Duizer et al., 2004; Cannon et al., 2006; Bozkurt et al., 2013), and HAV (Croci et al., 1999; Hewitt and Greening, 2004) in buffered cell culture media (Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and supplemented with antibiotics) . Among these studies, only one was related to the determination of the thermal inactivation kinetics of human norovirus surrogates in capillary tubes (Bozkurt et al., 2013), and they did not consider HAV. To our knowledge, there are no studies established to compare the thermal inactivation kinetics of human norovirus surrogates and HAV. To characterize the effect of heat treatment on inactivation behavior, mathematical modeling has been used to predict the number of survivors during thermal processing and to give detailed information about inactivation kinetics during treatments. Choice of the most appropriate model is crucial to gather correct information about thermal inactivation kinetic behavior. Recent studies conducted on thermal inactivation of human norovirus surrogates (Bozkurt et al., 2013; Bozkurt et al., 2014a; Bozkurt et al., 2014b; Seo et al., 2012; Tuladhar et al., 2012) revealed that the

Weibull model was statistically superior in describing the thermal inactivation kinetics of norovirus surrogates than the first-order model. A precise understanding of thermal inactivation kinetics is potentially useful for optimizing thermal treatments to eliminate the risk associated with foodborne pathogens while avoiding over-processing of the food material and thus optimal energy utilization. Therefore, generation of correct thermal process data and establishment of proper thermal processes for inactivating human norovirus surrogates and HAV are important both for consumers and industry. Therefore, the purpose of this study was (i) to characterize and compare the thermal inactivation behavior of human norovirus surrogates and HAV in buffered cell culture medium in 2 ml vials, (ii) to compare first-order, and Weibull models in describing the data in terms of selected statistical parameters, and (iii) to calculate z-values and activation energy for each model.

Material and Methods

Viruses and cell lines

Murine norovirus (MNV-1) was obtained from Dr. Skip Virgin (Washington Univ., St Louis, MO) and its host RAW 264.7 cells were obtained from the University of Tennessee, Knoxville. Feline calicivirus (FCV-F9) and its host cells (Crandell Reese Feline Kidney, (CRFK) were obtained from ATCC (Manassas, VA). Hepatitis A virus (HAV, strain HM175) and fetal monkey kidney (FRhK4) cells were kindly provided by Dr. Kalmia Kniel (University of Delaware).

CRFK, RAW 264.7, and FRhK4 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM-F12: HyClone Laboratories, Logan, UT)

supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1×Anti-Anti (Antibiotic-Antimycotic; Invitrogen, Grand Island, NY) at 37°C in an atmosphere containing 5% CO₂.

Propagation of viruses

CRFK, RAW 264.7, and FRhK4 cells with ~90% confluence in cell culture flasks were washed with phosphate-buffered saline (PBS; pH 7.4) twice before adding FCV-F9, MNV-1, and HAV stocks to their respective cell monolayers. The infected cells were then incubated until >90% cell lysis in a water jacketed CO₂ incubator at 37°C. All three viruses were recovered by centrifugation at 5,000 × g for 10 min, followed by filtration through 0.2-µm filters, aliquoted, and stored at -80°C until use as described before (Su et al., 2010).

Thermal treatment

Heat treatment was carried out in a circulating water bath (Haake model V26, Karlsruhe, Germany) in 2 ml screw-capped vials. Sterilized (121°C, 15 min) vials were carefully filled with 2 ml buffered cell culture medium (Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic-antimycotic) containing virus by using sterile pipettes in a biosafety cabinet. The filled vials were surface rinsed in 70% ethanol before immersion in a thermostatically controlled water bath. Water bath temperature was confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA) and by placing type-T thermocouples (Omega Engineering, Inc., Stamford, CT) in the geometric center of the water-bath. Another thermocouple probe was placed at the geometric center of a vial through the lid to

monitor the temperature of the buffered media. Thermocouples were connected to MMS3000-T6V4 type portable data recorder (Commtest Inc., New Zealand) to monitor temperature. Samples were heated at 50, 56, 60, 65, and 72°C for varying treatment times (0-60 min). The treatment time began (and was recorded) when the target internal temperature reached the designated temperature as described earlier (Bozkurt et al., 2013; Bozkurt et al., 2014a; Bozkurt et al., 2014b). Triplicate tubes were used for each temperature and time-point. After the thermal treatment, sample vials were immediately cooled in an ice water bath for 15 min to stop further thermal inactivation. The contents were transferred into a tube which contained 1.5 ml maintenance medium (Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, and 1% antibiotic-antimycotic) using a micro pipette. Un-heated virus suspensions were enumerated as controls.

Enumeration of survivors by infectious plaque assays

Thermally treated and control viral suspensions were diluted 1:10 in Dulbecco's Modified Eagle Medium (DMEM-F12) containing fetal bovine serum (10% for MNV-1 and 2% for FCV-F9 and HAV) and 1% antibiotic-antimycotic. Infectivity of each treated virus was evaluated using standardized plaque assays in comparison to untreated virus controls following the previously described procedures (Su et al., 2010). Viral survivors were enumerated as plaque forming units/ml (PFU/ml).

Modeling of inactivation kinetics

First-order kinetics

The traditional approach to describe the change in number of survivors over time for first-order kinetic model can be written as follows:

$$\log_{10} \frac{N(t)}{N_0} = -\frac{t}{D} \quad (1)$$

where $N(t)$ is the number of survivors after an exposure time (t) in PFU/ml and the initial population is N_0 (PFU/ml). D is the decimal reduction time in min (time required to kill 90% of viruses) and t is the treatment time (min).

The relationship between reaction constant (k) and the D value for the first order model can be expressed in the following equation:

$$D = \frac{\ln(10)}{k} = \frac{2.303}{k} \quad (2)$$

Weibull model

The Weibull model assumes that the survival curve is a cumulative distribution of lethal effects

$$\frac{N(t)}{N_0} = \exp\left(-\left(\frac{t}{\alpha}\right)^\beta\right) \quad (3)$$

where α (min^{-1}) and β (-) are the scale, and shape parameters, respectively.

As indicated by previous studies, the inverse of the of the scale factor (α) as a reaction rate constant k' (min^{-1}), the equation becomes (Fernandez, Collado, Cunha et al., 2002):

$$\frac{N(t)}{N_0} = \exp(-(k't)^\beta) \quad (4)$$

For the Weibull model, the time required to reduce the number of microorganisms by a factor 10 (analogous to the D-value) can be calculated by using the shape and scale parameters as shown in Eq (5);

$$t_D = \alpha \left(-\ln(10^{-D}) \right)^{1/\beta} \quad (5)$$

where D represents decades (*or log*) reduction of a microbial population. t_D has the stated meaning only when it refers to the treatment time starting at zero.

Arrhenius activation energy

The inactivation rate is primarily influenced by temperature, and the temperature dependence of the rate constant is typically described by the Arrhenius equation:

$$k = A \exp \left(-\frac{E_a}{RT} \right) \quad (6)$$

where A is a frequency factor which is constant, E_a is the activation energy (J/mole), R is the universal gas constant ($8.314 \text{ Jmole}^{-1}\text{K}^{-1}$), k is the rate constants (1/min), T is the absolute temperatures (K).

The obtained inactivation rate constants for each model were then fitted to an Arrhenius equation.

$$\ln k = \ln A - \frac{E_a}{RT} \quad (7)$$

The construction of $\ln k_{(T)}$ versus $1/T$, the slope of the curve will be a straight line which equals to activation energy. This concept has been used to calculate activation energy of microbial inactivation (Corradini and Peleg, 2007).

Statistical Analysis

Statistical and non-linear regression analyses were performed using SPSS Ver.11.0.1 statistical package. The statistical criteria applied to discriminate

(differentiate) between the kinetic models were R^2 (coefficient of determination), and standard errors (std. error) for each coefficient. The confidence level used to determine statistical significance was 95%.

Results and Discussion

The thermal inactivation of human norovirus surrogates and HAV in buffered medium was performed at 50-72°C in 2 ml vials, and thermal inactivation kinetics were determined using first-order and Weibull models. The inactivation parameters obtained from each model are shown in Table 3.1.

The D-values calculated from first-order model (50-72°C) were in the range of 19.21±0.70 to 0.21±0.01 min for FCV-F9, 36.28±3.21 to 0.25±0.01 min for MNV-1, and 56.22±1.95 to 0.88±0.11 min for HAV min (Table 3.1). For each virus, the temperature had a significant effect on D-values for the temperature range studied ($p<0.05$). In general, HAV was more resistant to thermal treatment than FCV-F9 and MNV-1 at all temperatures studied suggesting that it would require a more severe treatment than human norovirus surrogates for inactivation in buffered cell culture medium. In agreement with the present study, Gibson and Schwab (2011) investigated the thermal inactivation behavior of MNV-1, FCV-F9, and HAV at 50, and 60°C for various times (15-180 min) and concluded that HAV was more resistant to thermal treatment than human norovirus surrogates (MNV-1 and FCV-F9). Similarly, Sow et al., (2011) concluded that the thermal resistance of HAV was higher than MNV-1 at 85 and 90°C and suggested that HAV would be a good candidate as a surrogate for studies involving thermal inactivation of foodborne enteric viruses. Even though HAV is a non-enveloped RNA virus and

structurally similar to noroviruses, the compositional differences between these viruses might directly influence their stability. It has been stated that the ionic composition of media can influence the thermal stability of viruses in solution and that this effect can be different, even with closely related viruses (Roberts and Hart, 2000; Wallis et al., 1965). Besides HAV, the thermal resistance of resistance of MNV-1 was higher than FCV-1 at temperatures above 60°C. Cannon et al., (2006) also compared the thermal stability of norovirus surrogates (MNV-1 and FCV-F9) at 56, 63, and 72°C for 5-20 s using a capillary tube method and concluded that thermal resistance of MNV-1 was higher than FCV-F9 at 63 and 72°C. Bozkurt et al., (2013) determined the D-values MNV-1 and FCV-F9 in buffered medium (Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic-antimycotic) in 50 µl capillary tubes at 50-72°C. Although the D-values were lower than those obtained in the present study at 60, 65, and 72°C ($p < 0.05$), there was no statistical differences observed between the studies at 50 and 56°C ($p > 0.05$). The difference in results may be explained by different heat transfer rate due to sample sizes. In the capillary tube method, the temperature reaches the desired level almost instantly because of higher heat transfer rate; however in the 2 ml vial, there is a short come up time to achieve the desired temperature. Chung et al., (2007) investigated the influence of heat transfer in various size tubes (3, 13, 20 mm) on measured thermal inactivation parameters for *Escherichia coli*. They concluded that the increase in the tube size might contribute to differences in heat transfer rate and resulted with increased apparent heat resistance. In the present study, at temperatures above 60°C, the increase in sample size (compared to capillary tubes) resulted in longer come up times

of 116, 142, and 158 s for 60, 65, and 72°C, respectively. Thus the apparent heat resistance of the viruses increased. For industrial applications, the usage of larger sample size requires larger heating environment to avoid slow heat transfer rate. According to Stumbo (1973), the contribution of come up time (t_c) on the apparent heat resistance could be calculated by the addition of $0.4 \cdot t_c$ to the calculated D-value for that specific temperature. Since the contribution of the come up time increases with increasing temperature, apparent heat resistance of the viruses increased at temperatures above 60°C. However at 50 and 56°C, the effect of sample volume on heat resistance of viruses was not significant due to the slower inactivation rate and shorter come up times (54 s for 50°C and 66 s for 56°C). The change in observed differences between the two methods (capillary tube and 2 ml vial tube method) at temperatures above and below 60°C could be associated with the apparent inactivation rate. This might be explained by the structural changes that occur in the capsid during thermal treatment. It has been reported that the quaternary structure of the virus capsid was unaffected up to 60°C; however, above 60°C, the icosahedral capsid was significantly altered as inactivation of virus occurs at a faster rate above that temperature (Ausar et al., 2006). This hypothesis was also supported by other researchers (Bertrand et al., 2012; Bozkurt et al., 2013; Bozkurt et al., 2014a; Bozkurt et al., 2014b; Croci et al., 1999; Croci et al., 2012; Sow et al., 2011).

To investigate the applicability of the Weibull model, the shape and scale factors parameters were calculated and are shown in Table 3.1. The Weibull shape factor (β) ranges for the temperature studied (50-72°C) were 1.59 ± 0.06 to 0.14 ± 0.18 for FCV-F9,

2.23±0.27 to 0.62±0.38 for MNV-1, 1.98±1.13 to 0.67±0.16 for HAV. A shape factor >1 indicates that the remaining population becomes increasingly damaged, whereas a shape factor <1 indicates that the remaining population has the ability to adapt to applied stress (van Boekel, 2002). Cunha et al., (1998) indicated that the shape factor was a behavior index describing the kinetic patterns of the mechanism controlling the process studied and therefore should be independent of external factors. Consistent with Chung et al., (1998), the results of this study revealed that for each virus strain, the heating temperature apparently did not influence the shape parameter and could not be described by any model.

The scale factor (α) ranges for the temperatures studied (50-72°C) were 4.14±0.38 min for FCV-F9, 23.41±1.26 to 0.02±0.01 min for MNV-1, 20.26±18.50 to 0.36±0.07 min for HAV. In contrast to the shape factor, the scale parameter depends on the heating temperature and the change in scale factor describes the effect of heating environment on the inactivation. A second order polynomial model was established to quantify the influence of temperature on the scale factor. The relationship between scale factors and temperature for FCV-F9, MNV-1, and HAV, respectively, were;

$$\alpha = 0.0123T(^{\circ}C)^2 - 1.6954T(^{\circ}C) + 58.495 \quad R^2 = 0.92 \quad (8)$$

$$\alpha = 0.1078T(^{\circ}C)^2 - 14.061T(^{\circ}C) + 454.83 \quad R^2 = 0.90 \quad (9)$$

$$\alpha = 0.1289T(^{\circ}C)^2 - 16.915T(^{\circ}C) + 551.82 \quad R^2 = 0.93 \quad (10)$$

Parameters of the Weibull model (shape factor = β and scale factor = α) were used to calculate t_D value which was used as an analog to the D-value of the first order model when it refers to the treatment time starting at zero (Table 3.1). For the Weibull model,

the calculated time to destroy 1 log ($D=1$) for FCV-F9, MNV-1, and HAV were in the range of 13.27 ± 0.98 to 0.10 ± 0.01 min, 26.78 ± 3.12 to 0.09 ± 0.02 , 39.91 ± 23.09 to 1.03 ± 0.36 min, respectively for the range 50-72°C. For FCV-F9 and MNV-1, this indicates that at each temperature (50-72°C) over-processing would occur if the target was a one log reduction when the first-order model was used instead of the Weibull model (Table 3.1). For HAV, this result was observed at temperatures above 60°C. Since one log reduction as a target is rarely used in food industry, a six log reduction was calculated such that is often used for pasteurization. The time required to achieve a six log reduction for the first order model is equal to $6D$. Due to the nonlinear behavior of the Weibull model, the time required to achieve a six log reduction for the Weibull model is not $6t_{D=1}$ but is $t_{D=6}$. Based on the thermal inactivation data obtained from this study, for both models (first order and Weibull model) the required process time to achieve 6 log reduction (as in the case of processes such as pasteurization) at temperatures 50, 56, 60, 65, and 72°C were also calculated (Table 3.2). At 72°C, the time required to achieve six log reduction of FCV-F9, MNV-1, and HAV were 1.3 min, 1.5 min, and 5.3 min, respectively (Table 3.2). For the Weibull model, the treatment times were 1.42 min for FCV-F9, 2.1 min for MNV-1, and 7.0 min for HAV. For the studied temperature range (50-72°C), over processing occurs (if the target is six log reduction) if the Weibull model is used instead of the first order model. Therefore, the usage of Weibull model provides an impressive safety record for thermal inactivation of norovirus surrogates and HAV.

The temperature dependency of the inactivation rate constant (k and k') were fitted by the exponential Arrhenius function for both models (Table 3.3). The Weibull model gave

higher R^2 than the first-order model when the rate constants were fitted to the Arrhenius equation (Table 3.3). For the first order model, the estimated inactivation rate constants for the temperatures studied (50-72°C) were in the range of 36.11 ± 12.73 to 0.24 ± 0.02 min^{-1} for FCV-F9, 61.11 ± 34.69 to 0.04 ± 0.01 min^{-1} for MNV-1, and 2.85 ± 0.56 to 0.05 ± 0.03 min^{-1} for HAV. The temperature dependency of the inactivation rate constant for the first-order model was expressed by the second order polynomial model. The relationship between the inactivation rate constant and temperature for FCV-F9, MNV-1, and HAV respectively were;

$$k = 0.0291T^2(^{\circ}\text{C}) - 3.0809T(^{\circ}\text{C}) + 81.571 \quad R^2 = 0.97 \quad (11)$$

$$k = 0.0219T^2(^{\circ}\text{C}) - 2.2683T(^{\circ}\text{C}) + 58.952 \quad R^2 = 0.96 \quad (12)$$

$$k = 0.1418T^2(^{\circ}\text{C}) - 0.23T(^{\circ}\text{C}) + 0.1552 \quad R^2 = 0.98 \quad (13)$$

The estimated inactivation rate constants for the Weibull model for the temperatures studied (50-72°C) were in the range of 4.77 ± 0.23 to 0.05 ± 0.01 min^{-1} for FCV-F9, 4.00 ± 0.16 to 0.03 ± 0.01 min^{-1} for MNV-1, and 1.15 ± 0.14 to 0.02 ± 0.01 min^{-1} for HAV. A second order polynomial model was established to quantify the influence of temperature on the inactivation rate constant for the Weibull model. The relationship between inactivation rate constants and temperature for FCV-F9, MNV-1, and HAV respectively were;

$$k' = 0.1083T(^{\circ}\text{C}) - 11.221T(^{\circ}\text{C}) + 302.47 \quad R^2 = 0.99 \quad (14)$$

$$k' = 0.1747T^2(^{\circ}\text{C}) - 19.242T(^{\circ}\text{C}) + 527.46 \quad R^2 = 0.98 \quad (15)$$

$$k' = 0.0046T^2(^{\circ}\text{C}) - 0.4347T(^{\circ}\text{C}) + 10.285 \quad R^2 = 0.99 \quad (16)$$

When microorganisms are exposed to heat, they do not all receive the same dose of energy per unit time because at the microscopic level, the kinetic energy (speed of molecules) is distributed according to the Maxwell Boltzmann distribution. For an inactivation event to occur, the interacting molecules need a minimum amount of energy, the activation energy. The proportion of molecules that have kinetic energy above a certain critical level increases with temperature. According to this approach microorganisms would receive a certain amount of energy, and this energy causes the cause denaturation of target microorganism (Klotz et al., 2007).

The estimated activation energies for first order model were 195, 202, 171 kJ/mole for FCV-F9, MNV-1, and HAV, respectively. For the Weibull model, estimated activation energies for FCV-F9, MNV-1, and HAV were 225, 278, 182 kJ/mole, respectively (Table 3.4). For the same virus strain, the differences in activation energies between models occur due to their underlying mechanism. For first order model, there is a log linear relationship between energy required for inactivation and temperature. However, survival curves with shoulder and tail may require multiple “hits” before being inactivated. Due to the nonlinear nature of the Weibull model, it requires higher activation energy than first order model. The difference in activation energies between viral strains occur due to their temperature sensitivity. The results revealed that the inactivation of HAV is less temperature sensitive than the inactivation of FCV-F9 and MNV-1.

In addition to activation energies for both models, the z-values for inactivation of human norovirus surrogates and HAV in buffered medium were also calculated (Table

3.5). The z-values determined for FCV-F9, MNV-1, and HAV were $9.66 \pm 0.94^{\circ}\text{C}$, $9.16 \pm 1.12^{\circ}\text{C}$, and $14.50 \pm 2.93^{\circ}\text{C}$, respectively, using the Weibull model. For the first order model, z-values were $9.36 \pm 0.62^{\circ}\text{C}$, $9.32 \pm 0.47^{\circ}\text{C}$, and $12.49 \pm 0.20^{\circ}\text{C}$ for FCV-F9, MNV-1, and HAV, respectively. In terms of z-values determined for FCV-F9 and MNV-1, there were no significant differences observed between the present study and those reported by Bozkurt et al., (2013). Based on the thermal inactivation data obtained from this study, for both models (first order and Weibull model) the required process time to achieve 6 log reduction (as in the case of processes such as pasteurization) at temperatures 80, 85, 90, 95, and 100°C were calculated (Table 3.6). It is important to note that usage of different food samples and heating conditions might have different heating characteristics and validation of the recommended process conditions must be carried out before actual application of the process. At each temperature, the results obtained showed that HAV was the most heat resistant and required longer treatment times rather than the two tested human norovirus surrogates. Since those thermal inactivation data values were generated in buffered cell culture media, investigation of the thermal inactivation of these viruses in various food commodities is also needed. The precise understanding of the thermal inactivation behavior of foodborne enteric viruses would be useful for the food industry during integration of thermal processing to control foodborne enteric virus associated outbreaks.

Conclusion

The aim of this study was to investigate and compare the inactivation kinetics of human norovirus surrogates and HAV in buffered cell culture medium. The results

revealed that the Weibull model produced a better fit to the data than the traditional linear model for describing the thermal inactivation kinetics of human norovirus surrogates and HAV. Accurate model prediction of survival curves would be beneficial to the food industry in selecting optimum process conditions to obtain the desired level of inactivation. The results of this study will be useful to the food industry in designing thermal processes such as pasteurization to inactivate or control human norovirus surrogates and HAV, and thus prevent foodborne illness outbreaks.

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Appendix

Table 3.1. Coefficients of the first-order and Weibull models for the survival curves of feline calicivirus (FCV-F9), murine norovirus (MNV-1) and hepatitis A virus (HAV) during thermal inactivation.

Virus	T (°C)	Weibull distribution				First-order kinetics	
		β	α (min)	$t_{D=1}$ (min)	R^2	D (min)	R^2
FCV-F9	50	0.72±0.02	4.14±0.38	13.27±0.98	0.99	19.95±0.70	0.98
	56	1.59±0.06	2.79±0.11	4.05±0.09	0.99	6.37±0.59	0.93
	60	0.14±0.18	0.22±0.05	0.40±0.17	0.97	0.94±0.04	0.95
	65	0.70±0.03	0.11±0.02	0.35±0.05	0.99	0.72±0.01	0.97
	72	0.70±0.02	0.03±0.01	0.10±0.01	0.98	0.21±0.01	0.98
MNV-1	50	2.23±0.27	23.41±1.26	26.78±3.12	0.99	36.28±3.21	0.91
	56	0.62±0.38	0.66±0.43	2.34±0.43	0.98	3.74±0.68	0.92
	60	0.71±0.01	0.21±0.01	0.68±0.02	0.99	1.09±0.03	0.94
	65	0.71±0.06	0.12±0.03	0.39±0.07	0.99	0.77±0.03	0.96
	72	0.64±0.07	0.02±0.01	0.09±0.02	0.99	0.25±0.01	0.97
HAV	50	1.98±1.13	20.26±18.50	39.91±26.09	0.99	56.22±1.95	0.90
	56	1.25±0.77	3.80±0.73	11.11±8.73	0.98	8.40±0.43	0.93
	60	0.67±0.16	1.23±0.07	4.76±2.04	0.99	2.67±0.42	0.95
	65	0.68±0.12	0.73±0.09	2.56±0.32	0.97	1.73±0.98	0.95
	72	0.90±0.33	0.36±0.07	1.03±0.36	0.99	0.88±0.11	0.96

Table 3.2. Calculated process time to achieve 6 log reduction for the first-order and Weibull models of feline calicivirus (FCV-F9), murine norovirus (MNV-1) and hepatitis A virus (HAV) during thermal inactivation.

Model	Virus	Time (min)				
		Temperature (°C)				
		50°C	56°C	60°C	65°C	72°C
First order	FCV-F9	119.7	38.22	5.64	4.32	1.3
	MNV-1	217.68	22.44	6.54	4.32	1.5
	HAV	338.32	50.40	16.02	10.38	5.3
Weibull	FCV-F9	169.15	43.73	5.59	4.73	1.42
	MNV-1	244.11	29.66	8.47	4.84	2.1
	HAV	371.15	58.57	26.79	19.44	7.0

Table 3.3. Arrhenius inactivation rate constant of the first-order and Weibull models for the survival curves of feline calicivirus (FCV-F9), murine norovirus (MNV-1) and hepatitis A virus (HAV) in buffered cell culture medium during thermal inactivation.

Virus	T (°C)	Arrhenius model			
		First order model		Weibull model	
		k(min ⁻¹)	R ²	k(min ⁻¹)	R ²
FCV-F9	50	0.24±0.02	0.94	0.05±0.01	0.96
	56	0.36±0.01	0.89	0.16±0.01	0.97
	60	4.49±1.00	0.91	1.07±0.05	0.95
	65	9.30±1.72	0.92	1.39±0.02	0.98
	72	36.11±12.73	0.94	4.77±0.23	0.95
MNV-1	50	0.04±0.01	0.87	0.03±0.01	0.97
	56	2.26±1.83	0.89	0.27±0.05	0.96
	60	4.77±0.23	0.90	0.92±0.03	0.98
	65	8.70±2.25	0.91	1.39±0.06	0.98
	72	61.11±34.69	0.92	4.00±0.16	0.97
HAV	50	0.05±0.03	0.88	0.02±0.01	0.97
	56	0.27±0.05	0.91	0.12±0.01	0.96
	60	0.81±0.05	0.94	0.38±0.06	0.98
	65	1.38±0.17	0.93	0.76±0.51	0.95
	72	2.85±0.56	0.95	1.15±0.14	0.97

Table 3.4. The activation energies of the first-order and Weibull models for feline calicivirus (FCV-F9), murine norovirus (MNV-1) and hepatitis A virus (HAV).

Virus	Weibull distribution		First-order kinetics	
	E_a (kJ/mol)	R^2	E_a (kJ/mol)	R^2
FCV-F9	225	0.93	195	0.94
MNV-1	278	0.92	202	0.92
HAV	182	0.92	171	0.91

Table 3.5. The z-values of the first-order and Weibull models for feline calicivirus (FCV-F9), murine norovirus (MNV-1) and hepatitis A virus (HAV).

Virus	Weibull distribution		First-order kinetics	
	z value (°C)	R ²	z value (°C)	R ²
FCV-F9	9.66±0.94	0.91	9.36±0.62	0.92
MNV-1	9.16±1.12	0.95	9.32±0.47	0.90
HAV	14.50±2.93	0.92	12.49±0.20	0.90

Table 3.6. Estimated process time to achieve 6 log reduction for the first-order and Weibull models of feline calicivirus (FCV-F9), murine norovirus (MNV-1) and hepatitis A virus (HAV) during thermal inactivation.

Model	Virus	Time (s)				
		Temperature (°C)				
		80°C	85°C	90°C	95°C	100°C
First order	FCV-F9	11	3	1	1	1
	MNV-1	13	4	1	1	1
	HAV	72	29	11	5	2
Weibull	FCV-F9	12	4	1	1	1
	MNV-1	16	5	1	1	1
	HAV	112	50	23	11	5

*It is important to note that usage of different heating conditions might have different heating characteristics and validation of the recommended process conditions using steam must be carried out before actual application of the process.

CHAPTER IV
THERMAL INACTIVATION KINETIC MODELING OF HUMAN
NOROVIRUS SURROGATES IN BLUE MUSSEL (*MYTULIS*
***EDULIS*) HOMOGENATE**

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Abstract

Control of seafood-associated norovirus outbreaks has become an important priority for public health authorities. Due to the absence of human norovirus infectivity assays, cultivable surrogates such as feline calicivirus (FCV-F9) and murine norovirus (MNV-1) have been used to begin to understand thermal inactivation behavior. In this study, the effect of thermal treatment on inactivation of human norovirus surrogates in blue mussels was investigated at 50, 56, 60, 65, and 72°C for various times (0-6 min). The results obtained were analyzed using the Weibull and first order models. The Theil error splitting method was used for model comparison. This method splits the error in the predicted data into fixed and random error. This method was applied to select satisfactory models for determination of thermal inactivation of norovirus surrogates and kinetic modeling. The D-values calculated from the first-order model (50-72°C) were in the range of 0.07 to 5.20 min for FCV-F9 and 0.18 to 20.19 min for MNV-1. Using the Weibull model, the $t_{D=1}$ for FCV-F9 and MNV-1 to destroy 1 log ($D=1$) at the same temperatures were in the range of 0.08 to 4.03 min and 0.15 to 19.80 min, respectively. The z-values determined for MNV-1 were $9.91 \pm 0.71^\circ\text{C}$ ($R^2=0.95$) using the Weibull model and $11.62 \pm 0.59^\circ\text{C}$ ($R^2=0.93$) for the first-order model. For FCV-F9 the z-values were $12.38 \pm 0.68^\circ\text{C}$ ($R^2=0.94$) and $11.39 \pm 0.41^\circ\text{C}$ ($R^2=0.97$) for the Weibull and first-order models, respectively. The Theil method revealed that the Weibull model was satisfactory to represent thermal inactivation data of norovirus surrogates and that the

model chosen for calculation of thermal inactivation parameters is important. Knowledge of the thermal inactivation kinetics of norovirus surrogates will allow development of processes that produce safer shellfish products and improve consumer safety.

Key words: Murine norovirus, feline calicivirus, blue mussel (*Mytilus edulis*), thermal treatment, Weibull model, first-order model, Theil method, D-value, z-value.

Introduction

Human norovirus outbreaks associated with consumption of seafood are an important public health problem which are well documented and recognized internationally (Cliver, 1997). Iwatoma et al., (2010) investigated the epidemiology of seafood-associated infections in the United States from 1973 to 2006, and found that human norovirus was the third most commonly reported pathogen associated with seafood and the most common viral agent, causing 77.5% of outbreaks of viral illness. They also stated that 21.3% of seafood associated outbreaks, including those associated with bivalve mollusks (85%), fish (12.5%), and crustaceans (2.5%), were caused by viruses. It has been shown that bivalve mollusks such as clams, cockles, mussels, and oysters, are especially prone to virus transmission and they present an elevated hazard because they are filter feeders. The exposure to human fecal contamination in their growing environment results in retention and concentration of any microorganism present, including viruses. Afzal and Minor (1994) stated that while bacteria are excreted quickly from bivalve mollusks, viruses are known to be persistent and, as a result, can be concentrated by mussels when present in their environment. Although effective methods for the bacterial depuration of contaminated mussels exist, these methods are poorly effective for enteric viruses (de Medici et al., 2001). Also, as viruses do not multiply in food or in the environment, typical methods used to control bacterial growth in food products appear to be relatively ineffective against viruses (Jaykus, 2000). Thus, the presence of foodborne enteric viruses in bivalve mollusks constitutes a potential health risk for consumers and is an important concern for health authorities (Hewitt and Greening, 2004).

Current monitoring practices for mussel safety rely on bacteriological criteria that are not suitable for application to viruses. In general, mussels are prepared by cooking but they are often heated just until the shells open which is usually achieved at temperatures under 70°C for 47±5 s (Baert et al., 2007). It is evident that shell opening is not indicative of whether the product has reached the recommended internal temperature, and a minimum heating period is required to ensure that adequate temperature parameters are reached independently of whether the shells are opened (Hewitt and Greening, 2006), thus it is not sufficient for shellfish viral decontamination (Crocì et al., 1999). The investigation of thermal inactivation characteristics and generation of precise thermal process data is required. Since there is no specific Federal regulation covering the minimum time-temperature combinations for inactivating virus contaminated mussels, establishment of proper thermal processes for inactivating human norovirus in foods would seem to be essential for protecting public health.

Despite its importance in public health, there is little information on norovirus thermal inactivation characteristics because the virus is nonculturable in the laboratory and infectivity can only be assessed using human dose (feeding studies) experiments. Cultivable surrogates such as feline calicivirus (FCV-F9) and murine norovirus (MNV-1) have been used as human norovirus surrogates in survival studies (Hewitt and Greening, 2004).

