

Comparative Study of Thermal Kinetics for *Clostridium sporogenes* PA 3679 Inactivation using Glass Capillary Tube and Aluminum Tube Methods in Carrot Juice and Phosphate Buffer

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Sterilization is one of the most commonly used processing methods in the food industry for the low acid foods. Thermal treatment must be adequate to destroy spores of *Clostridium botulinum* to ensure the low acid food is microbiologically safe. *C. sporogenes* PA 3679 spores were used as a surrogate to validate the thermal inactivation methods to control *C. botulinum*. The objective of this study was to determine the thermal resistance of *C. sporogenes* PA 3679 in M/15 phosphate buffer (pH 7.0) and carrot juice (pH 6.2) at temperatures of 115.6, 118.3, and 121.1°C using glass capillary tubes and novel aluminum thermal death time (TDT) tubes. The differences in come up time and thermal inactivation parameters of *C. sporogenes* PA 3679 (1.0×10^7 spores/ml) were compared. In phosphate buffer, D115.6, D118.3, D121.1 values for glass capillary tubes were 2.60, 1.11, and 0.54 min and for the aluminum TDT tubes 2.20, 1.12 and 0.60 min, respectively. In carrot juice, D115.6, D118.3, D121.1 values were 2.12, 1.32, and 0.69 min for glass capillary tubes and 2.40, 1.36, and 0.71 min for the aluminum TDT tubes, respectively. The z-values in phosphate buffer were 8.06°C and 11.27°C, and in carrot juice were 9.75°C and 10.39°C for capillary tube and aluminum TDT tubes, respectively. The results of this study may be used to design a thermal sterilization process for carrot and carrot containing food products.

Key words: Thermal inactivation kinetics, *Clostridium sporogenes* PA 3679, glass capillary tube, thermal death time tube, phosphate buffer, carrot juice.

Clostridium sporogenes (*C. sporogenes*) PA3679 is a mesophilic spore forming bacterium¹ and has been widely used as a surrogate microorganism to validate thermal processes because it is one of the most resistant anaerobic food spoilage bacteria known² and has a greater thermal resistance than the target pathogen *Clostridium botulinum* (*C. botulinum*). Its biochemical and physiological properties are closely related to *C. botulinum*.^{3,4} Because of the deadly neurotoxin it produces, *C. botulinum* is the target pathogen in commercial sterilized low-acid

foods (pH > 4.6). Compared to vegetative cells, spores resist physical and chemical treatments, and can rapidly recover from dormancy and germinate under anaerobic conditions.⁵

Carrot (*Daucus carota* L.) is one of the most commonly consumed and well-known vegetables with rich nutritional value such as vitamins (A, D, B, E, C, and K) and minerals (calcium, potassium, phosphorus, sodium, and iron).⁶ It can be eaten raw, processed or manufactured into a variety of products including juices, dehydrated soups, baby foods, and can be utilized in many chilled and frozen meals.⁷ During September and October of 2006, a severe outbreak of botulism in Canada and USA which was associated with commercial chilled fresh carrot juice was reported.^{8,9}

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These cases have raised questions regarding the safety of fresh carrot juice. Temperature abuse of foods intended to be stored chilled has also been associated with foodborne botulinum.¹⁰

Foods with pH values greater than 4.6 (meat, dairy produce, soups, and vegetables), as opposed to high acid foods like fruits and fruit juices, need a predefined time-temperature regime to inactivate *C. botulinum* spores. Heat inactivation of clostridial spores can be affected by the composition of the medium, the time and temperature of the heat treatment, and inter-strain variation.¹¹ These factors could, in turn, affect the accuracy of calculated D-values and changes in temperature required to change the D-values (z-values). The difference in D-values in media is mostly due to a different heat resistance in experimental strains used by different investigators. Greater variation in D-values of *C. sporogenes* for foods, since foods vary in their chemical composition, moisture content, pH, and physical characteristics.¹² These factors are important to control since *C. botulinum* can grow at pH > 4.6 and $a_w > 0.85$ in an anaerobic environment.¹³ The acidity of the substrate or medium in which spores can germinate is an important factor in determining heat resistance. *C. sporogenes* are most resistant to heat at their optimal pH for growth of 7.0.^{14,15} Therefore, phosphate buffer with its neutral pH was chosen for this study a treatment medium as well as carrot juice which can be considered as a suitable substrate for low-acid vegetables, since it is widely used (as ingredient or on its own) by the industry in several minimally processed foods, so that a more realistic shelf life can be established.

