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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 noroviruses 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 to 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. The *z*-values determined for MNV-1 were 11.66 $\pm$ 0.42°C using the Weibull model and 10.98 $\pm$ 0.58°C for the first-order model and for FCV-F9 were 10.85 $\pm$ 0.67°C and 9.89 $\pm$ 0.79°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 to 7.56% and 11.99 to 21.01%, respectively, and for MNV-1, 3.10 to 7.01% and 13.14 to 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.

Human noroviruses are the leading cause of acute nonbacterial gastroenteritis worldwide because of their highly infectious nature and prevalence *(14)*. While epidemiological studies have shown presence of human norovirus in stools, the primary source for human infection is still unclear *(2, 23)*. 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 (MNV-1) and feline calicivirus (FCV-F9) have been used as surrogates to understand human norovirus behavior under laboratory conditions. MNV-1 and FCV-F9 both belong to the *Caliciviridae* family with single-stranded genomic RNA *(13)*. 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 *(30)*.

FCV-F9 is a respiratory virus and was the first animal virus surrogate used in laboratories to mimic human noroviruses *(15)*. It has been commonly used in studies involving leafy and green vegetables such as lettuce *(1, 18, 19, 21, 22, 26, 31, 42, 45)*, basil and parsley *(6, 7)*, cabbage *(1)*, and green onions *(18, 19, 22)*. It has been used to understand norovirus behavior in studies involving chemical disinfection *(1, 6, 21, 22, 42)*, inactivation by heat *(1, 42)*, freezing *(7)*, UV irradiation *(18, 19)*, determination of recovery efficiency *(18)*, and detection *(31)*.

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 *(44)*. 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 *(8)*. MNV-1 has been extensively used in studies involving lettuce *(11, 16, 21, 22, 25, 28, 29, 39, 42)* green onion *(2, 22)*, brussel sprouts and

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peas *(37),* iceberg lettuce *(3, 24),* parsley *(7, 32),* romaine lettuce *(12, 17)*, basil *(7),* and cabbage *(29).* As with FCV-9, it has been used in studies on chemical disinfection *(3, 21, 24, 29, 39, 42)*, inactivation by heat *(2, 42),* UV irradiation *(24)*, gamma irradiation *(17),* ozone *(22),* and freezing *(7),* and for studies on recovery efficiencies *(28, 32),* detection methodology *(37),* and stability *(11, 12, 16, 25, 42).*

Although several studies have been performed to investigate survival of norovirus surrogates in leafy vegetables, only a few *(2, 32, 34, 43)* have investigated survival in spinach and only one related to thermal inactivation. Baert et al. *(2)* investigated the efficiency of blanching (at 80°C for 1 min and 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. Therefore, 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 objectives of this study were (i) to determine thermal inactivation behavior of MNV-1 and 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 *(27).*

MATERIALS AND METHODS

Viruses and cell lines. MNV-1 were obtained from Dr. Skip Virgin (Washington University, St. Louis, MO), and its host Raw 264.7 cells were obtained from the University of Tennessee, Knoxville. FCV-F9 and its host, Crandell Reese feline kidney cells, were obtained from the American Type Culture Collection (Manassas, VA).

Propagation of viruses. FCV-F9 and MNV-1 stocks were prepared by inoculating FCV-F9 or MNV-1 onto confluent Crandell Reese feline kidney or RAW 264.7 cells, respectively, in 175-cm^2 flasks and incubating at 37°C and 5% CO_2 until >90% cell lysis was observed. The methods followed for the propagation of the viruses were as described in detail by Su et al. *(38).*