In the current literature, seafood such as cockles (Millard et al., 1987), mussels (Crocì et al., 1999; De Medici, et al., 2001; Baert et al., 2006; Johne et al., 2011), green shell mussels (Hewitt and Greening, 2006), marinated mussels (Hewitt and Greening,

2004), spiked molluscs (Croci et al., 2012), and soft shell clams (Sow et al., 2011) have been commonly used in studies involving detection, heat inactivation depuration, survival, persistence, and accumulation of human norovirus, hepatitis A, feline calicivirus, murine norovirus, and rotavirus. However, there are limited studies (Hewitt and Greening, 2006; Sow et al., 2011) involving thermal inactivation of human norovirus and/or surrogates in mussels. Thus, to our knowledge, there are limited thermal inactivation data for human norovirus surrogates in shellfish, and there is also no information on the thermal inactivation kinetics or models used to describe thermal inactivation. Some recent studies have evaluated different models to describe thermal inactivation kinetics of human norovirus surrogates in stool suspension (Tuladhar et al., 2012), and buffer (Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum) (Seo et al., 2012; Bozkurt et al., 2013). These studies revealed that the Weibull model was statistically superior in describing the thermal inactivation kinetics of norovirus surrogates than the first-order model.

Another method for analyzing and comparing different models was proposed by Henri Theil (Theil et al., 1966) and uses splitting of residual error into random and fixed sources. It was used by Harte et al., (2009) in determining the best model for inactivation of *E. coli* in various heating media. In this study, the Theil error splitting method is used as a tool for analyzing and determining the best model to describe thermal inactivation behavior of norovirus surrogates in blue mussels.

The objectives of this study were to (i) determine thermal inactivation behavior of murine norovirus (MNV-1) and feline calicivirus (FCV-F9) in blue mussels, (ii) compare

first-order and Weibull models for describing the data, and (iii) to evaluate model efficiency using the Theil method.

Material and methods

Viruses and cell lines

Murine norovirus (MNV-1) was obtained from Dr. Skip Virgin (Washington Univ., St Louis, MO) and its host Raw 264.7 cells were obtained from the University of Tennessee, Knoxville. Feline calicivirus (FCV-F9) and its host cells (Crandell Reese Feline Kidney, (CRFK) were obtained from ATCC (Manassas, VA).

Propagation of viruses

FCV-F9 and MNV-1 stocks were prepared by inoculating FCV-F9 or MNV-1 onto confluent CRFK or RAW 264.7 cells, respectively in 175 cm² flasks and incubating at 37°C and 5% CO₂ until >90% cell lysis was observed. The methods followed for the propagation of the viruses were as described in detail by Su et al. (2010).

Inoculation of mussel

Fresh blue mussel (*Mytilus edulis*) samples were purchased from a local seafood market. The blue mussels were reportedly harvested from the North Atlantic Coast during the winter season. The fresh mussel samples were shucked and homogenized using a Waring blender (Model 1063, Waring Commercial, USA) at maximum speed. Five ml of each virus stock with initial titers of 8.06 ± 1.24 log PFU/ml for FCV-F9 and 7.14 ± 1.12 log PFU/ml for MNV-1 were individually added to 25 g of mussel sample in a sterile beaker and held at 4°C for 24 h.

Thermal treatment

Heat treatment was carried out in a circulating water bath (Haake model V26, Karlsruhe, Germany) at selected temperatures ($50\text{-}72^{\circ}\text{C}\pm 0.1^{\circ}\text{C}$) at different times (0-6 min) in 2 ml vial glass tubes. Sterilized vials (2 ml) were carefully filled with inoculated homogenized mussels by using sterile pipettes in a biosafety cabinet. The filled vials were surface washed in 70% ethanol before immersion in a thermostatically controlled water bath. Water bath temperature was confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA) and by placing type-T thermocouples (Omega Engineering, Inc., Stamford, CT) in the geometric center of the water-bath. Another thermocouple probe was placed at the geometric center of a vial through the vial lid and in contact with the mussel sample to monitor the internal temperature. The thermocouples were connected to MMS3000-T6V4 type portable data recorder (Commtest Ins., New Zealand) to monitor temperature. Samples were heated at 50, 56, 60, 65, and 72°C for different treatment times (0-6 min). The treatment time began (and was recorded) when the target internal temperature reached the designated temperature. The come up times for each temperature were 98, 104, 140, 158, 172 s for 50, 56, 60, 65, and 72°C , respectively. Triplicate tubes were used for each time-point. After the thermal treatment, sample vials were immediately cooled in an ice water bath for 15 min to stop further thermal inactivation. The vial contents were collected in a sterile beaker using a sterile pipette. The remaining contents of the vials were washed with sterile autoclaved elution buffer (described below) by using sterile pipettes to flush out the entire sample and the virus extraction protocol was followed. Unheated virus suspensions from mussels were used as controls and enumerated.

Virus extraction

The method for virus extraction was performed as described in Baert et al. (2006) with some modifications. Inoculated and thermally treated mussels were washed with 12.5 ml of autoclaved elution buffer (1:6 ratio) containing 0.05 M glycine (which inhibits adsorption of negatively charged virus to the food surface in addition to blocking nonspecific binding) and 0.15 M NaCl (to assist precipitation), at pH 9.0 to allow the detachment of virus particles from the food matrix in the presence of an alkaline environment. After addition of the elution buffer to the thermally treated blue mussel samples, the pH was then adjusted to 9.0 using 10 M NaOH. Samples in the sterile beaker were kept shaking on a platform (120 rpm) for 20 min at 4°C. Samples were centrifuged at 10.000 x g for 15 min at 4°C, and the pH of the supernatant was adjusted to 7.2 to 7.4 using 6 N HCl (to precipitate viruses and proteins in the sample though it does not exclude all organic material).

Polyethylene glycol (PEG) 6000 (to precipitate viruses and proteins in the sample) and NaCl were added to obtain a final concentration of 6% PEG (w/v) and 0.3 M NaCl (while the mechanism is not completely understood, these mask charges and hydrophilic residues on the virus surface facilitating their precipitation or “falling out” of solution). These samples were placed on a shaking platform (120 rpm) overnight at 4°C and then centrifuged at 10.000 x g for 30 min at 4°C. The supernatant was discarded and the pellet dissolved in 2 ml phosphate buffered saline (PBS, pH 7.2) and mixed using a shaker for 20 min. Virus extracts were stored at -80°C until enumeration of MNV-1 and FCV-F9 by plaque assays.

Enumeration of survivors by infectious plaque assays

Thermally treated and control viral suspensions were diluted 1:10 in Dulbecco's Modified Eagle Medium (DMEM-F12) containing fetal bovine serum (10% for MNV-1 and 2% for FCV-F9) and 1% antibiotic-antimycotic. Infectivity of each treated virus was evaluated using standardized plaque assays in comparison to untreated virus controls following the previously described procedures by Su et al. (2010). Viral survivors were enumerated as plaque forming units/ml (PFU/ml).

Modeling of inactivation kinetics

First-order kinetics

The first-order kinetic model assumes a linear logarithmic reduction of the number of survivors over treatment time:

$$\log_{10} \frac{N(t)}{N_0} = -\frac{t}{D} \quad (1)$$

Where $N(t)$ is the number of survivors after an exposure time (t) in PFU/ml and the initial population is N_0 (PFU/ml). D is the decimal reduction time in min (time required to kill 90% of viruses) and t is the treatment time (min).

Weibull model

The Weibull model assumes that the survival curve is a cumulative distribution of lethal effects

$$\frac{N(t)}{N_0} = \exp\left(-\left(\frac{t}{\alpha}\right)^\beta\right) \quad (2)$$

where α and β are the scale and shape parameters, respectively.

For the Weibull model, the time required to destroy achieve a specific logarithmic reduction of virus can be evaluated by using shape and scale parameters as shown in Eq (3);

$$t_D = \alpha (-\ln(10^{-D}))^{1/\beta} \quad (3)$$

where D is the number of decimal reductions.

Error splitting method

Theil's error splitting method for analysis of predicted data in comparison with experimental data is established by means of calculating their difference into fixed and random error (Harte et al., 2009; Theil et al., 1966). The average error between predicted and experimental results is the sum of fixed and random error, while fixed error can be further split into bias and regression error. The bias fixed (B), regression fixed (R) and random errors (ε) are calculated for the predicted values of inactivation data using following equations.

$$B = (\hat{N}_{exp} - \hat{N}_{model})^2 \quad (4)$$

$$R = (S_{N_{exp}} - \hat{\beta}_1 \cdot S_{N_{exp}})^2 \quad (5)$$

$$\varepsilon = (1 - r^2) \cdot S^2_{N_{model}} \quad (7)$$

Total average error between q values of experimental data points ($N_{exp,i}$) and predicted data points ($N_{model,i}$) is given as:

$$\frac{1}{q} \sum_{i=1}^q (N_{exp,i} - N_{model,i})^2 = B + R + \varepsilon \quad (8)$$

Where $\hat{N}_{exp}$ and $\hat{N}_{model}$ are the average experimental and modeled inactivation values, calculated as:

$$\hat{N}_{exp} = \frac{\sum_{i=1}^q N_{exp,i}}{q} \quad (8)$$

$$\hat{N}_{model} = \frac{\sum_{i=1}^q N_{model,i}}{q} \quad (9)$$

Where $\hat{\beta}_1$ is the experimental slope of the linear regression between predicted and experimental inactivation values, calculated as follows:

$$\hat{\beta}_1 = \frac{\sum_{i=1}^q [(N_{exp,i} - \bar{N}_{exp}) \cdot (N_{model,i} - \bar{N}_{model})]}{\sum_{i=1}^q (N_{exp,i} - \bar{N}_{exp})^2} \quad (10)$$

The significance of each error is calculated as outlined in Harte et al. (2009). The bias fixed error is tested using Student's pairwise comparison for mean, with the null hypothesis that $\mu_{N_{model}} - \mu_{N_{exp}} = 0$. The defined hypothesis is not rejected if $\bar{N}_{exp} - \bar{N}_{model} \in \mp S_D \cdot t_{(q-1;\alpha/2)}$, where t is the Student's distribution value for $q-1$ degrees of freedom for a pairwise comparison set, α is the probability of type I error, and S_D is the standard deviation for the means. The regression fixed error is tested using Student's distribution test, with the null hypothesis that $\hat{\beta}_1 = 1$. The defined hypothesis is not rejected if $\hat{\beta}_1 \in \mp S_{\hat{\beta}_1} \cdot t_{(q-2;\alpha/2)}$, where is t the Student's distribution value for $q-2$ degrees of freedom for a pairwise comparison set, α is the probability of type I error, and $S_{\hat{\beta}_1}$ calculated as:

$$S_{\hat{\beta}_1} = \sqrt{\frac{\sum_{i=1}^q (N_{model,i} - \bar{N}_{model})^2 - \left[\sum_{i=1}^q [(N_{exp,i} - \bar{N}_{exp}) \cdot (N_{model,i} - \bar{N}_{model})]^2 / \sum_{i=1}^q (N_{exp,i} - \bar{N}_{exp})^2 \right]}{(q-2) \cdot \sum_{i=1}^q (N_{exp,i} - \bar{N}_{exp})^2}} \quad (11)$$

The random error is tested for normality with a 0 expected value and existing variance $\in \sim N(0, \sigma_s^2)$. Normality for random error term is tested using the Shapiro-Wilk

test (Shapiro and Wilk, 1965). The experimental and predicted data are compared by means of correlation of coefficient (r).

After error analysis the models were evaluated to select the best model that could satisfactorily predict inactivation values (Harte et al., 2009). The criteria used for determining a satisfactory model were: (i) minimizing the average squared difference between experimental and predicted inactivation values (fixed error which is combination of bias and regression errors), (ii) maximizing the contribution of fixed random error, and (iii) maximizing the coefficient of correlation between experimental and predicted inactivation values.

Data analysis and model evaluation

The statistical evaluation, linear and non-linear regression analyses were performed using SPSS Ver.11.0.1 Statistical package. The confidence level used to determine statistical significance was 95%.

Results and discussion

The initial titers of viruses stocks were 8.06 ± 1.24 log PFU for FCV-F9 and 7.14 ± 1.12 log PFU for MNV-1. After inoculation of blue mussel samples with virus stocks, recovered titers varied between 6.69 ± 0.57 to 7.60 ± 0.27 log PFU/ml for FCV-F9 and 6.28 ± 1.36 to 6.96 ± 1.12 log PFU/ml for MNV-1, respectively (Table 4.1). The variation in virus titer after inoculation could be due to losses that incur during the virus extraction steps.

In the heating studies with inoculated mussels, samples were heated to selected temperatures ($50-72^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$). The effect of thermal treatment on MNV-1 and FCV-F9

inactivation in mussels is shown Table 3.1. Thermal treatment at 50°C for 6 min resulted in a less than 1 log reduction (PFU/ml) for both norovirus surrogates (Table 4.1). The presence of certain food components in the heating medium, such as protein (12%) and fat (2%), may play a protective role against heat inactivation. Croci et al., (2012) suggested that the presence of fat and protein in the heating environment medium influences the heat inactivation rate by protecting the cell receptors or formation of viral aggregates. Although exposure to heat at mild temperatures (around 50°C) leads mainly to damage of the virus cell receptor binding site, the damage to capsid is only limited and it retains the ability to protect the nucleic acid from the environment and thus the virus remains infectious (Croci et al., 2012). This could be the reason for observing modest levels of inactivation at low temperatures (50 and 56 °C). However, inactivation above certain temperatures might be related to capsid protein unfolding and faster inactivation rates (Volkin et al., 1997). Several researchers (Croci et al., 1999, 2012, Sow et al., 2011, Bertrand et al., 2012, Bozkurt et al., 2013) also confirmed that inactivation of virus occurs at a faster rate above the temperature required for denaturation of protein ($T > 56^{\circ}\text{C}$). Ausar et al., (2006) also stated that during thermal treatment of human norovirus, the quaternary structure of the capsid was unaffected up to 60°C but, above 60°C, the icosahedral capsid was significantly altered. The results of this study also confirmed that faster virus inactivation occurred at higher temperatures (65, and 72°C) and total inactivation was achieved in less than 20 s for both norovirus surrogates (Table 4.1).

The D-values calculated from first-order model (50-72°C) were in the range of 19.80±8.86 to 0.15±0.03 min and 5.20±0.55 to 0.07±0.01 min for MNV-1 and FCV-F9 in blue mussels, respectively (Table 4.2). Parameters of the Weibull model (scale factor = β and shape factor = α) were used to calculate t_D value which was used as an analog to the D-value of the first order model (Table 4.2). For the Weibull model, the calculated time to destroy 1 log (D=1) for MNV-1 and FCV-F9 was in the range of 20.19±0.22 to 0.18±0.03 min and 5.20±0.55 to 0.07±0.01 min, respectively, for the temperature range 50-72°C. Temperature had a significant effect on both t_D - and D-values for the range from 50 to 72°C for both norovirus surrogates ($p<0.05$).

The Weibull shape factor (β) ranges for the temperature studied (50-72°C) were 2.68±0.31-0.04±0.03 for MNV-1, and 2.68±0.38-0.23±0.26 for FCV-F9. The scale factor (α) ranges for the temperature studied (50-72°C) were 0.03±0.01-9.06±0.81 for MNV-1, and 0.08±0.01-4.03±0.95 for FCV-F9. The heating temperature does not present any influence on the shape parameter and could not be described by any model tried. Conversely, the scale parameter depends on the heating temperature and the change in scale factor described the effect of heating environment on the inactivation. A second order polynomial model was established to quantify influence of temperature on scale factor. The relationship between scale factors and temperature for FCV-F9 and MNV-1, respectively were;

$$\alpha = 0.0071T(^{\circ}C)^2 + 0.974T(^{\circ}C) + 33.29 \quad R^2 = 0.998 \quad (12)$$

$$\alpha = 0.0322T(^{\circ}C)^2 - 4.31T(^{\circ}C) + 143.73 \quad R^2 = 0.976 \quad (13)$$

The viruses associated with outbreaks of foodborne illnesses involving shellfish consumption are human norovirus and HAV. Even though, HAV is a non-enveloped RNA virus, and structurally similar to noroviruses, their infection cycles, and their inactivation mechanisms are different. To provide a complete review on the studies associated with inactivation of foodborne enteric viruses in shellfish, studies that involved inactivation of HAV in shellfish were considered (Millard et al., 1987; Croci et al., 1999; Hewitt and Greening, 2006; Sow et al., 2011). It must be noted that in all of the previous studies on thermal inactivation of foodborne enteric viruses in shellfish, the focus was on total inactivation without consideration of thermal inactivation kinetic data (i.e., D- and z-values) (Millard et al., 1987; Croci et al., 1999; Hewitt and Greening, 2006; Sow et al., 2011). Millard et al., (1987) injected HAV virus into batches of live cockles (*Cerastoderma edule*) then applied thermal treatment by either immersion in water at temperatures ranging from at 85, and 100°C or using steam. An internal temperature of cockle meat at 85-90°C for 1 min was reported to be sufficient to completely inactivate HAV virus (Millard et al., 1987). However, Croci et al., (1999) investigated inactivation of HAV in homogenized mussels (*Mytilus galloprovincialis*) at 60, 80, and 100°C for various times and they suggested that treatments at 60°C for 30 min, 80°C for 10 min and an immersion at 100 for 1 min were not sufficient to inactivate HAV virus in mussels, and that it was necessary to extend the heat treatment at 100°C for 2 min to completely inactivate viruses in mussels. In another study, Hewitt and Greening, (2006) evaluated the effect of boiling and steaming on HAV in New Zealand Greenshell mussels (*Perna canaliculus*). These researchers injected human norovirus and HAV into

multiple sites in the gut region of individual mussels while still in the shell and then immersed mussels directly in water. They demonstrated a modest level of inactivation (1.5 log decrease in tissue culture infective dose (TCID₅₀)) at 63°C for 180 s for HAV but complete inactivation (3.5 log reduction) in 180 sec at 90-92°C. However, for both boiling (37 s, 180 s) and steaming (37 s, 180 s) experiments, no significant reduction in the real-time RT-PCR titer for HAV was observed for any of the heat treatments when compared with the unheated control group (Hewitt and Greening, 2006). Sow et al., (2011) also concluded that application of 90°C for 180 s was sufficient to obtain 5.47 log reduction in soft shell clams (*Mya arenaria*).

HAV was the virus that mainly used in these reported studies (Millard et al., 1987; Croci et al., 1999; Hewitt and Greening 2006; Sow et al., 2011) that requires longer heating times compared to norovirus surrogates due to its thermal stability (Sow et al., 2011). Since norovirus surrogates were used in the present study, the difference in thermal inactivation could be related to different susceptibility to heat treatment for different types of viruses. Other than virus strain, differences in results among studies may be explained the varying experimental approaches used. For example, Millard et al., (1987) and Hewitt and Greening (2006) immersed inoculated shellfish directly in water. However, due to heat conduction through the shell, it takes more time to reach center of the shellfish and thus a much longer come-up time. In the study by Sow et al., (2011), the amount of sample in the test vial (15 ml) was much greater than used in the present study (2 ml) which would result in increased come-up time. Also, as stated by Chung et al., (2007) the size of vial contributed to differences in D-values even for same strains of

microorganism. Use of blended mussels in the present study provided a more homogenous media for thermal treatment which would presumably result in shorter come-up times.

In addition to HAV, some of these studies (Hewitt and Greening, 2006 and Sow et al., 2011) also considered human norovirus (Hewitt and Greening, 2006), and the human norovirus surrogate (MNV-1) (Sow et al., 2011). There were no significant reductions in real time RT-PCR titer of human norovirus after thermal treatment for both boiling (37 s, 180 s) and steaming (37 s, 180 s) (Hewitt and Greening, 2006). However, the heat treatment at 90°C for 180 s resulted in greater than 5.47 log reduction of MNV-1.

The calculated z-value for MNV-1 using the Weibull model was $9.91 \pm 0.71^\circ\text{C}$ ($R^2=0.95$) which was lower ($p<0.05$) than that determined using the first-order model of $11.62 \pm 0.59^\circ\text{C}$ ($R^2=0.93$) (Figure 4.1). For FCV-F9, there was no statistical difference ($p>0.05$) between the z-values calculated using the Weibull ($12.38 \pm 0.68^\circ\text{C}$ ($R^2=0.94$)) or first-order models ($11.39 \pm 0.41^\circ\text{C}$ ($R^2=0.97$)) (Figure 4.2). In a previous study, Bozkurt et al., (2013) determined the z-values for MNV-1 in buffer as 9.19°C (Weibull) and 9.31°C (first-order) and for FCV-F9, 9.31°C (Weibull) and 9.36°C (first-order). In agreement with the present results for FCV-F9, there was no significant difference ($p>0.05$) between the models.

Koopman and Duizer (2004) in discussing foodborne viruses as an emerging pathogen issue, stated that with the exception of ultrahigh temperature, no method could completely inactivate (i.e., more than 3 log reduction) foodborne viruses and these viruses would pose a possible foodborne illness risk factor. Based on the thermal

inactivation data obtained from the present study, thermal treatment of blue mussels at 70, 80, and 90°C requires 131, 18, and 2 s, respectively, to achieve 6 log (PFU/ml) reduction of MNV-1 and at 70, 80, and 90°C requires 37, 5, and 1 s, respectively, to achieve a 6 log (PFU/ml) reduction of FCV-F9.

Since shellfish associated norovirus outbreaks are an important concern for public health authorities, establishment of the proper thermal inactivation data is crucial. For this purpose model efficiency was also investigated by the Theil method to provide precise information on the thermal inactivation behavior of norovirus surrogates. Both the Weibull and first-order models had high correlations, $r > 0.949$ (Table 4.3). The total error was highest for first-order model for both MNV-1, and FCV-F9. The total errors also were higher for the first-order model than the Weibull model for each temperature (Table 3.3). The regression fixed error for the Weibull model and first order model were in the range of 1.11% to 51.84%, and 5.8% to 93.5% for MNV-1 and 5.83% to 93.50%, and 91.4% to 96.4% for FCV-F9, respectively. For the first-order model, having a high regression fixed error indicates that the model consistently underestimated the inactivation values and thus it is not a satisfactory model. As a comparison, for each model at each temperature the regression fixed error was highest in first-order but the slope for experimental versus predicted inactivation was not significantly different for either model (Table 4.3). For the Weibull model, most of the total error was concentrated in random sources with normal distribution for both norovirus surrogates, while first-order had less than 20.4% in random sources. In general, both models had varying levels of random error with normal distribution (Table 3.3). The first-order model had the

highest bias fixed error, meaning that the model consistently overestimated the inactivation values at some points through inactivation curve and also had a very high random error and total error which meant it was not a satisfactory model. The Weibull model had the highest random error with very good correlation and low total error. Thus, it can be considered the most appropriate model to predict thermal inactivation values of norovirus surrogates for temperatures from 50°C to 65°C.

Conclusions

Results indicate that just as for other microorganisms, thermal inactivation of norovirus surrogates strongly depends on time-temperature interactions. Inactivation at higher temperature (65 and 72°C) has a faster inactivation rate compared to lower temperatures. The Theil error splitting method demonstrated that the Weibull model showed better thermal inactivation prediction than the first-order model. The correct understanding of the thermal inactivation behavior of norovirus surrogates could provide precise determination of the thermal process calculations to prevent foodborne viral outbreaks associated with consumption of mussels. The results obtained should contribute to the development of appropriate thermal processing protocols to ensure safety of seafood for human consumption.

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Appendix

Table 4.1. Effect of thermal treatment against feline calicivirus (FCV-F9) and murine norovirus (MNV-1) inactivation in mussel.

Virus strain	Temperature (°C)									
	50		56		60		65		72	
	Treatment	Recovered titer (log PFU/ml)	Treatment	Recovered titer (log PFU/ml)	Treatment	Recovered titer (log PFU/ml)	Treatment	Recovered titer (log PFU/ml)	Treatment	Recovered titer (log PFU/ml)
FCV-F9	Control	6.69±0.57	Control	7.34±0.03	Control	7.60±0.27	Control	7.50±0.14	Control	7.58±0.22
	0 min	6.09±0.17	0 min	5.47±0.20	0 s	4.00±0.69	0 s	2.54±0.14	0 s	3.41±0.07
	2 min	5.69±0.48	1 min	5.10±0.38	20 s	3.68±0.68	15 s	1.88±0.34	10 s	1.17±0.29
	4 min	5.44±0.45	2 min	4.72±0.20	40 s	3.11±0.75	30 s	ND	20 s	ND
	6 min	4.88±0.04	3 min	4.59±0.18	60 s	2.67±0.62	45 s	ND	30 s	ND
MNV-1	Control	6.28±0.12	Control	6.47±0.04	Control	6.74±0.07	Control	6.96±0.36	Control	6.72±0.08
	0 min	4.43±0.20	0 min	5.20±0.11	0 s	4.96±0.61	0 s	3.63±1.18	0 s	2.28±0.12
	2 min	4.38±0.21	1 min	5.02±0.29	20 s	4.83±0.54	15 s	2.95±1.06	10 s	1.33±0.29
	4 min	4.23±0.22	2 min	4.84±0.17	40 s	4.69±0.55	30 s	2.72±1.11	20 s	ND
	6 min	4.17±0.21	3 min	4.71±0.09	60 s	4.59±0.59	45 s	1.67±1.15	30 s	ND

Each treatment was replicated three times, and plaque assays for evaluating the inactivation of the viruses were carried out in duplicate.
 ND = Not detected (limit of detection was 1 log PFU/ml)

Table 4.2. Coefficients of the first-order and Weibull models for the survival curves of murine norovirus (MNV-1) and feline calicivirus (FCV-F9) during thermal inactivation.

Virus strain	T (°C)	Weibull distribution			First-order kinetics		
		β	α (min)	t_d (min)	R^2	D (min)	R^2
MNV-1	50	0.04±0.03	9.06±0.81	19.80±8.86	0.96	20.19±0.22	0.95
	56	0.20±0.15	2.46±0.36	11.44±12.16	0.97	6.12±0.81	0.91
	60	0.38±0.02	1.15±0.05	3.01±1.26	0.99	2.64±0.15	0.97
	65	2.68±0.31	0.22±0.05	0.42±0.03	0.94	0.41±0.03	0.93
	72	1.88±0.06	0.03±0.01	0.15±0.03	0.98	0.18±0.03	0.99
FCV-F9	50	0.23±0.26	2.40±1.77	4.03±0.95	0.98	5.20±0.55	0.90
	56	0.42±0.15	1.02±0.55	3.13±0.24	0.97	3.33±0.43	0.92
	60	1.37±0.40	0.43±0.24	0.67±0.18	0.98	0.77±0.20	0.95
	65	2.02±0.34	0.09±0.06	0.31±0.10	0.93	0.33±0.03	0.97
	72	2.68±0.38	0.01±0.01	0.08±0.01	0.99	0.07±0.01	0.99

Table 4.3. Error analysis for both models used to predict the inactivation of murine norovirus (MNV-1) and feline calicivirus (FCV-F9).

Virus strain	Model	T (°C)	Bias		Regression		Random		Total error	r
			%	<P	%	<P	%	<P		
MNV-1	FO	50	42.865	0.167	50.186	0.812	6.949	0.972	0.002	0.975
		56	73.781	0.492	5.831	0.589	20.389	0.972	0.004	0.954
		60	44.308	0.252	48.862	0.685	6.830	0.972	0.002	0.985
		65	2.809	0.647	93.504	0.741	3.686	0.972	0.147	0.964
		72	-	ND	-	ND	-	ND	-	0.995
	W	50	0.224	0.930	51.837	0.704	47.939	0.883	0.0006	0.980
		56	4.101	0.871	1.108	0.848	94.791	0.584	0.0001	0.985
		60	0.736	0.839	14.247	0.875	85.018	0.837	0.0001	0.995
		65	0.980	0.884	10.921	0.849	88.009	0.898	0.021	0.970
		72	-	ND	-	ND	-	ND	-	0.990
FCV-F9	FO	50	2.086	0.387	96.384	0.729	1.530	0.972	0.996	0.949
		56	1.253	0.508	97.529	0.635	1.218	0.972	0.491	0.959
		60	7.854	0.111	91.373	0.380	0.773	0.972	1.378	0.975
		65		ND		ND		ND		0.985
		72		ND		ND		ND		0.995
	W	50	0.756	0.696	4.817	0.606	94.427	0.525	0.003	0.990
		56	0.295	0.944	29.353	0.802	70.352	0.742	0.004	0.985
		60	0.165	0.851	27.549	0.802	72.285	0.749	0.007	0.990
		65		ND		ND		ND		0.964
		72		ND		ND		ND		0.995

*FO: First order model

W: Weibull model

ND: Not determined

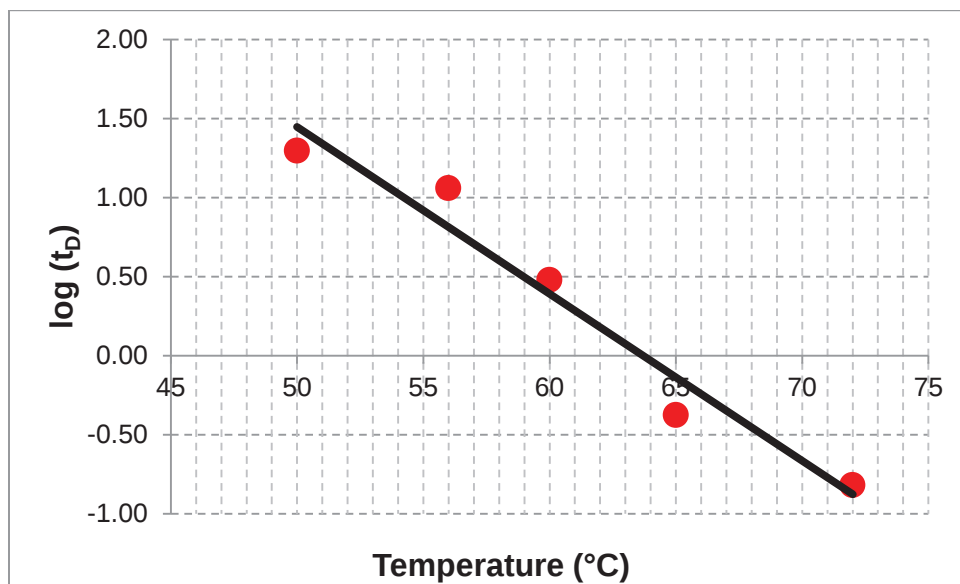
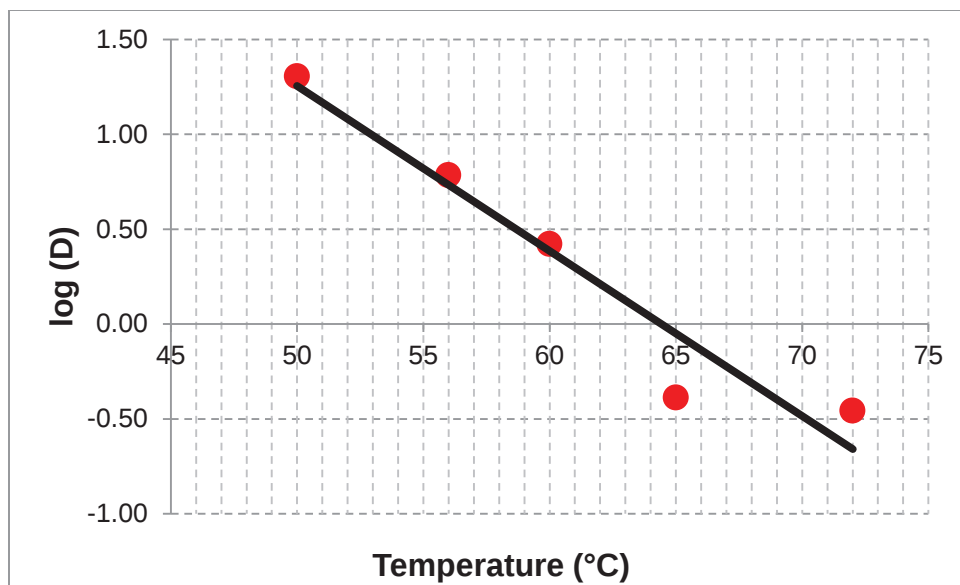
A**B**

Figure 4.1. Thermal death time curves of murine norovirus (MNV-1) for the (A) Weibull model ($R^2=0.95$) and (B) first-order model ($R^2=0.93$).