The two most popular methods to measure heat resistance in the range 60-135 °C are the end-point method and the multiple point method.⁵ Several types of multiple-point method have been reported in studies on the heat resistance of *C. sporogenes* among them are the thermal death time (TDT) method, the capillary tube method, and the thermoresistometer.¹⁴ A thermoresistometer has the advantage of providing short come-up times but the extensive control of residence times through direct exposure of a spore suspension in a liquid sample to a steam heating medium is needed.¹⁶⁻¹⁹ Computerization of thermal death time experiments has also been recommended

by Hachigan.²⁰ The TDT method is an indirect heating method suggested by Pflug²¹ as an objective tool for laboratory and manufacturing use to validate sterilization processes.¹ One of the major problems of heating spore suspensions is compensating for the time it takes for heat transfer through the container wall. TDT tubes and TDT cans are widely used to determine the value in food products while thermoresistometer and capillary methods are used only for liquid suspension.^{13,22} Luechapattananorn *et al.*³, al-Holy *et al.*²³ developed novel aluminum TDT tubes validating dielectric heating processes with inoculated pack studies. The capillary tube method is a modification of a TDT method and has been used to study the kinetics of inactivation of *C. sporogenes* and *Listeria monocytogenes*.^{14,24} It has the advantage of providing a much shorter come up times than other methods, and requires lower volumes of highly concentrated spore suspensions. Although widely differing values are reported in published studies for the heat resistance of *C. sporogenes* a D-value of *C. sporogenes* at 121°C range between 0.1 and 1.5 min with z-value ranging between 7.8 – 11.1 °C.^{22,25,26} depending upon media, strain, and experimental method employed. The method that determines the Thermal death time is also important which may affect the come-up time, heating uniformity, or heat distribution during thermal inactivation experiments.

The aim of this study was to determine the thermal resistance of the *C. sporogenes* in a M/15 phosphate buffer (pH 7.0) and a food matrix (carrot juice as its natural pH (6.2) by using the novel aluminum TDT tubes and the classical capillary glass tube at 115.6, 118.3, and 121.1 °C based on the comparison of the experimental methods.

MATERIAL AND METHODS

Spore preparation

The *C. sporogenes* PA 3679 (NFPA nr S.C. 218) spore suspension in phosphate buffer (1.6 x 10⁸ colony forming units (CFU)/mL) were obtained from the Technical Service Center of National Food Processors Association (NFPA) now Food Products Association in Dublin, CA. The identity of the spores was confirmed microscopically upon

receipt. The spore suspension was enumerated and the initial concentration was verified. Any vegetative cells that may have been present in the spore suspension were inactivated by heating at 90 °C for 10 min.

Preparation of phosphate buffer

M/15 phosphate buffer (pH 7.0) was prepared by mixing of Na₂HPO₄ (5.675g), KH₂PO₄ (3.63g) in 1000 ml of distilled water. The buffer was dispensed in 100-mL quantities and sterilized by autoclaving at 121 °C for 20 min. The buffer was refrigerated until use.

Preparation of carrot juice

Carrots (*Daucus carota L. var. Sativa*) were purchased from a local store. After being washed thoroughly, they were peeled and washed again. The juice was prepared using a Hamilton Beach® juice extractor. Homogenate was filtered. The juice obtained was dispensed in 100 ml volumes and sterilized following a tyndallization process, which involved heating at 80 °C for 1 h on three consecutive days and storing at room temperature between heatings.²⁷ After autoclaving (121 °C for 15 min) the juice was kept in refrigerator until use.