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 blender (Waring Model 1063, Waring Commercial, USA) to homogenize the sample. Five milliliters of each of the 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, Thermo Haake, Karlsruhe, Germany) was used to maintain a constant temperature (50 to 72°C ± 0.1°C) of the water bath during each experiment. Water bath temperature was confirmed with a mercury-in-glass 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 a MMS3000-T6V4–type portable data recorder (Commtest Inc., Christchurch, New Zealand) to monitor temperature. Samples were heated at 50, 56, 60, 65, and 72°C for different treatment times (0 to 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 the 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. *(3)* 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 nonspecific virus adsorption to the food matrix during extraction and facilitate the flocculation of norovirus surrogate particles on polyethylene glycol molecules), and 0.05 M glycine (to reduce nonspecific 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 × 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 polyethylene glycol precipitation of the virus particles). Polyethylene glycol 6000 (used for precipitation of viruses at high ionic concentrations without precipitation of other organic materials) and NaCl was added to obtain a final concentration of 10% polyethylene glycol 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 × *g* for 30 min at 4°C. The supernatant was discarded and the pellet dissolved in 1 ml of phosphate-buffered saline. 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 in spinach were diluted 1:10 in Dulbecco's modified Eagle medium 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. *(38).* Viral survivors were enumerated as PFU per milliliter.

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

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

where $N(t)$ is the number of survivors after an exposure time (t) in PFU per milliliter and the initial population is N_0 (PFU per

milliliter). D is the decimal reduction time in minutes (time required to kill 90% of viruses), and t is the treatment time (minutes).

Modeling of inactivation kinetics: Weibull model. The Weibull probability density function (equation 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 *(41)*. 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 (equation 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

$$\frac{N(t)}{N_0} = \exp\left(-\left(\frac{t}{\alpha}\right)^{\beta}\right) \quad (4)$$

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

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

where D is the number of decimal reductions.

Uncertainty analysis. Uncertainty analysis is a parameter that is associated with the result of measurement and used to characterize the dispersion of the values that could reasonably be attributed to the measurement *(20)*. 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 *(25)*. 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 *(27)*.

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, and u_a and u_b are standard uncertainties for a and b, respectively.

The relative standard uncertainty of personal counting is described as

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

where w_z is the relative standard uncertainty of personal accounting, 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_y is defined in equation 8

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

where $\sigma^2_{x_k}$ 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

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

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 is the relative standard uncertainty of one volume measurement, and V is the sum of all portions.

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

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

Data analysis and model evaluation. The statistical evaluation, linear, and nonlinear regression analyses were performed using SPSS ver.11.0.1 statistical package. The comparison test (analysis of variance and 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 $\pm$ 0.97 log PFU/ml for FCV-F9 and 7.40 $\pm$ 1.12 log PFU/ml for MNV-1. After inoculation of spinach with virus stocks, recovered titers varied between 5.60 $\pm$ 0.19 to 7.18 $\pm$ 0.12 log PFU/ml for MNV-1 (Table 1) and 6.4 $\pm$ 0.07 to 7.32 $\pm$ 0.06 log PFU/ml for FCV-F9, respectively (Table 2). The percentage recovery observed for MNV-1 was 75 to 97%, and for FCV-F9 it was 78 to 89%. These percentage recoveries were similar to that described by Baert et al. *(2)* who attributed greater adsorption of the virus to the smooth surface of the spinach, which could account allow for more efficient elution. In another study, Shieh et al. *(34)* found that hepatitis A virus was reduced by only 1 log over 4 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 *(3)*.

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 1 and 2). Also, MNV-1 (Table 1) and FCV-F9 (Table 2) survival decreased as temperature and time increased. Thus, temperature is the critical factor that determines the efficiency of thermal treatment against norovirus surrogate inactivation. As can be seen in Tables 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

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

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

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

TABLE 2. *Effect of thermal treatment on feline calicivirus (FCV-F9) inactivation in spinach*

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

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

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 nonenveloped, positive-stranded RNA viruses that are surrounded by protein shell (capsid) formed by units known as capsomers *(13)*. Due to this protein shell, inactivation of virus is faster after reaching the temperature required for the denaturation of protein (>56°C). Bertrand et al. *(4)* 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 and 60°C. A recent study by Bozkurt et al. *(5)* 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. *(36)* 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 *(36)*. Croci et al. *(10)* 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 *(5, 33, 40)* on application of different models to determine thermal inactivation kinetics of norovirus surrogates. Seo et al. *(33)* suggested the use of the Weibull model to describe the effect of temperature (24 to 85°C), pH (2 to 7), and NaCl concentration (3.3 to 6.3%) on the inactivation kinetics of MNV-1 rather than first-order model. Tuladhar et al. *(40)* also stated that the Weibull model provided the best fit to describe thermal stability of most of structurally variable viruses. Bozkurt et al. *(5)* concluded that the use of the Weibull model gave better fit compared with 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 3). The shape factor intervals for the studied temperature (50 to 72°C) were 0.51 ± 0.02 to 1.42 ± 0.04 for MNV-1 and 0.53 ± 0.05 to 2.09 ± 0.37 for FCV-F9. The results revealed that the shape factor values were significantly influenced by virus strain and temperature (Table 3); however, there was no correlation and/or trend with treatment temperature. The findings of the present study are consistent with Bozkurt et al. *(5)* 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.