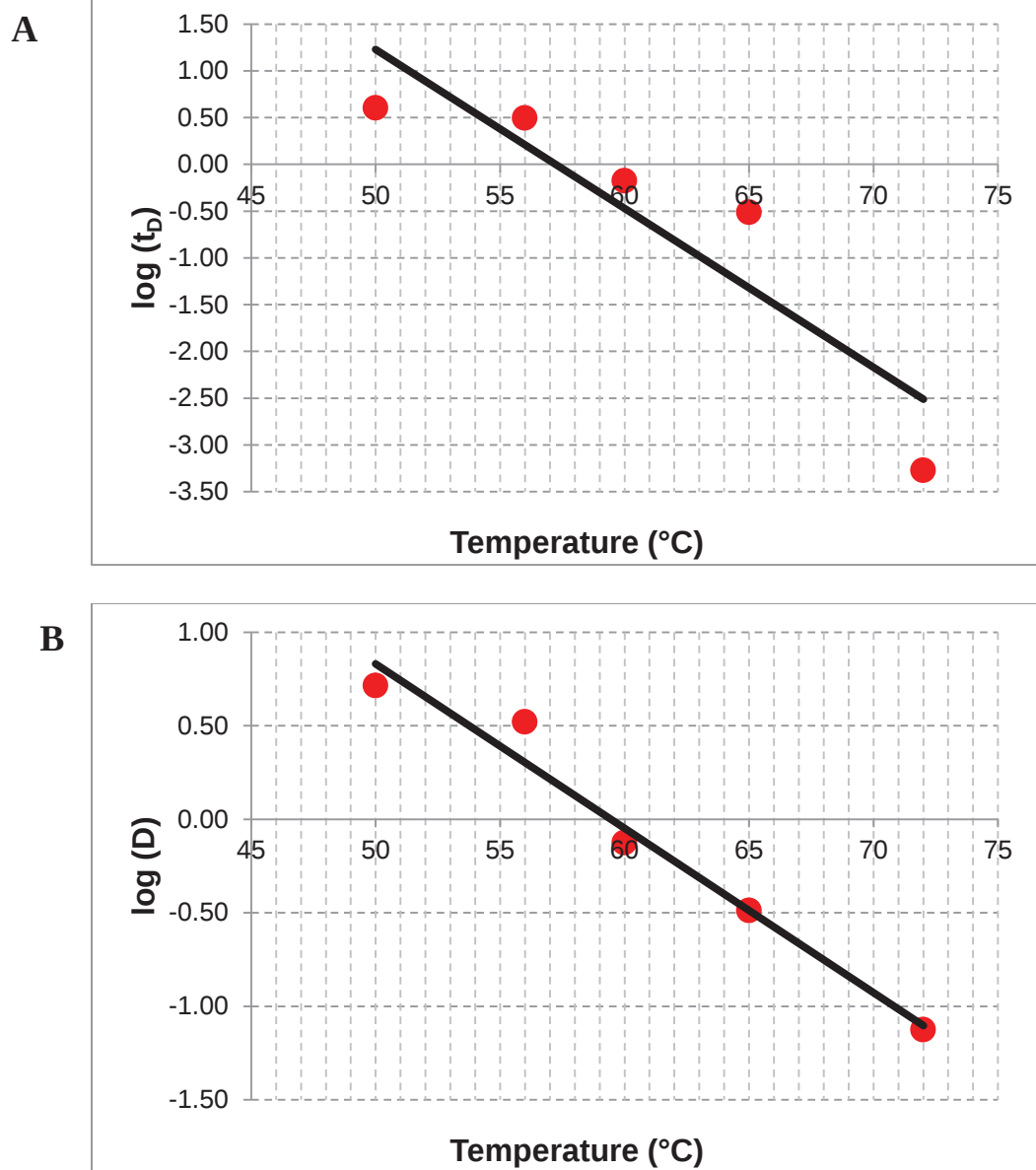


Figure 4.2. Thermal death curves of feline calicivirus (FCV-F9) for the (A) Weibull model ($R^2=0.87$) and (B) first-order model ($R^2=0.97$).

CHAPTER V
DETERMINATION OF THERMAL INACTIVATION KINETICS OF
HEPATITIS A VIRUS IN BLUE MUSSEL (*MYTULIS EDULIS*)
HOMOGENATE

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Abstract

Hepatitis A virus (HAV) is a foodborne enteric virus responsible for outbreaks of hepatitis associated with consumption of shellfish. The objectives of this study were to determine the thermal inactivation behavior of HAV in blue mussels, compare first-order and Weibull models to describe the data, calculate Arrhenius activation energy for each model, and evaluate model efficiency by using selected statistical criteria. The D-values calculated from the first-order model (50-72°C) ranged from 1.07 to 54.17 min for HAV. Using the Weibull model, the t_D for HAV to destroy 1 log ($D=1$) at the same temperatures was 1.57 to 37.91 min. At 72°C, the required treatment time to achieve 6 log reduction was 7.49 min for the first-order model and 8.47 min for the Weibull model. The z-values calculated for HAV were $15.88 \pm 3.97^\circ\text{C}$ ($R^2=0.94$) using the Weibull model and $12.97 \pm 0.59^\circ\text{C}$ ($R^2=0.93$) for the first-order model. The calculated activation energies for the first-order model and the Weibull model were 165 and 153 kJ/mole, respectively. Results revealed that the Weibull model was more appropriate to represent the thermal inactivation behavior of HAV in blue mussels for the data being analyzed. The correct understanding of the thermal inactivation behavior of HAV could provide precise determination of the thermal process calculations to prevent foodborne viral outbreaks associated with consumption of contaminated mussels.

Key words: Hepatitis A virus, blue mussels (*Mytilus edulis*), thermal treatment, Weibull model, first order model, Arrhenius model, activation energy, D-value, z-value.

Introduction

Contamination of bivalve shellfish with viruses and other agents can occur because they obtain their food by filtering small particles. In the process of filter feeding, bivalve shellfish may also concentrate and retain human pathogens derived from the environment (Lees, 2000). Epidemiological evidence suggests that human enteric viruses are the most common pathogens transmitted by shellfish. Hepatitis A virus (HAV) causes a severe viral infection linked to shellfish consumption resulting in a serious debilitating disease and occasionally death (Iwamoto et al., 2010). HAV can remain infectious within shellfish tissues for as long as three weeks (Kingsley and Chen, 2009). Even though the linkage of HAV infection to shellfish consumption was established approximately 50 years ago, HAV outbreaks associated with seafood have been, and currently remain, a serious public health concern. While an efficacious vaccine has reduced the overall incidence of HAV in the United States and elsewhere, shellfish-associated outbreaks still occur (Crocini et al., 1999; Fleet et al., 2000; Lees, 2000; Shieh et al., 2007).

HAV is a non-enveloped RNA virus, structurally similar to noroviruses, enteroviruses and astroviruses. Numerous studies have addressed the high stability of HAV under denaturing environmental conditions compared to other non-enveloped RNA viruses (Deboosere et al., 2010). Due to its resistance to thermal treatment, a cell culture adapted HAV strain would seem to be a relevant indicator in studies aimed at developing thermal inactivation strategies for most enteric viruses (Bidawid et al., 2000; Butot et al., 2008, 2009; Deboosere et al., 2004), especially since efforts to cultivate human norovirus have been unsuccessful (Bidawid et al., 2000; Deboosere et al., 2004). However, it is very

likely that more than one viral surrogate would be necessary based on behavior to different stressors or inactivation treatments. There are few strains of HAV (HM-175, HAS-15, MBB 11/5) which have been used for inactivation studies that are cell-culture adaptable and can be maintained using fetal rhesus monkey kidney (FRhK-4) and/or human fetal lung fibroblast (MRC-5) cells (Martin and Lemon, 2006; Reiner et al., 1992).

Heating appears to be the most effective measure for the inactivation of HAV (Deboosere et al., 2004). In general, mussels or other shellfish are prepared by cooking but generally they are heated only until the shells open, which is usually achieved at temperatures under 70°C for 47 ± 5 s (Baert et al., 2007). Clearly, shell opening is not indicative of whether the product has reached the recommended internal temperature. To assure food safety, a minimum temperature and heating time are required and these are independent of whether the shellfish has opened (Hewitt and Greening, 2006). Thus, shell-opening is not a sufficient indicator for viral inactivation and does not ensure shellfish safety (Crocì et al., 1999).

There have been studies on the thermal inactivation of HAV in seafood such as cockles (Millard et al., 1987), mussels (Crocì et al., 1999, 2005) green shell mussels (Hewitt and Greening, 2006), and clams (Cappellozza et al., 2012; Sow et al., 2011). Although research has been done on thermal inactivation of HAV in mussels, reported results are inconsistent, most likely because of the thermal processing conditions utilized. For example, Millard et al. (1987) reported that an internal temperature of cockle meat at 85-90°C for 1 min was sufficient to inactivate HAV (ca. 4 log TCID₅₀/ml). However, Crocì et al. (1999) concluded that immersion at 100°C for 1 min was not sufficient to

inactivate HAV in homogenized Mediterranean mussels (*Mytilus galloprovincialis*) and that it was necessary to extend the heat treatment to 100°C for 2 min for complete inactivation (5.5 log TCID₅₀/ml). In another study, Hewitt and Greening (2006) stated that thermal treatments at 90-92°C for 3 min were sufficient to achieve a 3.5 log reduction of HAV in New Zealand greenshell mussels (*Perna canaliculus*). Similarly, Hewitt and Greening (2006) and Sow et al. (2011) concluded that application of 90°C for 3 min was sufficient to obtain a 5.47 log reduction of HAV in soft shell clams (*Mya arenaria*). In contrast, Cappellozza et al. (2012) reported that 90°C for 10 min was required to inactivate 5.43 logs of HAV in Manila clams (*Ruditapes philippinarum*). Due to the variable results in the literature, there appears to be a need to utilize precise thermal inactivation conditions to establish the minimal thermal process conditions required to obtain a safe product.

Mathematical models to predict thermal inactivation of foodborne pathogens assist in developing adequate thermal processes. Recent studies conducted on thermal inactivation of human norovirus surrogates (Bozkurt et al., 2013, 2014; Seo et al., 2012; Tuladhar et al., 2012) revealed that the Weibull model was statistically superior in describing the thermal inactivation kinetics of norovirus surrogates than the first-order model. To provide a valid prediction, determination of appropriate selection criteria and correct interpretation of these selected criteria are as important as model construction. The selection criteria used to determine goodness of fit include coefficient of determination, correlation factor, predicted versus observed data, root mean square error, and percentage variance (Black et al., 2010; Harte et al., 2009). To our knowledge, there

are no studies on the thermal inactivation kinetics of HAV in blue mussels. Generation of precise thermal process data and the establishment of proper thermal processes for inactivating HAV in mussels would seem to be important both for consumers and industry. Therefore, the purpose of this study was (i) to characterize the thermal inactivation behavior of HAV in blue mussels (*Mytilus edulis*), (ii) to compare first-order and Weibull models in describing the data in terms of selected statistical parameters, and (iii) to calculate z-values and activation energy for each model.

Material and methods

Viruses and cell lines

Hepatitis A virus (HAV: strain HM175) and fetal monkey kidney (FRhK4) cells were kindly provided by Dr. Kalmia Kniel (University of Delaware). FRhK4 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM-F12: HyClone Laboratories, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1×Anti-Anti (Antibiotic-Antimycotic; Invitrogen, Grand Island, NY) at 37°C in an atmosphere containing 5% CO₂.

Propagation of viruses

FRhK4 cells with ~90% confluence in cell culture flasks were washed with phosphate-buffered saline (PBS; pH 7.4) twice before adding HAV stocks to its host-cell monolayers. The infected cells were then incubated until >90% cell lysis in a water jacketed CO₂ incubator at 37°C. Viruses were recovered by centrifugation at 5,000 × g for 10 min, followed by filtration through 0.2-μm filters, aliquoted, and stored at -80°C until use.

Inoculation of mussel

Fresh blue mussels (*Mytilus edulis*) were purchased from a local seafood market. The blue mussels were harvested from the North Atlantic Coast during the winter season. The fresh mussel samples were shucked and homogenized using a Waring blender (Model 1063, Waring Commercial, USA) at maximum speed. Since the primary objective of this study was to investigate the interaction of the virus and heat, homogenized blue mussel samples were used to obtain a uniform food matrix and homogenous temperature distribution. Five-ml of virus stock (HAV) with an initial titer of 7.04 ± 1.34 log PFU/ml were added to 25 g of mussels in a sterile beaker and held at 4°C for 24 h. The inoculated blue mussel sample without heat treatment was used as a control and enumerated.

Thermal treatment

Heat treatment was carried out in a circulating water bath (Thermo Haake, Haake model V26, Karlsruhe, Germany) at selected temperatures ($50-72^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$) at different times (0-6 min) in 2 ml vial glass tubes. Sterilized vials (2 ml) were carefully filled with inoculated homogenized mussels using sterile pipettes in a biosafety cabinet. The filled vials were rinsed in 70% ethanol before immersion in a thermostatically controlled water bath. Water bath temperature was confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA) and by placing type-T thermocouples (Omega Engineering, Inc., Stamford, CT) in the geometric center of the water bath. Another thermocouple probe was placed at the geometric center of a vial through the vial lid and in contact with the mussel sample to monitor the internal temperature. The thermocouples

were connected to a MMS3000-T6V4 type portable data recorder (Commtest Ins., Christchurch, New Zealand) to monitor temperature. Samples were heated at 50, 56, 60, 65, and 72°C for different treatment times (0-6 min). The come up times (CUT; time to reach target temperature) at 50, 56, 60, 65, and 72°C were 104, 113, 154, 166, 187 s, respectively. The treatment time began (and was recorded) when the target internal temperature reached the designated temperature. A sample was taken to enumerate HAV prior to heating, when the sample reached target temperature ($t = 0$) and at all sampling time points. Triplicate tubes were used for each time and temperature point. After the thermal treatment, sample vials were immediately cooled in an ice water bath for 15 min to stop further thermal inactivation. The vial contents were collected in a sterile beaker using a sterile pipette. The remaining contents of the vials were washed with elution buffer by using sterile pipettes to flush out the entire sample and the virus extraction protocol was followed.

Inoculated mussels without heat treatment were used as controls and HAV enumerated. The titer of the control for each temperature is indicated in Table 5.1.

Virus extraction

The method for virus extraction was performed as described by Baert et al. (2007) with some modifications. Virus extraction and enumeration was measured for the inoculated sample before thermal treatment and following each thermal treatment. Inoculated and thermally treated mussels were washed with 12.5 ml of elution buffer (1:6 ratio) containing 0.05 M glycine (Fisher Scientific, BP381-5, USA) and 0.15 M NaCl (Fisher Scientific, S671-500), at pH 9.0 to allow the detachment of virus particles from

the food matrix in the presence of an alkaline environment. The pH was then adjusted to 9.0 using 10 M NaOH (Sigma-Aldrich, S80-45, USA). Samples in the sterile beaker were put on a shaking platform (120 rpm) and kept for 20 min at 4°C. Sample were centrifuged at $10,000 \times g$ for 15 min at 4°C (Eppendorf centrifuge, Model 5804R, USA), and the pH of the supernatant was adjusted to 7.2 to 7.4 using 6 N HCl (Sigma-Aldrich, H17-58) to improve the PEG precipitation of the virus particles. Polyethylene glycol (PEG) 6000 (Fisher Scientific, A17541-0B) and NaCl were added to obtain a final concentration of 6% PEG (w/v) and 0.3 M NaCl. These samples were placed on a shaking platform (120 rpm) overnight at 4°C and then centrifuged at $10,000 \times g$ for 30 min at 4°C (Eppendorf centrifuge, Model 5804R, USA). The supernatant was discarded and the pellet dissolved in 2 ml PBS and put on a shaker for 20 min to homogenize. Virus extracts were stored at -80°C until enumeration of plaques using HAV plaque assays.

Enumeration of survivors by infectious plaque assays

Thermally treated and control viral suspensions were diluted 1:10 in Dulbecco's Modified Eagle Medium (DMEM-F12) containing fetal bovine serum (2%) and 1% antibiotic-antimycotic. Infectivity of each treated virus was evaluated using standardized plaque assays in comparison to untreated virus controls following the previously described procedures by Su et al. (2010). Viral survivors were enumerated as plaque forming units/ml (PFU/ml).

Modeling of inactivation kinetics

First-order kinetics

The first-order kinetic model assumes a linear logarithmic reduction of the number of survivors over treatment time. The model for the inactivation of microorganisms can be written as follows:

$$-\frac{dN}{dt} = kN \quad (1)$$

where N is the number of survivors at time t and k is the first order rate constant with a unit of min^{-1} . The integration from $t=0$ to $t=t$, yields to Eq. (2).

$$\frac{N(t)}{N_0} = e^{-kt} \quad (2)$$

where N_0 is the initial number of the microorganisms. The slope of survival curve will always be a straight line with slope k . The time to reduce the population by 1 log cycle (D-value) will be equal to:

$$D = \frac{\ln(10)}{k} = \frac{2.303}{k} \quad (3)$$

Substitution of Eq. (3) into Eq. (2) yields first order survival model;

$$\log_{10} \frac{N(t)}{N_0} = -\frac{t}{D} \quad (4)$$

where $N_{(t)}$ is the number of survivors after an exposure time (t) in PFU/ml and the initial population is N_0 (PFU/ml). D is the decimal reduction time in min (time required to kill 90% of viruses) and t is the treatment time (min).

Weibull model

The Weibull model assumes that the survival curve is a cumulative distribution of lethal effects

$$\frac{N(t)}{N_0} = \exp\left(-\left(\frac{t}{\alpha}\right)^\beta\right) \quad (5)$$

where α (min^{-1}) and β are scale shape parameters for Weibull model, respectively.

As indicated by previous studies, the inverse of the of the scale factor (α) as a reaction rate constant k' (min^{-1}), the equation becomes (Fernandez et al., 2002):

$$\frac{N(t)}{N_0} = \exp(-(k't)^\beta) \quad (6)$$

For the Weibull model, the time required to reduce the number of microorganisms by 90% (analogous to the D-value) can be calculated by using shape and scale parameters as shown in Eq. (9) (van Boekel, 2002);

$$t_D = \alpha \left(-\ln(10^{-D})\right)^{1/\beta} \quad (7)$$

where D represents 90% reduction of a microbial population. t_D is valid only when it refers to the treatment time starting at zero.

Arrhenius activation energy

The inactivation rate is primarily influenced by temperature, and the temperature dependence of the rate constant is typically described by the Arrhenius equation:

$$k = A \exp\left(-\frac{E_a}{RT}\right) \quad (8)$$

where A is a frequency factor which is constant, E_a is the activation energy (J/mole), R is the universal gas constant ($8.314 \text{ Jmole}^{-1}\text{K}^{-1}$), k is the rate constants (1/min), and T is the absolute temperature (K).

The obtained inactivation rate constants for each model were then fitted to an Arrhenius equation.

$$\ln k = \ln A - \frac{E_a}{RT} \quad (9)$$

The construction of $\ln k_{(T)}$ versus $1/T$, the slope of the curve will be a straight line which equals to activation energy. This concept has been used to calculate activation energy of microbial inactivation (Corradini and Peleg, 2005).

Statistical analysis

Statistical and non-linear regression analyses were performed using SPSS Ver.11.0.1 statistical package. The statistical criteria applied to discriminate among the kinetic models were R^2 (coefficient of determination), r (correlation coefficient), root mean square error (RMSE, the lower the better), and standard errors (std. error) for each coefficient. In addition to r (correlation coefficient), root mean square error (RMSE), and standard errors (std. error), the percentage of variance (%V) accounted for by the model (based on number of terms):

$$\%V = \left[1 - \frac{(1-R^2)(n-1)}{n-N_T-1} \right] \times 100 \quad (10)$$

where R^2 is the correlation coefficient, n is the number of data points and N_T is the number of model equation terms. This coefficient takes into account the complexity of the model and the population of data used to describe it. As the number of observations n increases, the number of terms (N_T) has less of an effect on the model fitness.

The confidence level used to determine statistical significance was 95%.

Results and discussion

After inoculation of blue mussel samples with HAV stocks, the unheated titer recovered was 6.73 ± 1.27 log PFU/ml of homogenate for the control. Survivor curves of

HAV in blue mussels at different temperatures (50-72°C) are shown in Figure 5.1. As temperature and/or treatment times increased, virus inactivation also increased. During the CUT lethality also occurred. At 50°C, the amount of reduction was 0.43 log PFU/ml during CUT. There was an increase in the log reduction during CUT with increasing temperature to a maximum of 1.1 log PFU/ml during CUT at 72°C. As stated by Chung et al. (2007) the size of heating vessel contributed to differences in CUT and D-values even for the same strain of microorganism. Since the reduction in number of survivors during CUT is important to determine precise thermal process conditions, the CUT should be taken into account in designing appropriate thermal processes. The shapes of the inactivation curves were characterized by an initial drop in viral counts followed by a tailing behavior. Visual inspection of these survival curves indicated that a nonlinear model would describe these data better than a linear model (Figure 5. 1).

The D-values calculated from first-order model (50-72°C) were in the range of 54.17 ± 4.94 to 1.07 ± 0.24 min (Table 5.1). The temperature had a significant effect on D-values for the temperature range studied ($p < 0.05$). To understand the relationship between inactivation rate and temperature, it is necessary to examine the underlying inactivation mechanism during thermal treatment. Hirneissen et al. (2013) stated that the mechanism of heat inactivation of viruses occurs due to changes in the capsid of virus particles. The virus capsid is the protein coat that encloses the viral genome and any other components necessary for virus structure or function and is also responsible for binding to the host. Croci et al. (2012) stated that, exposure to mild temperatures (ca. 50°C) leads mainly to damage to the viral receptor binding site through structural changes in the

capsid protein that does not allow binding and thus cause low levels of inactivation. Higher inactivation rates at increased temperatures ($>56^{\circ}\text{C}$) may be due to denaturation of capsid proteins. At higher temperatures, alteration of tertiary structure occurs and therefore, the capsid does not play a protective role against degradation of nucleic material (Katen et al., 2013). This hypothesis is supported by previous research (Bertrand et al., 2012; Bozkurt et al., 2013, 2014; Croci et al., 1999, 2012; Sow et al., 2012; Volking et al., 1997).

Although several studies have been performed to investigate thermal inactivation of HAV in mussels (Croci et al., 1999, 2005; Hewitt and Greening, 2006), no studies were found on the calculation of thermal inactivation parameters. While valuable empirical information was gathered in these studies, there was no thermal kinetic information generated and thus designing an adequate thermal process outside the limits of the studies is impossible.

Temperature had a significant effect on both t_D and D-values for the range 50 to 72°C ($p<0.05$). Parameters of the Weibull model (scale factor = β and shape factor = α) were used to calculate the t_D value which was used as an analog to the D-value of the first order model (Table 5.1). For the Weibull model, the calculated time to destroy 1 log ($D=1$) for HAV ranged from 37.91 ± 6.95 to 1.57 ± 1.04 min for the temperature range 50 to 72°C . Calculation of the time needed for six log reduction (often used as a target for processes such as pasteurization) for first-order model is 6 times the D-value ($6D$). However, time needed for six log reduction for the Weibull model is not $6t_{D=1}$ but it is $t_{D=6}$, this is the consequence of nonlinear behavior. Based on the thermal inactivation data

obtained from the present study, at 72°C, the required treatment time to achieve 6 log reduction of HAV, was 7.49 min for first-order model and 8.47 min for Weibull model. This indicates over-processing if Weibull model is used instead of first-order model. These over-processing phenomena could explain the impressive safety record of the Weibull model, especially in canning industry where over-processing for *Clostridium botulinum* is widely practiced (Corradini et al., 2005).

For further investigation of the Weibull model, temperature dependence of the parameters may be evaluated. The Weibull shape (β) and scale factors (α) ranges for the temperature studied (50-72°C) were 1.02 ± 0.45 to 0.53 ± 0.19 , and 16.91 ± 9.38 to 0.32 ± 0.05 , respectively. van Boekel (2002) reviewed 55 thermal inactivation studies on microbial vegetative cells and concluded that, in most cases, shape factors were clearly independent of heating temperature whereas scale factors could be a function of heating environment. Thus, a change in scale factor described the effect of heating environment on the inactivation. The present study is consistent with those findings in that temperature did not influence the shape parameter. The results of this study revealed that, at 50°C, a monotonic downward concave (shoulder) behavior was observed with a shape factor of 1.02 ± 0.45 . At temperatures higher than 50°C, monotonic upward concave (tailing) behavior was observed with shape factors range of 0.43 ± 0.02 to 0.65 ± 0.09 , but no relationship was observed between the temperature and shape parameters. However, the scale parameter was dependent on heating temperature. A second order polynomial model was established to quantify influence of temperature on scale factor. The relationship between scale factors and temperature was:

$$\alpha = 0.0651T^2(^{\circ}C) - 44.138T(^{\circ}C) - 7483.4 \quad R^2 = 0.902$$

The temperature dependency of the inactivation rate constant (k and k') were fitted by the exponential Arrhenius function for both models (Table 5.2). The Weibull model gave higher R^2 than the first-order model, when the rate constants were fitted to the Arrhenius equation (Table 5.2). For first order model, estimated inactivation rate constants were in the range of 0.04 ± 0.01 to $2.21 \pm 0.44 \text{ min}^{-1}$ for the temperature range $50-72^{\circ}C$. The temperature dependency of inactivation rate constant for first-order model was expressed by second order polynomial model;

$$k = 0.0037T^2(^{\circ}C) - 0.3579T(^{\circ}C) + 8.6101 \quad R^2 = 0.99$$

The estimated inactivation rate constants for Weibull model for the temperatures studied ($50-72^{\circ}C$) were 0.04 ± 0.01 to $2.21 \pm 0.44 \text{ min}^{-1}$. A second order polynomial model was established to quantify influence of temperature on inactivation rate constant for Weibull model. The relationship between inactivation rate constants and temperature was;

$$k' = 0.0079T^2(^{\circ}C) - 0.8385T(^{\circ}C) - 22.306 \quad R^2 = 0.97$$

When living organisms at the microscopic level are exposed to heat, they do not all receive the same dose of energy per unit time. For an inactivation event to occur, the interacting molecules need a minimum amount of energy, the activation energy (Klotz et al., 2007). This energy causes denaturation in the target organism. According to first order model, there is a log linear relationship between energy required for inactivation and temperature. The calculated activation energies for the first-order model and the

Weibull model based on inactivation rate constants are shown in Figure 5.2. The activation energy obtained from first order model was 153 kJ/mole while for the Weibull model, the activation energy was 165 kJ/mole. Inactivation curves with shoulders and/or tailing are usually explained on the basis that more than one critical target may require more than one hit before being inactivated.

In addition to activation energies for both models, the z-values were also calculated. The z-values for HAV were $15.88 \pm 3.97^{\circ}\text{C}$ ($R^2=0.94$) using the Weibull model and $12.97 \pm 0.59^{\circ}\text{C}$ ($R^2=0.93$) for the first-order model (Figure 5.3). Using the parameters generated in the present study, an industrial thermal process for whole mussels could be estimated. The reported CUT for whole New Zealand greenshell mussels (*Perna canaliculus*) in boiling water (100°C) was 240 s (Hewitt and Greening, 2006). According to Stumbo (1973), the contribution of CUT (t_c) to the apparent lethality of a process can be calculated by addition of $0.4 \cdot t_c$ (in min) to the calculated process time for that specific temperature. For whole mussels, based on the CUT determined by Hewitt and Greening (2006), and using the thermal inactivation parameters obtained from the present study, the required process times to achieve a 6 log reduction of HAV in mussels in boiling water (100°C) would be 2.7 and 3.2 min for the first order and Weibull models, respectively.

To compare the goodness-of-fit of the first order and Weibull models, the coefficient of determination (R^2), correlation factor (r), root mean square error (RMSE), and percentage variance (%V) values were calculated (Table 5.3). The Weibull model consistently produced the best fit for all the survivor curves. For the survivor curves at 50

to 72°C, the Weibull had R^2 values of 0.91-0.96, correlation factor values of 0.95-0.99, RMSE values of 0.01 to 0.04, and percentage variance values of 88 to 95%. Accurate model prediction of survival curves would be beneficial to the food industry in selecting the optimum combinations of temperature and time to obtain the desired levels of inactivation. The present results revealed that the Weibull model could be successfully used to describe thermal inactivation of HAV in blue mussels.

Conclusions

The heat resistance of HAV was greatly affected by temperatures from 50 to 72°C. The application of higher temperatures likely caused both denaturation of nucleic material and capsid protein resulting in significantly decreased D- and t_D values. The z-values obtained from the first order and Weibull models were $12.97 \pm 0.59^\circ\text{C}$ ($R^2=0.93$) and $15.88 \pm 3.97^\circ\text{C}$ ($R^2=0.94$), respectively. The calculated activation energies for the first-order model and the Weibull model were 165 and 153 kJ/mole, respectively. Precise information on the thermal inactivation of HAV in mussels was generated enabling more reliable thermal process calculations to control and/or inactivate the virus in potentially contaminated mussels and thus prevent foodborne illness outbreaks.

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Appendix

Table 5.1. Coefficients of the first-order and Weibull models for the survival curves of Hepatitis A (HAV) in mussel during thermal inactivation.

T (°C)	Weibull distribution				First-order kinetics	
	β	α (min)	$t_{D=1}$ (min)	R^2	D (min)	R^2
50	1.02±0.45	16.91±9.38	37.91±6.95	0.91	54.17±4.94	0.89
56	0.50±0.02	1.97±0.04	10.43±0.49	0.99	9.32±3.26	0.91
60	0.65±0.09	2.13±0.33	7.73±0.20	0.99	3.25±0.72	0.90
65	0.43±0.02	0.96±0.13	6.73±0.30	0.99	2.16±0.17	0.86
72	0.53±0.19	0.32±0.05	1.57±1.04	0.96	1.07±0.24	0.91

Table 5.2. Arrhenius inactivation rate constant of the first-order and Weibull models for the survival curves of Hepatitis A (HAV) in mussel during thermal inactivation.

T (°C)	Arrhenius model			
	First order model		Weibull model	
	k(min ⁻¹)	R ²	k(min ⁻¹)	R ²
50	0.04±0.01	0.88	0.08±0.05	0.92
56	0.27±0.11	0.87	0.51±0.01	0.99
60	0.63±0.13	0.83	0.48±0.07	0.99
65	1.07±0.09	0.79	0.65±0.05	0.99
72	2.21±0.44	0.86	3.18±0.50	0.97

Table 5.3. Statistical comparison of the first-order and Weibull models for the survivor curves of Hepatitis A (HAV).