Heat Treatment Procedure

Thermal resistance of *C. sporogenes* PA 3679 was determined in M/15 phosphate buffer (pH 7.0) and carrot juice (pH 6.2) at temperatures of 115.6, 118.3, and 121.1 °C using glass capillary tubes (KIMAX-51 glass melting point capillary tubes 0.8-1.1 mm in diameter and 100 mm long; Kimax, Kimble / Kontes, Vineland, NJ) and aluminum TDT tubes.²³ Glass tubes were covered with aluminum foil and sterilized at 135 °C, then centrally filled with 0.04 ml of spore suspension in M/15 phosphate buffer or carrot juice at ca. 1.0x10⁶ CFU/ml by using a thin micropipet tip. Both ends of the capillary tube were heat sealed, and precautions were taken to assure that the injected suspension was not heated during this process. The spore inoculated samples also were transferred to sterile aluminum TDT tubes. Both sample containing glass and aluminum TDT tubes were kept in an ice bath at 0±0.2°C until they were heat treated. Three glass capillary tubes were randomly selected for each heat treatment. Then the glass and aluminum tubes were completely immersed in VWR Model 1157 programmable circulator oil bath (VWR Science Products, West Chester, PA) at the

designated treatment temperatures. The sample temperature was monitored and recorded by inserting a T-type thermocouple (Omega, Stamford, CT) attached to a data logger (Delta-T devices, Cambridge, UK) into a control glass capillary tube (sealed with silicon) and aluminum TDT tubes containing buffer or carrot juice without spores. The thermal come-up time was recorded when the target temperature (115.6°C, 118.3°C, and 121.1°C) was attained. Immediately after the sample temperature reached the set temperature (± 0.2°C), the heating time was recorded and the first set of tubes (t=0) was removed from the oil bath. Thereafter, samples in glass capillary tubes or aluminum TDT tubes corresponding to the each treatment were removed at intervals of 20, 40, and 60 s for 115.6, 118.5, and 121 °C, respectively. After heating in the oil bath, the samples were immersed promptly into an ice bath at 0.0±0.2°C. Unheated control samples were activated by heating in a test tube for 10 min in a water bath set at 90°C.

Microbiological analysis

Heat treated and cooled glass capillary tubes were sanitized by submersion in 70% ethyl alcohol for one min. Alcohol was removed by placing the tube on sterile filter paper. Each capillary tube was then placed in a sterile disposable test tube containing 4 ml of freshly prepared 0.1% peptone water (Difco, Detroit, MI). The capillary tube was crushed inside the test tube using a glass rod with a flat end. Heat treated and cooled aluminum TDT samples were diluted with sterile peptone water. Serially diluted samples were pour plated in duplicates onto Shahidi Ferguson Perfringens (SFP) agar (Difco Laboratories, Inc, Detroit, MI). The plates were incubated at 37 °C for 48 h in anaerobic jars using Anaerogen (Oxoid, Ogdensburg, NY) and GasPak (VWR International, Brisbane, CA); an anaerobic atmosphere generator and anaerobic indicator, respectively. After incubation, plates were counted, and the average number of surviving colony forming units (CFU) per ml of samples was calculated. Experiments were conducted in triplicates.

Calculation of D- and z-values

The decimal reduction time (D-value) quantify heat resistance and D represents the probability of a cell escaping death per unit time, and can be defined as “the heating time in minutes to give an expected inactivation of 90% of

organisms, or a 10% survival".²⁸ The probability of inactivating spores with equal heat resistance for any given thermal process can be defined in equation (1) where the number of survivors N at any given time t can be expressed in the following probabilistic model.⁵

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

Where: N equals the number of survivors, N_0 is the number of viable organisms at time zero and D the decimal reduction time (D -value).

Survivor curves were constructed by plotting \log_{10} number of survivors at each temp vs. heating times. The D -values for the *C. sporogenes* PA 3679 at each temperature were calculated by taking the negative inverse of the survivor curve slope value in semi-log coordinates using by Equation 1.

The z value (the change in temperature required for a 10-fold change in the D -value) of *C. sporogenes* spores was estimated by plotting the $\log_{10} D$ values versus temperatures (Microsoft Excel 2003 Software (Microsoft Inc., Redmond, WA) and calculated by Equation 2.²⁹

$$z = \frac{T_2 - T_1}{\log_{10} D_{T1} - \log_{10} D_{T2}} \quad \dots(2)$$

Statistical analysis

The \log_{10} numbers of the survivors at each temperature were plotted against the time. The best fit line was extrapolated, and the D -values were determined. The z -values were determined by plotting the calculated $\log D$ -values against the corresponding temperatures. Each single number is an average of at least three replicate experiments.