TABLE 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*[a]

		Weibull distribution				First-order kinetics	
Virus strain	T (°C)	β	α (min)	$t_{D=1}$ (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

[a] Different letters indicate a significant difference among parameters (β, α, t_D-value, D-value) and within each virus ($P < 0.05$).

Parameters of the Weibull model (β and α) were used to calculate the t_D-value that was used as an analog to the D-value of the first-order model (Table 3). The D-values calculated from first-order model (50 to 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 model, 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 to 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. *(5)* reported t_D- and D-values for MNV-1 and FCV-F9 in buffer solution (Dulbecco's modified Eagle medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic) for the temperature range between 50 and 72°C. Because 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 the 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- and D-values were significantly higher than the t_D- and D-values of this study ($P < 0.05$).

For FCV-F9, the t_D- 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. *(4)* 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 with the buffer solution. This 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 *(10)*. 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. Because 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. *(4)* 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 *(9)*. To understand thermal inactivation of viruses in food, temperature and matrix interaction should be considered together *(4)*.

In a study by Baert et al. *(3)*, the effect of blanching on the survival of MNV-1 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 removed, and the spinach was kept in

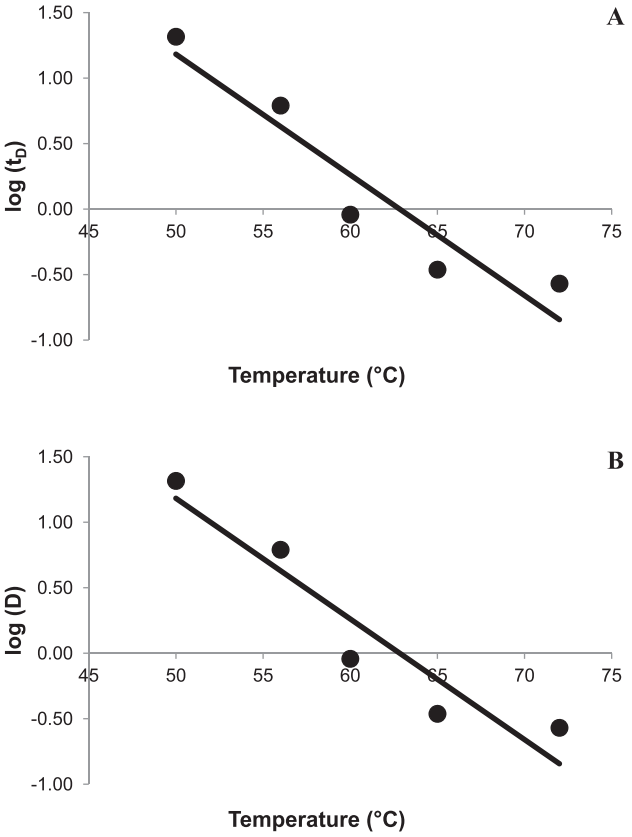

FIGURE 1. *Thermal inactivation curves of murine norovirus (MNV-1) for the (A) Weibull model (*$R^2 = 0.97$*) and (B) first-order model (*$R^2 = 0.96$*).*