	T(°C)	RMSE	R²	r	%V
First order model	50	0.05	0.89	0.94	85
	56	0.14	0.91	0.95	88
	60	0.11	0.90	0.95	87
	65	0.14	0.86	0.93	81
	72	0.18	0.91	0.95	88
Weibull model	50	0.04	0.91	0.95	88
	56	0.01	0.99	0.99	99
	60	0.01	0.99	0.99	99
	65	0.01	0.99	0.95	99
	72	0.04	0.96	0.98	95

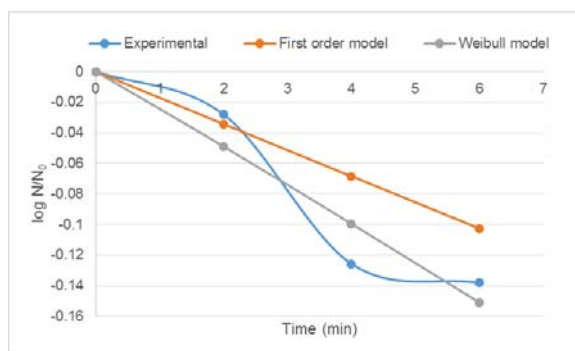
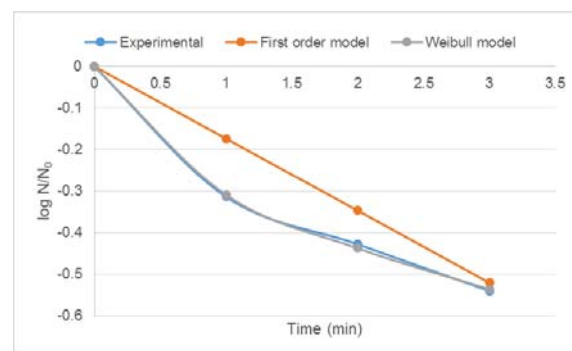
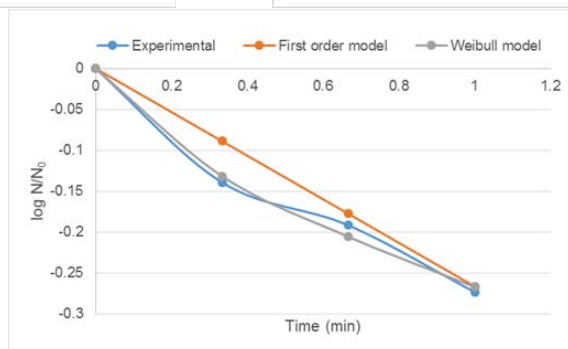
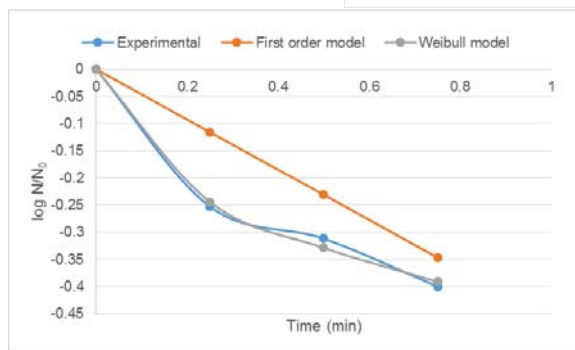
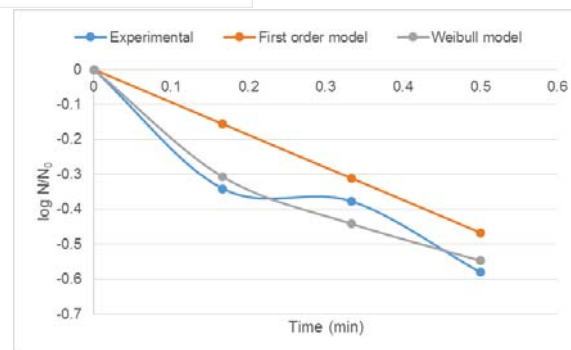
A**B****C****D****E**

Figure 5.1. Survival curves of Hepatitis A in blue mussel at different temperatures [A]50°C; [B]56°C; [C]60°C; [D]65°C; [E]72°C.

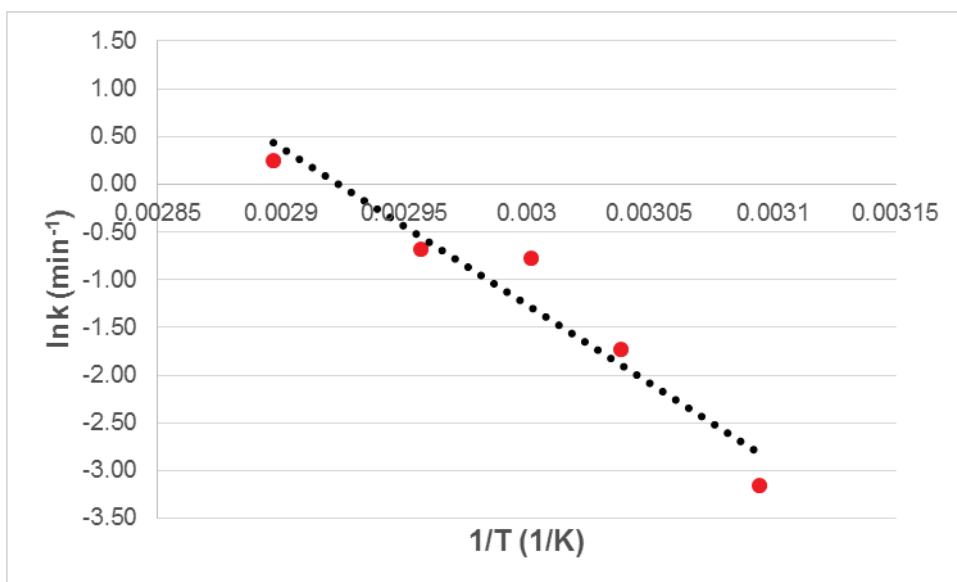
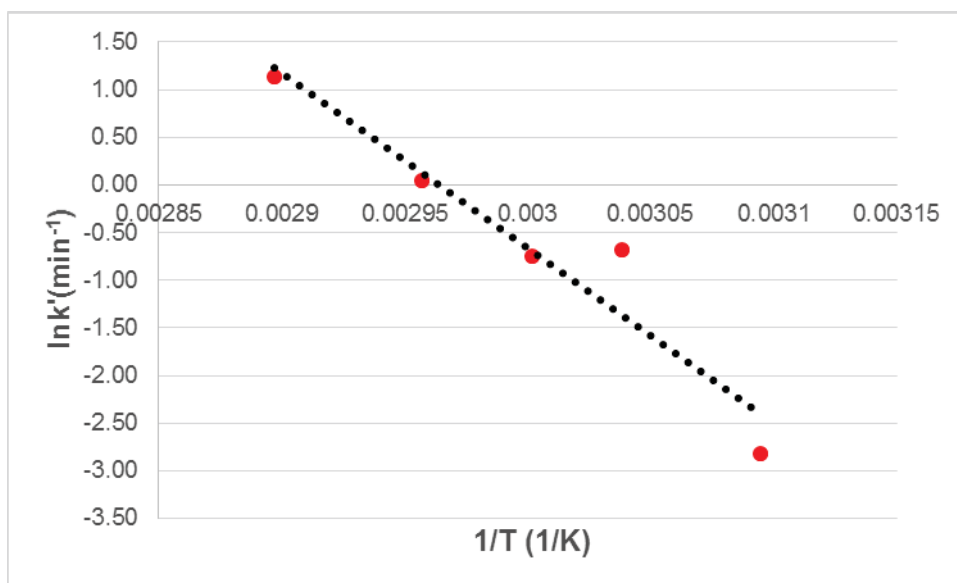
A**B**

Figure 5.2. Arrhenius plot for inactivation rate constant versus temperature for the (A) First order model ($R^2=0.93$), and (B) Weibull model ($R^2=0.93$).

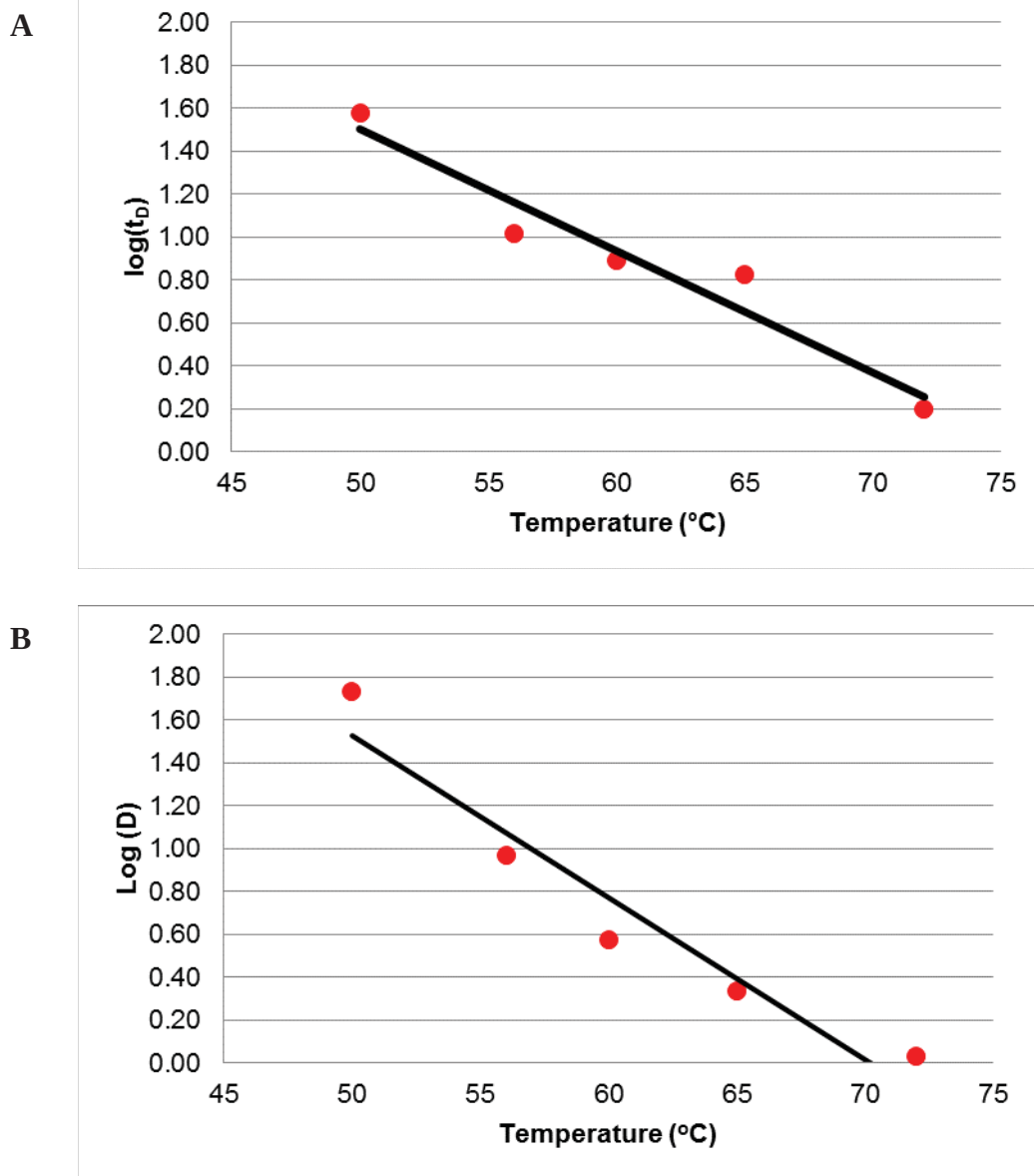


Figure 5.3. Thermal death time curves of Hepatitis A for the [A] Weibull model ($R^2=0.94$) and [B] first order model ($R^2=0.93$).

CHAPTER VI
THERMAL INACTIVATION OF HUMAN NOROVIRUS
SURROGATES IN SPINACH AND MEASUREMENT OF ITS
UNCERTAINTY

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Abstract

Leafy greens, including spinach, have potential for human norovirus transmission through improper handling and/or contact with contaminated water. Inactivation of norovirus prior to consumption is essential to protect public health. Because of the inability to propagate human norovirus *in vitro*, murine norovirus (MNV-1) and feline calicivirus (FCV-F9) have been used as surrogates to model human norovirus behavior under laboratory conditions. The objectives of this study were to determine thermal inactivation kinetics of MNV-1 and FCV-F9 in spinach, compare first-order and Weibull models, and measure the uncertainty associated with the process. D-values were determined for viruses at 50, 56, 60, 65 and 72°C in 2 ml vials. The D values calculated from the first-order model (50-72°C) ranged from 0.16 to 14.57 min for MNV-1 and 0.15 to 17.39 min for FCV-9. Using the Weibull model, the t_d for MNV-1 and FCV-F9 to destroy 1 log ($D=1$) at the same temperatures ranged from 0.22 to 15.26 and 0.27 to 20.71 min, respectively. z-values determined for MNV-1 were $11.66 \pm 0.42^\circ\text{C}$ using the Weibull model and $10.98 \pm 0.58^\circ\text{C}$ for the First-order model and for FCV-F9 were $10.85 \pm 0.67^\circ\text{C}$ and $9.89 \pm 0.79^\circ\text{C}$, respectively. There was no difference in D- or z-value using the two models ($p > 0.05$). Relative uncertainty for dilution factor, personal counting and test volume were 0.005%, 0.0004% and ca. 0.84%, respectively. The major contribution to total uncertainty was from the model selected. Total uncertainties for FCV-F9 for the Weibull and First-order models were 3.53-7.56% and 11.99-21.01%, respectively, and for MNV-1, 3.10-7.01% and 13.14-16.94%, respectively. Novel and precise information on

thermal inactivation of human norovirus surrogates in spinach was generated enabling more reliable thermal process calculations to control noroviruses. The results of this study may be useful to the frozen food industry in designing blanching processes for spinach to inactivate or control noroviruses.

Key words: Murine norovirus, feline calicivirus, Weibull model, first-order model, spinach, D-value, z-value, uncertainty measurement, thermal inactivation.

Introduction

Human noroviruses are the leading cause of acute non-bacterial gastroenteritis worldwide because of their highly infectious nature and prevalence (Donaldson et al., 2008). While epidemiological studies have shown presence of human norovirus in stools, the primary source for human infection is still unclear (Baert et al., 2008; Koopmans and Duizer, 2004). Contaminated water and food are recognized as sources for human norovirus transmission. Leafy greens, shellfish, and ready-to-eat foods (i.e., no lethality step prior to consumption) may be associated with human norovirus transmission throughout improper handling and/or contact with contaminated water. The proper inactivation of human norovirus in foods prior to consumption is essential to protect public health.

Despite its importance in public health, human norovirus biology is not well understood. This is most likely due to the absence of cell culture systems for propagation and/or lack of animal models. Due to the inability to propagate human norovirus *in vitro*, cultivable murine norovirus and feline calicivirus have been used as surrogates to understand human norovirus behavior under laboratory conditions. Murine norovirus (MNV-1) and feline calicivirus (FCV-F9) both belong to the *Caliciviridae* family with single-stranded genomic RNA (Dimmock et al., 2001). These norovirus surrogates are used based on the assumption that they can mimic characteristics of human noroviruses. FCV-F9 and MNV-1 are both widely used in environmental and food safety research (Richards, 2012).

FCV-F9 is a respiratory virus and was the first animal virus surrogate used in laboratories to mimic human noroviruses (Doultree et al., 1999). It has been commonly used in studies involving leafy and green vegetables such as lettuce (Allwood et al., 2004; Fino and Kniel, 2008a, 2008b; Fraisse et al., 2011; Hirneisse et al., 2011; Mattison et al., 2007; Rutjes et al., 2006; Wang et al., 2012; Zhou et al., 2011), basil and parsley (Butot et al., 2008, 2009), cabbage (Allwood et al., 2004), and green onions (Fino and Kniel, 2008a, 2008b; Hirneisse et al., 2011). It has been used to understand norovirus behavior in studies involving chemical disinfection, inactivation by heat, freezing, UV irradiation, determination of recovery efficiency and detection.

MNV-1 is another potential human norovirus surrogate that has similar size, shape, buoyant density, and genomic organization to human norovirus with a closer genetic relation (Wobus et al., 2006). MNV-1 is known to be relatively resistant and stable to environmental factors such as high and low pH, organic solvents, dry and wet conditions (Cannon et al., 2006). MNV-1 has been extensively used in studies involving lettuce (Deboosere et al., 2012; Fallahi and Mattison, 2011; Fraisse et al., 2011; Hirneisse et al., 2011; Li et al., 2012; Park et al., 2010; Predmore et al., 2011; Takahashi et al., 2011; Wang et al., 2012), green onion (Baert et al., 2008; Hirneisse et al., 2011), brussels sprouts and peas (Stals et al., 2011), iceberg lettuce (Baert et al., 2009; Li et al., 2011), parsley (Butot et al., 2009; Sanchez et al., 2012), Romaine lettuce (DiCaprio et al., 2012; Feng et al., 2011), basil (Butot et al., 2009), and cabbage (Predmore et al., 2011). As with FCV-9, it has been used in studies on chemical disinfection, inactivation by heat, UV

irradiation, gamma irradiation, ozone, and freezing, and for studies on recovery efficiencies , detection methodology, and stability .

Although several studies have been performed to investigate survival of norovirus surrogates in leafy vegetables, only a few (Baert et al., 2008; Sanchez et al., 2012; Shieh et al., 2009; Ward et al., 1987) have investigated survival in spinach and only one related to thermal inactivation. Baert et al., (2008) investigated the efficiency of blanching (at 80°C for 1 min, then at 4°C for 1 min) on survival of MNV-1 during spinach processing. However, the researchers did not consider come up time for the blanch process and they did not specify the final temperature of the spinach after their treatment. Thus, no thermal inactivation kinetics was established. Thus, to our knowledge, there are no reported studies on the thermal inactivation kinetics of norovirus surrogates in spinach.

The objective of this study was (i) to determine thermal inactivation behavior of murine norovirus (MNV-1) and feline calicivirus (FCV-F9) in spinach, (ii) to compare first-order and Weibull models in describing the data in terms of selected statistical parameters, and (iii) to measure all uncertainties that are associated with the process. The latter objective was undertaken to determine a quantitative indication of analytic variability of the results to enhance the validity of data (Niemela, 2002).

Material and Methods

Viruses and cell lines.

Murine norovirus (MNV-1) were obtained from Dr. Skip Virgin (Washington Univ., St Louis, MO) and its host Raw 264.7 cells were obtained from the University of

Tennessee, Knoxville. Feline calicivirus (FCV-F9) and its host, Crandell Reese Feline Kidney (CRFK) cells were obtained from ATCC (Manassas, VA).

Propagation of viruses

FCV-F9 and MNV-1 stocks were prepared by inoculating FCV-F9 or MNV-1 onto confluent CRFK or RAW 264.7 cells, respectively in 175 cm² flasks and incubating at 37°C and 5% CO₂ until >90% cell lysis was observed. The methods followed for the propagation of the viruses were as described in detail by Su et al., (2010).

Inoculation of spinach

Frozen chopped spinach samples purchased from a local grocery store were chosen to eliminate any background contamination. The chopped spinach samples were blended using a Waring blender (Model 1063, Waring Commercial, USA) to homogenize the sample. Five-ml of each virus stocks with initial titers of 8.19 ± 0.97 log PFU/ml for FCV-F9 and 7.40 ± 1.12 log PFU/ml for MNV-1 were individually added to 25 g of spinach sample in a sterile beaker and held at 4°C for 24 h.

Thermal treatment

Sterilized vials (2 ml) were filled carefully with inoculated homogenized spinach using a sterile pipet in a biosafety cabinet, and filled vials were rinsed in 70% ethanol before immersion in a thermostatically controlled water bath. An open bath circulator (Haake model V26, Karlsruhe, Germany) was used to maintain a constant temperature ($50-72^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$) of the water bath during each experiment. Water bath temperature was confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA) and by placing type-T thermocouples (Omega Engineering, Inc., Stamford, CT) in the

geometric center of the water bath. Another thermocouple was placed at the geometric center through the lid of a sealed vial and was in contact with the spinach sample to monitor the internal temperature. The thermocouples were connected to MMS3000-T6V4 type portable data recorder (Commtest Ins., New Zealand) to monitor temperature. Samples were heated at 50, 56, 60, 65, and 72°C for different treatment times (0-6 min). The treatment time began when the target internal temperature reached the designated temperature. The come up times for each temperature were 24, 32, 41, 57, and 100 s for 50, 56, 60, 65, and 72°C, respectively and the treatment time started after the desired temperature was reached for each temperature. Triplicate tubes were used for each time-point. After the thermal treatment, sample vials were immediately cooled in an ice water bath for 15 min to stop further thermal inactivation. The vial contents were removed from the vials with a sterile pipet and inside of the vials were washed with elution buffer (12.5 ml) using a sterile pipet to flush out the remaining sample. The unheated virus suspensions from spinach were used as controls and enumerated.

Virus extraction

The method for virus extraction was performed as described in Baert et al., (2008) with some modifications. Inoculated and thermally treated spinach were washed with 12.5 ml of elution buffer (1:6 ratio) containing 0.1 M Tris-HCl, pH 9.5 (to elute the virus particles from the spinach sample in the presence of an alkaline environment), 3% beef extract powder (to reduce non-specific virus adsorption to the food matrix during extraction and facilitate the flocculation of norovirus surrogate particles on polyethylene glycol (PEG) molecules), and 0.05 M glycine (to reduce non-specific virus adsorption to the food

matrix during extraction). The pH was then adjusted to 9.5 using 10 M NaOH. Samples in the sterile beaker were then kept shaking on a shaking platform (120 rpm) for 20 min at 4°C. Samples were then transferred into a sterile stomacher bag with a filter compartment and stomached at high speed for 60 s. The filtrate obtained was centrifuged at 10,000 x g for 15 min at 4°C, and the pH of the supernatant was adjusted to 7.2 to 7.4 using 6 N HCl (to improve the PEG precipitation of the virus particles). Polyethylene glycol (PEG) 6000 (used for precipitation of viruses at high ionic concentrations without precipitation of other organic materials) and NaCl were added to obtain a final concentration of 10% PEG and 0.3 M NaCl. These samples were placed on a shaking platform (120 rpm) overnight at 4°C and then centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was discarded and the pellet dissolved in 1 ml PBS. Virus extracts were stored at -80°C until enumeration of plaques using MNV-1 and FCV-F9 plaque assays.

Enumeration of survivors by Infectious Plaque Assays

Thermally treated and control viral suspensions were diluted 1:10 in Dulbecco's Modified Eagle Medium (DMEM-F12) containing fetal bovine serum (10% for MNV-1 and 2% for FCV-F9) and 1% antibiotic-antimycotic. Infectivity of each treated virus was evaluated using standardized plaque assays in comparison to untreated virus controls following the previously described procedures by Su et al., (2010). Viral survivors were enumerated as plaque forming units/ml (PFU/ml).

Modeling of inactivation kinetics

First-order kinetics

The first-order kinetic model assumes a linear logarithmic reduction of the number of survivors over treatment time:

$$\log_{10} \frac{N(t)}{N_0} = -\frac{t}{D} \quad (1)$$

Where $N(t)$ is the number of survivors after an exposure time (t) in PFU/ml and the initial population is N_0 (PFU/ml). D is the decimal reduction time in min (time required to kill 90% of viruses) and t is the treatment time (min).

Weibull model

The Weibull probability density function (Eq.2) was used to describe the time for desired amount of inactivation,

$$f(t) = \frac{\beta}{\alpha} \left(\frac{t}{\alpha}\right)^{\beta-1} \times \exp\left(-\left(\frac{t}{\alpha}\right)^{\beta}\right) \quad (2)$$

where α and β are the scale and shape parameters, respectively. A value for $\beta < 1$ indicates that the remaining cells have the ability to adapt to the applied stress, whereas $\beta > 1$ indicates that the remaining cells become increasingly damaged (van Boekel, 2002). A change in scale parameter has the same effect on the distribution as a change of abscissa scale.

To investigate the effect of each parameter on Weibull distribution, cumulative distribution function was also considered (Eq.3)

$$F(t) = \exp\left(-\left(\frac{t}{\alpha}\right)^{\beta}\right) \quad (3)$$

The application of Weibull cumulative distribution function to survival inactivation kinetics assumes that the survival curve is a cumulative distribution of lethal effects

$$\frac{N(t)}{N_o} = \exp\left(-\left(\frac{t}{\alpha}\right)^\beta\right) \quad (4)$$

For Weibull model, the time required to destroy desired amount of logarithmic reduction could be evaluated by using shape and scale parameters as shown in Eq (5);

$$t_D = \alpha - \ln(10^{-D})^{1/\beta} \quad (5)$$

where D is the number of decimal reductions.

Uncertainty analysis. Uncertainty analysis is a parameter which is associated with the result of measurement and used to characterize the dispersion of the values that could reasonably be attributed to the measurement (Forster, 2003). Combined relative uncertainty in microbiological experiments could arise from enumeration steps for microorganisms including dilution factor, personal counting, total test portion volume, and, in the case of usage of empirical models, uncertainty that comes from model estimation . In the present study, relative uncertainty for dilution factor, personal counting, total test portion volume and model were considered separately and total uncertainty was calculated as a function of all the individual relative uncertainties using the method described by Niemela (2002).

The relative standard uncertainty of dilution factor may be described by:

$$w_f^2 = \frac{1}{(a+b)^2} \left[u_b^2 + \left(\frac{b}{a}\right)^2 u_a^2 \right] \quad (6)$$

where w_f is the relative standard uncertainty of dilution factor, a is the suspension transfer volume, b is the dilution blank volume, u_a and u_b are standard uncertainties for a and b respectively.

The relative standard uncertainty of personal counting is described as:

$$w_z^2 = \frac{2}{n} \sum_{i=1}^n \left(\frac{z_1 - z_2}{z_1 + z_2} \right)^2 \quad (7)$$

where w_z is the relative standard uncertainty of personal accounting, and z is number of colonies counted and n is the number of plates.

The relative standard uncertainty of presumptive calculation or the uncertainty of each presumptive w_x is defined in Eq. (8)

$$w_x^2 = \sum_{k=1}^n \frac{1}{\sigma_{x_k}^2} (x_k - \hat{x}_k)^2 \quad (8)$$

Where $\sigma_{x_k}^2$ is the variance, x_k is the estimated plaques count and $\hat{x}_k$ is the mean of the experimental plaques count.

The relative standard uncertainty of the total test portion volume is defined as:

$$w_V^2 = \frac{nv^2w_v^2}{V^2} \quad (9)$$

where w_v is the relative standard uncertainty of the total test portion volume, n is the number of plaques in plates, v is the volume of one portion, w_V the relative standard uncertainty of one volume measurement, and V is the sum of all portion.

The combined relative uncertainty is expressed in Eq. (10) as functions of relative standard uncertainty of dilution factor, personal counting, presumptive calculation and total test portion volume:

$$W_{Total}^2 = \sqrt{W_f^2 + W_z^2 + W_x^2 + W_V^2} \quad (10)$$

Data analysis and model evaluation

The statistical evaluation, linear and non-linear regression analyses were performed using SPSS Ver.11.0.1 Statistical package. The comparison test (ANOVA, Post Hoc test) was carried out to analyze the effects of time on survival ratio. The confidence level used to determine statistical significance was 95%.

Results and Discussion

The initial titers of viruses stocks were around 8.19 ± 0.97 log PFU/ml for FCV-F9 and 7.40 ± 1.12 log PFU/ml for MNV-1. After inoculation of spinach with virus stocks, recovered titers varied between 5.60 ± 0.19 to 7.18 ± 0.12 log PFU/ml for MNV-1 (Table 6.1) and 6.4 ± 0.07 to 7.32 ± 0.06 log PFU/ml for FCV-F9, respectively (Table 6.2). The percentage recovery observed for MNV-1 was 75-97% and for FCV-F9 it was 78-89%. These percentage recoveries were similar to that described by Baert et al., (2008) who attributed greater adsorption of the virus to the smooth surface of the spinach which could account for/allow for more efficient elution. In another study, Shieh et al., (2009) found that HAV was reduced by only 1 log over four weeks of storage in spinach. Thus, because foodborne viruses apparently have extended survival on leafy vegetables, they could be the source of foodborne illness outbreaks as produce is often eaten raw or lightly cooked (Baert et al., 2009).

In the heating studies with inoculated spinach, the difference between the control and 0 min treatment showed that the number of virus survivors decreased with increasing temperature during come up time for both surrogates (Tables 6.1 and 6.2). Also, MNV-1 (Table 6.1) and FCV-F9 (Table 6.2) survival decreased as temperature and time increased. Thus, temperature is the critical factor which determines the efficiency of

thermal treatment against norovirus surrogate inactivation. As can be seen in Table 1 and 2, 56°C seems to be a critical inactivation temperature for norovirus surrogates where the D-value decreases. The survival behavior of norovirus surrogates (D and t_D -values) below and above this temperature (56°C) were significantly different ($p < 0.05$). This behavior may be related to the capsid structure of the viruses and its stability. FCV-F9 and MNV-1 are both non enveloped, positive stranded RNA virus that are surrounded by protein shell (capsid) formed by units known as capsomers (Dimmock et al., 2001). Due to this protein shell, inactivation of virus is faster after reaching the temperature required for the denaturation of protein ($> 56^\circ\text{C}$). Bertrand et al., (2012) reviewed 76 viral studies to investigate the influence of temperature on enteric viruses in food and water and concluded that a faster virus inactivation rate occurred at the temperatures between 50°C and 60°C. A recent study by Bozkurt et al., (2013) concluded that 56°C was the critical temperature for the thermal inactivation of human norovirus surrogates (FCV-F9 and MNV-1), which is in agreement with the present study. Sow et al., (2011) proposed that the inactivation of viruses by heat was associated with structural changes in the capsid structure. Heat causes possible conformational changes to viral proteins, such as disruption of attachment to receptors (Sow et al., 2011). Croci et al., (1999) also concluded that thermal inactivation of virus occurs through coagulation and breakdown of the capsomers.

Choice of the most appropriate model is crucial to gather correct information about thermal inactivation kinetic behavior of norovirus surrogates. In the current literature, there are limited studies (Bozkurt et al., 2013; Seo et al., 2012; Tuladhar et al.,

2012) on application of different models to determine thermal inactivation kinetics of norovirus surrogates. Seo et al., (2012) suggested the use of the Weibull model to describe the effect of temperature (24-85°C), pH (2-7) and NaCl concentration (3.3 to 6.3%) on the inactivation kinetics of murine norovirus rather than first-order model. Tuladhar et al., (2012) also stated that the Weibull model provided the best fit to describe thermal stability of most of structurally variable viruses. Bozkurt et al., (2013) concluded that the use of the Weibull model gave better fit compared to the first order model to describe thermal inactivation behavior of MNV-1 and FCV-F9.

The shape and scale factors are parameters obtained from the Weibull model (Table 6.3). The shape factor intervals for the studied temperature (50-72°C) were 0.51 ± 0.02 - 1.42 ± 0.04 for MNV-1 and 0.53 ± 0.05 - 2.09 ± 0.37 for FCV-F9. The results revealed that the shape factor values were significantly influenced by virus strain and temperature (Table 6.3); however, there was no correlation and/or trend with treatment temperature. The findings of the present study are consistent with Bozkurt et al., (2013) who concluded both FCV-F9 and MNV-1 had monotonic upward concave (tailing) curve behavior ($\beta < 1$) and monotonic downward concave (shoulder) behavior ($\beta > 1$) depending on the temperature.

Parameters of the Weibull model (β and α) were used to calculate t_D value which was used as an analog to the D-value of the first order model (Table 6.3). The D-values calculated from first-order model (50-72°C) were in the range of 14.57 ± 2.89 to 0.16 ± 0.11 min and 17.39 ± 2.24 to 0.15 ± 0.10 min for MNV-1 and FCV-F9, respectively. As an analogy, for the Weibull the calculated time to destroy 1 log ($D=1$) for MNV-1 and FCV-

F9 was in the range of 15.26 ± 3.27 to 0.22 ± 0.12 min and 20.71 ± 3.26 to 0.27 ± 0.14 min, respectively, for the temperature range 50-72°C (Table 3). Although temperature had a significant effect on t_D and D-values for the range from 50 to 60°C ($p < 0.05$), there was no change in the values at 65 and 72°C ($p > 0.05$).

Bozkurt et al., (2013) reported t_D values and D-values for murine norovirus (MNV-1) and feline calicivirus (FCV-F9) in buffer solution (Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic-antimycotic) for the temperature range between 50-72°C. Since there is an inconsistency in the current literature about thermal inactivation behavior data for norovirus surrogates, this study was chosen as a basis for comparison because the same temperature intervals were used.

For MNV-1, although their reported D-values were lower than those obtained in this study at 60°C, statistical differences were not observed between both studies for temperatures at 56, 65, and 72°C ($p > 0.05$). In terms of calculated t_D -value, there were no statistical differences observed at 56, 60, 65, and 72°C for MNV-1 ($p > 0.05$). For MNV-1, at 50°C, both the calculated t_D -value and D-values were significantly higher than the t_D -value and D-value of this study ($p < 0.05$).

For FCV-F9, the t_D -values and D-values were lower than those obtained in this study at 60°C, where statistically significant differences were observed ($p < 0.05$). At 50, 56, and 72°C the D-values were not significant different ($p > 0.05$), whereas for t_D -values, the differences were statistically significant ($p < 0.05$) between these studies for FCV-F9.