The standard deviation was determined. The data analysis was conducted using SAS (SAS Institute, Cary, NC). Analysis of variance method was performed using PROC GLM procedure in SAS software ($P < 0.05$).

RESULTS AND DISCUSSION

Come-up time of the test methods

Figure 1 shows a typical come-up time of phosphate buffer and carrot juice in a TDT tubes and glass capillary tubes in oil bath set 121°C. Within the experimental ranges of the study, thermal treatment had a come-up time (CUT) of approximately 9-10 s and to 14-14.9s for glass and TDT tubes at 121 °C. Although the test cell CUT time was still larger than the typical D -value of the microorganisms, but the CUT was reasonably short for the sample size. The thermal CUT was recorded when the target temperature was attained (Table 1). The reproducible come up time of 9 ± 1 sec (Figure 1) for phosphate buffer was necessary.

Experimentally measured CUT for the carrot juice in the test cell was 12 s, which was shorter than that for the aluminum tubes.

Similar come up times for each treatment temperature were observed at the three treatment temperatures, possibly due to the narrow temperature range (3.3°C). Capillary tubes provide a relatively short come up times compared to other methods. For example, Al-Holy *et al.*²³ used Al tubes for salmon caviar and found that the CUTs were significantly shorter in the Al TDT tubes (82.7 s) than in the glass tubes (181.7 s) at 60 °C. Short come-up time could reduce thermal lag time and

Table 1. Come-up times and heat resistance of *C. sporogenes* PA 3679 in phosphate buffer and carrot juice

	Temp (°C)	Come-up time (s)		D-value ^a	
		Glass capillary tubes (1-3mm)	Al-TDT tubes	Glass capillary tubes	Al-TDT tubes
Phosphate buffer (pH 7.0)	115.6	8.20	12.2	2.60	2.20
	118.3	8.50	13.1	1.11	1.12
	121.1	9.00	14.9	0.54	0.60
Carrot juice (pH 6.2)	115.6	8.50	12.6	2.12	2.40
	118.3	8.90	13.4	1.32	1.36
	121.1	9.28	14.0	0.69	0.71

^a Average standard deviation of three measurements

increase accuracy for determining the heat resistance of *Listeria monocytogenes*.³⁰ In other study, come up times of greater than 8 sec at 135°C sec were found by Stern and Proctor.³¹ However, the glass tubes used in this study are capillary glass tubes sized 1-3mm, therefore shorter come up time was expectable.

Thermal resistance of *C. sporogenes* PA 3679 spores

Spores of the *C. sporogenes* PA 3679 suspended in phosphate buffer and carrot juice were tested for heat resistance over the temperature range 115.6-121.1 °C. Figure 2 and 3 show the survival curves of *C. sporogenes* PA 3679 spores in a phosphate buffer

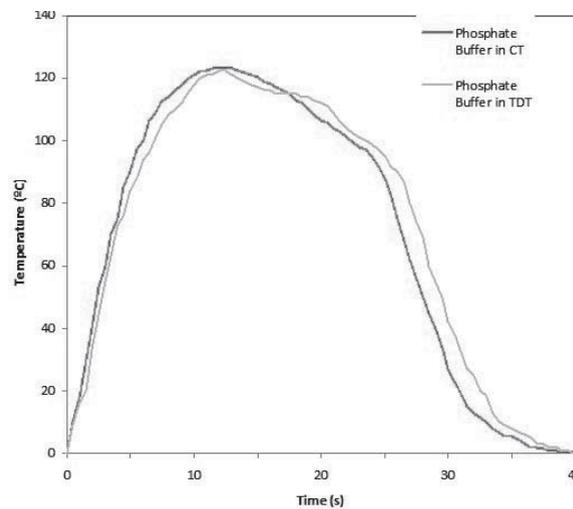


Fig. 1. Time-temperature history for inactivation of *C. sporogenes* PA 3679 in capillary tubes and TDT contain phosphate buffer or carrot juice at 121.1 °C