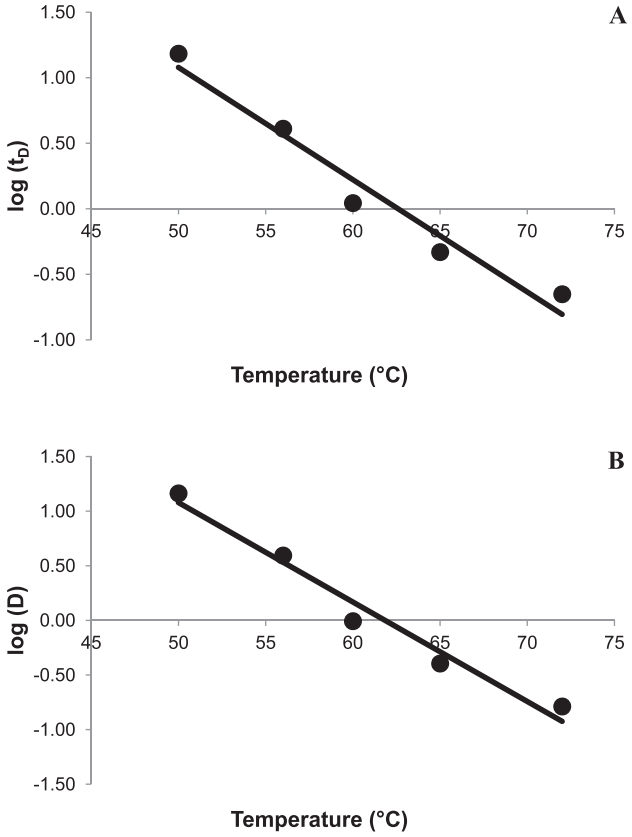

FIGURE 2. *Thermal inactivation curves of feline calicivirus (FCV-F9) for the (A) Weibull model (*$R^2 = 0.93$*) and (B) first-order model (*$R^2 = 0.90$*).*

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 Figures 1A and 1B and 2A and 2B. The calculated *z*-values for MNV-1 were 11.66 $\pm$ 0.42°C ($R^2 = 0.97$) and 10.98 $\pm$ 0.58°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°C [$R^2 = 0.93$]) or first-order model (9.89 $\pm$ 0.79°C [$R^2 = 0.90$]). In a previous study, Bozkurt et al. *(5)* 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, 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. 3A and 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 *(35),* industrial blanching conditions for spinach include use of steam as a heating medium for 120 to 180 s. Using the information generated in the present study and the thermal parameters of Singh *(35)* as a basis, the blanching of spinach in water at 100°C for 120 to 180 s under atmospheric conditions will provide

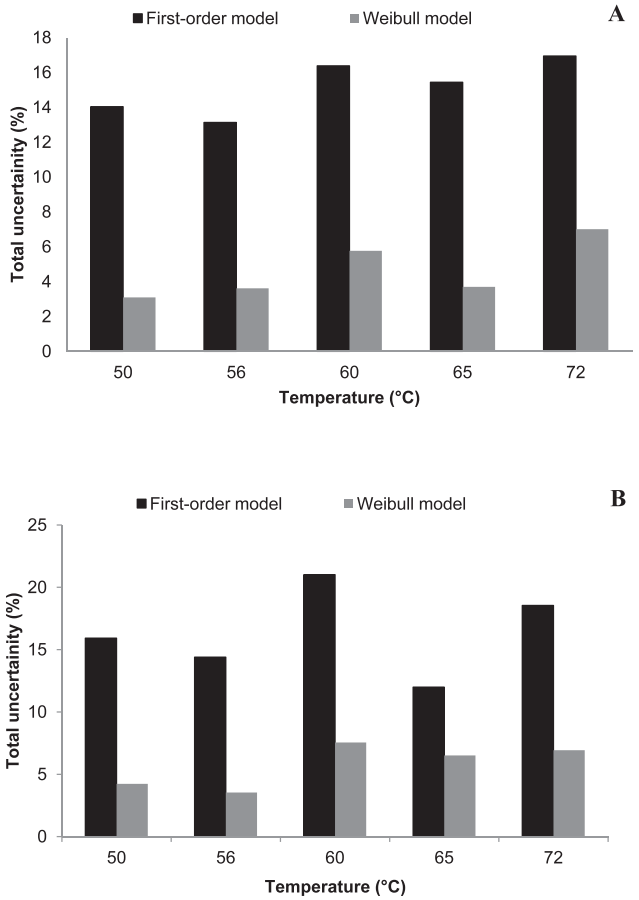

FIGURE 3. *Total combined uncertainty values for (A) murine norovirus (MNV-1) and (B) feline calicivirus (FCV-F9).*

greater than 7-log reduction of both norovirus surrogates using either model. Note that the 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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