The differences in results may be explained by the compositional differences of buffer solution and spinach, because the environment in which viruses are found

influences their sensitivity to thermal inactivation. Bertrand et al., (2012) concluded that the presence of a complex matrix will lead to faster protein denaturation for virus inactivation. According to the product description, the composition of frozen chopped spinach included sodium (0.1%), carbohydrates (3.7%), protein (2.5%), and moisture (93.6%) thus providing a complex matrix compared to the buffer solution. This then might explain the more rapid inactivation of norovirus surrogates in spinach than in buffer solution. Also, the buffer solution contained 10% fetal bovine serum, with the protein content being higher than spinach. The presence of protein in the environment may protect the virus from the action of heat (Croci et al., 1999). In general, for the lowest treatment temperature (50°C) which was below the critical point (56°C), the high protein content of the buffer solution resulted in greater resistance to the thermal treatment than spinach for both norovirus surrogates. Since the greatest denaturation was expected at higher temperatures (65 and 72°C), there were no differences observed between results for buffer and spinach for either norovirus surrogates. For temperatures in between the extremes (56, and 60°C), the resistance of norovirus surrogates varied with virus strain and the interaction was not clear. Bertrand et al., (2012) stated that viral inactivation was dependent on the interaction between temperature and matrix type. Although, the presence of a complex matrix leads to faster protein denaturation, the influence of temperature might affect inactivation. Another potential factor for differences between the studies was that capillary tubes were used for heating the buffer while 2 ml vials were used for spinach. Differences in container size can potentially lead to differences in come-up time and thus differences in D-value (Chung et al., 2007). To

understand thermal inactivation of viruses in food, temperature and matrix interaction should be considered together (Bertrand et al., 2012).

In a study by Baert et al., (2008), the effect of blanching on the survival of murine norovirus during the production process of spinach was investigated. Virus was inoculated into spinach (50 g) with 300 ml of potable water (80°C) and held for 1 min. The water was then removed and the spinach was kept in ice water (4°C) for 1 min. The conclusion was that the D-value for this process was around 0.40 min. These researchers did not consider come up time and they did not mention the final temperature of spinach leaves. In contrast, in the present study, the temperature of the spinach in the vial was monitored using a thermocouple.

The z-value curves of both norovirus surrogates (MNV-1 and FCV-F9) for the first order model and Weibull model are given in Figs.6.1 a-b and 6.2 a-b. The calculated z-values for MNV-1 were $11.66 \pm 0.42^{\circ}\text{C}$ ($R^2=0.97$) and $10.98 \pm 0.58^{\circ}\text{C}$ ($R^2=0.96$) for the Weibull and first-order models, respectively, and with no statistical difference ($p>0.05$). For FCV-F9, there also was no statistical difference ($p>0.05$) between z-values using the Weibull ($10.85 \pm 0.67^{\circ}\text{C}$ ($R^2=0.93$)) or first-order model ($9.89 \pm 0.79^{\circ}\text{C}$ ($R^2=0.90$)). In a previous study, Bozkurt et al., (2013) determined the z-values for MNV-1 in buffer as 9.19 (Weibull) and 9.31°C (first-order) and for FCV-F9, 9.31°C (Weibull) and 9.36°C (first-order). In agreement with the present results, there was no significant difference ($p>0.05$) between the models for either norovirus surrogate.

The estimation of total uncertainties for this or any study gives precise information about the experimental procedures. In the present study, relative uncertainty

for dilution factor, personal counting, total test portion volume and presumptive calculation were considered separately and total uncertainty was calculated as a function of all these individual relative uncertainties (Fig. 6.3a-6.3b). The relative uncertainty for dilution factor, personal counting and test portion volume were 0.005%, 0.0004% and ca. 0.84%, respectively. Results revealed that the greatest contribution to total estimated uncertainty was from the model selected. Total uncertainties of FCV-F9 for the first order model were 15.93%, 14.40%, 21.01%, 11.99%, and 18.54%, and for the Weibull model were 4.23%, 3.53%, 7.56%, 6.52%, and 6.94% for 50, 56, 60, 65, and 72°C, respectively. For MNV-1, the estimated total uncertainty for the Weibull model were 3.10%, 3.62%, 5.77%, 3.70%, and 7.01%, and for the first order model were 14.04%, 13.14%, 16.38%, 15.44%, and 16.94% for 50, 56, 60, 65, and 72 °C, respectively. The results showed that the selection of the right model and the consideration of total uncertainties are crucial to describe the thermal inactivation behavior of norovirus surrogates. It also could be stated that the appropriateness of Weibull model was confirmed using total estimated uncertainty analysis.

Proper inactivation of human noroviruses in spinach before freezing is desirable to improve microbiological safety. No time and temperature recommendations were found in the literature for inactivation of noroviruses in spinach. According to Singh (2005), industrial blanching conditions for spinach include use of steam as a heating medium for 120-180 s. Using the information generated in the present study and the thermal parameters of Singh (2005) as a basis, the blanching of spinach in water at 100°C for 120-180 s under atmospheric conditions will provide greater than 7 log reduction of

both norovirus surrogates using either model. It is important to note that use of steam as a heating medium and immersion in water at 100°C have different heating characteristics and validation of the recommendation using steam must be carried out before actual application of the process. In conclusion, novel and precise information on thermal inactivation of norovirus surrogates in spinach was generated enabling more reliable thermal process calculations to control and/or inactivate the virus. Consideration of uncertainty measurements, which allow quantitative indication of analytic variability for any result, enhanced the validity of represented data.

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Appendix

Table 6.1. Effect of thermal treatment on murine norovirus (MNV-1) inactivation in spinach.

Temperature (°C)									
50°C		56°C		60°C		65°C		72°C	
Treatment	Recovered titer log (PFU/ml)	Treatment	Recovered titer log (PFU/ml)	Treatment	Recovered titer log (PFU/ml)	Treatment	Recovered titer log (PFU/ml)	Treatment	Recovered titer log (PFU/ml)
Control	6.18±0.12	Control	6.18±0.12	Control	5.76±0.14	Control	5.60±0.19	Control	5.77±0.14
0 min	6.05±0.09	0 min	5.36±0.09	0 min	5.17±0.09	0 min	3.59±0.19	0 min	4.08±0.06
2 min	5.99±0.10	1 min	4.54±0.07	1 min	3.49±0.26	20 s	2.53±0.20	20 s	2.56±0.06
4 min	5.69±0.10	2 min	4.40±0.08	2 min	2.40±0.25	40 s	1.94±0.18	40 s	ND
6 min	5.71±0.13	3 min	4.36±0.06	3 min	2.15±0.16	60 s	ND	60 s	ND

Each treatment was replicated three times, and plaque assays for evaluating the inactivation of the viruses were carried out in duplicate.
 ND = Not detected (limit of detection was 1 log(PFU/ml)).

Table 6.2. Effect of thermal treatment on feline calicivirus (FCV-F9) inactivation in spinach.

Temperature (°C)									
50°C		56°C		60°C		65°C		72°C	
Treatment	Recovered titer log (PFU/ml)	Treatment	Recovered titer log (PFU/ml)	Treatment	Recovered titer log (PFU/ml)	Treatment	Recovered titer log (PFU/ml)	Treatment	Recovered titer log (PFU/ml)
Control	7.15±0.02	Control	7.06±0.04	Control	7.32±0.06	Control	6.94±0.17	Control	6.40±0.07
0 min	6.84±0.09	0 min	6.13±0.10	0 min	6.43±0.07	0 min	3.28±0.15	0 min	4.55±0.06
2 min	6.63±0.23	1 min	5.53±0.44	1 min	6.14±0.02	20 s	2.79±0.22	20 s	3.23±0.10
4 min	6.59±0.04	2 min	5.31±0.27	2 min	4.87±0.02	40 s	1.18±0.21	40 s	ND
6 min	6.47±0.02	3 min	5.06±0.18	3 min	2.68±0.41	60 s	ND	60 s	ND

Each treatment was replicated three times, and plaque assays for evaluating the inactivation of the viruses were carried out in duplicate.
 ND = Not detected (limit of detection was 1 log (PFU/ml)).

Table 6.3. Coefficients of the first-order and Weibull models for the survival curves of murine norovirus (MNV-1) and feline calicivirus (FCV-F9) during thermal inactivation.

Virus strain	T (°C)	Weibull distribution			First-order kinetics		
		β	α (min)	t_D (min)	R^2	D (min)	R^2
MNV-1	50	1.12±0.04 ^A	7.42±2.16 ^A	15.26±3.27 ^A	0.999	14.57±2.89 ^A	0.878
	56	1.50±0.05 ^B	2.66±0.87 ^B	4.09±0.65 ^B	0.998	3.29±0.96 ^B	0.813
	60	0.51±0.02 ^C	0.24±0.08 ^C	1.11±0.26 ^C	0.993	0.98±0.24 ^C	0.908
	65	0.64±0.03 ^D	0.13±0.09 ^C	0.47±0.29 ^D	0.999	0.40±0.22 ^D	0.974
	72	1.42±0.04 ^B	0.14±0.08 ^C	0.22±0.12 ^D	0.999	0.16±0.11 ^D	0.977
FCV-F9	50	0.58±0.17 ^A	5.17±1.42 ^A	20.71±3.26 ^A	0.999	17.39±2.24 ^A	0.921
	56	0.53±0.05 ^A	1.42±0.25 ^B	6.17±0.97 ^B	0.985	5.83±1.12 ^B	0.933
	60	1.37±0.26 ^B	0.54±0.13 ^C	0.91±0.36 ^C	0.999	0.78±0.35 ^C	0.890
	65	2.09±0.37 ^C	0.31±0.08 ^D	0.35±0.12 ^D	0.987	0.27±0.12 ^D	0.914
	72	1.19±0.08 ^B	0.14±0.09 ^E	0.27±0.14 ^D	0.999	0.15±0.10 ^D	0.977

* Different letters indicate a significant difference among parameters (β , α , t_D , D) and within each virus ($p<0.05$).

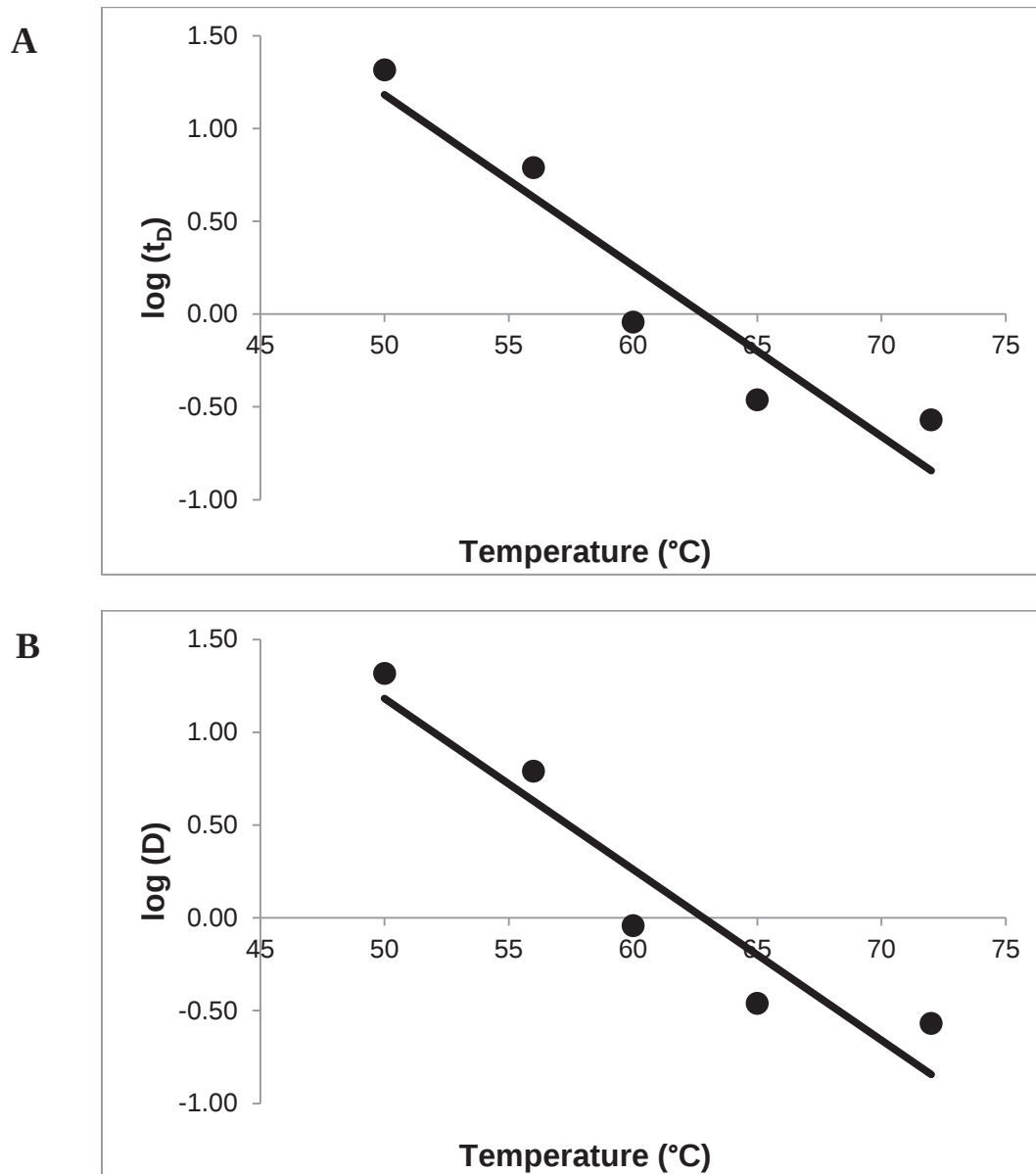


Figure 6.1. Thermal inactivation curves of murine norovirus (MNV-1) for the [A] Weibull model ($R^2=0.97$) and [B] first-order model ($R^2=0.96$).

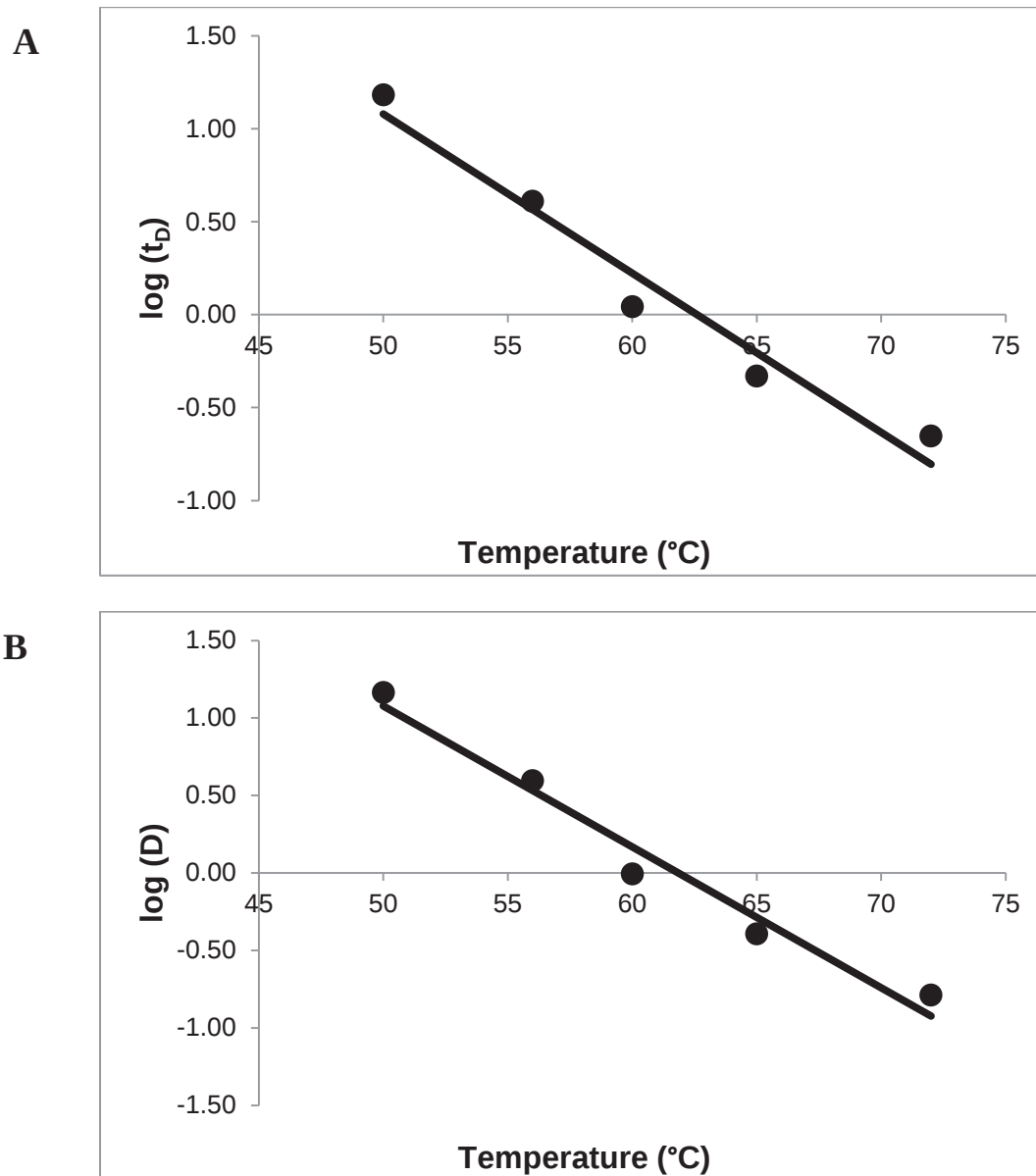


Figure 6.2. Thermal inactivation curves of feline calicivirus (FCV-F9) for the [A] Weibull model ($R^2=0.93$) and [B] first-order model ($R^2=0.90$).

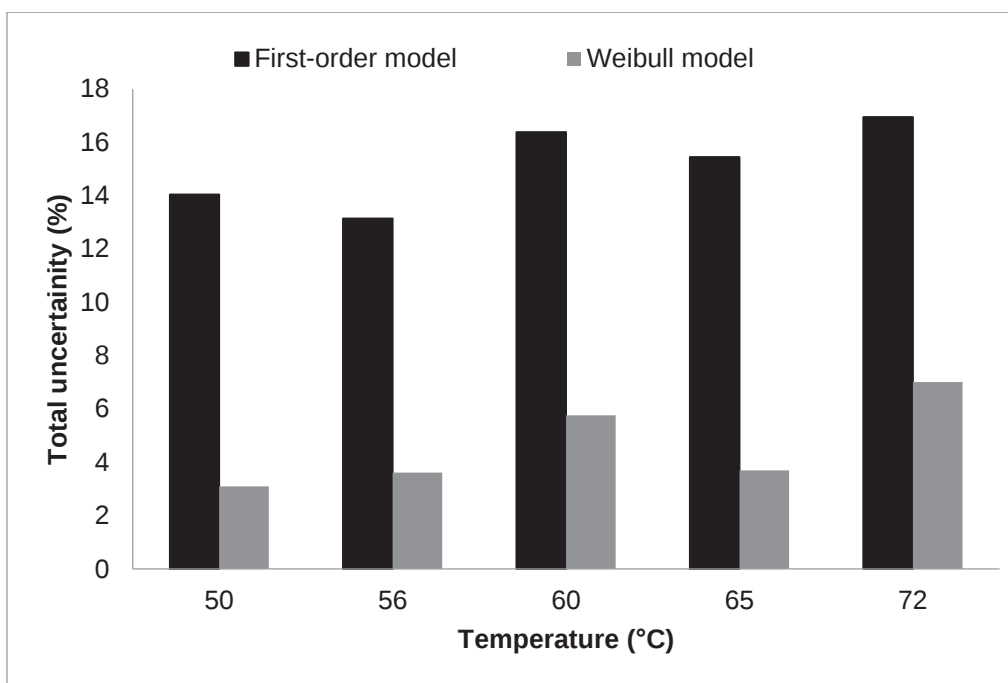
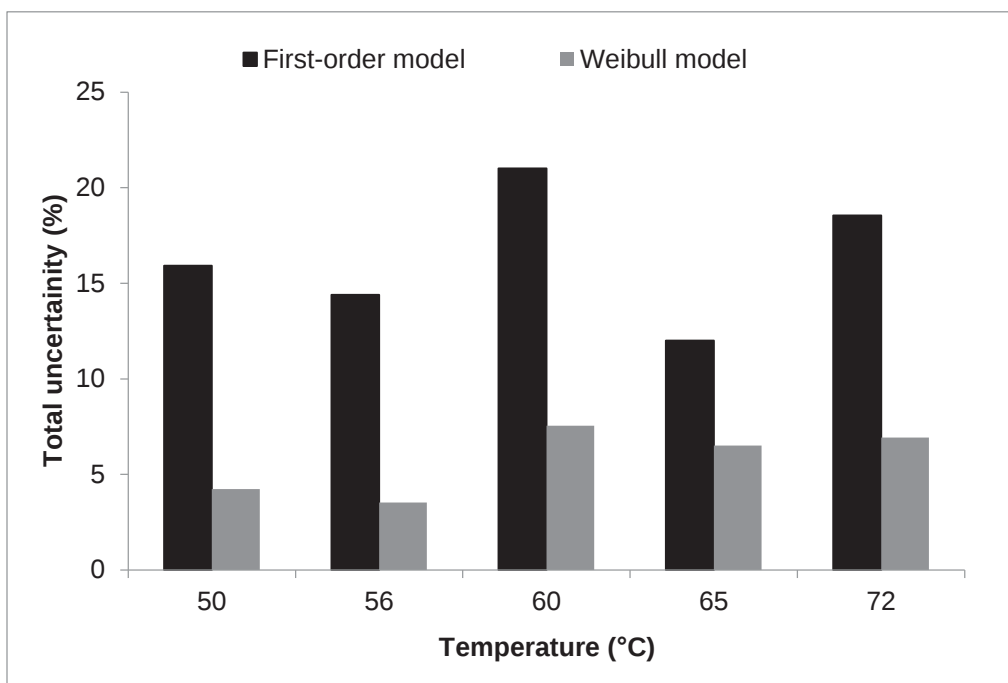
A**B**

Figure 6.3. Total combined uncertainty values for [A] murine norovirus (MNV-1) and [B] feline calicivirus (FCV-F9).

CHAPTER VII
THERMAL INACTIVATION KINETICS OF HEPATITIS A VIRUS
IN SPINACH

Abstract

Leafy vegetables have been recognized as important vehicles for the transmission of foodborne viral pathogens. To control hepatitis A viral foodborne illness outbreaks associated with mildly heated (e.g., blanched) leafy vegetables such as spinach, generation of adequate thermal processes is important both for consumers and the food industry. Therefore, the objectives of this study were to determine the thermal inactivation behavior of hepatitis A virus in spinach, compare first-order and Weibull models to describe the data, calculate Arrhenius activation energy for each model, and evaluate model efficiency using selected statistical criteria. The D-values calculated from the first-order model (50-72°C) ranged from 34.40 ± 4.08 to 0.91 ± 0.12 min for HAV. Using the Weibull model, the $t_{D=1}$ for HAV (time to destroy 1 log) at the same temperature range was 37.08 ± 3.37 to 0.93 ± 0.09 min. The z-values determined for HAV were $15.07 \pm 1.63^\circ\text{C}$ and $13.92 \pm 0.87^\circ\text{C}$ for the Weibull model and the first-order model, respectively. The calculated activation energies for the first-order model and the Weibull model were 162 and 151 kJ/mole, respectively. Using the information generated in the present study and the thermal parameters of industrial blanching conditions for spinach as a basis (100°C for 120-180 s), the blanching of spinach in water at 100°C for 120-180 s under atmospheric conditions will provide greater than 6 log reduction of HAV using either model. The results of this study may be useful to the frozen food industry in designing blanching conditions for spinach to inactivate or control hepatitis A virus.

Key words; spinach, thermal inactivation, Weibull and first-order model, D and z value, Arrhenius activation energy.

Introduction

Hepatitis A virus (HAV) is the leading cause of acute viral hepatitis which may occasionally be fatal. Thus, it constitutes a serious concern for public health authorities (Coudray-Meunier et al., 2013). Even though the effectiveness of HAV vaccine is high and overall HAV cases have declined, outbreaks still continue to occur (Kingsley and Chen, 2009). HAV is able to replicate in the human gastro-intestinal tract and is dispersed by shedding in high concentrations into the stool. The stability of HAV with regard to several physical stresses, such as low pH and elevated temperatures, contributes significantly to its persistence in the environment. Transmission of these viruses occurs by the fecal-oral route, primarily through direct person-to-person contact, but they are also efficiently transmitted by ingestion of contaminated drinking water or contaminated food (D'Souza et al., 2007).

The foods most likely to be contaminated by HAV are leafy vegetables, fruits, shellfish and ready-to-eat foods (i.e., those with no lethality step prior to consumption) (CDC, 2014a). Gould et al. (2013) investigated the surveillance of foodborne disease outbreaks in the United States from 1998 to 2008, and found that among individual food categories, leafy vegetables were the second most commonly reported food vehicle associated with foodborne illness, accounting for 13% of outbreaks. Leafy vegetables are often consumed raw or mildly heated (e.g., blanched) and thus may become vehicles for viral transmission if contamination occurs anywhere from farm to fork (Brassard et al., 2011). Therefore, for mildly heated leafy vegetables, such as those blanched prior to freezing, the application of a precise thermal process to inactivate HAV would improve the microbiological safety of the products. Recent foodborne outbreaks of HAV in

frozen berries and pomegranate kernels also underline the need to investigate proper means to inactivate this virus in food products that will be frozen (CDC, 2014b).

In the current literature, several studies have investigated the survival of HAV in leafy vegetables such as lettuce (Bidawid et al., 2000, 2001; Croci et al., 2002; Fino and Kniel, 2008; Fraisse et al., 2011), green onions (Fino and Kniel, 2008; Laird et al., 2011; Sun et al., 2012), and parsley (Butot et al., 2008). Yet, there are limited studies (Hida et al., 2013; Jones et al., 2009; Shieh et al., 2009) involving the survival of HAV in spinach. Among these studies, only Shieh et al. (2009) investigated the survival of HAV in spinach during storage. They examined the survival behavior of HAV at refrigeration temperature ($5.4 \pm 1.2^{\circ}\text{C}$) for up to 42 days. To our knowledge, there have been no thermal inactivation kinetics established for inactivation of HAV in spinach.

The first step in designing any thermal process is defining the thermal resistance of the target pathogen (Solomon et al., 2002). The choice of the most appropriate model is crucial to gather correct information about the thermal inactivation kinetic behavior of HAV. Although several studies have been performed to investigate the efficiency of the Weibull model to describe the inactivation behavior of human norovirus surrogates (Bozkurt et al., 2013, 2014a, 2014b; Seo et al., 2012; Tuladhar et al., 2012), there are limited studies (Bozkurt et al., 2014c) involving the Weibull model to describe the inactivation behavior of HAV. Bozkurt et al. (2014c) showed that the Weibull model was statistically superior in describing the thermal inactivation kinetics of HAV than the first-order model for blue mussel homogenate. A precise understanding of thermal inactivation kinetics is potentially useful for optimizing thermal treatments to eliminate the risk

associated with foodborne pathogens while avoiding over-processing of the food material and thus resulting in optimal energy utilization. The objectives of this study were to (i) determine thermal inactivation behavior of hepatitis A virus in spinach, (ii) compare first-order and Weibull models to describe the data, (iii) calculate Arrhenius activation energy for each model, and (iv) evaluate model efficiency by using selected statistical criteria.

Materials and Methods

Viruses and cell lines

Hepatitis A virus (HAV: strain HM175) and fetal monkey kidney (FRhK4) cells were kindly provided by Dr. Kalmia Kniel (University of Delaware). FRhK4 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM-F12: HyClone Laboratories, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1×Anti-Anti (Antibiotic-Antimycotic; Invitrogen, Grand Island, NY) at 37°C in an atmosphere containing 5% CO₂.

Propagation of viruses

FRhK4 cells with ~90% confluence in cell culture flasks were washed with phosphate-buffered saline (PBS; pH 7.4) twice before adding HAV stocks to these cell monolayers. The infected cells were then incubated until >90% cell lysis in a water jacketed CO₂ incubator at 37°C. All viruses were recovered by centrifugation at 5,000 × g for 10 min, followed by filtration through 0.2-µm filters, aliquoted, and stored at -80°C until use.

Inoculation of spinach

Frozen chopped spinach samples were purchased from a local grocery store. The chopped spinach samples were blended using a Waring blender (Model 1063, Waring Commercial, USA) to homogenize the sample. Five-ml of HAV stock with initial titers of 7.34 ± 1.28 log PFU/ml was added to 25 g of spinach sample in a sterile beaker and held at 4°C for 24 h.

Thermal treatment

Homogenized inoculated spinach (6 ml) was added to moisture barrier plastic vacuum bags (13 cm x 19 cm) using a sterile pipet in a biosafety cabinet. The inoculated samples were vacuum sealed in to -100 kPa with a Multivac A300/16 vacuum-packaging unit (Sepp Haggemuller KG, Wolfertschwenden, Germany) and the bags were flattened. To monitor the internal temperature of the spinach, a thermocouple was placed at the geometric center of an uninoculated control package of spinach. The sealed bags were placed into a holding unit, rinsed with 70% ethanol, and then the holding unit with the bags was immersed in a thermostatically controlled ($\pm 0.1^\circ\text{C}$) circulating water bath (Haake model V26, Karlsruhe, Germany). Water bath temperature was confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA) and by placing type-T thermocouples (Omega Engineering, Inc., Stamford, CT) in the geometric center of the water bath. The thermocouples were connected to a MMS3000-T6V4 type portable data recorder (Commtest Ins., New Zealand) to monitor temperature. Samples were heated at 50, 56, 60, 65, and 72°C for different treatment times (0-6 min). The treatment time began when the internal temperature reached the designated target temperature. Triplicate bags were used for each time-point. After the thermal treatment, sample bags

were immediately cooled in an ice water bath for 15 min to stop further thermal inactivation. The bags were washed again with ethanol before removal of the contents. Bags were placed in a biosafety cabinet and aseptically cut with sterilized (121°C for 15 min) scissors. Bag contents were removed with a sterile pipet and inside of the bags were washed with elution buffer (15 ml) using a sterile pipet to remove the remaining sample. The un-heated virus suspensions from spinach were used as controls and enumerated (Table 7.1).

Virus extraction

The method for virus extraction was performed as described in Baert et al. (2008) with some modifications. Inoculated and thermally treated spinach was washed with 15 ml of elution buffer (2:5 ratio) containing 0.1 M Tris-HCl, pH 9.5, 3% beef extract powder, and 0.05 M glycine. The pH was then adjusted to 9.5 using 10 M NaOH. Samples in the sterile beaker were then kept shaking on a shaking platform (120 rpm) for 20 min at 4°C. Samples were then transferred into a sterile stomacher bag with a filter compartment and stomached at high speed for 60 s. The filtrate obtained was centrifuged at 10,000 x g for 15 min at 4°C, and the pH of the supernatant was adjusted to 7.2 to 7.4 using 6 N HCl. Polyethylene glycol (PEG) 6000 and NaCl were added to obtain a final concentration of 10% PEG and 0.3 M NaCl. These samples were placed on a shaking platform (120 rpm) overnight at 4°C and then centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was discarded and the pellet dissolved in 1 ml PBS. Extracts containing the virus were stored at -80°C until the HAV plaque assay.

Enumeration of survivors by plaques assays

Thermally treated and control viral suspensions were diluted 1:10 in Dulbecco's Modified Eagle Medium (DMEM-F12) containing fetal bovine serum (2%) and 1% antibiotic-antimycotic. Infectivity of each treated virus was evaluated using standardized plaque assays in comparison to untreated virus controls following the previously described procedures (Su et al., 2010). Viral survivors were enumerated as plaque forming units/ml (PFU/ml).