(pH 7.0) and carrot juice (pH 6.2) at three temperatures (115.6, 118.3, and 121.1°C) determined with the aluminum TDT and capillary tube method, respectively. Based on the linear portion of these survivor curves, D values were calculated and z values were obtained. Table 1 summarizes the D values and z values for *C. sporogenes* in carrot juice and phosphate buffer. The D-values of *C. sporogenes* PA 3679 in glass capillary tubes were 2.60 (1.78) ($R^2=0.95$), 1.11 ($R^2=0.87$), and 0.54 ($R^2=0.96$) min for phosphate buffer and 2.12, 1.32, and 0.69 min for carrot juice; and the D-values for the aluminum TDT tubes, 2.20, 1.12, and 0.60 min for phosphate buffer and 2.20, 1.36, and 0.71 min for carrot juice at 115.6, 118.3, and 121.1 °C, respectively. The D-values obtained with the glass capillary tube for both carrot juice and phosphate buffer were not significantly different from those by the novel Aluminum TDT tubes ($P>0.05$). The estimated z-values in phosphate buffer (pH 7.0) were 8.06 °C and 11.27 °C and in carrot juice were 9.75 °C and 10.39 °C for capillary tube and aluminum TDT tubes, respectively. The z-values obtained for the glass capillary tube and aluminum TDT tubes were also not significantly different

($P>0.05$).

Although the z-values fall within the range of z-values reported by Stumbo²² for *C. sporogenes*, it is lower than the one (12°C) reported by Luechapattanaporn *et al.*³¹ using aluminum TDT tubes and by others.³² It appears that the thermal resistance of *C. sporogenes* in phosphate buffer was lower than that in carrot juice. This is probably due to the products' different pH, water activity, and nature of constituents.³³

The heat resistance of bacteria in foods using different methods have been studied by many researchers. Nevertheless, lower or similar heat resistance was reported for *C. sporogenes* PA 3679 in mushroom extract, asparagus puree and mashed potato as compared to reference buffers.^{34,35,3,32} For example, Chung *et al.*³² compared D-values of *C. sporogenes* spores in phosphate buffer and mashed potato using the newly designed test cell method against glass capillary tube (3mm) and TDT tubes. They observed a D-value at 121.1 °C of *C. sporogenes* PA 3679 spores in a phosphate buffer as 0.88 min. and a z-value of 10.04 °C in mashed potato.

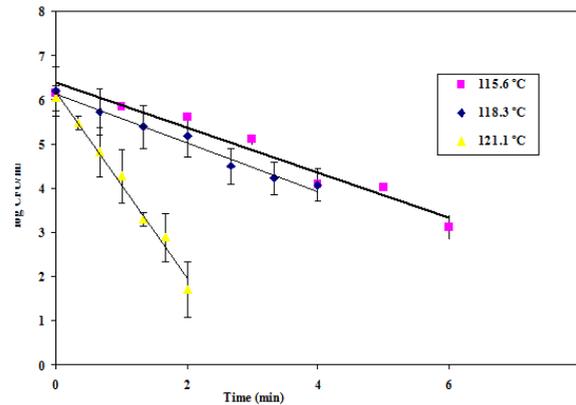


Fig. 2. Survival curves of *Clostridium sporogenes* PA 3679 spores in a phosphate buffer (pH 7.0) at 115.6, 118.3, and 121.1 °C temperature via the capillary tube method