Modeling of inactivation kinetics

First-order kinetics

The traditional approach to describe the change in number of survivors over time for first-order kinetic model can be written as follows:

$$\ln N_{(t)} = \ln N_0 - kt \quad (1)$$

where $N_{(t)}$ is the number of survivors after an exposure time (t) in PFU/ml and the initial population is N_0 (PFU/ml), and k as the first-order rate constant (1/s). This equation is then rearranged into:

$$\log_{10} \frac{N(t)}{N_0} = -\frac{t}{D} \quad (2)$$

where D is the decimal reduction time ($D=2.303/k$, units in min or s) and is thus actually a reciprocal first-order rate constant. The semi-logarithmic curve resulting when $\log N(t)/N_0$ is plotted vs. time is frequently referred to as the survival curve.

Weibull model

Experimental data were fitted in decimal logarithmic form as follows:

$$\frac{N(t)}{N_0} = \exp \left(- \left(\frac{t}{\alpha} \right)^\beta \right) \quad (3)$$

where α (min^{-1}) and β (-) are the scale, and shape parameters, respectively.

As indicated by previous studies, the inverse of the of the scale factor (α) as a reaction rate constant k' (min^{-1}), the equation becomes (Fernandez et al., 2002):

$$\frac{N(t)}{N_0} = \exp(-(k't)^\beta) \quad (4)$$

For the Weibull model, the time required to reduce the number of microorganisms by a factor 10 (analogous to the D-value) can be calculated by using the shape and scale parameters as shown in Eq (5);

$$t_D = \alpha - \ln(10^{-D})^{1/\beta} \quad (5)$$

where D represents decades (*or log*) reduction of a microbial population. t_D has the stated meaning only when it refers to the treatment time starting at zero.

Arrhenius activation energy

The inactivation rate is primarily influenced by temperature, and the temperature dependence of the rate constant is typically described by the Arrhenius equation:

$$k = A \exp\left(-\frac{E_a}{RT}\right) \quad (6)$$

where A is a frequency factor which is constant, E_a is the activation energy (J/mole), R is the universal gas constant ($8.314 \text{ Jmole}^{-1}\text{K}^{-1}$), k is the rate constants (1/min), T is the absolute temperatures (K). The construction of $\ln k_{(T)}$ versus $1/T$, the slope of the curve will be a straight line which equals to activation energy. This concept has been used to calculate activation energy of microbial inactivation (Corradini and Peleg, 2005).

Statistical Analysis

Statistical and non-linear regression analyses were performed using the SPSS Ver.11.0.1 statistical package. The statistical criteria applied to discriminate

(differentiate) between the kinetic models were R^2 (coefficient of determination), and standard errors (std. error) for each coefficient. The confidence level used to determine statistical significance was 95%.

Results and Discussion

The recovered titer of unheated inoculated spinach (control) ranged from 6.20 ± 0.79 to 5.08 ± 0.61 log PFU/ml. The difference between the control and 0 min treatment showed the log reduction during come up time (time to reach target temperature) (Table 7.1). The come up times for each temperature were 28, 31, 35, 40, 44 s for 50, 56, 60, 65, and 72°C, respectively, thus the flattened plastic vacuum bags allowed for the desired temperature to be reached in less than one minute. Bozkurt et al. (2014b) investigated thermal inactivation of human norovirus surrogates (feline calicivirus and murine norovirus) in spinach using 2 ml vials and reported the come up time as 100 s at 72°C. Since, heat transfer rate increases with increased heat transfer area, use of the vacuum sealed bags decreased the come up time in comparison to the 2 ml vials. Log reductions during come up times (50-72°C) were in the range of 0.47 to 0.89 log PFU/ml. Hence, the reduction in number of survivors during come up time was important and should be a consideration to achieve the desired amount of reduction and to design appropriate thermal processes.

The effect of thermal treatment on HAV inactivation in spinach is shown in Table 7.1. At 50°C, heating for 6 min resulted in a less than 1 log reduction (PFU/ml) of HAV. At the highest temperatures used (65 and 72°C) inactivation rate was increased. At lower temperatures (<56°C), it has been suggested that damage occurs to the viral receptor

through structural changes in the capsid protein that interferes with binding and causes low level inactivation, while at higher temperatures, alteration of the tertiary structure of the capsid proteins occur leading to eventual release and degradation of nucleic material (Ausar et al., 2006; Croci et al., 2012; Wigginton et al., 2012). This might be the reason for the observation of a lower reduction rate at mild temperatures ($<56^{\circ}\text{C}$) compared with a greater reduction rate at higher temperatures ($>65^{\circ}\text{C}$) as observed for the thermal inactivation of HAV in spinach. This hypothesis was also supported by other researchers (Bertrand et al., 2012; Bozkurt et al., 2014a, 2014b, 2014c; Croci, et al., 1999; Pollard 1960; Song et al., 2011; Sow et al., 2011; Volking et al., 1997).

The thermal inactivation kinetics of HAV in spinach at $50\text{-}72^{\circ}\text{C}$ as determined using first-order and Weibull models are shown in Table 7.2. The D-values calculated from the first-order model ($50\text{-}72^{\circ}\text{C}$) were in the range of 34.40 ± 4.08 to 0.91 ± 0.12 min. Parameters of the Weibull model (scale factor = β and shape factor = α) were used to calculate t_D value which was defined as the time to destroy 1 log of HAV and was used as an analog to the D-value of the first order model. For the Weibull model, the calculated time to destroy 1 log ($D=1$) for HAV ranged from 37.08 ± 3.37 to 0.93 ± 0.09 min for the temperature range 50 to 72°C .

The Weibull shape factor (β) ranges for the temperature studied ($50\text{-}72^{\circ}\text{C}$) were 1.49 ± 0.31 to 0.96 ± 0.07 (Table 2). At 50°C , the inactivation curve was a monotonic downward concave (shoulder) and had a shape factor of 0.96 ± 0.07 . At temperatures higher than 50°C , monotonic upward concave (tailing) behavior was observed with shape factors ranging from 1.25 ± 0.46 to 1.49 ± 0.31 (Table 7.2). A shape factor greater than one

indicates that the remaining population becomes increasingly damaged, whereas a shape factor less than one indicates that the remaining population has the ability to adapt to applied stress (van Boekel, 2002). In another study, Cunha et al. (1998) indicated that the shape factor was a behavior index describing the kinetic patterns of the mechanism controlling the process studied and therefore should be independent of external factors. Similarly, Couvert et al. (2006) also concluded that the shape factor of any microbial population should be independent of heating conditions. Consistent with those studies (Couvert et al., 2006; Cunha et al., 1998), the results of this study revealed that the heating temperature apparently did not influence the shape parameter and could not be described by any model.

The scale factor (α) ranges for the temperatures studied (50-72°C) were 14.89 ± 1.13 min for HAV. Couvert et al., (2005) investigated the effect of environmental factors on Weibull parameters and they concluded the scale parameter depends on the heating temperature and the change in scale factor describes the effect of heating environment on the inactivation. Like the classical D-value, scale factor decreases with increasing temperature, and a second order polynomial model was established to quantify influence of temperature on scale factor. The relationship between scale factors and temperature for HAV was;

$$\alpha = 0.054T(^{\circ}C)^2 - 7.19T(^{\circ}C) + 238 \quad R^2 = 0.95 \quad (7)$$

To understand the impacts of temperature on HAV inactivation rate, the Arrhenius correlation between inactivation rate constants and temperatures over the range 50-72°C was developed. Therefore, temperature dependency of the inactivation rate constant (k

and k') were fitted by the exponential Arrhenius function for both models (Table 7.3).

The estimated inactivation rate constants for the temperatures studied (50-72°C) were in the range of 1.11 ± 0.18 to $0.03 \pm 0.01 \text{ min}^{-1}$ for the first-order model and 1.92 ± 0.29 to 0.07 ± 0.01 for the Weibull model. According to data in Table 7.3, the inactivation was relatively high at 72°C, with inactivation rate constants 37 and 27 times greater than that of 50°C experiments, for the first-order and Weibull model, respectively. Results for the present study revealed that temperature had a significant effect on estimated inactivation rate constants for both models. The relationship between the inactivation rate constant and temperature for first-order model and Weibull model respectively were;

$$k = 0.0496T(^{\circ}C) - 2.6011 \quad R^2 = 0.90 \quad (8)$$

$$k' = 0.0903T(^{\circ}C) - 4.6453 \quad R^2 = 0.94 \quad (9)$$

The activation energy is the minimum amount energy that required to initiate an inactivation event to occur and cause denaturation of target organism (Klotz et al., 2007). Based on inactivation rate constants, the calculated activation energies for the first-order model and the Weibull model are shown in Figure 7.1. The activation energy obtained from the first order model was 162 kJ/mole while for the Weibull model, the activation energy was 151 kJ/mole. Bozkurt et al. (2014c) reported that the activation energy for HAV in blue mussel homogenate was 165 and 153 kJ/mole for the first-order model and the Weibull model, respectively. In agreement with the present results for HAV, the reported activation energies for both studies were not statistically different for either model ($p > 0.05$). Since, the magnitude of activation energy indicates the amount of energy that is required for an inactivation event to occur, application of the first-order would

result in over-processing. This over-processing phenomena could explain the impressive safety record of the first-order model for many years, especially in canning industry where over-processing for *Clostridium botulinum* is widely practiced (Corradini and Peleg, 2005).

In addition to activation energies for both models, the z-values were also calculated. The z-values for HAV were $15.07 \pm 1.63^{\circ}\text{C}$ using the Weibull model and $13.92 \pm 0.87^{\circ}\text{C}$ for the first-order model (Fig. 7.2). Bozkurt et al. (2014c) determined the z-values for HAV in blue mussel homogenate (*Mytilus edulis*) as $15.88 \pm 3.97^{\circ}\text{C}$ for Weibull model and $12.97 \pm 0.59^{\circ}\text{C}$ for the first-order model. Unlike D values, the z-value is not a function of the heating environment, it represents the temperature required for the thermal destruction curve to change by one log cycle. Therefore, it should be same for the same microorganism under different environments. The results of present study were consistent with those of the study by Bozkurt et al. (2014c) who reported statistically similar z-values for HAV ($p > 0.05$).

The detailed thermal data obtained from this study will be useful to eliminate or decrease the risk associated with the consumption of spinach contaminated with HAV. Koopmans and Duizer (2004) classified the risks of infection for the consumer if viruses are present before processing. The risks were classified into four grades as negligible risk, low risk, medium risk and high risk. According to this classification, any treatment that would results in at least 4 log reduction of viruses could have negligible risk of infection. Thus, achieving a 6 log reduction (often used as a target for processes such as pasteurization) could be considered as a safe food process. Calculation of the time needed

for six log reduction for first-order model is 6 times the D-value ($6D$), however the time needed for six log reduction for the Weibull model is not $6t_{D=1}$ but $t_{D=6}$, which is a consequence of nonlinear behavior. Even though there was a difference between required process time calculations for each model the results were not significant at any temperature (50-72°C) ($p>0.05$). Based on the thermal inactivation data obtained from the present study, the treatment time required to achieve 6 log reductions were 90, 19, and 4 s at 80, 90, and 100°C, respectively, for both models.

The industrial blanching process time for spinach could be estimated by using the thermal data obtained from this study. According to Singh (2005), industrial blanching conditions for spinach include use of steam as a heating medium for 120-180 s. Using the information generated in the present study and the thermal parameters of Singh (2005) as a basis, the blanching of spinach in water at 100°C for 120-180 s under atmospheric conditions will provide greater than a 6 log reduction of HAV using either model. It is important to note that use of steam as a heating medium and immersion in water at 100°C have different heating characteristics and validation of the recommendation using steam must be carried out before actual application of the process.

Conclusion

The result of this study revealed that inactivation rate constants were higher at high temperatures in a comparison to mild temperatures. The z-values determined for HAV were $15.07 \pm 1.63^\circ\text{C}$ and $13.92 \pm 0.87^\circ\text{C}$ for the Weibull model and the first-order model, respectively. The calculated activation energies for the first-order model and the Weibull model were 162 and 151 kJ/mole, respectively. Accurate model prediction of

survival curves will be beneficial to the food industry in selecting optimum process conditions to obtain the desired level of inactivation. Based on the thermal inactivation data obtained from the present study, the application of industrial blanching conditions (100°C for 15-20s) for spinach will provide a > 6 log reduction of HAV using either model. The results of this study will be useful to the frozen food industry in designing blanching conditions for spinach to inactivate or control HAV.

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Appendix

Table 7.1. Effect of thermal treatment on hepatitis A virus (HAV) inactivation in spinach.

Temperature (°C)									
50		56		60		65		72	
Treatment	Recovered titer (log PFU/ml)	Treatment	Recovered titer (log PFU/ml)	Treatment	Recovered titer (log PFU/ml)	Treatment	Recovered titer (log PFU/ml)	Treatment	Recovered titer (log PFU/ml)
Control	6.20±0.79	Control	6.43±0.97	Control	6.37±1.02	Control	5.20±0.84	Control	5.08±0.61
0 min	5.69±0.12	0 min	5.96±0.52	0 min	5.80±0.37	0 s	4.53±0.14	0 s	4.19±0.43
2 min	5.63±0.12	1 min	5.85±0.47	1 min	5.60±0.39	20 s	4.34±0.28	20 s	3.94±0.37
4 min	5.58±0.09	2 min	5.74±0.44	2 min	5.41±0.41	40 s	4.24±0.30	40 s	3.60±0.30
6 min	5.51±0.11	3 min	5.59±0.45	3 min	5.11±0.49	60 s	4.04±0.32	60 s	3.06±0.37

*Each treatment was replicated three times, and plaque assays for evaluating HAV inactivation were carried out in duplicate.

Table 7.2. Coefficients of the first-order and Weibull models for hepatitis A virus (HAV) in spinach during thermal inactivation.

T (°C)	Weibull distribution				First-order kinetics	
	β	α (min)	$t_{D=1}$ (min)	R^2	D (min)	R^2
50	0.96±0.07 ^A	14.89±1.13 ^A	37.08±3.38 ^A	0.97	34.40±4.08 ^A	0.97
56	1.25±0.46 ^{AB}	3.36±0.43 ^B	7.08±1.55 ^B	0.98	8.43±1.72 ^B	0.97
60	1.27±0.48 ^{AB}	2.12±0.33 ^C	4.41±1.19 ^B	0.99	4.55±0.82 ^C	0.98
65	1.18±0.56 ^{AB}	0.90±0.42 ^D	1.96±0.35 ^C	0.99	2.30±0.82 ^D	0.94
72	1.49±0.31 ^B	0.53±0.09 ^D	0.93±0.09 ^D	0.99	0.91±0.14 ^E	0.96

^{A-E} Different letters indicate a significant difference among parameters (β , α , t_D -value, D-value) when compared within each column ($P<0.05$).

Table 7.3. Arrhenius inactivation rate constant of the first-order and Weibull models for hepatitis A virus (HAV) in spinach during thermal inactivation.

T (°C)	Arrhenius model			
	First order model		Weibull model	
	k (min ⁻¹)	R ²	k' (min ⁻¹)	R ²
50	0.03±0.01 ^A	0.94	0.07±0.01 ^A	0.95
56	0.12±0.02 ^B	0.91	0.30±0.04 ^B	0.92
60	0.22±0.04 ^C	0.95	0.48±0.08 ^C	0.96
65	0.54±0.29 ^C	0.91	1.37±0.83 ^D	0.95
72	1.11±0.18 ^D	0.92	1.92±0.29 ^D	0.97

^{A-D} Different letters when compared within each column indicate significant differences among parameters (k, and k') ($P < 0.05$).

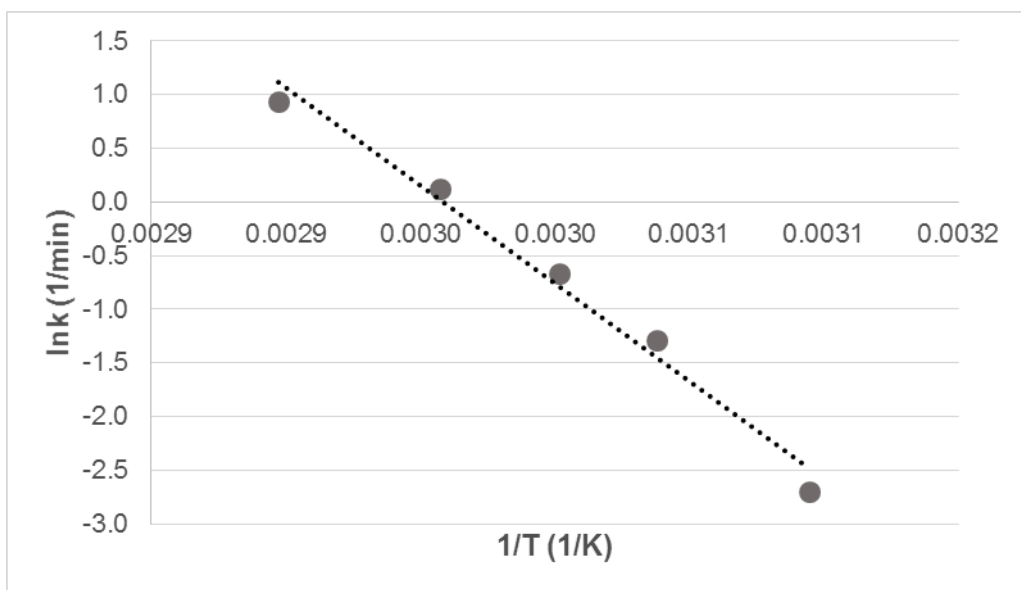
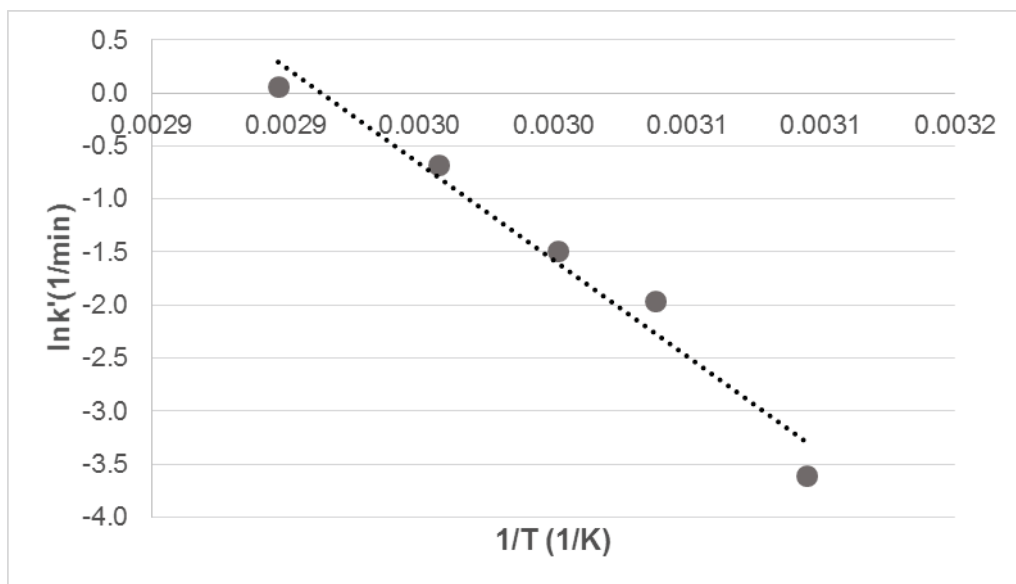
A**B**

Figure 7.1. Arrhenius plot of the inactivation rate constant versus temperature for the [A] First order model and [B] Weibull model.

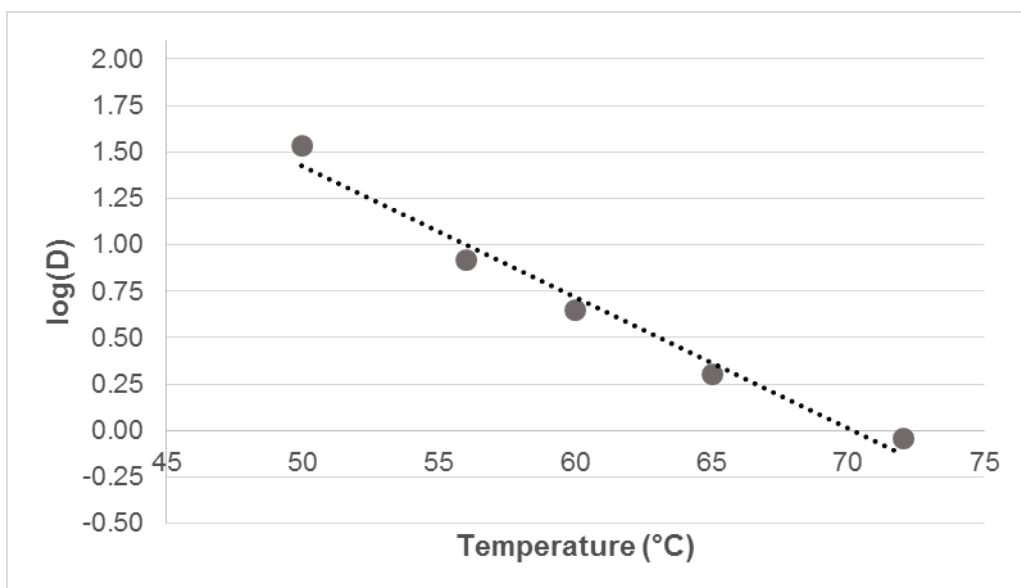
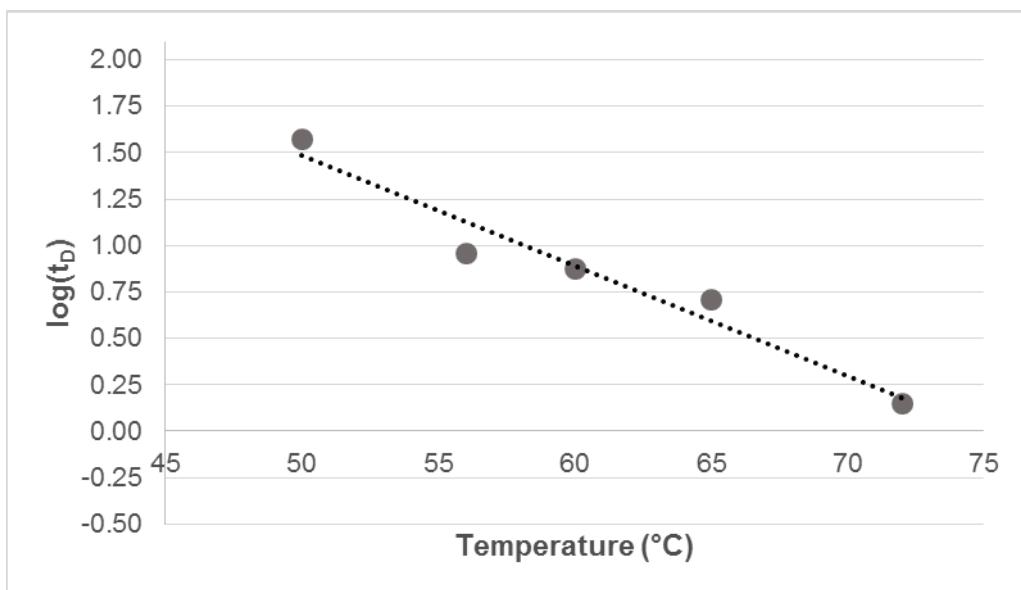
A**B**

Figure 7.2. Thermal death time curves of hepatitis A virus for the A] First order model and [B] Weibull model.

CHAPTER VIII
THERMAL INACTIVATION KINETICS OF HUMAN NOROVIRUS
SURROAGETS AND HEPATITIS A VIRUS IN TURKEY DELI
MEAT

Abstract

Human noroviruses and hepatitis A virus (HAV) have been implicated in several foodborne outbreaks linked to the consumption of pre-sliced ready to eat deli meats. The proper inactivation of these viruses prior to consumption is essential to protect public health. Therefore, the objectives of this research were to determine the thermal inactivation kinetics of human norovirus surrogates (murine norovirus (MNV-1), and feline calicivirus (FCV-F9) and HAV in turkey deli meat, compare first-order and Weibull models to describe the data, calculate Arrhenius activation energy for each model, and evaluate model efficiency using selected statistical criteria. The D-values calculated from the first-order model (50-72°C) ranged from 0.14 ± 0.01 to 9.94 ± 3.93 min for FCV-F9, 0.22 ± 0.01 to 21.01 ± 0.77 min for MNV-1, and 1.01 ± 0.14 to 42.08 ± 5.57 min for HAV. Using the Weibull model, the $t_{D=1}$ (time to destroy 1 log) for FCV-F9, MNV-1 and HAV at the same temperatures ranged from 0.13 ± 0.03 to 11.93 ± 5.13 , 0.25 ± 0.05 to 17.82 ± 1.78 , and 1.0 ± 0.09 to 31.10 ± 19.89 min, respectively. The z-values determined for FCV-F9, MNV-1, and HAV were $11.24 \pm 1.46^\circ\text{C}$, $11.46 \pm 1.44^\circ\text{C}$, and $15.08 \pm 2.62^\circ\text{C}$, respectively, using the Weibull model. For the first order model, z-values were $11.90 \pm 1.0^\circ\text{C}$, $10.91 \pm 1.25^\circ\text{C}$, and $12.83 \pm 1.67^\circ\text{C}$ for FCV-F9, MNV-1, and HAV, respectively. For the Weibull model, estimated activation energies for FCV-F9, MNV-1, and HAV were 216 ± 34 , 234 ± 33 , and 151 ± 15 kJ/mole, respectively, while the calculated activation energies for the first order model were 181 ± 16 , 196 ± 5 , and 167 ± 9 kJ/mole, respectively. Knowledge of the thermal inactivation kinetics of norovirus surrogates and

HAV will allow the development of processes that produce safer food products and improve consumer safety.

Key words: human norovirus surrogates, hepatitis A virus, turkey deli meat, Weibull model and first order model, D- value and z value, activation energy.

Introduction

Foodborne enteric viruses are the leading cause of gastroenteritis in humans, globally. In particular, human noroviruses and hepatitis A virus (HAV) are the most important foodborne viral pathogens with regard to the number of outbreaks and people affected (illnesses reported). Scallan et al., (2011) investigated the foodborne illnesses acquired in the United States, and reported that a large number of outbreaks are caused by human noroviruses (58%), and high hospitalization and death rates are associated with HAV infection (32 and 2%, respectively). In another study, Gould et al., (2013) investigated the surveillance of foodborne disease outbreaks in the United States from 1998 to 2008, and found that among the individual food categories, poultry accounted for the most commonly reported food vehicle, causing 17 % of outbreaks of foodborne illness.

According to the U.S. Food and Drug Administration (FDA) and U.S. Department of Agriculture Food Safety and Inspection Service (FSIS), deli meat posed the highest per annum risk of illness and death among poultry products (USDA-FSIS, 2001). Since, ready-to-eat (RTE) meat and poultry deli products can be consumed without further cooking, these products should be free of non-spore-forming pathogens at the end of the cooking process. However, they can become contaminated before packaging in the final retail wrap and pose a safety thread for public health (Murphy et al., 2004; Houben and Eickenhausen, 2006). The viral contamination of RTE and prepared foods most frequently comes from poor hand-washing practices of food handlers after toilet use, as fecal material can be left on hands or even under nails, which then can come in contact with

food products (Jaykus 2000). Handling cooked products with bare hands has been identified as a major factor for pathogen transfer to RTE foods (Bryan 1995), and there is presumed to be a direct correlation between the number of pathogenic organisms on a food employee's hands and the probability of microbial transfer from hands to cooked food products (Restaino and Wind 1990). Since the contamination most likely occurs at the surface, it seems advisable to decontaminate the outer layer of products. Post package pasteurization technologies including thermal treatment, irradiation, and exposure to ultrahigh pressure could be practiced as one of the efficient post-processing decontamination techniques to eliminate risk associated with surface contamination (Houben and Eckenhausen, 2006).

One of the challenges for the industry would be to assess the required time and temperature combination which will depend on viral characteristics. It's known that foodborne enteric viruses are reported to be more heat resistant than most other non-spore forming food pathogens (Bozkurt et al., 2013, Bozkurt et al., 2014a, 2014b, 2014c); thus, processing recommendations based on data for non-spore forming bacteria may not eliminate similar numbers of foodborne enteric viruses. Since, there is no specific Federal regulation covering the minimum time-temperature combinations for inactivating virus contaminated deli meat, establishment of proper thermal processes for inactivating human norovirus and HAV in turkey deli meat would seem to be essential for protecting public health.

Even though, the importance of human noroviruses in public health is well-documented, there is little information on the thermal inactivation characteristics of

human noroviruses and wild type strain of HAV due to the lack of appropriate cell culture systems. Therefore, viral surrogates have been commonly used based on the assumption that they can mimic the viruses they represent. Two cultivable animal caliciviruses, feline calicivirus (FCV-F9) and murine norovirus (MNV-1) have been extensively used as human norovirus surrogates in inactivation studies (Hewitt and Greening, 2004; Richards, 2012). There are few strains of HAV (HM-175, HAS-15, MBB 11/5) that are cell-culture adaptable and used in research for inactivation studies (Martin and Lemon, 2006; Reiner et al., 1992).

Knowledge of the thermal inactivation data (D- and z-values) for a particular microorganism makes it possible to design thermal processes that target specific organisms (Houben and Eckenhausen, 2006). For this purpose, mathematical modelling has been used with different thermal processes to predict the number of survivors during thermal processing and to give detailed information about inactivation kinetics during treatments (Peleg and Cole, 1998). The use of a first-order model is more common in the food processing industry (Peleg, 1999). However, this model may not always be applicable, and nonlinear behavior may also be observed. Thus, the choice of the most appropriate model is crucial to gather correct information about the thermal inactivation kinetic behavior of the target pathogen. Recent studies conducted on thermal inactivation of human norovirus surrogates (Bozkurt et al., 2013, 2014a, 2014b; Seo et al., 2012; Tuladhar et al., 2012) and HAV (Bozkurt et al., 2014c) revealed that the Weibull model was statistically superior in describing the thermal inactivation kinetics of norovirus surrogates and HAV than the first-order model.

To our knowledge, there have been no thermal inactivation kinetics established for inactivation of human norovirus surrogates and HAV in turkey deli meat. Thus, generation of correct thermal process data and establishment of proper thermal processes for inactivating human norovirus surrogates and HAV in turkey deli meat are important both for consumers and industry. Therefore, the objectives of this study were (i) to determine the thermal inactivation behavior of human norovirus surrogates and HAV in turkey deli meat, (ii) to compare first-order, and Weibull models in describing the data in terms of selected statistical parameters, and (iii) to calculate z-values and activation energy for each model.

Material and Methods

Viruses and cell lines

Murine norovirus (MNV-1) was obtained from Dr. Skip Virgin (Washington Univ., St Louis, MO) and its host RAW 264.7 cells were obtained from the University of Tennessee, Knoxville. Feline calicivirus (FCV-F9) and its host cells (Crandell Reese Feline Kidney, (CRFK) were obtained from ATCC (Manassas, VA). Hepatitis A virus (HAV: strain HM175) and fetal monkey kidney (FRhK4) cells were kindly provided by Dr. Kalmia Kniel (University of Delaware).

As described before (Bozkurt et al., 2014a), CRFK, RAW 264.7, and FRhK4 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM-F12: HyClone Laboratories, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1×Anti-Anti (Antibiotic-

Antimycotic; Invitrogen, Grand Island, NY) at 37°C in an atmosphere containing 5% CO₂.

Propagation of viruses

CRFK, RAW 264.7, and FRhK4 cells with ~90% confluence in cell culture flasks were washed with phosphate-buffered saline (PBS; pH 7.4) twice before adding FCV-F9, MNV-1, and HAV stocks, respectively to their host-cell monolayers. The infected cells were then incubated until >90% cell lysis in a water jacketed CO₂ incubator at 37°C. All three viruses were recovered by centrifugation at $5,000 \times g$ for 10 min, followed by filtration through 0.2- μ m filters, aliquoted, and stored at -80°C until use as described before (Su et al., 2010).

Inoculation of turkey deli meat

Turkey deli meat was purchased from a local market and cut into circular pieces (diameter=3 cm) prior to virus inoculation. One-hundred μ l of each virus stocks (FCV-F9, MNV-1, and HAV) with initial titers of 7.12 ± 0.89 log PFU/ml for FCV-F9, 6.85 ± 1.23 log PFU/ml for MNV-1, and 7.27 ± 1.46 log PFU/ml for HAV were individually used to aseptically inoculate the surface of deli meat, and allowed to dry at room temperature for 30 min under the biosafety cabinet.

Thermal treatment

The inoculated turkey deli meat (6 g) was placed into moisture barrier plastic vacuum bags (13 cm x 19 cm) using sterile plastic forceps in a biosafety cabinet. The inoculated samples were vacuum sealed to -100 kPa with a Multivac A300/16 vacuum-

packaging unit (Sepp Haggemuller KG, Wolfertschwenden, Germany) and the bags were flattened. To monitor the internal temperature of the sample, a thermocouple was placed at the geometric center of an uninoculated control package of turkey deli meat. The sealed bags were placed into a holding unit, rinsed with 70% ethanol, and then the holding unit with the bags was immersed in a thermostatically controlled ($\pm 0.1^{\circ}\text{C}$) circulating water bath (Haake model V26, Karlsruhe, Germany). The water bath temperature was confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA) and by placing type-T thermocouples (Omega Engineering, Inc., Stamford, CT) in the geometric center of the water bath. The thermocouples were connected to a MMS3000-T6V4 type portable data recorder (Commtest Ins., New Zealand) to monitor temperature. Samples were heated at 50, 56, 60, 65, and 72°C for different treatment times (0-6 min). The treatment time began when the internal temperature reached the designated target temperature. Triplicate bags were used for each time-point. After the thermal treatment, sample bags were immediately cooled in an ice water bath for 15 min to stop further thermal inactivation. The bags were washed again with ethanol before removal of the contents. Bags were placed in a biosafety cabinet and aseptically cut with sterilized (autoclaved at 121°C for 15 min) scissors. Bag contents were removed with a sterile pipet and the inside of the bags were washed with elution buffer (15 ml) using a sterile pipet to remove the remaining sample. The un-heated virus suspensions from turkey deli meat were used as controls and enumerated (Table 1).

Virus extraction

The method for virus extraction was performed as described in Bozkurt et al., (2014a)

with some modifications. Inoculated and thermally treated turkey deli meat was washed with 15 ml of elution buffer (2:5 ratio) containing 0.15 M NaCl and 0.05 M glycine. The pH was then adjusted to 9.0 using 10 M NaOH. Samples in the sterile beaker were then kept shaking on a shaking platform (120 rpm) for 20 min at 4°C. Samples were centrifuged at 10,000 x g for 15 min at 4°C, and the pH of the supernatant was adjusted to 7.2 to 7.4 using 6 N HCl. Polyethylene glycol (PEG) 6000 and NaCl were added to obtain a final concentration of 6% PEG and 0.3 M NaCl. These samples were placed on a shaking platform (120 rpm) overnight at 4°C and then centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was discarded and the pellet dissolved in 2 ml PBS and put on a shaker for 20 min to homogenize.

Extracts containing the individual viruses were stored at -80°C until enumeration of plaques using HAV, MNV-1 and FCV-F9 plaque assays.

Enumeration of survivors by infectious plaque assays

Thermally treated and control viral suspensions were diluted 1:10 in Dulbecco's Modified Eagle Medium (DMEM-F12) containing fetal bovine serum (10% for MNV-1 and 2% for FCV-F9 and HAV) and 1% antibiotic-antimycotic. Infectivity of each treated virus was evaluated using standardized plaque assays in comparison to untreated virus controls following the previously described procedures (Su et al., 2010). Viral survivors were enumerated as plaque forming units/ml (or PFU/g).

Modeling of inactivation kinetics
First-order kinetics

The traditional approach to describe the change in number of survivors over time for first-order kinetic model can be written as follows:

$$\ln N_{(t)} = \ln N_0 - kt \quad (1)$$

where $N_{(t)}$ is the number of survivors after an exposure time (t) in PFU/g and the initial population is N_0 (PFU/g), and k as the first-order rate constant (1/s). This equation is then rearranged into:

$$\log_{10} \frac{N(t)}{N_0} = -\frac{t}{D} \quad (2)$$

where D is the decimal reduction time ($D=2.303/k$, units in min or s) and is thus actually a reciprocal first-order rate constant as described before (Bozkurt et al., 2013; Bozkurt et al., 2014a, b). The resulting semi-logarithmic curve when $\log N(t)/N_0$ is plotted vs. time is frequently referred to as the survival curve.

Weibull model

Experimental data were fitted in decimal logarithmic form as follows:

$$\frac{N(t)}{N_0} = \exp\left(-\left(\frac{t}{\alpha}\right)^\beta\right) \quad (3)$$

where α (min^{-1}) and β (-) are the scale, and shape parameters, respectively.

As indicated by previous studies, the inverse of the of the scale factor (α) as a reaction rate constant k' (min^{-1}), the equation becomes (Fernandez et al., 2002):

$$\frac{N(t)}{N_0} = \exp(-(k't)^\beta) \quad (4)$$

For the Weibull model, the time required to reduce the number of microorganisms by a factor 10 (analogous to the D-value) can be calculated by using the shape and scale parameters as shown in Eq (5);

$$t_D = \alpha - \ln(10^{-D})^{1/\beta} \quad (5)$$

where D represents decades (*or log*) reduction of a microbial population. t_D has the stated meaning only when it refers to the treatment time starting at zero as described before (Bozkurt et al., 2013; Bozkurt et al., 2014a, b).

Arrhenius activation energy

The inactivation rate is primarily influenced by temperature, and the temperature dependence of the rate constant is typically described by the Arrhenius equation:

$$k = A \exp\left(-\frac{E_a}{RT}\right) \quad (6)$$

where A is a frequency factor which is constant, E_a is the activation energy (J/mole), R is the universal gas constant ($8.314 \text{ Jmole}^{-1}\text{K}^{-1}$), k is the rate constant (1/min), T is the absolute temperature (K). The construction of $\ln k_{(T)}$ versus $1/T$, the slope of the curve will be a straight line which equals to activation energy. This concept has been used to calculate activation energy of microbial inactivation (Corradini and Peleg, 2005).

Statistical Analysis

Statistical and non-linear regression analyses were performed using SPSS Ver.11.0.1 statistical package. The statistical criteria applied to discriminate (differentiate) between the kinetic models were R^2 (coefficient of determination) and standard error (std. error) for each coefficient. The confidence level used to determine statistical significance was 95%.

Result and Discussion

The initial titers of viruses stocks were 7.12 ± 0.89 log PFU/ml for FCV-F9, 6.85 ± 1.23 log PFU/ml for MNV-1, and 7.27 ± 1.46 log PFU/ml for HAV. After inoculation of turkey deli meat samples with virus stocks, recovered titers were 6.96 ± 0.62 log PFU/g for FCV-F9, 5.77 ± 0.42 log PFU/g for MNV-1, 6.86 ± 0.57 log PFU/g for HAV, respectively. The variation in virus titer after inoculation could be due to losses associated with the virus extraction steps.

In the heating studies with inoculated turkey deli meat, samples were heated up to selected temperatures ($50\text{-}72^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$). At 50°C , heating for 6 min resulted in a less than 1 log reduction (PFU/ml) for all viral surrogates. For all viruses, the degree of inactivation was dependent on the temperature and treatment time. As temperature magnitudes and/or treatment time increased, the degree of inactivation also increased. At the highest temperatures used (65 and 72°C), total inactivation was achieved in less than 30 s only for norovirus surrogates (FCV-F9 and MNV-1), but not for HAV. Since the various components of the virus such as capsid, and nucleic acid have widely different values of entropy and enthalpy among different viruses. Therefore, the degradation rate of viruses would be different (Pollard, 1960). It has been suggested that exposure to mild temperatures ($<56^{\circ}\text{C}$) mainly leads to damage of the viral receptors through structural changes in the capsid protein that interferes with binding and causes low level inactivation, while at higher temperatures alteration of tertiary structure occur leading to eventual degradation of nucleic material (Ausar et al., 2006; Croci et al., 2012; Wigginton et al., 2012). This might be the reason for the observation of a lower reduction rate at mild temperatures ($<56^{\circ}\text{C}$) compared with a greater reduction rate at higher

temperatures ($>65^{\circ}\text{C}$). This hypothesis was also supported by other researchers (Bertrand et al., 2012; Bozkurt et al., 2014a, 2014b, 2014c; Croci, et al., 1999; Pollard 1960; Song et al., 2011; Sow et al., 2011; Volking et al., 1997).

The D-values calculated from first-order model ($50\text{-}72^{\circ}\text{C}$) were in the range of 9.94 ± 3.93 to 0.14 ± 0.01 min for FCV-F9, 21.01 ± 0.77 to 0.22 ± 0.01 min for MNV-1, and 42.08 ± 5.57 to 1.01 ± 0.14 min for HAV min (Table 8.1). For each virus, the temperature had a significant effect on D-values for the temperature range studied ($p<0.05$). In general, HAV was more resistant to thermal treatment than FCV-F9 and MNV-1 at all temperatures studied suggesting that it would require a more severe treatment than human norovirus surrogates for inactivation in turkey deli meat.

To investigate applicability of the Weibull model, the shape and scale factors parameters were calculated and are shown in Table 8.1. The Weibull shape factor (β) ranges for the temperature studied ($50\text{-}72^{\circ}\text{C}$) were 1.80 ± 0.98 to 0.33 ± 0.01 for FCV-F9, 1.72 ± 0.15 to 1.03 ± 0.41 for MNV-1, 1.40 ± 0.43 to 0.79 ± 0.41 for HAV. A shape factor greater than one indicates that the remaining population becomes increasingly damaged, whereas a shape factor less than one indicates that the remaining population has the ability to adapt to applied stress (van Boekel, 2002). In his review paper, van Boekel (2002) evaluated data from 55 different studies of thermal inactivation of vegetative cells, and in 48 of them he reported independency of shape factor to temperature. Moreover, Cunha et al. (1998) also reported the independence of shape factor on external factors, because the parameter was a behavior index which showed the kinetic pattern of the mechanism controlling the process studied. Consistent with Cunha et al., (1998), the

results of this study revealed that for each virus strain, the heating temperature apparently did not influence the shape parameter and could not be described by any model.

The scale factor (α) ranges for the temperatures studied (50-72°C) were 3.83±2.47 to 0.01±0.01 min for FCV-F9, 8.63±0.38 to 0.15±0.08 min for MNV-1, 17.57±6.01 to 0.62±0.06 min for HAV. In contrast to the shape factor, the scale parameter depends on the heating temperature and the change in scale factor describes the effect of heating environment on the inactivation. The results revealed that temperature had a significant effect on the scale factor (α) values ($p<0.05$). A second order polynomial model was established to quantify influence of temperature on scale factor. The relationship between scale factors and temperature for FCV-F9, MNV-1, and HAV, respectively, were;

$$\alpha = 0.0145T(^{\circ}C)^2 - 1.9265T(^{\circ}C) + 63.568 \quad R^2 = 0.93 \quad (8)$$

$$\alpha = 0.0284T(^{\circ}C)^2 - 3.8319T(^{\circ}C) + 129.04 \quad R^2 = 0.99 \quad (9)$$

$$\alpha = 0.0528T(^{\circ}C)^2 - 7.2224T(^{\circ}C) + 246.76 \quad R^2 = 0.99 \quad (10)$$

Parameters of the Weibull model (shape factor = β and scale factor = α) were used to calculate t_D value which was used as an analog to the D-value of the first order model, when it refers to the treatment time starting at zero (Table 8.1). For the Weibull model, the calculated time to destroy 1 log ($D=1$) for FCV-F9, MNV-1, and HAV were in the range of 11.93±5.13 to 0.13±0.03 min, 17.82±1.78 to 0.25±0.05, 31.10±19.89 to 1.00±0.09 min, respectively for the range 50-72°C.

The temperature dependency of the inactivation rate constant (k and k') were fitted by the exponential Arrhenius function for both models (Table 8.2). The Weibull model gave higher R^2 than the first-order model when the rate constants were fitted to the Arrhenius

equation (Table 8.2). For the first order model, estimated inactivation rate constants for the temperatures studied (50-72°C) were in the range of 16.52±1.81 to 0.25±0.08 min⁻¹ for FCV-F9, 10.73±0.73 to 0.11±0.01 min⁻¹ for MNV-1, and 2.31±0.32 to 0.06±0.01 min⁻¹ for HAV. Results for the present study revealed that temperature had a significant effect on estimated inactivation rate constants for both models. The temperature dependency of the inactivation rate constant for first-order model was expressed by the second order polynomial model. The relationship between inactivation rate constant and temperature for FCV-F9, MNV-1, and HAV respectively were;

$$k = 0.045T(^{\circ}C)^2 - 4.7797T(^{\circ}C) + 126 \quad R^2 = 0.99 \quad (11)$$

$$k = 0.0366T^2(^{\circ}C) - 4.0183T(^{\circ}C) + 109 \quad R^2 = 0.98 \quad (12)$$

$$k = 0.0056T^2(^{\circ}C) - 0.5743T(^{\circ}C) + 14.878 \quad R^2 = 0.99 \quad (13)$$

The estimated inactivation rate constants for the Weibull model for the temperatures studied (50-72°C) were in the range of 79.86±14.44 to 0.21±0.08 min⁻¹ for FCV-F9, 8.37±3.06 to 0.12±0.01 min⁻¹ for MNV-1, and 1.62±0.16 to 0.06±0.02 min⁻¹ for HAV. A second order polynomial model was established to quantify influence of temperature on inactivation rate constant for the Weibull model. The relationship between inactivation rate constants and temperature for FCV-F9, MNV-1, and HAV respectively were;

$$k' = 0.03215(^{\circ}C) - 36.03T(^{\circ}C) + 1002 \quad R^2 = 0.93 \quad (14)$$

$$k' = 0.0277T^2(^{\circ}C) - 3.0296T(^{\circ}C) + 82.615 \quad R^2 = 0.98 \quad (15)$$

$$k' = 0.0026T^2(^{\circ}C) - 0.2467T(^{\circ}C) + 5.7569 \quad R^2 = 0.98 \quad (16)$$

The estimated activation energies for first order model were 181±16, 196±5, 167±9 kJ/mole for FCV-F9, MNV-1, and HAV, respectively. For the Weibull model,

estimated activation energies for FCV-F9, MNV-1, and HAV were 216 ± 34 , 234 ± 33 , 151 ± 15 kJ/mole, respectively (Table 8.3). Bozkurt et al., (2014c) reported that the activation energy for FCV-F9, MNV-1 and HAV in cell culture media (Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic-antimycotic) were 225, 278, and 182 kJ/mole, for Weibull model, and 195, 202, and 171 kJ/mole for the first-order model, respectively. In agreement with the present results for FCV-F9, MNV-1, and HAV, the reported activation energies for both studies were not statistically different for either model ($p>0.05$).

In addition to activation energies for both models, the z-values for inactivation of human norovirus surrogates and HAV in buffer were also calculated (Table 8.4). The z-values determined for FCV-F9, MNV-1, and HAV were $11.24\pm1.46^{\circ}\text{C}$, $11.46\pm1.44^{\circ}\text{C}$, and $15.08\pm2.62^{\circ}\text{C}$, respectively, using the Weibull model. For the first order model, z-values were $11.90\pm1.0^{\circ}\text{C}$, $10.91\pm1.25^{\circ}\text{C}$, and $12.83\pm1.67^{\circ}\text{C}$ for FCV-F9, MNV-1, and HAV, respectively. Bozkurt et al., (2014c) also determined the z-values for FCV-F9, MNV-1 and HAV in cell culture media (Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic-antimycotic) as $9.66\pm0.94^{\circ}\text{C}$, $9.16\pm1.12^{\circ}\text{C}$, and $14.50\pm2.93^{\circ}\text{C}$ for the Weibull model, and $9.36\pm0.62^{\circ}\text{C}$, $9.32\pm0.47^{\circ}\text{C}$, and $12.49\pm0.20^{\circ}\text{C}$ for the first-order model, respectively. Unlike D-values, the z-value is not a function of the heating environment, it represents the temperature required for the thermal destruction curve to change by one log cycle. Therefore, it should be same for the same microorganism under different environments. The results of present study were

consistent with those of the study by Bozkurt et al., (2014c) who reported statistically similar z-values for FCV-F9, MNV-1, and HAV ($p>0.05$).

The detailed thermal data obtained from this study will be useful to eliminate or decrease the risk associated with the consumption of turkey deli meat contaminated with human norovirus or HAV. Koopmans and Duizer (2004) classified the risks of infection for the consumer if viruses are present before processing. The risks were classified into four grades as negligible risk, low risk, medium risk and high risk. According to this classification, any treatment that would result in at least 4 log reduction of viruses could have negligible risk of infection. Thus, achieving a 7 log reduction (often used as a pasteurization criteria for deli meat) could be considered as a safe food process. For the first order model, the extent of inactivation is simple multiplication of D, while with Weibull model it cannot be computed from t_D (both α and β are needed). This means that D values are determined from the linear part of the overall survivor curve whereas t_D value takes into account everything that happens up to reaching the survival decimal reduction point (Peleg and Cole, 1998). This might lead to the possibility of under or over estimation of thermal destruction times when the single t_D value is considered as in the case of conventional D value. It should also be noted that as with the log linear approach where 7 D is equal to $7 \times D$, t_D is not $7 \times t_{D=1}$ but $t_{D=7}$. Based on the thermal inactivation data obtained from this study, for both models (first order and Weibull model) required process time to achieve 7 log reduction at temperatures 80, 85, 90, 95, and 100°C were calculated (Table 8.5). Results revealed that at each temperature, HAV was the most heat resistant strain and required longer treatment times rather than human norovirus

surrogates. The treatment time required to achieve 7 log reductions of HAV in turkey deli meat at temperatures 80, 85, 90, 95, and 100°C were 125, 54, 23, 10, and 5 s for first-order model and 71, 37, 20, 10, and 6 s for Weibull model, respectively. For these processes at higher temperatures, employing first-order predictions would lead to unnecessary over-processing, that offers no additional safety but surely damages/decreases the product quality.

The industrial pasteurization process time for turkey deli meat could be estimated by using the thermal data obtained from this study. According to Pulsfus (2006), industrial pasteurization process conditions for turkey deli meat include use of hot water at 200°F as a heating medium for 3-5 min. Using the information generated in the present study and the thermal parameters of Pulsfus (2006), as a basis, the pasteurization of turkey deli meat in hot water at 200°F for 3-5 min under atmospheric conditions will provide greater than a 7 log reduction of human noroviruses or HAV using either model. Since those thermal inactivation data values were generated in turkey deli meat, investigation of the thermal inactivation of these viruses in various food commodities is also needed. The precise understanding of thermal inactivation behavior of foodborne enteric viruses would be useful for the food industry during integration of thermal processing to control foodborne enteric viruses associated outbreaks.

Conclusion

The result of this study revealed that inactivation rate constants were higher at high temperatures in comparison to mild temperatures. The z-values determined for FCV-F9, MNV-1, and HAV were $11.24 \pm 1.46^\circ\text{C}$, $11.46 \pm 1.44^\circ\text{C}$, and $15.08 \pm 2.62^\circ\text{C}$,

respectively, using the Weibull model. For the first order model, z-values were $11.90 \pm 1.0^{\circ}\text{C}$, $10.91 \pm 1.25^{\circ}\text{C}$, and $12.83 \pm 1.67^{\circ}\text{C}$ for FCV-F9, MNV-1, and HAV, respectively. For the Weibull model, estimated activation energies for FCV-F9, MNV-1, and HAV were 216 ± 34 , 234 ± 33 , and 151 ± 15 kJ/mole, respectively, while the calculated activation energies for the first order model were 181 ± 16 , 196 ± 5 , and 167 ± 9 kJ/mole, respectively. Based on the thermal inactivation data obtained from the present study, the application of industrial pasteurization conditions (200°F for 3-5 min) for turkey deli meat will provide a >7 log reduction of HAV and human norovirus surrogates using either model. Accurate model prediction of survival curves will be beneficial to the food industry in selecting optimum process conditions to obtain the desired level of inactivation. The results of this study will be useful to the food industry in designing pasteurization conditions for turkey deli meat to inactivate or control HAV and/or human norovirus surrogates.

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Appendix

Table 8.1. Coefficients of the first-order and Weibull models for the survival curves of feline calicivirus (FCV-F9), murine norovirus (MNV-1) and hepatitis A virus (HAV) in turkey deli meat during thermal inactivation.

Virus strain	T (°C)	Weibull distribution			First-order kinetics		
		β	α (min)	t_d (min)	R^2	D (min)	R^2
FCV-F9	50	0.85±0.46	3.83±2.47	11.93±5.13	0.99	9.94±3.93	0.94
	56	0.59±0.11	0.70±0.15	2.96±0.25	0.99	3.03±0.16	0.94
	60	0.90±0.27	0.30±0.11	0.79±0.08	0.99	0.82±0.09	0.96
	65	1.80±0.98	0.28±0.18	0.45±0.15	0.97	0.43±0.09	0.89
	72	0.33±0.01	0.01±0.01	0.13±0.03	0.99	0.14±0.01	0.90
MNV-1	50	1.16±0.10	8.63±0.38	17.82±1.78	0.98	21.01±0.77	0.97
	56	1.11±0.14	3.13±0.33	6.71±0.61	0.99	7.30±0.80	0.99
	60	1.03±0.41	1.13±0.15	2.78±0.53	0.99	2.74±0.59	0.98
	65	1.72±0.15	0.52±0.01	0.85±0.02	0.96	0.94±0.02	0.94
	72	1.67±1.00	0.15±0.08	0.25±0.05	0.99	0.22±0.01	0.96
HAV	50	1.40±0.43	17.57±6.01	31.10±19.89	0.99	42.08±5.57	0.97
	56	1.09±0.43	8.87±3.08	20.77±11.68	0.98	20.62±2.39	0.96
	60	0.88±0.46	2.55±0.39	8.55±3.95	0.99	5.91±1.33	0.94
	65	0.79±0.41	1.03±0.22	4.48±3.51	0.99	2.27±0.40	0.95
	72	1.75±0.04	0.62±0.06	1.00±0.09	0.99	1.01±0.14	0.93

Table 8.2. Arrhenius inactivation rate constant of the first-order and Weibull models for the survival curves of feline calicivirus (FCV-F9), murine norovirus (MNV-1) and hepatitis A virus (HAV) in turkey deli meat during thermal inactivation.

Virus		Arrhenius model			
strain	T (°C)	First order model		Weibull model	
		k(min ⁻¹)	R ²	k'(min ⁻¹)	R ²
FCV-F9	50	0.25±0.08	0.91	0.21±0.08	0.98
	56	0.76±0.23	0.92	1.46±0.59	0.98
	60	2.82±0.01	0.92	3.58±1.22	0.97
	65	5.54±0.04	0.86	5.31±4.22	0.96
	72	16.52±1.81	0.88	79.86±14.44	0.98
MNV-1	50	0.11±0.01	0.96	0.12±0.01	0.97
	56	0.32±0.04	0.97	0.32±0.03	0.99
	60	0.87±0.17	0.98	0.89±0.11	0.98
	65	2.46±0.05	0.93	1.91±0.03	0.96
	72	10.73±0.73	0.95	8.37±3.06	0.98
HAV	50	0.06±0.01	0.97	0.06±0.02	0.99
	56	0.11±0.01	0.90	0.12±0.04	0.97
	60	0.40±0.10	0.90	0.40±0.06	0.98
	65	1.03±0.19	0.93	1.00±0.20	0.99
	72	2.31±0.32	0.90	1.62±0.16	0.99

Table 8.3. The activation energies of the first-order and Weibull models for feline calicivirus (FCV-F9), murine norovirus (MNV-1) and hepatitis A virus (HAV).

Virus strain	Weibull distribution		First-order kinetics	
	E _a (kJ/mol)	R ²	E _a (kJ/mol	R ²
FCV-F9	216±34	0.93	181±16	0.98
MNV-1	234±33	0.95	196±5	0.99
HAV	151±15	0.95	167±9	0.97

Table 8.4. The z values of the first-order and Weibull models for feline calicivirus (FCV-F9), murine norovirus (MNV-1) and hepatitis A virus (HAV).

Virus strain	Weibull distribution		First-order kinetics	
	z value (°C)	R ²	z value (°C)	R ²
FCV-F9	11.24±1.46	0.99	11.90±1.0	0.98
MNV-1	11.46±1.44	0.99	10.91±1.25	0.99
HAV	15.08±2.62	0.98	12.83±0.67	0.97

Table 8.5. Estimated process time to achieve 7 log reduction for the first-order and Weibull models of feline calicivirus (FCV-F9), murine norovirus (MNV-1) and hepatitis A virus (HAV) in turkey deli meat during thermal inactivation.

Model	Virus strain	Time (s)				
		Temperature (°C)				
		80°C	85°C	90°C	95°C	100°C
First order	FCV-F9	14	6	3	1	1
	MNV-1	18	7	3	1	1
	HAV	125	54	23	10	5
Weibull	FCV-F9	9	4	2	1	1
	MNV-1	11	4	2	1	1
	HAV	71	37	20	10	6

CHAPTER IX
STRUCTURAL ANALYSIS OF THERMALLY TREATED
HEPATITIS A VIRUS IN BUFFERED CELL CULTURE MEDIA

Abstract

The precise understanding of the viral inactivation mechanism is highly desired to develop new strategies and/or to improve existing methods. To determine whether thermal treatment causes any structural changes to the virus, hepatitis A virus (HAV) was thermally treated at different temperatures (50-72°C) for different treatment times (0-2 min). The objective of this study were to (i) investigate the effect of thermal treatment (50-72°C) on viral structure by comparing and analyzing Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM) images, and (ii) determine the mechanism of virus inactivation during thermal treatment. The results of both TEM and AFM revealed that significant changes in virus structure occurred after thermal treatment. Even though TEM was useful to gain insight about virus inactivation, AFM provided a better approach for visualizing structural changes of HAV after thermal treatment. Based on these results, the proposed mechanism for thermal inactivation of HAV include disruption in capsid structure and degradation of the viral proteins. The degree of disruption that occurred in capsid was also found to be increased with increasing temperature. This study will provide useful knowledge about structural changes of HAV during thermal treatment and will be useful to understand inactivation mechanism of HAV during thermal treatment.

Key words: thermal inactivation, hepatitis A virus, atomic force microscopy, transmission electron microscopy.

Introduction

Thermal inactivation is among the most widely used and reliable food processing methods. The main goal of thermal processing is to inactivate the pathogenic microorganisms and produce a safe product with enhanced shelf life (Escudero-Abarca et al., 2014). A precise understanding of the mechanism of microbial inactivation by heat is potentially useful for optimizing heat treatments to eliminate foodborne disease and spoilage risk associated with common and emerging strains while avoiding over processing of the food material. It's also known that thermal inactivation of microorganisms is associated with irreversible damages to the cell structure (Lee and Kaletunc, 2002). Although the mode of action during thermal inactivation of bacteria is a well-known (Russel, 2003; Shapiro and Cowen 2012), the patterns of macromolecular changes that induce the inactivation of foodborne viruses during heat treatment are still not clearly known.

Among foodborne viruses, it's known that hepatitis A virus (HAV) requires longer exposure to heat rather than other foodborne enteric viruses due to its thermal stability (Sow et al., 2011; Coudray-Meunier et al., 2013). Due to its resistance to thermal treatment, a cell culture adapted HAV strain would seem to be a relevant indicator in studies aimed to understand thermal inactivation behavior of most enteric viruses (Deboosere et al., 2004; Bidawid et al., 2008), especially since efforts to propagate human norovirus and wild type strain of HAV in routine laboratory cell culture or primary tissue cultures have been unsuccessful (Duizer et al, 2004; Richards 2012).

In the current literature, limited studies have been performed to investigate thermal inactivation of HAV (Bozkurt et al., 2014d; Croci et al., 1999; Hewitt and Greening, 2004) in buffered cell culture media (Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and supplemented with antibiotics). To our knowledge, there are no studies established to investigate structural analysis of HAV in buffered cell culture media during thermal treatment. Therefore, the mechanism of viral inactivation during thermal treatment is poorly understood and to date the effect of thermal treatment on viral capsid and genomic RNA has not been determined yet.

Transmission electron microscopy (TEM) and atomic force microscopy (AFM) are tools that have been commonly used for characterization and identification of nano-sized biological structures. They both have a significant importance in virology. TEM is indispensable for structural studies and virus identification, but is also used for particle counts (Ackermann and Heldal, 2010). AFM enables researchers not only to observe structural details of cells but also to measure the nanoscale chemical and physical properties of cells and the localization and properties of individual molecules (Muller and Dufrene, 2008, 2011; Muller et al., 2009). Due to its ability to observe single microbial cells at nanometer resolution, to monitor structural dynamics in response to environmental changes or chemicals, and to detect and manipulate single-cell surface constituents, AFM provides new insight into the structure-function relationships of cell structure and is having an increasingly important impact in the field of virology (Xing et al., 2014). Thus, the objective of this study were (i) investigate effect of thermal

treatment (50-72°C) on viral structure by comparing TEM and AFM techniques, and (ii) determine the mechanism of virus inactivation during thermal treatment.

Material and methods

Viruses and cell lines

Hepatitis A virus (HAV: strain HM175) and fetal monkey kidney (FRhK4) cells were kindly provided by Dr. Kalmia Kniel (University of Delaware). FRhK4 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM-F12: HyClone Laboratories, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1×Anti-Anti (Antibiotic-Antimycotic; Invitrogen, Grand Island, NY) at 37°C in an atmosphere containing 5% CO₂.

Propagation of viruses

FRhK4 cells with ~90% confluence in cell culture flasks were washed with phosphate-buffered saline (PBS; pH 7.4) twice before adding HAV stocks to these cell monolayers. The infected cells were then incubated until >90% cell lysis in a water jacketed CO₂ incubator at 37°C. All viruses were recovered by centrifugation at 5,000 × g for 10 min, followed by filtration through 0.2-μm filters, aliquoted, and stored at -80°C until use.

Thermal treatment

Heat treatment was carried out in a circulating water bath (Haake model V26, Karlsruhe, Germany) in 2 ml screw-capped vials. Sterilized (121°C, 15 min) vials were carefully filled with 2 ml buffer-cell culture media (Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic-antimycotic) containing

virus by using sterile pipettes in a biosafety cabinet. The filled vials were surface rinsed in 70% ethanol before immersion in a thermostatically controlled water bath. Water bath temperature was confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA) and by placing type-T thermocouples (Omega Engineering, Inc., Stamford, CT) in the geometric center of the water-bath. Another thermocouple probe was placed at the geometric center of a vial through the lid to monitor the temperature of the buffer media. Thermocouples were connected to MMS3000-T6V4 type portable data recorder (Commtest Inc., New Zealand) to monitor temperature. Samples were heated at 50, 56, 60, 65, and 72°C for different treatment times (0-2 min). The treatment time began (and was recorded) when the target internal temperature reached the designated temperature as described earlier (Bozkurt et al., 2013, 2014a, 2014b). Triplicate tubes were used for each temperature and time-point. After the thermal treatment, sample vials were immediately cooled in an ice water bath for 15 min to stop further thermal inactivation.

Sample preparation

For high-resolution images, samples should be sterile and free from bacteria and other large particles. This is achieved by filtration through membrane filters of 0.2 µm pore size. The purification of viruses is also required because proteins and salts interfere with staining and resolution, therefore their amounts must be reduced to acceptable levels. For microscopic examination, the common method of purification is washing with buffer followed with ultracentrifugation (Ackermann and Heldal, 2010). The virus stock with an initial titer of (7.34 ± 1.28 log PFU/ml) washed in a buffered cell culture media

(Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM-F12; HyClone Laboratories, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1×Anti-Anti (Antibiotic-Antimycotic; Invitrogen, Grand Island, NY) and it was centrifuged at 25,000 x g (fixed-angle rotor) for 1 h at 4°C, and supernatant was discarded. The pellet was dissolved in 50µl DEPC treated water.