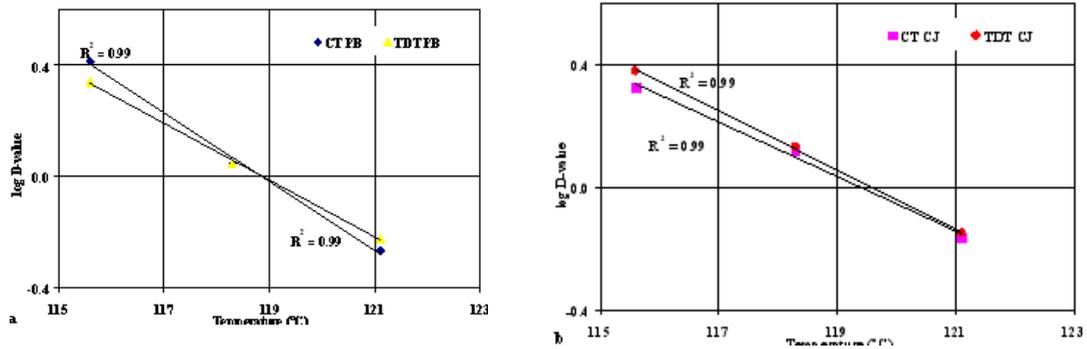


Fig. 3. Thermal death time curve of *C. sporogenes* PA 3679 via the capillary tube method (CT) and TDT tube method as a function of temperature in (a) phosphate buffer (PB) (pH 7.0) and (b) carrot juice (CJ) (pH 6.2)

Luechapattanaporn *et al.*³ obtained the D-values (min) of 1.8, 1.1 and 0.62 at 115.6, 118.3, and 121.1 °C, respectively using aluminum TDT tubes for mashed potato samples. Cameron *et al.*¹⁴ compared the D-values of *C. sporogenes* spores in a phosphate buffer using the capillary tube method with the D-values in pea puree by the TDT can method. The authors found that the D-values in pea puree with TDT cans were nine times higher than that of the capillary tube method (6.4, 4.0, and 2.6 min, at 115.6, 118.3, 121.1 °C, respectively) with a phosphate buffer at the same pH and the z-value of TDT can was slightly higher than capillary tubes. Al-Holy *et al.*²³ reported the heat resistance of *L. innocua* in salmon caviar by aluminum tubes and glass tubes. In another study, D- and z-value were 1.07 min and 9.33 °C for *C. sporogenes*, respectively, in a neutral phosphate buffer.³⁶ To the best of our knowledge, no direct comparison of bacterial spore

heat resistance by capillary tube methods with other test methods in real food matrices exists.

In the present study, z-values of 11.27 °C or 10.39 °C in carrot juice either for glass capillary tube or TDT tubes at 115–121.1 °C were generally higher than literature data for most of other bacterial endospores, usually around $z = 10$ °C. However, Santos and Zarzo,³⁷ reported that z-value for *C. sporogenes* PA 3679 was 8.99 °C at the highest temperature range (130–145 °C) and 23.5 °C at the lowest range (121–145 °C) measured in Sorensen's phosphate buffer. The results of this study and the literature show that, z-values may be higher or lower than the universally accepted 10 °C. This is possibly because the spores had a different thermal resistance. The discrepancy may be due to differences in the physical characteristics and chemical composition of food substrates such as pH, solid content and water activity as well as

strain choice, temperature range and heating method (i.e. dry or moist heat).³⁷

CONCLUSION

The capillary and TDT tube methods can be useful for determine decimal reduction times (D-value) and z-values for *C. sporogenes* PA 3679 and validating thermal processes. D-values of *C. sporogenes* PA 3679 using glass capillary tube method were 2.60, 1.10 and 0.54 min in phosphate buffer and 2.12, 1.32, and 0.69 min in carrot juice at 115.6, 118.3 and 121.1°C, respectively; with a z-value of 8.01°C and 11.27°C in phosphate buffer (pH=7.0) and in carrot juice (pH=6.2), respectively. D-values for TDT tubes, 2.20, 1.12, and 0.60 min in phosphate buffer and 2.40, 1.36, and 0.71 min in carrot juice at 115.6, 118.3 and 121.1°C, respectively; with a z-value of 9.75°C in phosphate buffer 10.39° for carrot juice. This z- value was lower than reported in some other recent studies indicating that strains of *C. sporogenes* PA 3679 have different heat resistance. The results of this study may be used as an aid to predict the processing time required at specified temperatures to achieve a certain number of log-cycle reductions of *Clostridium* spores when heated in carrot and carrot containing food products. Automation and evaluation of a number of different strains should be included in further studies to provide better processing parameters for producing higher quality and safer food products.

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