Transmission electron microscopy:

Negative-staining electron microscopy of samples was performed to determine whether thermal treatment damages the virus particles. The principle of negative staining is to mix the particles to be examined with an electron-dense solution of a metal salt of high molecular weight and small molecular size. Aliquots (20µl) of thermally treated or untreated samples were fixed in copper grids (Electron Microscopy Sciences, Hatfield, PA) and negatively stained with 0.5% phosphotungstic acid. Virus particles were visualized by Zeiss Libra 200 MC transmission electron microscope (TEM) at 60-200 kV at the Advanced Microbiology and Imaging Center at University of Tennessee, Knoxville. Images were captured on a MegaView III side-mounted charge-coupled-device (CCD) camera (Soft Imaging System, Lakewood, CO), and figures were processed using ImageJ Software (Image Processing Systems, San Jose, CA).

Atomic force microscopy:

The effect of thermal treatment on the HAV particles was examined by AFM. The samples were prepared by applying 5µl of viral suspensions without treatment (control) or thermally treated onto a clean mica surface. The samples was air dried for 5 minutes, then the substrate rinsed once with water to remove salt crystals. The stream of dry

nitrogen was used to dry samples before analysis. The dried sample on the mica surface was fixed on a glass slide with a carbon tape.

Atomic force microscopy (AFM) was carried out in the dynamic mode (AC) using a Asylum Research MFP3D microscope equipped with the Nanoscope IIIa electronic Device (Digital Instruments-Veeco, Santa Barbara, CA) at the Advanced Microbiology and Imaging Center at University of Tennessee, Knoxville. Nanoprobes cantilevers made of silicon (NCH, Digital Instruments) with a spring constant of 0.05 N/m and a resonance frequency of 276 kHz were used. The oscillation amplitude was 15–20 nm. Images were treated with the Digital Nanoscope Software (Version 4.43r2, Digital Instruments) for 3D representation. AFM observations were done with control and thermally treated HAV virus.

Results and discussion

To gain mechanistic insight about viral inactivation of HAV in buffered cell culture medium, thermal treatment at 50, 56, 60, 65, and 72°C was applied for different treatment times (0-2 min) (Figure 1A-E).

Based on electron micrographs applied to un-treated HAV (control), the size of the virus particles was between 30 to 34 nm. After thermal treatment at 50°C for 2 min, the average diameter of the particles was 27 nm. Even though this value still corresponds to the inside diameter of HAV capsid (27-32 nm), it could be considered as a slight change in virus structure when compared to untreated control virus. In addition to decrease in particle size, the deformation around capsid was also observed (Figure 1A, B). The similar structural change in capsid was also observed after thermal treatment at 56°C for

1 min (Figure 1C). It has been reported that at mild temperatures ($<56^{\circ}\text{C}$), the destruction of the viral receptor and structural changes in the capsid might cause the inactivation by disrupting the specific structures needed to recognize and bind the host cells (Wigginton et al., 2012).

After treatment for 1 min at 60°C , the disruption around capsid structure was observed (Figure 1D). This structural change might be associated with the degradation of the viral capsid protein by heat. Pollard (1960) discussed the theory of virus inactivation during thermal treatment and he concluded that structural alterations in viral protein occur due to the differential expansion of the various parts of the virus under the action of heat. Heat disrupt the hydrogen bonding and destroy the space relationship that is necessary to keep the structural integrity of viral proteins mode of action. It is quite possible that the various components of the virus such as capsid, and nucleic acid have widely different values of entropy and enthalpy. Therefore, the degradation rate and/or amount of these component would be different (Pollard, 1960).

After treatment for 20 s at 65, and 72°C , the structural deformations around capsid structure were also observed (Figure 1E, F). This might be explained by the structural changes that occur in the capsid during thermal treatment. It has been reported that the quaternary structure of the virus capsid was unaffected up to 60°C ; however, above 60°C , the icosahedral capsid was significantly altered as inactivation of virus occurs at a faster rate above that temperature (Ausar et al., 2006). This hypothesis was also supported by other researchers (Bertrand et al., 2012; Bozkurt et al., 2013; Bozkurt

et al., 2014a; Bozkurt et al., 2014b; Bozkurt et al., 2014c; Croci et al., 1999; Croci et al., 2012; Sow et al., 2011).

Even though electron micrograph was helpful to observe any structural changes that occurred during thermal treatment, it was hard to identify these changes. Therefore, the three dimensional structures of HAV have also been investigated to identify any structural changes that occurred during thermal treatment (Figure 2A-F).

According to height measurements that obtained from AFM technique, the average heights were 31.20 ± 1.52 nm for the untreated virus particles, 24.70 ± 1.23 nm for lower-temperature-inactivated virus particles (50, 56, and 60°C), and 19.28 ± 1.24 nm for the higher-temperature-inactivated virus particles (65, and 72°C) (Figure 2A-F). It is also obvious from these images (Figure 2A-E) that the surfaces of untreated virus particles have a rather smooth appearance. In contrast, the surfaces of the heat-inactivated virus particles are rather rough-looking as shown by the AFM images.

The structures of viruses observed by AFM are entirely consistent with TEM, but AFM was provided detailed information about structural changes of HAV after thermal treatment. At lower temperatures (50, 56, and 60°C), the decrease in virion diameter (Figure 2A-D), and at high temperatures (65, and 72°C) the disruption of capsid was observed (Figure 2E-F). The degree of disruption that occurred in capsid was also increased with increasing temperature. After 20 s at 65, and 72°C, the spherical particles of HAV unravel, as seen in Figure 2(E) and (F), and assume a different three dimensional conformation. They also consist of small domains, presumably individual secondary structural elements, or groups of these, distributed in a different manner. This study also

confirmed that viral RNA levels remained unchanged regardless of time-temperature treatment combination when evaluated by real time RT-PCR (data not shown). After thermal treatment, the disrupted capsid aggregates around nucleic acid and protects their genetic material, the single-stranded positive sense RNA (Dimmock et al., 2001). Thus, the reason of unchanged viral RNA level might be associated with the tendency of enteric viruses to aggregate and/or protecting nucleic acid from heat. It is possible to expect that at high temperatures ($>75^{\circ}\text{C}$), altered capsid structure might facilitates access into interior protein, and in addition to loss of binding ability also causes to damage of nucleic acid.

Based on the data obtained from this study, the mechanism of heat inactivation of viruses is thought to be due to changes in the capsid of the virus particle, thus avoids binding and becomes inactivated. At studied temperature ($50\text{-}72^{\circ}\text{C}$), this damage to capsid was only limited and it retains the ability to protect the nucleic acid from the environment due to its tendency to aggregate.

Conclusions

The virus capsid encloses the viral genome and any other components necessary to virus structure or function and also responsible for binding to the host, the mechanism of thermal inactivation of viruses is associated with the changes in the capsid of the virus. The data obtained from this study did show that at thermal treatment of HAV was resulted with the structural changes in capsid and denaturation of the viral proteins. The amount of inactivation was increased with increasing temperature. This study provided useful mechanistic insights about viral inactivation during thermal treatment. A better

understanding of the mechanism of viral inactivation will guide the proper application of thermal process in industry.

Acknowledgment

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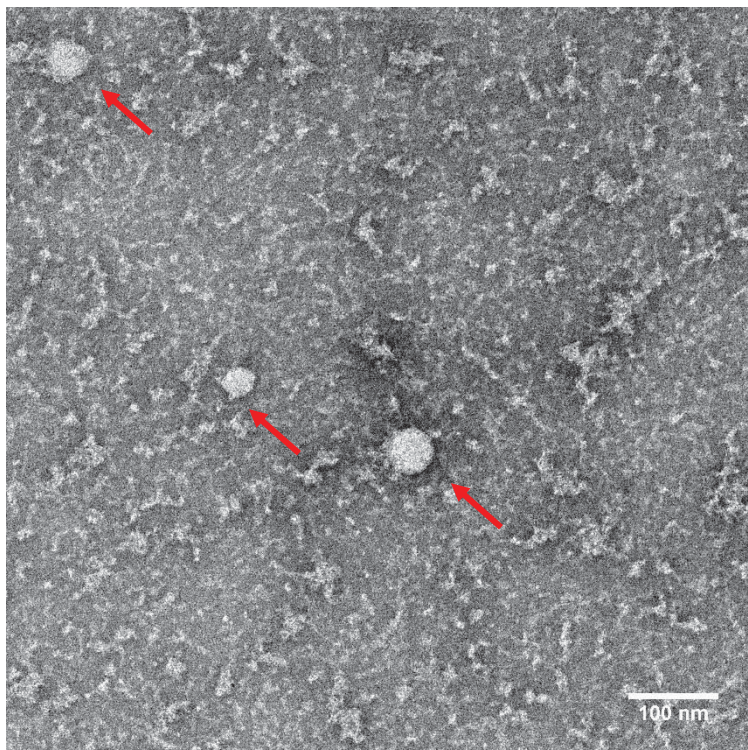
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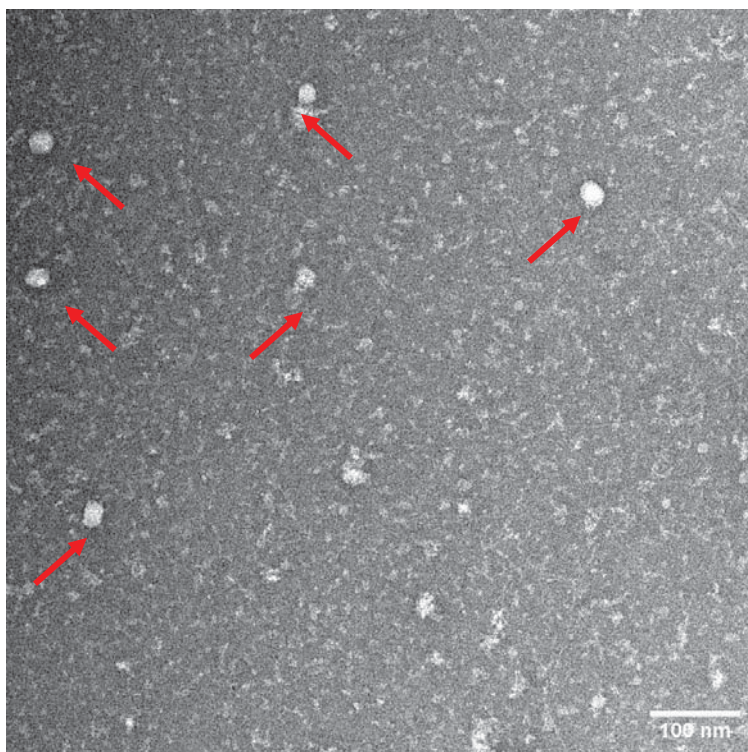
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Appendix

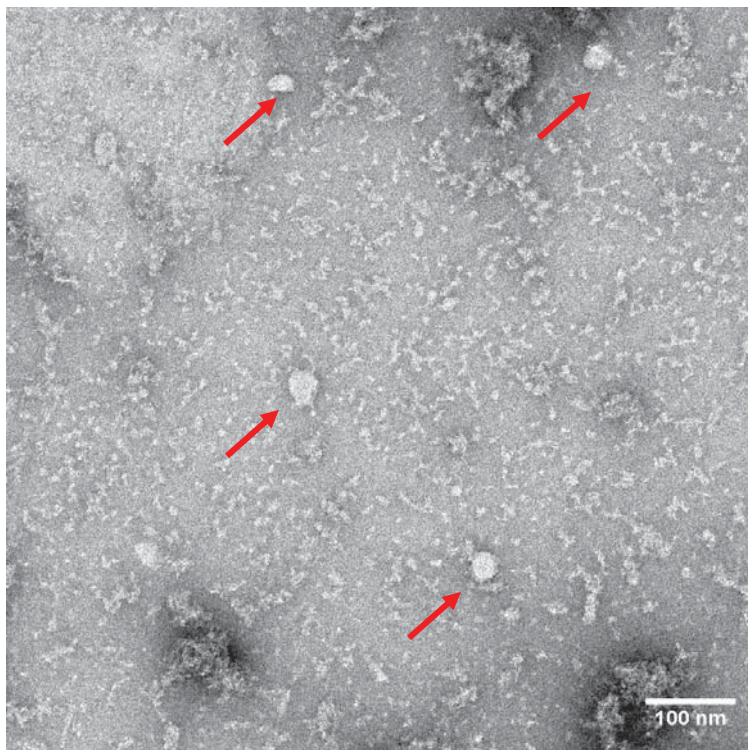


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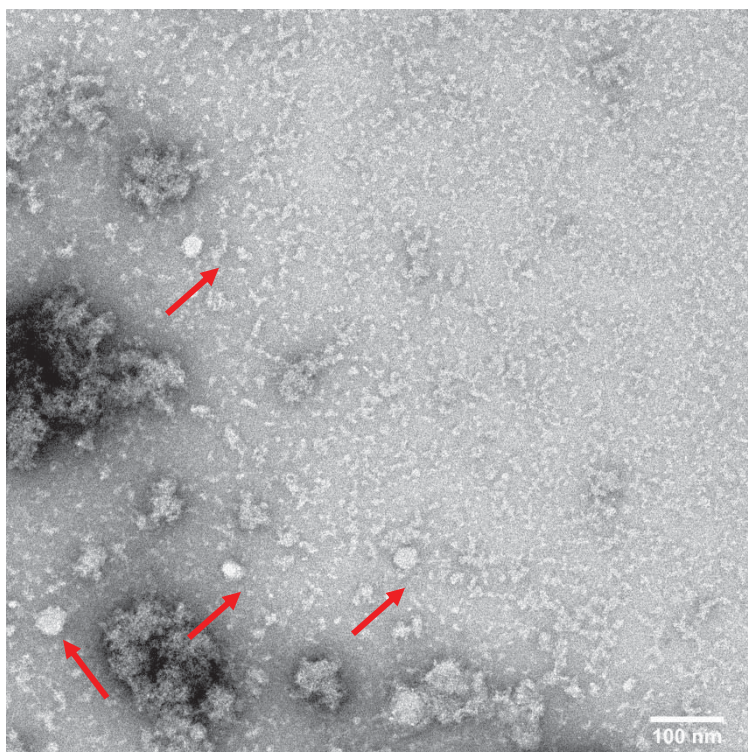


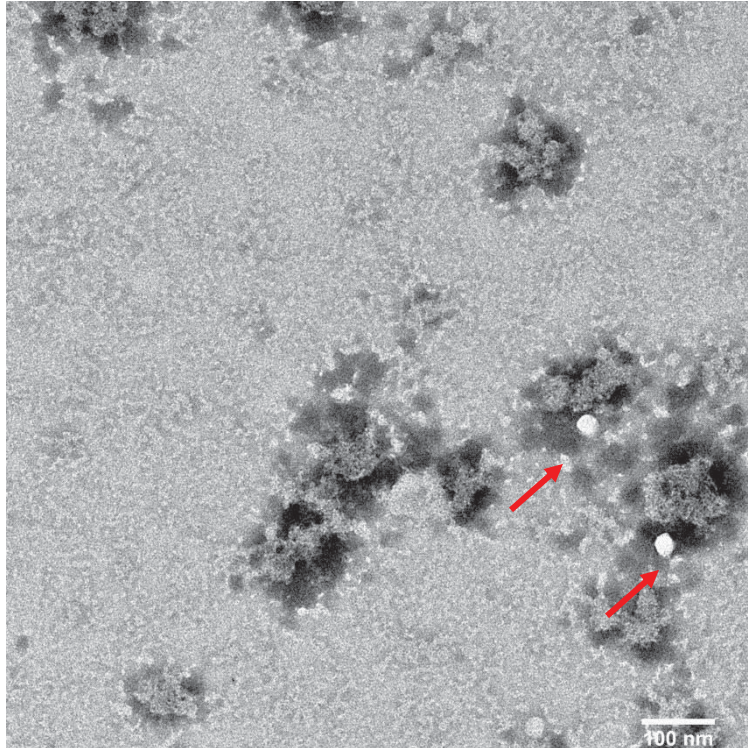
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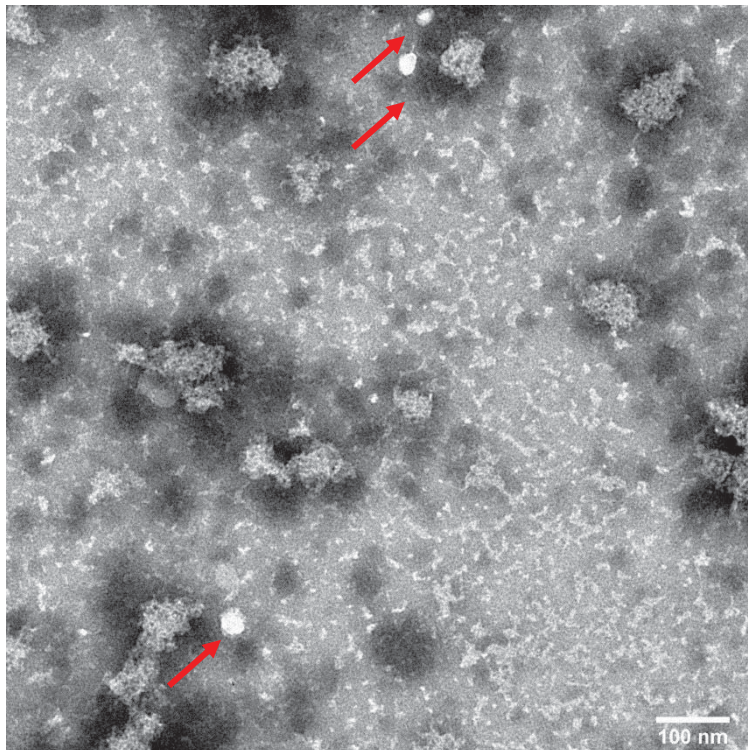


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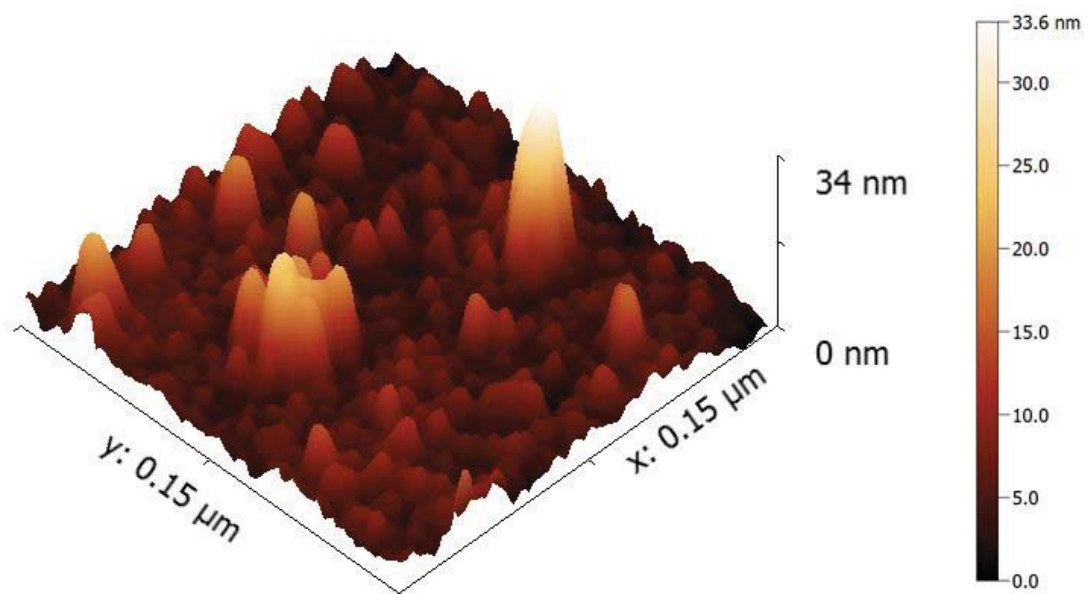
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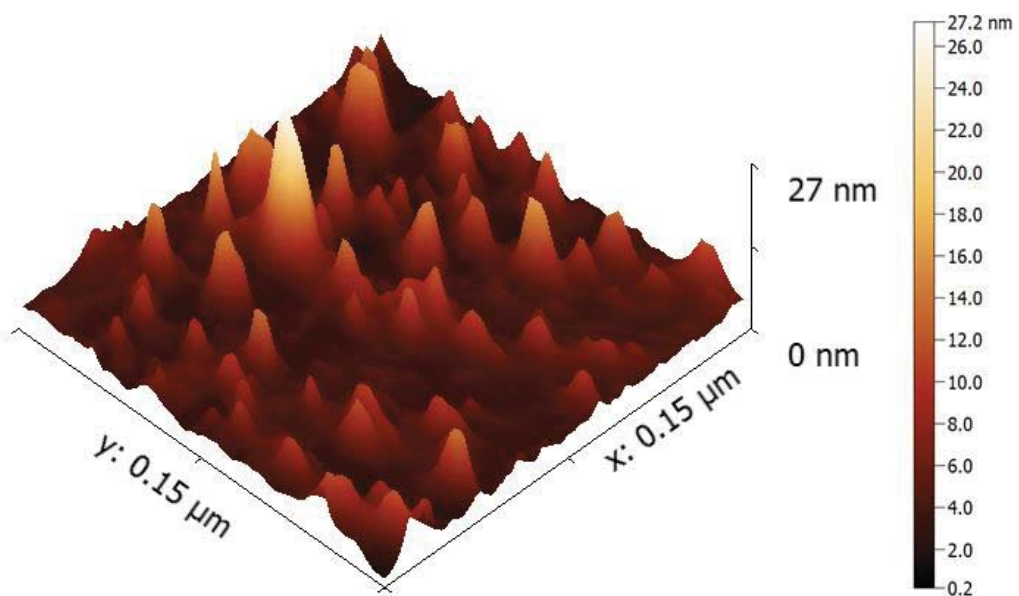
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Figure 9.1. Electron microscopy images of heat treated HAV. [A] control; [B] 50°C for 2 min; [C] 56°C for 1 min; [D] 60°C for 1 min; [E] 65°C for 20 s; [F] 72°C for 20s.

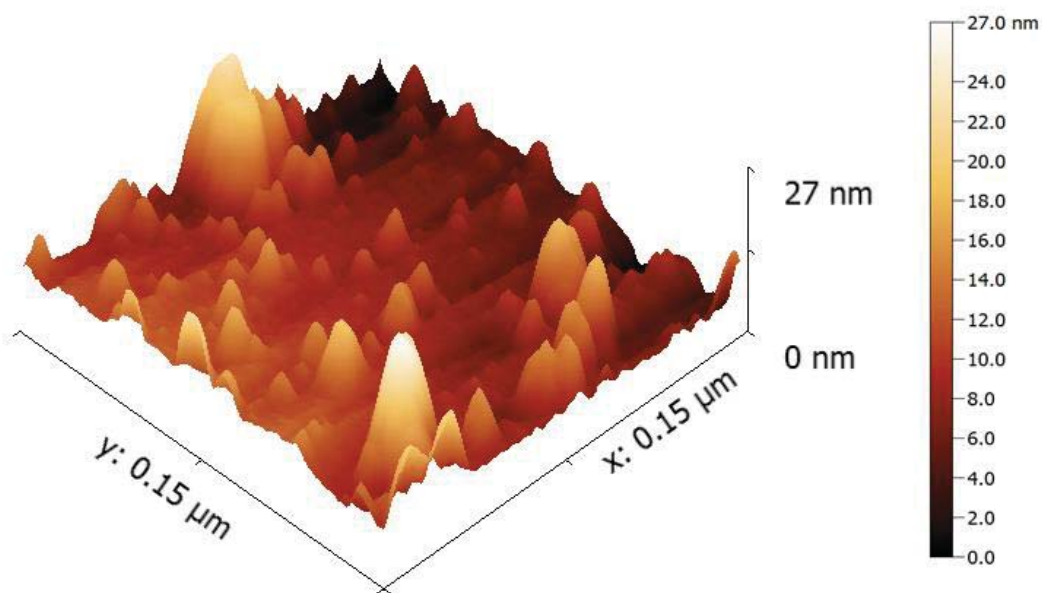
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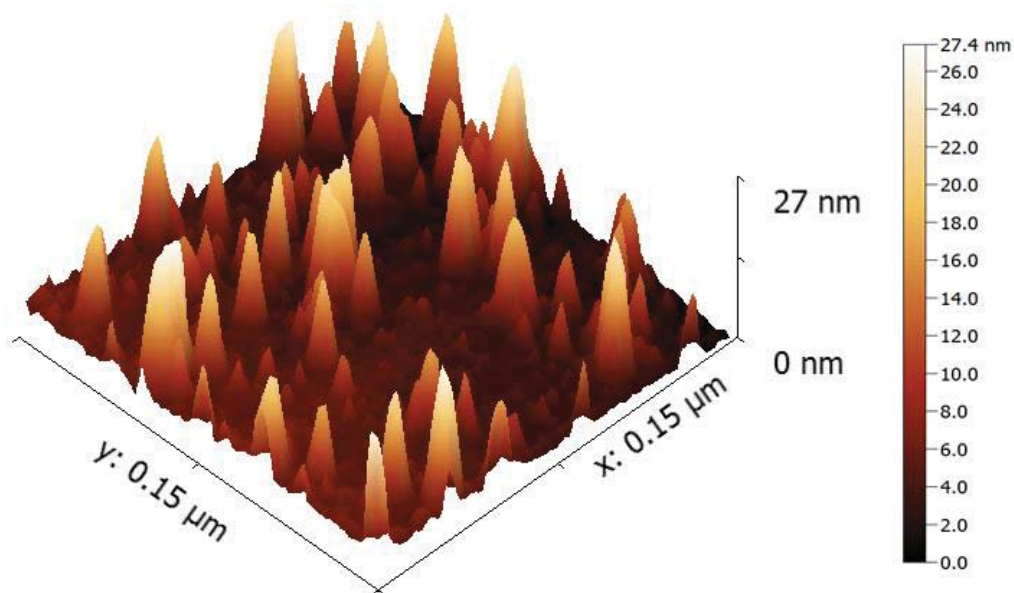
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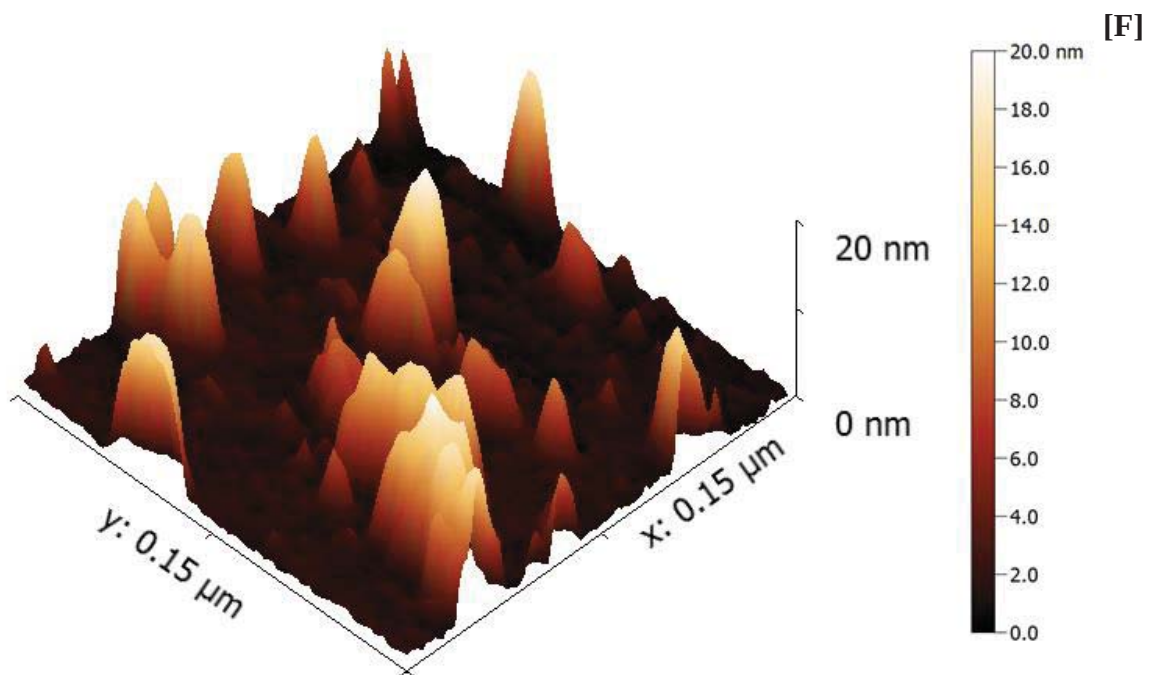
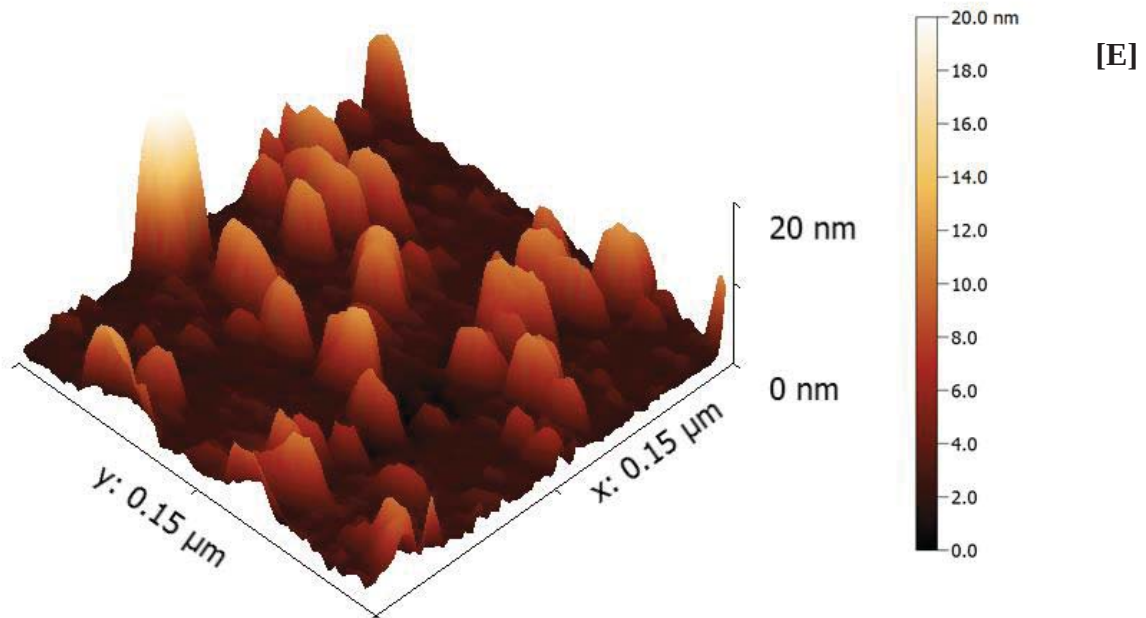


Figure 9.2. Atomic force microscopy images of heat treated HAV. [A] control; [B] 50°C for 2 min; [C] 56°C for 1 min; [D] 60°C for 1 min; [E] 65°C for 20 s; [F] 72°C for 20s.

CONCLUSION

Foodborne enteric viruses are more found to be heat resistant than most other foodborne non-sporeforming bacterial pathogens; thus, processing recommendations based on data for vegetative bacterial pathogens may not eliminate similar numbers of foodborne enteric viruses. Therefore, the correct understanding the thermal inactivation behavior of human norovirus and hepatitis A virus has great importance for integration of thermal processing. Since human noroviruses and HAV are the leading cause of acute gastroenteritis, the correct/accurate characterization of the thermal inactivation behavior of these viruses is essential for the food process industry. The result of this study should contribute to the development of appropriate thermal processing protocols to ensure safety of food for human consumption.

VITA

Hayriye Bozkurt Cekmer was born in Manisa on June 30, 1983. She was raised in Izmir as a youngest daughter of her family, who has three daughters and one son. She attended grade school, middle school, high school, and university in Izmir. She attended Ege University in Izmir and received her B.S. in Food Engineering in 2007 and an M.S. in Food Engineering in 2009. In 2011, she was awarded a Fulbright Doctoral Scholarship to study in University of Tennessee, Knoxville. Hayriye will obtain her doctorate degree in Food Science and Technology, major Food Microbiology at the University of Tennessee, Knoxville, in May 2014.